New candidates for CD4 T cell pathogenicity in experimental neuroinflammation and multiple sclerosis

Nicola Hoppmann,1 Christiane Graetz,1 Magdalena Paterka,1 Laura Poisa-Beiro,1 Catherine Larochelle,1 Maruf Hasan,1 Christina M. Lill,1,2,3 Frauke Zipp1,* and Volker Siffrin1,*

*These authors contributed equally to this work.

Multiple sclerosis is a chronic autoimmune demyelinating disease of the central nervous system, which is thought to be triggered by environmental factors in genetically susceptible individuals leading to activation of autoreactive T lymphocytes. Large multi-centre genome-wide association studies have identified multiple genetic risk loci in multiple sclerosis. In this study, we investigated T cell transcriptomic changes in experimental autoimmune encephalomyelitis, an animal model for multiple sclerosis. We correlated these findings with the multiple sclerosis risk genes postulated by the most recent Immunochip analysis and found that multiple sclerosis susceptibility genes were significantly regulated in experimental autoimmune encephalomyelitis. Our data indicate that nine distinct genes associated with multiple sclerosis risk, Bach2, Il2ra, Irf8, Mertk, Odf3b, Plek, Rgs1, Sdc30a7 and Thada, can be confirmed to be differentially regulated in pathogenic CD4+ T cells. During the effector phase within the inflamed CNS, CD4+ T cells undergo comprehensive transformation and we identified key transcription factors and signalling networks involved in this process. The transformation was linked to metabolic changes with the involvement of liver X receptor/retinoid X receptor signalling and cholesterol biosynthesis, which might control the T cell effector function in the central nervous system. Thus, our study confirms the involvement of multiple sclerosis risk genes in the pathophysiology of the animal model and sheds light on additional disease-relevant inflammatory networks.

1 Department of Neurology, Focus Program Translational Neuroscience (FTN) and Immunotherapy (FZI), Rhine-Main Neuroscience Network (rmn²), University Medical Centre of the Johannes Gutenberg University Mainz, Langenbeckstr. 1, 55131 Mainz, Germany
2 Neuropsychiatric Genetics Group, Department of Vertebrate Genomics, Max Planck Institute for Molecular Genetics, Ihnesstr. 63-73, 14195 Berlin, Germany
3 Platform for Genome Analytics, Institutes for Neurogenetics & Integrative and Experimental Genomics, University of Lübeck, Maria-Goeppert-Str. 1, 23562 Lübeck, Germany

Correspondence to: Dr Volker Siffrin,
Klinik und Poliklinik für Neurologie,
Universitätsmedizin Mainz,
Johannes-Gutenberg Universität Mainz,
Langenbeckstr. 1,
55131 Mainz, Germany
E-mail: siffrinv@gmx.de
New candidates in MS pathogenesis

**Introduction**

Multiple sclerosis is a chronic autoimmune disease of the CNS that affects mainly young adults in countries of the Western world. Multiple sclerosis is likely caused by a combination—and interaction—of a few environmental and dozens to hundreds of heritable risk factors. As early as in the 1970s, genetic variation in the major histocompatibility complex (MHC) region was identified to increase the risk of multiple sclerosis (Bertrams and Kuwert, 1972; Naito et al., 2005). The most evident question that arises from the identification of the nearest gene, these multiple sclerosis risk genes were subjected to gene ontology as well as network analyses, which showed an evident overrepresentation of immune cell-associated, and in particular T cell-associated, genes (IMSGC and WTCCC2, 2011; IMSGC, 2013b). Taken together, the currently identified common multiple sclerosis risk variants (single nucleotide polymorphisms, SNPs) explain ~28% of the sibling recurrence risk (IMSGC, 2013b). Following annotation of the identified multiple sclerosis risk variants to the nearest gene, these multiple sclerosis risk genes were subjected to gene ontology as well as network analyses, which showed an evident overrepresentation of immune cell-associated, and in particular T cell-associated, genes (IMSGC and WTCCC2, 2011; IMSGC, 2013b). These findings underline the primary role of immune processes in the pathophysiology of multiple sclerosis.

The most evident question that arises from the identification of these multiple sclerosis susceptibility genes concerns their biological function. Because of the scarcity of tissue samples, especially from the CNS of multiple sclerosis patients, the possibility of transcriptome analysis in the human system is very limited, and thus, disease models can be helpful in gaining insights into the pathophysiology underlying the human disease. A widely used animal model for multiple sclerosis is experimental autoimmune encephalomyelitis (EAE), which—despite having several limitations—reproduces specific aspects of multiple sclerosis pathology and has helped to understand different processes and cellular mechanisms relevant to the human disease (Gold et al., 2006; Batoulis et al., 2011). It has become clear that in the animal model the cytokine interleukin (IL) 23 is crucial for EAE induction through its role in propagating the pro-inflammatory Th17 phenotype (Cua et al., 2003; Langrish et al., 2005), which has also been associated with disease activity in the human disease (Tzartos et al., 2008; Brucklacher-Waldert et al., 2009; Montes et al., 2009).

In *vitro* primed IL17-producing cells undergo massive changes in the course of adoptive transfer EAE, including cytokine and transcription factor expression (Bending et al., 2009; Lee et al., 2009). These cells, also referred to as ‘ex-Th17 cells’, have characteristics of Th1 cells, e.g. IFNG production, but differ from classical Th1 cells (Kurschus et al., 2010; Hirota et al., 2011). There is continued debate about what factors render a T cell encephalitogenic. T cell phenotypes are determined through environmental cues and the expression of master transcription factors. Signal transducer and activator of transcription (STAT) 1 and STAT4 are needed for expression of T-box 21 (Tbx21, also known as Tbet) to drive classical Th1 cells that express the cytokine IFNG, whereas STAT3 activates the RAR-related orphan receptor c (Rorc, encoding ROR-gamma t), leading to a Th17 cell phenotype with high IL17 production (Kanno et al., 2012). Although these master transcription factors have been shown to be involved in EAE induction (Ivanov et al., 2006; Yeh et al., 2011), the complex transcriptional regulations that coordinate a fatal autoimmune T cell attack within the CNS are not known.

In this study, we performed comparative gene expression analysis of EAE-inducing T cells, *in vitro* primed Th17 cells, and naïve T cells to identify crucial transcriptional events and molecular targets in the cascade of differentiation, invasion into the CNS, and effector phase within the CNS. Furthermore, we performed transcriptional network analysis and compared our murine T cell gene expression data set to putative multiple sclerosis risk genes, which we selected based on their proximity [UCSC genome browser (hg19), dbSNP138] to the multiple sclerosis risk SNPs proposed by the most recent multiple sclerosis genome-wide association study (GWAS; IMSGC and WTCCC2, 2011; IMSGC, 2013b), in order to highlight genes that are potentially implicated in both multiple sclerosis and experimental neuroinflammation. We found that disproportionately high numbers of multiple sclerosis susceptibility genes were differentially expressed during T cell differentiation and EAE, of which we identified nine to be strong candidates for playing a role in T cell pathogenicity.

**Materials and methods**

**Animals**

C57BL/6 and B6.2d2 mice were purchased from the Central Animal Facility Institution of the University of Mainz.
Experimental autoimmune encephalomyelitis

Active and adoptive transfer EAE experiments were performed as previously described (Siffrin et al., 2010). Briefly, for active EAE, C57BL/6 mice were immunized subcutaneously with myelin oligodendrocyte glycoprotein peptide (250 μg MOG35-55; Research Genetics) emulsified in complete Freund’s adjuvant (Difco Laboratories) and supplemented with heat-inactivated Mycobacterium tuberculosis (Difco Laboratories) pertussis toxin (200 ng; Sigma Aldrich) intraperitoneally injected at the time of immunization and 5 d later. For adoptive transfer EAE, naive SPF animals. A negative (CD4 untouched) sort was followed by a positive sort (anti-CD62L beads, Miltenyi Biotec). Purity was checked by fluorescence-activated cell sorting staining and populations with a purity of >93% were used for the microarray and >97% for quantitative real-time PCR analysis. The remaining cells were largely CD11b-expressing cells in adoptive as well as active EAE (CD11c+, CD8+, CD45R+ cells were <1%).

RNA isolation

Total RNA was isolated using the RNeasy® Kit (Qiagen) according to the manufacturer’s protocol. Isolated RNA was further cleaned of possible genomic DNA by treatment with DNase I (Roche). Quality and integrity of total RNA preparation was confirmed using a NanoDrop™ Spectrophotometer (Thermo Scientific).

cDNA synthesis was performed by reverse transcription of total RNA using the SuperScript III First Strand Synthesis System and random hexamer primers (Invitrogen) following the manufacturer’s instructions.

Quantification by real-time PCR

Amplification primers for real-time PCR analysis were designed using Beacon Designer 8 Software (PREMIER Biosoft International) according to the manufacturer’s guidelines and subsequently tested for amplification efficiency and specificity. Sequences for primers are listed in Supplementary Table 1. Real-time PCR was performed using iQ SYBR® Green supermix (BioRad Laboratories) in an iCycler® Real Time Detection System (BioRad). Relative changes in gene expression were determined using the ΔΔCt method (Livak and Schmittgen, 2001) with Eef1a1 as the reference gene.

Statistical analysis

All data were analysed using PRISM6 (Graphpad software). Data are presented as mean ± SEM from three independent experiments. Statistical analysis of the data was conducted using a parametric test (one-way ANOVA) followed by Tukey’s multiple comparison test.

Isolation of cells from the CNS of EAE animals

Isolation of CD4+ T cells was done at the disease peak and at comparable disease stages for all EAE types and sample acquisitions (i.e. for active EAE: mean clinical score ± SEM 2.5/0.22, mean disease duration ± SEM 4.9/0.9 days, n = 7; for adoptive transfer EAE for array data: mean clinical score ± SEM 2.5/0.49, mean disease duration ± SEM 4.7/0.4 days, n = 7; for adoptive transfer EAE for quantitative PCR: mean clinical score ± SEM 2.45/0.19, mean disease duration ± SEM 4.5/0.3, n = 30). Animals were lethally anaesthetized and perfused with 50 ml phosphate-buffered saline. The brain and spinal cord were isolated, cut into small pieces, and digested in Iscove’s modified Dulbecco’s medium substituted with 360 U/ml collagenase, 200 U/ml DNase, and 5 μg/ml collagenase/dispase (all R&D). After incubation for 30 min at 37°C under continuous rotation, the CNS tissue was minced through a cell strainer and washed with cold Iscove’s modified Dulbecco’s medium. Mononuclear cells were separated on a 40–70% Percoll gradient and collected from the interphase. CD4+ T cells were enriched by magnetic bead isolation (anti-CD4+ beads, clone L3T4, Miltenyi Biotec). Purity was checked by fluorescence-activated cell sorting staining and populations with a purity of >93% were used for the microarray and >97% for quantitative real-time PCR analysis. The remaining cells were largely CD11b-expressing cells in adoptive as well as active EAE (CD11c+, CD8+, CD45R+ cells were <1%).
Microarray construction

All procedures necessary for microarray measurements, starting at RNA isolation until first data analysis steps, were carried out at Miltenyi Biotec. RNA was isolated using standard RNA extraction protocols (NucleoSpin® RNA II). Quality was checked via the Agilent 2100 Bioanalyzer platform (Agilent Technologies).

One hundred nanograms of each total RNA sample was used for the linear T7-based amplification step. To produce Cy3-labelled cRNA, the RNA samples were amplified and labelled using the Agilent Low Input Quick Amp Labelling Kit (Agilent Technologies) following the manufacturer's protocol. Hybridization to Agilent Whole Mouse Genome Oligo Microarrays 4x44K V2 was performed according to Agilent Gene Expression Hybridization Kit protocol (Agilent Technologies). Fluorescence signals of the hybridized Agilent Microarrays were detected using Agilent’s Microarray Scanner System (Agilent Technologies).

Microarray data analysis

The Agilent Feature Extraction Software (FES) was used to read out and process the microarray image files. The raw and normalized data are available online at the NCBI GEO database (Edgar et al., 2002), accession number GSE57098 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE57098). To determine differential gene expression, FES-derived output data files were further analysed using the Rosetta Resolver gene expression data analysis system (Rosetta BioSoftware) and gene expression ratios were calculated by dividing sample signal intensity through control signal intensity. For the detection of differentially expressed genes, P-values were calculated based on signal intensity variances using an error-model based hypothesis test (Weng et al., 2006). Putative candidate genes with a fold-change > 2 and signal intensity based P-value < 0.01 (Supplementary Table 2) were used for signalling pathway analysis using Ingenuity IPA Software (www.ingenuity.com). Agilent’s probe identifiers and corresponding homologene IDs were annotated with HomoloGene (NCBI: http://www.ncbi.nlm.nih.gov/homologene/). For intronic (54 SNPs), 3’UTR variant (two SNPs), 5’UTR variant (one SNP), downstream (one SNP) and intergenic (47 SNPs) SNPs, all genes within 50 kb of either side were considered. Additionally, for intergenic SNPs with no gene within this span, the nearest gene in each direction, no matter how far away, was considered. For exonic (five SNPs) SNPs, only the corresponding implemented gene was annotated. The overlap of multiple sclerosis risk genes with differentially expressed genes identified in the murine microarray study was analysed with the Venn analysis tool (http://bioinfogp.cnb.csic.es/tools/venny/). It should be noted that the functionally relevant gene might be located further away. To statistically control for the significance of the overlap between multiple sclerosis risk genes and differentially expressed genes in the various group comparisons, a null distribution was calculated as a random sampling. The null distribution gives the probability of finding x relevant genes among m differentially expressed genes by pure chance in a population of n genes containing k relevant genes. This is a hypergeometric distribution whose probability mass function is given by: (Johnson et al., 2005)

\[
Pr(X = x) = \binom{k}{x} \binom{n-k}{m-x} / \binom{n}{m}
\]

(1)

The probability of observing x of the relevant genes in a set of m random drawn genes without replacement (the cumulative distribution function) was calculated by

\[
Pr(X ≥ x) = 1 - \sum_{i=0}^{x-1} \binom{k}{i} \binom{n-k}{m-i} / \binom{n}{m}
\]

(2)

which was computed using the SAS function CDF('HYPER', x - 1, n, k, m).

Results

Transcriptomic analysis of encephalitogenic T cells in EAE

First, we investigated the overall change in gene expression of encephalitogenic Th17 cells at different time points (Fig. 1). Four different cell populations were analysed: (i) naïve (CD4+ CD62L+), myelin-specific (2d2) T cells before differentiation (T naïve); and (ii) after in vitro Th17 differentiation (Th17iv). Adoptive transfer EAE was induced by injecting these in vitro-primed (2d2) Th17 cells into lymphopenic Rag1−/− mice. At the peak of EAE; (iii) 2d2 Th17 cells were isolated from the CNS of these mice (Th17eae) (Fig. 1A); additionally, we isolated (iv) CD4+ T cells from the CNS of C57BL/6 mice with active MOG 35-55 immunization (CD4eae) (Fig. 1B). Flow cytometric analysis of the Th1 and Th17 lineage markers IFNG and IL17, respectively, revealed that T naïve did not show important numbers of these cytokine producers, whereas Th17iv comprised IL17 single producers and CD4eae and Th17eae included IL17 and IFNG single- and double-producers (Fig. 1C). Differential gene expression was analysed using Agilent Whole Mouse Genome Oligo Microarrays 4x44K V2. To evaluate the results of the gene expression analysis, we focused first on the expression of typical surface markers, cytokines and transcription factors of Th17 cells (Table 1). Th17iv showed a strong increase in classical Th17 surface markers, e.g. Il1r1, Il13ra1 and Il23r,
in the cytokines Il17a, Il17f, Il21, granulocyte-macrophage colony-stimulating factor (Csf2, encoding GM-CSF) and Il22, as well as the master transcription regulator Rorc (Table 1). Upregulation of typical Th1 markers was not observed for Th17iv versus naïve T cells, which suggests that these cells have a clear-cut Th17 phenotype (Table 1). Comparing T naïve and Th17 naïve cells, there was differential regulation of Th1- and Th17-related gene expression, which supports the concept of Th17 cell plasticity. The CNS-isolated Th17 naïve cells continued to strongly express Il17a and Il21 and even increased expression of Csf2 and Il13ra1. The expression of other typical Th17 lineage markers such as Il1r1, Il23r, Il17f, Il22 and Rorc decreased, whereas Th1 lineage markers such as Ifng, Interferon-gamma receptor (Ifngr) and the transcription factor Tbx21 increased. Interestingly, we found Stat1 and Stat4, which are important for the Th1 differentiation process, to be down- instead of upregulated. A direct comparison of Th17 naïve to Th17 naïve, we found transcripts encoding many chemokines (Cxcl1, Cxcl2, Ccl1, Ccl2, Ccl7, Ccl8, Ccl12) for the recruitment of mononuclear cells to the site of inflammation, the pleiotropic cytokine Il2 important in Th1, Th2 and Treg cell differentiation, and transcription factors (Cebpa, Fos, Fosb) involved in proinflammatory gene expression and T cell development. The expression levels of these genes were very similar in both Th17 naïve and CD4 naïve cells, highlighting the role of Th17 cells in EAE pathology (Fig. 2A).

**Network analysis revealed distinct signalling pathways regulated in EAE**

To identify genes possibly involved in T cell pathogenicity and plasticity, we analysed the overall change in gene expression of Th17iv cells and Th17 naïve cells directly: 39,429 probes that cover 30,905 genes were plotted on the microarray. Only genes with a significant microarray signal intensity P-value < 0.01 and an expression fold-change >2 were considered to be differentially expressed candidate genes and included in the analysis. Among the top 100 genes that were upregulated in the transition of Th17iv to Th17 naïve, we found transcripts encoding many chemokines (Cxcl1, Cxcl2, Ccl1, Ccl2, Ccl7, Ccl8, Ccl12) for the recruitment of mononuclear cells to the site of inflammation, the pleiotropic cytokine Il2 important in Th1, Th2 and Treg cell differentiation, and transcription factors (Cebpa, Fos, Fosb) involved in proinflammatory gene expression and T cell development. The expression levels of these genes were very similar in both Th17 naïve and CD4 naïve cells, highlighting the role of Th17 cells in EAE pathology (Fig. 2A).

The probes differentially expressed between classical (in vitro) Th17 cells and CD4 naïve T cells included from the CNS of EAE-affected animals (Th17iv versus Th17 naïve, Fig. 2B) were matched to 4385 genes, excluding duplicate
genes and genes without a primary sequence name, leading to 2258 up- and 2127 downregulated genes between Th17iv and Th17eae, which indicates a strong plasticity of gene expression of pathogenic Th17 cells in vivo.

To investigate signalling networks and the key transcriptional regulators underlying the differences identified between Th17iv and Th17eae in more detail, we evaluated the transcriptional changes between Th17iv and Th17eae using Ingenuity IPA Software (Content version: 18841524; Release Date: 2014-06-24). IPA mapped the 5788 Agilent microarray sequence codes of the candidate genes (fold change > 2; signal intensity P-value < 0.01) to a defined gene and excluded duplicate genes from the analysis, resulting in a total of 4303 analysed genes. The genes were associated with key signalling networks according to Gene Ontology terms and experimental observations on the basis of known connections from primary immune cells in wild-type mice. The ‘immune cells’ criterion was chosen in order to identify new signalling pathways, possibly as yet unknown, in T cells. The top three canonical pathways revealed by the unbiased IPA analysis were ‘communication between the innate and adaptive immune system’ (P = 1.0 × 10^-10), ‘altered T cell and B cell signalling in rheumatoid arthritis’ (P = 1.1 × 10^-9), and ‘LXR/RXR activation’ (P = 1.6 × 10^-8) (Fig. 2C). Analogous evaluations of the CD4eae versus Th17iv showed strikingly similar results (Supplementary Fig. 5) with a strong overlap of Th17iv versus Th17eae and Th17iv versus CD4eae, which underlines the relevance of these findings also for wild-type active EAE.

Two of the three most significant signalling networks underlying the transformation of Th17iv cells to Th17eae cells are displayed in Fig. 3. Most of the genes associated with Networks 1 and 3 were upregulated, which resulted in generally increased regulation of cellular function and maintenance, haematological system development, inflammatory response and tissue morphology and cellular development. Increased expression of the chemokines Cxcl2, Cxcl3, Cxcl9, Cxcl10, and cytokines Il1b, Il6, Il15, Il12b, Il18, Il23a and Il1b1 in cooperation with the transcription factors Irf1, Irf7, Rel, and Hhex contributed to an inflammatory environment by recruiting and activating leukocytes through interaction with cells from the innate immune system via the toll-like receptors Tlr2, Tlr3, Tlr4, and Tlr7 and co-stimulatory molecules Cd40, Cd80, and Cd86 (Fig. 3A). Therefore, a potential increase in the communication between the innate and the adaptive immune system could be observed. There was a downregulation of the Th17-associated factors Rorc, Batf, Il17f and Il22, in combination with increased expression of the cytokine Il9 and the transcription factor Spi1 (encoding PU.1), which might be relevant for Th17 cell transformation (Fig. 3B).

### Table 1: Regulation of Th1 and Th17 associated factors in T cells at distinct points in EAE

<table>
<thead>
<tr>
<th>Th cell properties</th>
<th>Th17iv versus Tnaive</th>
<th>Th17eae versus Tnaive</th>
<th>Th17eae versus Th17iv</th>
<th>CD4eae versus Tnaive</th>
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<tr>
<td></td>
<td>Fold change</td>
<td>Fold change</td>
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**Top:** The expression of Th17 cell associated factors was highly increased in vitro differentiated Th17 cells, which only slightly decreased in CD4⁺ T cells isolated from the CNS of adoptively transferred EAE animals. These factors were also highly expressed in CD4⁺ T cells from actively immunized mice. **Bottom:** The expression of Th1 cell phenotypic markers was not activated in Th17 differentiation but increased in Th17eae and CD4eae cells.

**Legend:**
- signal intensity P < 0.01; FC < 5.0
- signal intensity P < 0.01; FC > 10 ≤ 50.
- signal intensity P < 0.01; FC > 1.5 ≤ 10.
- no significant expression change.
- signal intensity P < 0.01; FC < −1.5 ≥ 10.
- signal intensity P < 0.01; FC < −10 ≤ −50.
- signal intensity P < 0.01; FC < −50.

TF = transcription factor; FC = fold change.
Figure 2 Identification of candidate genes and signalling pathways in the transition of in vitro-differentiated Th17 cells to ex-Th17 cells in the course of EAE. (A) Heat-map illustration of the 100 most regulated genes between Th17eae and Th17iv cells analysed in all groups. Most of the genes highly expressed in Th17eae cells were similarly upregulated in CD4eae cells (yellow colour). Family-tree analysis of the top-ordered genes shows significant changes in gene expression between Th17eae and Th17iv cells. (B) Ingenuity Pathway Analysis (IPA) of the top regulated genes revealed that the most significant pathways include Cellular Function and Maintenance, Cellular Movement, Immune Cell Trafficking, Cell-To-Cell Signaling and Interaction, Cellular Function and Maintenance, Inflammatory Response, and more. (C) Table showing the top network functions and canonical pathways with their respective P-values and ratios.
Various putative multiple sclerosis risk genes (IMSGC and WTCCC2, 2011; IMSGC, 2013b), were involved in these developmental processes depicted in both signalling networks, including Rgs1, Mertk, Cd80, Cd86, Il12b, Hhex (Network 1) and Baff (Network 3). Interestingly, these signalling networks provide further evidence for extensive metabolic changes occurring in the Th17 transformation process. This includes the activation of the canonical signalling pathways implicated in cholesterol biosynthesis via activation of liver X receptor/retinoid X receptor (LXR/RXR) signalling and its downstream targets Abcg1 and Abcg1, Apoe and Srebf1/Srebf2 (Fig. 3B and D, Supplementary Fig. 1). Taken together, our results indicate that in the transition from Th17iv cells to Th17eae, T cells increase metabolic activities in EAE. In addition, the Th17 cell transition seems to involve lymphocyte developmental restructuring and the activation of specific networks not normally associated with Th17.

Confirmation of the key transcription regulators identified by array analysis

Focusing on transcription factors, as these are the key regulators of signalling networks at the very top of activation cascades, we found Cebpa, Fos, Klf4, NfATc1, and Spi1 to belong to the most differentially regulated transcription factors, which have also been associated with prominent signalling networks (Figs 2A and B, 3 and Supplementary Fig. 4) in our analysis. We validated the microarray results in new adoptive transfer EAE experiments using quantitative real-time PCR (Fig. 4).

Cebpa, Fos, Klf4, NfATc1 and Spi1 were weakly expressed in Th17iv cells and highly upregulated in Th17eae cells in the microarray data set, which was also observed in the quantitative real-time PCR analysis, although the upregulation of NfATc1 did not reach statistical significance. Expression levels of Cebpa and Spi1 were similarly low in Tnaïve and Th17iv cells in the microarray as well as the quantitative real-time PCR analysis, indicating that it does not play a major role in the maintenance of either naïve or Th17 cell status. Expression of Fos, Klf4, and NfATc1 were increased to be naïve T cells compared to Th17iv, which reached statistical significance for Fos and Klf4 in the quantitative real-time PCR analysis. Overall, microarray and quantitative real-time PCR analysis showed that expression of all of these transcription factors increased in Th17eae relative to Th17iv, which suggests a role in T cell effector function and pathogenicity. Interestingly, expression levels of Fos and Klf4 were increased in naïve T cells compared to Th17iv, and were even comparable to Th17eae cells in the case of Fos, suggesting that these factors might be implicated in T cell reorganization processes through depolarization towards a ‘naïve’ T cell phenotype.

Regulation of multiple sclerosis susceptibility genes in the murine T cell gene expression data set

An important goal of our study was to assess to what extent multiple sclerosis susceptibility genes are regulated at different time points in our CD4+ T cell-based EAE model. In the most recent analysis of susceptibility variants for multiple sclerosis, 110 SNPs were reported to be associated with multiple sclerosis (IMSGC, 2013b). We mapped these SNPs to 220 human genes as described in detail in the ‘Materials and methods’ section. Two hundred and nine of the 220 genes were represented on the murine whole genome microarray used in this study (Supplementary Tables 3 and 4). We analysed their expression ratios for our sample groups and found 75/209 potential multiple sclerosis susceptibility genes regulated for Tnaïve versus Th17iv, 90/209 for Tnaïve versus Th17eae, 62/209 for Th17iv versus Th17eae and 87 for Tnaïve versus CD4eae (overview in Table 2; Supplementary Table 4 for detailed description of gene regulation in multiple sclerosis susceptibility genes). Using the null distribution we confirmed that these results exceed the overlap numbers of randomly chosen genes by pure chance (P-values in Table 2).

Based on these candidate genes, we aimed to pinpoint multiple sclerosis risk genes possibly involved in T cell pathogenicity by first evaluating the differential gene expression for Th17eae versus Th17iv, Th17eae versus Tnaïve, and CD4eae versus Tnaïve, and subsequently selecting the genes common to these three comparisons (Fig. 5A). As a starting point, we validated the expression of only the nearest gene by quantitative PCR. Using this approach, we identified 15 multiple sclerosis susceptibility genes that potentially play a role in T cell pathology in our model (Supplementary Table 4): Bach2, Cd86, Ets1, Hhex, Il12b, Il2ra, Il22ra1, Irf8, Mertk, Odf3b, Plek, Ptger4, Rgs1, Slc30a7, and Thada. We excluded genes from further analysis that were expressed slightly above/at background signal intensity level and statistically not significantly

Figure 2 Continued

| Figure 2 Continued group these 100 regulated genes into related subpopulations. (B) Intensity log plots showed that 5342 probes were differentially downregulated and 4470 probes upregulated, as marked by the red line (signal intensity \( P < 0.01 \)) in the comparison of Th17eae cells to Th17iv cells. Cebpa, fos, Klf4, Irf8, Spi1 (Sppil), Rel, and NfATc1 belonged to the most upregulated transcription factors in this comparison. (C) The unbiased Ingenuity Pathway Analysis (IPA) revealed that massive transcriptional changes occur in the transition from Th17iv to Th17eae cells. Underlying signalling networks are associated with functions such as cellular function and maintenance, cellular movement, immune cell trafficking \( (P = 1.0 \times 10^{-33}) \) and canonical pathways including communication between the innate and the adaptive immune system \( (P = 1.0 \times 10^{-10}) \) among others. |
Figure 3  Prominent signalling networks and associated canonical pathways involved in the transition of Th17iv cells to Th17eae cells in EAE. (A) The most significant signalling Network 1 ($P = 1.0 \times 10^{-13}$) was associated with changes in cellular function and maintenance, cellular movement and immune cell trafficking. Key molecules upregulated in this network and involved in the regulation of these changes were the transcription factors Hhex, Irf1, and Irf7, Rel, Nr3c1, the toll-like receptors Tlr2, Tlr3, Tlr4, and Tlr7, co-stimulatory molecules Cd40, Cd80, and Cd86, Merkt, Rgs1, the cytokines, Ifnb1, Il1b1, Il6, Il12b, Il18, and Il23a, and the chemokines Cxcl2, Cxcl3, Cxcl9, Cxcl10, and Ccl2. Many factors, associated with the canonical signalling pathway of communication between the innate and adaptive immune systems ($P = 1.0 \times 10^{-14}$) were represented and upregulated in signalling Network 1 (marked in light blue). (B) The signalling Network 3 ($P = 1.0 \times 10^{-13}$) is associated with changes in cellular movement, immune cell trafficking and inflammatory response. It involved the upregulation of Spi1 (PU.1) and I9 signalling and downregulation of the Th17 phenotype associated factors Rorc, Batf, Il22 and Il17a. (C) LXR/RXR signalling ($P = 1.6 \times 10^{-5}$), necessary for metabolic changes and cholesterol biosynthesis and efflux, was found to be enhanced in Th17eae cells compared to Th17iv cells, as indicated also by LXR/RXR target gene regulation Abca1, Abcg1, Lpl, Apoe, and Srebfl. These regulations are similar to known metabolic processes from macrophages or hepatocytes.
regulated in any of the comparisons (Ii12b, Ii22ra1). We validated nine of 13 selected candidate genes using quantitative real-time PCR in independent adoptive transfer EAE experiments (Fig. 5B). Of the nine candidate genes, Ii2ra and Odf3b were strongly expressed in Th17 cells and downregulated to (Ii2ra) or below (Odf3b) naïve T cell expression levels during EAE. The transcriptional repressor Bach2, the zinc transporter Slc30a7 (encoding ZnT7) and Thada were downregulated in Th17 cells and continued to be expressed at low levels in Th17eae cells. The modulator of G-protein coupled receptors Rgs1 was strongly upregulated in Th17 cells isolated from the CNS of diseased mice.

Table 2: Probability of multiple sclerosis risk gene enrichment in differentially expressed T cell genes

<table>
<thead>
<tr>
<th>Name</th>
<th>n</th>
<th>m1</th>
<th>m2</th>
<th>m4</th>
<th>m3</th>
<th>m5</th>
<th>m6</th>
<th>MS risk genes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of genes</td>
<td>30,905</td>
<td>5,749</td>
<td>7,044</td>
<td>4,385</td>
<td>6,991</td>
<td>1,793</td>
<td>4,808</td>
<td>209</td>
</tr>
<tr>
<td>L = Number of overlapping genes with MS risk genes (k)</td>
<td>209</td>
<td>75</td>
<td>90</td>
<td>62</td>
<td>87</td>
<td>21</td>
<td>65</td>
<td>209</td>
</tr>
<tr>
<td>P Probability L ≥ 1</td>
<td>n.a.</td>
<td>2.6 × 10^-9</td>
<td>1.0 × 10^-10</td>
<td>5.8 × 10^-9</td>
<td>6.0 × 10^-10</td>
<td>1.0 × 10^-2</td>
<td>4.2 × 10^-9</td>
<td>n.a.</td>
</tr>
</tbody>
</table>

In the various comparisons, the multiple sclerosis risk genes were disproportionately enriched (significant P-value: α = 0.01, Bonferroni corrected for multiple comparisons). Fourteen of 223 relevant multiple sclerosis risk genes were not represented on the murine microarray (n), leading to a total of 209 multiple sclerosis risk genes. Seventy-five of 209 (Tnaive versus Th17iv; P = 2.6 × 10^-9), 90/209 (Tnaive versus Th17eae; P = 1.0 × 10^-10), 62/209 (Th17iv versus Th17eae; P = 5.8 × 10^-10), 87/209 (Tnaive versus CD4eae; P = 6.0 × 10^-10), 21/209 (CD4eae versus Th17eae; P = 0.103, n.s.; control), 65/209 (CD4eae versus Th17iv; P = 4.2 × 10^-9) multiple sclerosis risk genes were differentially expressed in the individual data sets. 

I = number of differential expressed murine genes (m) overlapping with 209 multiple sclerosis risk genes (k); L = number of m randomly chosen genes of n genes on the array overlapping with 209 multiple sclerosis risk genes; MS = multiple sclerosis.
Figure 5 Validated multiple sclerosis-associated genes involved in T cell pathology. (A) We compared the 209 putative multiple sclerosis risk genes [± 50 kb, UCSC genome browser (hg19), dbSNP138 annotation], to the differentially expressed candidate genes from our comparisons (Th17ae vs. Th17iv, Th17ae vs. Tnaı̈ve, CD4ae vs. Tnaı̈ve, CD4ae vs. Th17ae, CD4ae vs. Th17iv). (B) Fifteen genes found in common to all of these comparisons were identified as genes potentially involved in T cell effector function. Thirteen of these 15 genes were chosen for replication and expression pattern of nine candidates were validated in three independent quantitative real-time PCR experiments. Depicted are inter-microarray normalized signal intensities and mRNA expression levels as determined by quantitative real-time PCR analysis relative to the housekeeping gene Eef1a1 and normalized to naïve T cells. Values are illustrated as mean ± SEM pooled from three independent experiments; P-values: *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001. MA = microarray; ns = not significant; vs. = versus.
involved in deeper T cell plasticity processes. In the direct defining genes, we identified genes that are potentially the proposed STAT1-independent Ifng active and adoptive transfer EAE model, which endorses Plek (encoding pleckstrin), and the transcription factor Irf8. In the microarray data set, Cd86, Evi5, Hhex and Ptger4 were all significantly upregulated in Th17eae cells and possibly involved in metabolic changes, pathogenicity, and the generation of a proinflammatory microenvironment, but this upregulation could not be validated in quantitative real-time PCR analysis (Supplementary Fig. 2).

Since the publication of the multiple sclerosis GWAS in 2011 (IMSGC and WTCCC2, 2011), some gene annotations have changed, leading to a slightly different multiple sclerosis risk gene list in our study. In our expression analysis of multiple sclerosis susceptibility genes, which was proposed by the IMSGC and WTCCC2 (2011) but are not included in the current multiple sclerosis risk gene list (Supplementary Table 3), we observed differential expression of Il12rb1, Myb and Vcam1, but not of Cd80 (Supplementary Fig. 3).

Taken together, nine multiple sclerosis susceptibility genes were found and validated to be differentially regulated in murine T cells, in particular in the transition from in vitro-generated Th17 cells to encephalitogenic CD4+ T cells.

**Discussion**

T cells are regarded as the main players in the induction and persistence of the demyelinating autoimmune disease multiple sclerosis. The exact causes of multiple sclerosis are still unknown, but it is assumed that environmental factors trigger the onset in individuals with a genetic predisposition by activating self-reactive, supposedly myelin-specific T lymphocytes of the Th1 and/or Th17 cell phenotypes (Siffrin et al., 2007). We analysed whole transcriptional changes between naïve T cells isolated from non-immunized mice (Tnaı̈ve) and in vitro-generated Th17 cells (Th17iv) that were adoptively transferred into lymphopenic Rag1-/- mice and re-isolated from the CNS at the peak of disease (Th17eae). This model yields very pure ex vivo pathogenic T cells, which are relevant to the proinflammatory and damage-inducing aspects of the disease. We identified signalling networks and therein new molecular targets involved in chronic neuroinflammation, e.g. transcription factors and multiple sclerosis susceptibility genes.

Pathogenic Th17 cells, re-isolated from EAE-affected mice, exhibited substantial transcriptomic changes in comparison to in vitro Th17 cells. In line with previous observations (Lexberg et al., 2010; Peck and Mellins, 2010; Hirota et al., 2011), Th17eae cells showed distinct differences to classical Th1 cells concerning, for example, Stat expression pattern. We showed here that Tbx21 expression increased independently of Stat1 and Stat4 in the active and adoptive transfer EAE model, which endorses the proposed STAT1-independent Ilng expression in active EAE (Yeh et al., 2011b). Apart from the lineage defining genes, we identified genes that are potentially involved in deeper T cell plasticity processes. In the direct comparison of Th17iv and Th17eae cells, we found upregulation of the transcription factors Cebpa (C/EBPA), Fos, Klf4, Nfatc1, and Spi1 (PU.1), which are involved in early T cell development or in homeostasis of naïve T cells (Nunomura et al., 2000; Rothenberg and Dionne, 2002; Wen et al., 2011; Patra et al., 2013). It is commonly accepted that CD4+ T cell lineage commitment is not as restricted in its determination as previously thought. It has been described that cytokine profiles and expression of effector molecules can be modulated in a certain range (Chang et al., 2005; Hegazy et al., 2010). In this respect, Th17 cells showed strong plasticity as they can be reprogrammed into different lineages in various differentiation states (Zhu and Paul, 2010; Kanno et al., 2012). Herein, we found a re-upregulation of critical transcription factors in Th17eae cells that are associated with naïve T cells or even earlier thymocyte developmental states and downregulated during T cell differentiation, thus providing a basis for new lineage commitments (Zhu and Paul, 2010). KLF4 is essential (together with OCT3/4, SOX2, and c-MYC) for stem cells to maintain their pluripotency (Takahashi and Yamanaka, 2006), while it is downregulated upon thymic progenitor cell activation and differentiation (Wen et al., 2011). KLF4 has been shown to directly bind the Il17a promoter to activate Il17 expression (An et al., 2011), thus influencing T cell differentiation and effector function. Signalling network analysis further suggested a contribution of the transcription factors PU.1 (gene product of Spi1) and C/EBPA in T cell plasticity and encephalitogenicity. PU.1 plays a central role in haematopoiesis, where it regulates gene expression through its interaction with other transcription factors including C/EBPA, IRF4, IRF8, and c-JUN. PU.1 was also detected in Th2 cells, in which its expression was associated with distinct Th2 subpopulations (Chang et al., 2005) and PU.1 was found to be essential for the expression of Il9, the master cytokine of Th9 cells, which is associated with allergic inflammation (Soroosh and Doherty, 2009; Carotta et al., 2010). Thus, according to our data, PU.1 might also have an important role in Th17 subpopulations in EAE lesions.

Furthermore, we correlated the matches from our transcriptomic T cell analysis with putative multiple sclerosis risk genes selected based on physical proximity to the multiple sclerosis risk SNPs that were proposed by the most recent multiple sclerosis GWAS and Immunochip analyses (IMSGC and WTCCC2, 2011; IMSGC, 2013b). This approach highlights interesting target genes that might be shared in the human disease and the murine model disease, which means that common mechanisms in CNS inflammation can be identified. Our findings underline previous work that the local pathophysiology in lesions in the murine model recapitulates many aspects of the human CNS pathology and that similar effector mechanisms are present in multiple sclerosis and EAE (Simmons et al., 2013). One limitation of our study is that mechanisms relevant to the induction of the disease might not be represented.
For most of the multiple sclerosis risk loci, the effect of the identified sequence variants on gene expression, protein function, and possible involvement in disease pathophysiology, e.g. via T cell-mediated immune responses, has not been determined. We found the transcription factor Bach2 to be strongly expressed in naïve T cells but weakly expressed in differentiated Th17 cells and in Th17eae cells, which underscores its role in the maintenance of a naïve state by suppression of effector memory-related genes (Tsukumo et al., 2013). Interestingly, Tsukumo et al. (2013) suggested that calcium-binding protein S100A is a putative direct target of BACH2 and found elevated levels of S100A in Bach2−/− mice. We also found increased expression of S100a1 in Th17iv cells compared to naïve T cells, which was further enhanced in Th17eae cells, therefore negatively correlating to Bach2 expression. S100A was proposed to increase inflammation and autoimmunity as its expression was correlated with the severity of inflammatory diseases including rheumatoid arthritis and atherosclerosis (Ehrchen et al., 2009). Thus, the observed downregulation of Bach2 in combination with increased levels of S100a1 is most likely a relevant feature of autoimmune neuroinflammation. The interferon-regulatory factor (IRF) family has been described to be deeply involved in T cell differentiation processes (Zhang et al., 2012). We found that the proposed multiple sclerosis risk gene Irf8 was upregulated upon acquisition of T cell pathogenicity in vivo. Interestingly, Irf8 has been described to inhibit Th17 cell differentiation without affecting Th1 or Th2 differentiation, and thus, increased expression of Irf8 in Th17eae cells might contribute to the transition process towards a Th1-like phenotype (Ouyang et al., 2011). In addition, Irf8 expression has been linked to IFNG-dependent induction of the death receptor Fas (Yang et al., 2007), which might be necessary for the restriction of the T cell response by apoptosis.

Besides transcription factor modulation, we identified important transcriptional regulation of crucial receptor signalling pathways, e.g. G-protein coupled receptor, death receptors and tyrosine kinase receptor signalling. A noteworthy finding was the detection of an upregulation of Mertk (encoding the receptor tyrosine kinases MER). Increased expression levels of membrane-bound as well as MER (MERTK) and the family member AXL have been found in homogenates from established multiple sclerosis lesions (Weiniger et al., 2009). However, Tyro3, Axl and Mertk (TAM) triple knockout mice exhibited systemic autoimmune diseases, including increased production of the pro-inflammatory cytokine TNFA, impaired integrity of the blood–brain barrier, glial activation and protein degradation, associated with accumulation of autoantibodies and autoreactive lymphocyte infiltration into the brain (Li et al., 2013). Similarly, we found that the modulator of G-protein coupled receptors Rgs1 is strongly correlated with T cell pathogenicity. Rgs1 is associated with a variety of T cell-mediated pathologies including coeliac disease, type 1 diabetes and multiple sclerosis (Gibbons et al., 2011). A contribution to T cell pathology is supported by recent findings showing that increased expression of RGS1 and other family members correlates with T cell activation and a slower in vivo migration of regulatory T cells (Agenes et al., 2005). We identified the multiple sclerosis risk gene Plek (pleckstrin/p47), which represents a major substrate of protein kinase C (PKC) (Lian et al., 2009) as a further potential modulator of cell signalling. Pleckstrin contributes in the PKC signalling cascade for secretion of proinflammatory cytokines TNFA and IL1B in phagocytes and its deregulation is associated with another autoimmune disorder (type 1 diabetes) (Ding et al., 2007).

Concerning cell surface molecules, we observed an increased cytokine receptor expression for IL2 (II2ra) in Th17iv cells that was reduced to naïve T cell expression levels in Th17eae cells, suggesting a negative regulatory role in the encephalitogenic process. A number of SNPs at the IL2RA locus have been associated with an increased risk of various autoimmune diseases including type 1 diabetes (Lowe et al., 2007) and Graves’ disease (Brand et al., 2007). Of note, the SNPs rs2104286 and rs12722489 were associated with increased levels of soluble IL2RA in the serum of multiple sclerosis patients (Maier et al., 2009). As soluble IL2RA can sequester IL2 and reduce IL2 responsiveness, reduced levels of IL2 might hamper the effectiveness of CD56+ regulatory natural killer cells to kill autoreactive CD4+ T cells, which has conversely been demonstrated to be a potent mechanism for reducing relapses in relapsing-remitting multiple sclerosis in clinical trials using the humanized anti-IL2RA monoclonal antibody daclizumab (Bielekova, 2013). Moreover, the SNP rs2104286 seems to confer a higher risk of developing multiple sclerosis by increasing the surface expression of IL2RA (Hartmann et al., 2014). IL2 signalling via STAT5 has recently been demonstrated to lead to an increase of GM-CSF-producing T cells (Noster et al., 2014) and the presence of the SNP showed a positive association with multiple sclerosis severity (Hartmann et al., 2014). These recent publications underline the importance of GM-CSF producing T cells in multiple sclerosis for T cell pathogenicity, which had been suggested by recent work in EAE (Codarri et al., 2011; El-Behi et al., 2011). This is supported by our findings of an elevation of II2ra in Th17iv and strongly increased CsF2 production in Th17eae and CD4eae, which might be sequential steps in the acquisition of T cell pathogenicity.

Three of our confirmed candidates have been barely investigated to date and their role in immune-cell function is unclear. Slc30a7 encodes ZnT7, which transports zinc from the cytosol into the Golgi apparatus (Kirschke and Huang, 2003) and thus contributes to cellular zinc homeostasis and enzyme function in the secretory pathway (Kambe, 2011). THADA is a molecule that has been associated with death receptor signalling based on two-hybrid experiments (Puduvalli and Ridgway, GenBank accession reference note) as well as with neoplasms (Drieschner et al., 2007), which might indicate that its downregulated
expression in Th17eae cells could also contribute to inhibition of apoptosis. Thirdly, Odf3b is a gene that encodes for a cytoskeletal coiled-coil protein, which has predominant importance for shear stress resistance in spermatocytes (Petersen et al., 2002). Its expression and potential function in immune cells is not yet clear.

In addition to these individual targets, our analysis of the overall transcriptional changes of Th17 cells in EAE revealed that the transition process of pathogenic Th17 cells was accompanied by modulation of distinct signalling networks. We observed increased expression of the Toll-like receptors (TLRs) Tlr2, Tlr4, Tlr6, and Tlr7 and the co-stimulatory molecules Cd40, Cd80 and Cd86. Toll-like receptors (TLRs) belong to the group of pattern recognition receptors (PRR) and are widely expressed on antigen presenting cells (APCs) for antigen-unspecific pathogen recognition. Our data are supported by other findings showing that TLRs are expressed on activated and memory CD4+ T cells, resulting in direct effects of danger-associated molecular patterns to mediate functional responses (Crellin et al., 2005; Mansson et al., 2006), possibly by acting as co-stimulatory molecules in T cell receptor (TCR) signalling to promote survival of activated CD4+ T cells (Gelman et al., 2004; Komai-Koma et al., 2004). Interestingly, naive CD4+ T cells from patients with secondary progressive multiple sclerosis showed enhanced T lymphocyte TLR expression and TLR-TCR signalling, which has been associated with a faster relapsing-remitting course of multiple sclerosis (Zastepa et al., 2014).

The third signalling network is centred around the heterodimer LXR/RXR, which belongs to the nuclear receptors. Nuclear receptors have an important role in cell metabolism but also in autoimmune inflammatory processes, which has been shown for the glucocorticoid receptor and the vitamin D receptor (Smolders et al., 2008; Tait et al., 2008; Guillot et al., 2010; Schweingruber et al., 2012). LXR/RXR is involved in the modulation of cholesterol metabolism, which has been suggested to be a promising therapeutic approach for multiple sclerosis (Yang and Chi, 2014) as increased mRNA levels of NR1H2 (LXR-β) were upregulated in peripheral blood mononuclear cells of multiple sclerosis patients relative to healthy controls (Giorelli et al., 2007). Furthermore, higher serum levels of cholesterol, low-density lipoprotein, and triglycerides were associated with worsening disability in multiple sclerosis patients (Weinstock-Guttman et al., 2011). Cholesterol is needed for membrane microdomain/lipid raft organization, guiding T cell polarization and effector function, e.g. by directly influencing the intensity of calcium and NFAT signalling (Izepei et al., 2013). The LXR/RXR regulates genes involved in cholesterol and fatty acid metabolism, such as ABCA1, ABCG1, SREBF1 (formerly known as SREBP-1c), and fatty acid synthase (Tontonoz and Mangelsdorf, 2003). In Th17eae cells, increased expression of LXR/RXR and its downstream targets Lpl, Abca1 and Abcg1 was observed, which is associated with cholesterol transport, whereas SREBF1, necessary for cholesterol biosynthesis, was downregulated. Reciprocal activation of LXR and SREBP transcriptional pathways are essential for cell proliferation and clonal expansion of T cells, and thus, acquired immune responses (Bensinger et al., 2008). Interestingly, forced expression of SREBF-1a and SREBF-1c inhibited Th17 cell differentiation, while the knockdown of either isoform of SREBF1 led to increased Th17 cell differentiation (Cui et al., 2011). Thus, the observed modulation of genes involved in cholesterol homeostasis in Th17eae cells suggests that metabolic factors contribute to the expansion and reorganization of proinflammatory CD4+ Th17 cells in vivo. However, the overall role of LXR/RXR in EAE remains unclear. Treatment of MOG35-55-immunized mice with LXR agonists ameliorated EAE, which could be associated with lower levels of IL17A in the CNS and lymph nodes, and lower levels of IL17A and IL23R in splenocytes. This might be due to an independent anti-inflammatory effect of LXR signalling on antigen presenting cells (macrophages, monocytes) by inhibition of TLR signalling (Castrillo et al., 2003). Distinct roles of LXR/RXR signalling in innate and adaptive immune cells might underline these findings and the opposite effects.

In summary, our investigations of the overall transcriptional changes occurring in naïve T cells, in vitro differentiated Th17 cells and pathogenic CD4+ T cells isolated from two different EAE models improve our understanding of signalling cascades and key molecular mechanisms controlling T cell plasticity and pathogenicity over the course of EAE. The substantial overlap of differentially expressed genes in the EAE model with multiple sclerosis susceptibility genes supports the potential relevance of findings in the animal model to the human disease. Thus, further functional analysis of these targets might help to establish new and more specific therapeutic approaches to control autoimmune neuroinflammation.

**Acknowledgements**

The authors would like to thank Andreas Zymny, Christin Lieflander, Heike Ehrengard, and Christine Oswald for reading the manuscript, and Miltenyi Biotech for providing Microarray technology and supporting the analysis. The authors further would like to thank Dr Irene Schmidtmann, IMBEI Mainz, Germany for programming the simulation with SAS.

**Funding**

German Research Foundation (DFG): GRK1043 to N.H.; SFB-TRR128 to F.Z. (B4), V.S. (B9); SFB 1080 to F.Z. (B6) and ‘Gemeinnützige Hertie-Stiftung’ to V.S.

**Supplementary material**

Supplementary material is available at Brain online.


New candidates in MS pathogenesis

BRAIN 2015: 138: 902–917 | 917


