

Gene-expression profiling of the early stages of MOG-induced EAE proves EAE-resistance as an active process[☆]

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Abstract

Experimental autoimmune encephalomyelitis (EAE) induced by myelin oligodendrocyte glycoprotein (MOG) is a well-established animal model of multiple sclerosis (MS) in rodents. It reflects the wide spectrum of disease pathology and serves as a valuable tool for studying the pathogenesis and for testing new therapies of MS. In order to identify genes responsible for resistance to and modulation of the disease, we compared the mRNA expression profile of more than 12,000 genes by DNA microarray technique in lymph nodes of the highly EAE-susceptible mouse strain C57Bl/6 (B6) and the resistant strain C57Bl/10.S (B10). The disease onset in B6 mice was day 15. We identified 84 genes that were up-regulated more than two-fold in B10 mice compared to vehicle-treated controls, whereas only two genes were up-regulated in B6 mice after 7 and 15 days post-immunization (p.i.), respectively. We were able to match five up-regulated genes in B10 mice to known quantitative trait loci (QTLs), which control for EAE susceptibility. Only 17, respectively 5, genes were down-regulated at both time points in B10 and B6 mice. Tests for immunoreactivity to MOG (T cell proliferation and interferon- γ (IFN- γ) secretion) revealed no stronger immune response in B6 compared to B10 mice supporting the hypothesis of an immunosuppressive effect as a target to prevent EAE in the B10 mice. We conclude that resistance to EAE (and possibly to MS) is an active process mediated by multiple genes up-regulated in peripheral lymphatic organs of resistant animals. Thus, monitoring of the expression of these new candidate genes may serve as a tool for the disease progression and the pharmaceutical treatment.

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1. Introduction

Multiple sclerosis (MS) is known to be a polygenic disease driven by a dysregulation of the immune system

Abbreviations: EAE, experimental autoimmune encephalomyelitis; EST, expressed sequence tag; IFN- γ , interferon- γ ; MBP, myelin basic protein; MHC, major histocompatibility complex; MNC, mononuclear cells; MOG, myelin oligodendrocyte glycoprotein; MS, multiple sclerosis; QTL, quantitative trait locus; PCR, polymerase chain reaction; p.i., post-immunization.

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leading to an autoimmune response against one or several antigens of the cerebral white matter. Experimental autoimmune encephalomyelitis (EAE) serves as a mouse model to study the etiology, pathogenesis as well as new therapeutic approaches of MS (Steinman, 1999). EAE induced by peptides of the myelin oligodendrocyte glycoprotein (MOG) is arguably the best animal model of MS in rodents so far. It reflects the wide spectrum of disease pathology and serves as a valuable tool to study the pathogenesis and to test new therapeutical approaches (Storch et al., 1998; Dahlman et al., 1999). Susceptibility to EAE has been linked to the major histocompatibility complex (MHC) locus (H2) (Encinas et al., 1996). However, expression of the MHC-linked haplotype, such as H2b and H2s, is not necessarily sufficient to determine EAE susceptibility indicating

important non-MHC genetic contributions. According to the widely accepted view of the pathogenetic process of the disease, T helper lymphocytes with reactivity to components of the myelin sheath migrate to the central nervous system, where resident antigen-hosting cells, which are of macroglial origin as well as antigen-presenting microglia, reactivate them. This reactivation is followed by invasion of cytotoxic T cells, B cells, and macrophages and the release of inflammatory mediators leading eventually to demyelination and axonal loss (Trapp et al., 1998; Lucchinetti et al., 2000; Neumann et al., 2002).

We previously determined mRNA expression profiles of the target tissues, i.e. inflamed spinal cords of EAE mice, during disease progress and were able to identify groups of genes linked to the disease that mostly reinforced the immune-based and inflammatory nature of the disease (Ibrahim et al., 2001; Mix et al., 2002). Inflammation resulted in a profile of increased gene expression of immune-related molecules, extracellular matrix and cell adhesion molecules, molecules involved in cell division and transcription and differential regulation of molecules involved in signal transduction, protein synthesis and metabolism. Many of those genes mapped to known EAE-linked quantitative trait loci (QTLs) and were therefore considered as putative candidate genes for susceptibility to EAE. To continue this work and to complete the picture of gene expression during the initiation phase of the disease, we took advantage of EAE resistant strains carrying EAE MHC susceptibility haplotypes, i.e. C57Bl/10.S (B10) mice, and the related, but susceptible C57Bl/6 (B6) mice. We identified the gene expression profile of genes responsible for susceptibility, respectively resistance, to the disease by investigating the early induction phase of the disease, which is mainly characterized by activation of myelin-reactive T cells and non-myelin reactive bystander cells in the peripheral immune system. A second motivation to choose peripheral immune cells for investigation was the availability of these cells in consecutive studies on MS patients, whereby genes determining the susceptibility, respectively resistance, to autoimmune demyelination and eventually the course of the disease may be verified in the human immune system.

We analyzed the mRNA-expression profile in lymph nodes of B10 and B6 mice at two time points after disease induction, a day 7 post-immunization (p.i.), when the anti-MOG immune response was established, and at day 15 p.i., when the first clinical signs of EAE appeared. More than 12,000 genes or expressed sequence tags (ESTs) were investigated by gene chip technology. To highlight possible differences in the strength of the anti-myelin immune response, we assessed proliferation and interferon- γ (IFN- γ) secretion of lymph node cells in response to the immunizing MOG 35–55 peptide at the same time points.

2. Materials and methods

2.1. Animals, antigen, immunization and assessment of disease

C57Bl/6 (B6) mice were obtained from Harlan-Winkelmann (Borchen, Germany) and C57Bl/10.S (B10) mice were obtained from the Jackson Laboratories (Maine, USA), both strains were kept under standard conditions at the animal facility of the University of Rostock. Seven-week-old mice were immunized subcutaneously with 150 μ g of rat MOG 35–55 peptide in complete Freund's adjuvant (CFA). Mice received an intraperitoneal injection of 500 ng pertussis toxin at day 1 post-immunization (p.i.). Clinical scores were assessed blinded before immunization (day 0) and thereafter daily until day 28 p.i. Severity of paresis was graded as follows: 0, normal; 1, flaccid tail; 2, moderate paraparesis; 3, severe paraparesis 4, tetraparesis. Mice were sacrificed 7 and 15 days p.i., which corresponded to full establishment of immune response to the immunizing MOG 35–55 peptide and onset of first signs of disease, respectively (for experimental design, see Fig. 1). Control mice were immunized with CFA only. Lymph nodes were dissected and immediately transferred into Fast-prepRNA tubes (Bio101, Vista, CA) with 500 μ l of lysis buffer (Qiagen, Hilden, Germany) for RNA preparation or used for cell culture experiments. Experiments were approved by the authorities of the state of Mecklenburg-Vorpommern, Germany.

2.2. Isolation of MNC from lymph nodes and cell culture

For proliferation assays and measurement of IFN- γ , popliteal, preperitoneal, inguinal, mesenteric and axillary lymph nodes were removed under aseptic conditions. Single cell suspensions of mononuclear cells (MNC) of pooled lymph nodes from individual mice were prepared. The cells were washed three times in culture medium before being suspended to 2×10^6 MNC/ml in round-bottomed 96-well polystyrene microtiter plates (Nunc, Copenhagen, Denmark) in a total volume of 200 μ l. The culture medium consisted of RPMI 1640 with Glutamax-II (Gibco BRL, Life Technologies, Karlsruhe, Germany) supplemented with 50 IU/ml penicillin, 60 μ g/ml streptomycin (Gibco) and 5% inactivated fetal bovine serum (Gibco) without mercaptoethanol. For lymphocyte stimulation, 10 μ l aliquots of MOG 35–55 peptide were added to cultures at a final concentration of 1–50 μ g/ml or 10 μ l of concanavalin A (ConA) (Difco, Detroit, MI) at a final concentration of 4 μ g/ml. These concentrations had optimal stimulator effects as assessed in preliminary experiments. Cells were incubated at 37 °C in humidified air with 5% CO₂ for 72 h. For proliferation assay, cultures were done in triplicates, and for ELISA measurements of IFN- γ in duplicates.

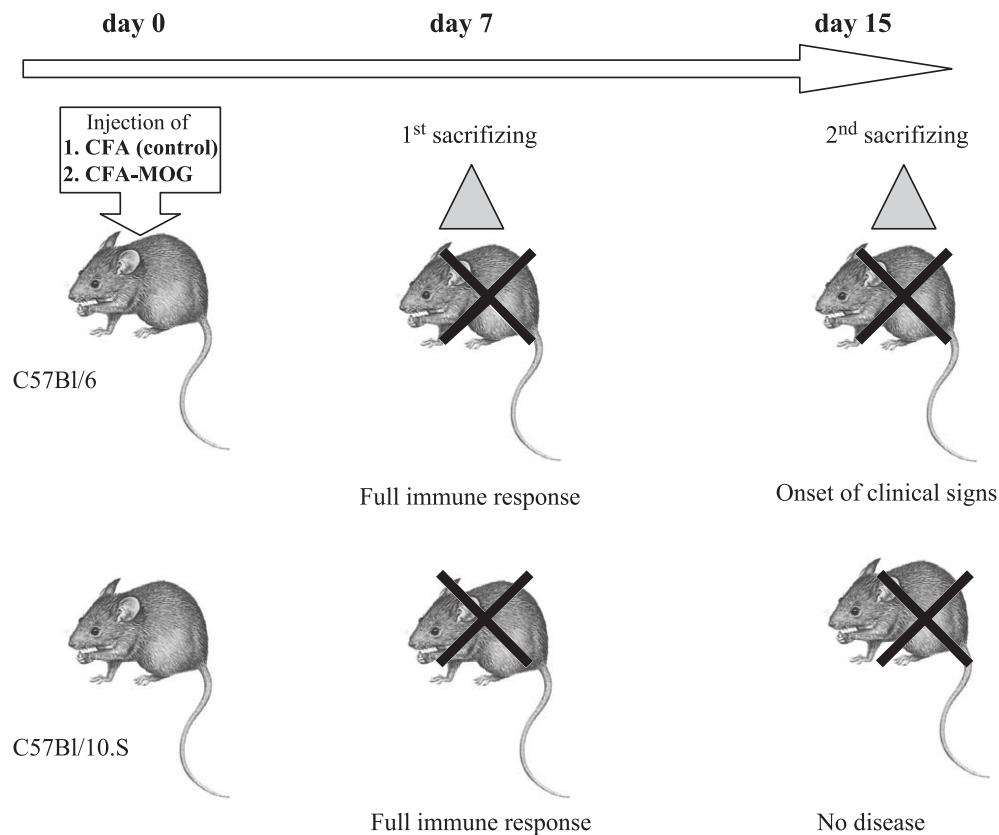


Fig. 1. Scheme of the experimental design for investigation of gene expression and immune response in lymph nodes of EAE-resistant (C57Bl/10.S=B10) and EAE-susceptible (C57Bl/6=B6) mice, respectively, during the early phase after immunization with MOG 35–55 peptide.

2.3. Proliferation assay

After 60 h of incubation, cells were pulsed with $10 \mu\text{l}$ ^3H -methylthymidine ($1 \mu\text{Ci}/\text{well}$, Amersham Pharmacia Biotech, Freiburg, Germany) and cultured for additional 12 h. Cells were harvested onto glass fiber filters (Titertek, Skatron, Lierbyen, Norway). ^3H -thymidine incorporation was analyzed in a liquid β -scintillation counter (Wallac 1214, LKB, Bromma, Sweden). The results were measured as counts per minute (cpm).

2.4. ELISA for measurement of IFN- γ

After 72 h of incubation supernatants were collected from the lymph node cell cultures, aliquoted and snap-frozen in two aliquots at -80°C . Concentrations of IFN- γ in the supernatants were determined by the Cytoscreen Immunoassay Kit (BioSource, Camarillo, CA) according to the manufacturer's instructions.

2.5. Sample preparation and oligonucleotide array hybridization

For all experiments, mRNA of three animals per group was pooled and investigated. First, total RNA was extracted from homogenized lymph nodes pooled from each individ-

ual animal as described for lymph node cell cultures (see above) using RNA extraction kit following the manufacturer's instructions (Qiagen). RNA concentrations were determined photometrically (260, 280 nm). Then RNA was pooled in equal amounts of each animal. RNA probes were labeled according to the supplier's instructions (Affymetrix, Santa Clara, CA). Analysis of gene expression was carried out with the Mu74Av2 gene chip (Affymetrix) with a capacity of more than 12,000 probe sets. Hybridization and washing of gene chips was done as previously described (Böttcher et al., 2003). The fluorescence levels were acquired by laser scanning (Hewlett-Packard Gene ScannerTM).

2.6. Analysis of microarray data

We used the Affymetrix Microarray Suite 5.1 (Affymetrix) to process raw microarray probe set data and to generate expression values (signal), detection and change calls and associated p -values for every transcript presented on the arrays. Wilcoxon's signed rank test was used to generate transcript detection and change p -values. Qualitative calls, such as "present", are derived from these p -values. Data were scaled based on the total intensity (for details see www.affymetrix.com, Statistical Algorithms Reference Guide, Part No. 701110 Rev1). The data were given as fold changes of expression levels at days 7 and 15 compared to

the expression level at day 0. Our recent microarray experiments (Ibrahim et al., 2001; Böttcher et al., 2003; Pahnke et al., 2004) and published work by others (Whitney et al., 2001; Lock et al., 2002; Mycko et al., 2003; Nicot et al., 2003; Stürzebecher et al., 2003) showed that a change of two-fold in the expression level is a cutoff to consider expression changes to be significant. The suggested detection p -values ($p < 0.04$) would enable us to regard even lower fold-changes but would otherwise massively increase the number of regulated genes. Due to this fact, we commonly use a two-fold change as cutoff to gain approximately 400–500 probe sets of interest. Thus, we lower the detection p -value ($p < 0.01$) and gain higher stringency of our results (for details, see www.affymetrix.com, Fine Tuning Your Data Analysis, Part No. 701138 Rev2). An Access2002 database (Microsoft) was used for semiautomatic search of gene function and pathway relations in online-databases (S.O.U.R.C.E., PubMed, OMIM, EnsEMBL, GeneCard, SwissProt, PubGene, GeneLynx, EuGene).

2.7. Quantification of mRNA levels using kinetic PCR analysis

Expression verification was performed by semiquantitative RT-PCR using the LightCycler™ equipment (Roche, Mannheim, Germany) as described previously (Ibrahim et al., 2001; Mix et al., 2002). Briefly, to degrade contaminating genomic DNA DNase I digestion of the purified RNA preparation (see oligonucleotide array hybridization) was

performed. For RT reaction, 2 µg RNA was incubated with 100 ng random hexanucleotides (Gibco) for 10 min at 70 °C. A mixture containing 100 units superscript MMLV reverse transcriptase (Gibco), 3 mM MgCl₂, 50 mM Tris–HCl (pH 8.3), 75 mM KCl, 10 mM DTT, 0.5 mM dNTPs and 40 U RNasin (MBI Fermentas, St. Leon-Rot, Germany) was added and the sample was incubated at 42 °C for 50 min. The reaction was stopped by denaturation at 95 °C for 5 min. One tenth of the RT reaction was added as template to a 20 µl polymerase chain reaction (PCR) master mix in glass capillaries (Roche) including 2.0 U Taq polymerase (Amersham), 125 µM dNTPs (Roche), 5 pmol of each primer, 2 pmol fluorescein labeled and 4 pmol LCRed640 labeled probes, 50 mM KCl, 10 mM TrisHCl (pH 8.3), 2.5 mM MgCl₂, 0.5 g/l bovine serum albumin (BSA, New England BioLabs, Beverly, MA) and 50 mg/l dimethylsulfoxide. For oligonucleotide sequences of the probes and primers of five candidate genes, refer to Table 1. To standardize gene transcript levels, the housekeeping gene β -actin was quantified in parallel samples as an external control. Cycling parameters were as followed: 60 s at 95 °C; 40 × (1 s at 95 °C; 10 s at 55 °C); 12 s at 72 °C. Incremental synthesis of specific amplicons was measured online at the annealing step in each cycle. LightCycler™ quantification software was used to calculate crosspoints of amplification slopes and deduce the cDNA content in a semiquantitative manner, whereby the crosspoints correspond inversely to the cDNA content. The results of five candidate genes were normalized to the β -actin signals and

Table 1
Sequences of primers and detection probes used for semiquantitative RT-PCR in the LightCycler™

Name	Labeling	Sequence
Cebpa fw		ACCgCCTTggAAAgTCACA
Cebpa rv		CCgACTCCATgggggAgTTA
Cebpa FL	3'FL	AAggCgCCAgtAggATggTgCCT FL
Cebpa LC	5'LC-RED640	LC Red640-CTgggTCTTAgtAgCCCgCCTTCTC p
Thra fw		GAAgAgTgACCgCACAgATTg
Thra rv		GCCAAATCgAACAgCATCTCgT
Thra FL	3'FL	CATCATgAggATgAACAggAACCGg-FL
Thra LC	5'LC-RED640	LC Red640-CCAgtAgTgCCgCTTTAAgtAgTg p
Brcal fw		CgAAAATCTTAgAgTgTCCgATCT
Brcal rv		CCTCCTCATTCAAACgCTCAC
Brcal FL	3'FL	GCTTgCTgAAgAgCTgCTgAgAATAATg-FL
Brcal LC	5'LC-RED640	LC Red640-CTgCTTTggAgCTTgACACgggAAT p
Tim fw		GCTTTCCAgCTgTCCCTgT
Tim rv		CCACTgTCgAgggAgCCAT
Tim FL	3'FL	CCgTgAACCAgAAAgCgTTTgTg-FL
Tim LC	5'LC-RED640	LC Red640-gCTgCTATTCTggAAgAACACCgCA p
Swap70 fw		CATggACCgTCAgACCgTA
Swap70 rv		CTTCTCTTATCTgAggCACTgAT
Swap70 FL	3'FL	AACCCTgCTTCAATACATCTAAAATAAgC-FL
Swap70 LC	5'LC-RED640	LC Red640-CATTgAAgACTTCgTTAATggCCATAg p
β -Actin fw		ACC CAC ACT gTg CCC ATC TA
β -Actin rv		gCC ACA ggA TTC CAT ACC CA
β -Actin FL	3'FL	gCC ACg CTC ggT CAg gAT CTT CAT-FL
β -Actin LC	5'LC-RED640	LC Red640-Agg TAg TCT gTC Agg TCC Cgg CCA p

Abbreviations refer to the following meanings: fw, forward primer; rv, reverse primer; FL, fluorescein; LC, LC-Red640; p, dephosphorylated.

presented as differences of crosspoints between day 0 (no immunization) and day 7 (full immune response), respectively day 15 (onset of disease). Thereby positive values correspond to gene up-regulation and negative values to gene down-regulation (Ibrahim et al., 2001).

2.8. Western blot

Lymph nodes were dissected, pooled, counted and lysed in ice-cold lysis buffer according to standard procedure (Jaster et al., 1996). The cell lysates were centrifuged ($17,000 \times g$, 5 min, 20 °C) and supernatants were used for further processing within the next 12 h. The protein concentration in the samples was determined with the BCA-reagent-kit (Pierce, Rockford, IL). The lysates were adjusted to 5 µg protein per µl with 4 × Laemmli-buffer (2% SDS, 10% glycerol, 5 mM EDTA, 62.5 mM Tris, 0.025% bromophenol blue, 5% β-mercaptoethanol). Subsequently, proteins were denaturated at 95 °C for 5 min. Gel electrophoresis was run at 150–200 V for 1–1.5 h using a Criterion-Cell (BioRad, München, Germany) and precasted polyacrylamide gradient gels (4–15%); 30 µg protein per lane was loaded. The gel was then equilibrated in transfer buffer (48 mM Tris, 39 mM glycine, 1.3 mM SDS, 20% methanol) and blotted onto a nitrocellulose membrane (Hybond-ECL, Amersham) in a semidry blot chamber (BioRad). Nonspecific binding sites were blocked by shaking the membrane at 20 °C in phosphate buffered saline (PBS) containing 1% BSA and 0.1% Tween-20 for 2 h. The primary antibodies goat anti-mouse c-Erb-A (Santa Cruz Biotechnology Santa Cruz, CA) (sc-10819) diluted 1:200, or rabbit anti-mouse Timeless (Alpha Diagnostic International, San Antonio, TX) diluted 1:500 were added to the blocking solution and the membrane incubated overnight at 4 °C. Subsequently, the membrane was washed with PBS and three times in PBS containing 0.1% Tween 20. The secondary antibody (donkey-anti-goat or anti-rabbit IgG peroxidase-conjugated, diluted 1:1000 [Dako, Glostrup Denmark]) was added to the blocking solution and the membrane was incubated at 20 °C for additional 2 h. Proteins were detected by using the ECL detection reagents (Amersham) according to the manufacturer's instructions. For molecular weight estimation, the prestained broad range (BioRad) protein marker was used.

3. Results

3.1. Both susceptible and resistant strains mount a robust immune response to MOG peptide

In order to control for amount of immune response to the immunizing MOG 35–55 peptide in EAE susceptible and resistant mice lymph node reactivity was assessed in proliferation and IFN-γ secretion assays at days 7 and 15 p.i. (Fig. 2). There was no difference in the proliferative

and cytokine response to the mitogenic stimulant ConA in EAE resistant B10 mice compared to EAE susceptible B6 mice, however with regard to the specific anti-MOG response DNA synthesis rate and IFN-γ release were higher in B10 mice as compared to B6 mice. From day 7 to day 15 p.i., the mitogen-induced proliferation decreased and the mitogen-induced IFN-γ release increased in both mouse strains, but to a higher extent in B6 mice. The proliferative and cytokine responses to MOG 35–55 peptide increased from day 7 to day 15 p.i. in both mouse strains, but on a higher level in B10 mice. In summary, both mouse strains exerted a strong and increasing immune response to the immunizing MOG 35–55 peptide, a higher proliferation capacity and a higher IFN-γ production in the EAE resistant B10 mice.

3.2. Analysis of gene expression in lymph nodes of EAE mice: more genes are up-regulated in resistant B10 mice

Oligonucleotide microarrays representing more than 12,000 genes were used to determine gene expression profiles of lymph nodes of EAE mice with fully established immune response to the immunizing MOG 35–55 peptide (day 7 p.i.) and after appearance of first clinical signs (day 15 p.i.). Genes with at least two-fold up- or down-regulation were compared in MOG-treated and CFA-only-treated mice (day 0), analyzed, clustered and visualized (Fig. 3). Fewer genes were expressed (present call) in nonimmunized EAE susceptible B6 mice (Fig. 3B, day 0) than in nonimmunized EAE resistant B10 mice (Fig. 3A, day 0). With regard to changes over time more genes were down-regulated at day 7 in B6 mice than in B10 mice (443 vs. 92), whereas the number of up-regulated genes were nearly the same in both mouse strains (245 vs. 221). Of special interest were the genes up-regulated at day 15 compared to day 0. Here the largest differences between the two mouse strains were observed. In B10 mice, 84 genes showed a sustained up-regulation at days 7 and 15, and 92 genes showed no change at day 7, but up-regulation at day 15. In B6 mice, the corresponding values were 2 and 47, respectively. Early down-regulation (day 7) and consecutive up-regulation (day 15) was seen for only 1 gene in B10 mice and for 19 genes in B6 mice. The identification of all genes up-regulated at day 15 compared to day 0 is given as supplementary information (made available on-line at doi:10.1016/j.jneuroim.2004.03.007). The analysis of genes grouped tentatively according to their expected functions revealed no obvious differences between the two mouse strains, except a relative dominance of genes related to cell metabolism, mitochondrial function, protein transport and secretion in B10 mice. The two genes with prolonged up-regulation in B6 mice are cathepsin C and the transcription factor β-enolase repressor 1; the single gene with early down-regulation and consecutive up-regulation in B10 mice is the ets-related protein 81. Only a

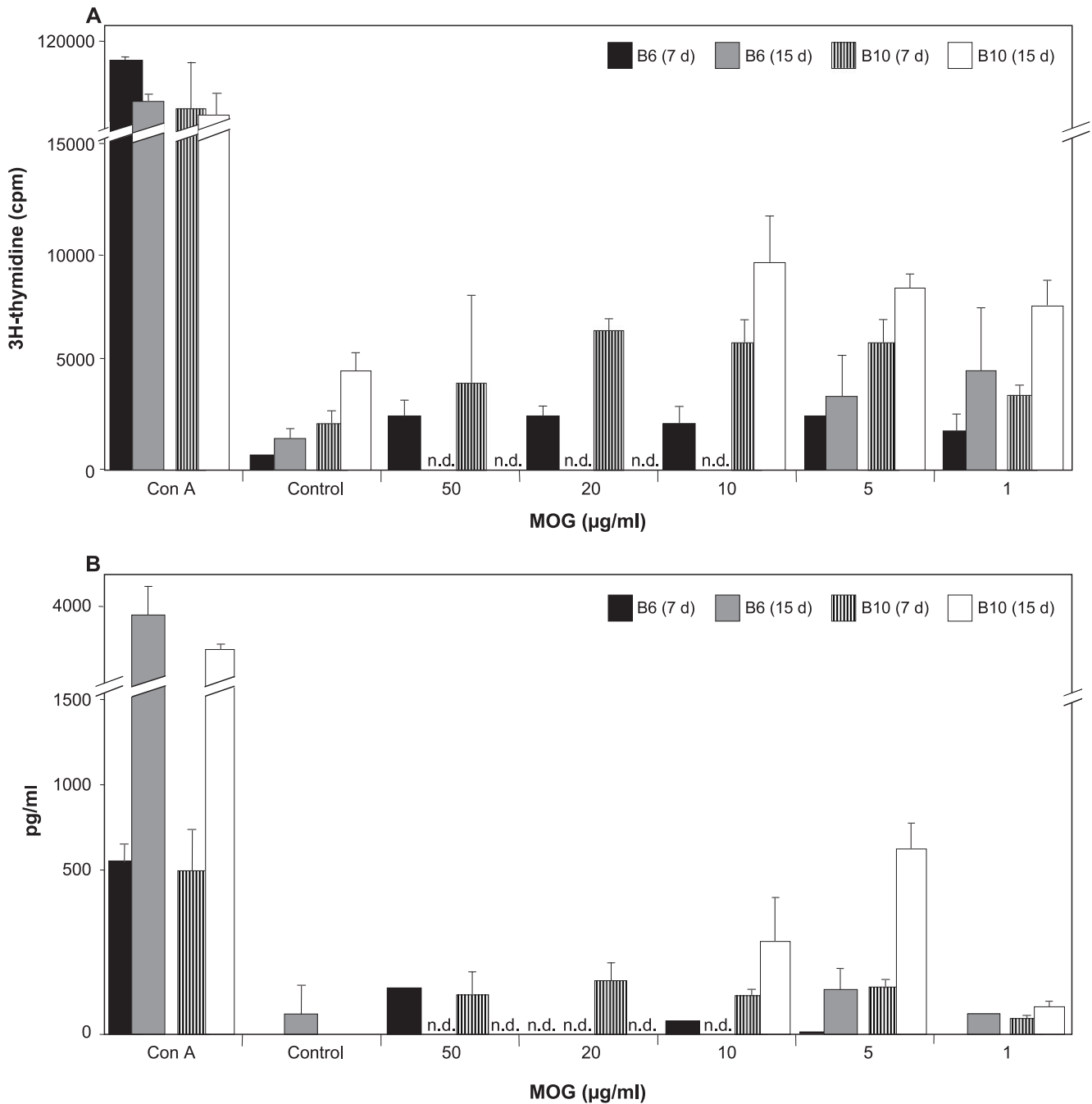


Fig. 2. Immune response in lymph nodes of EAE-susceptible (B6) and EAE-resistant (B10) mice, respectively, at time points two and three (days 7 and 15 p.i., see Fig. 1) as assessed in lymphocyte cultures with the mitogen concanavalin A (ConA) and the immunizing MOG 35–55 peptide at different concentrations: (A) proliferation (DNA synthesis) as determined by ^3H -thymidine incorporation and (B) IFN- γ production as determined by ELISA of culture supernatants.

few genes related to the immune response, especially immunoglobulin genes, were up-regulated in EAE lymph nodes compared to lymph nodes of CFA-only-treated controls in both mouse strains. However, five genes, which were selectively up-regulated in B10 mice either at day 7 or at day 15 compared to day 0, mapped to known EAE susceptibility loci (Table 2). These genes are regarded as candidate genes for linkages with EAE resistance. Additionally, the relative expression changes of

these genes were verified by quantitative RT-PCR (see below).

3.3. Analysis of expression of genes mapping to EAE-linked loci

We used gene expression profiling to combine expression with linkage analysis for the identification of new susceptibility genes for EAE. Therefore, we analyzed

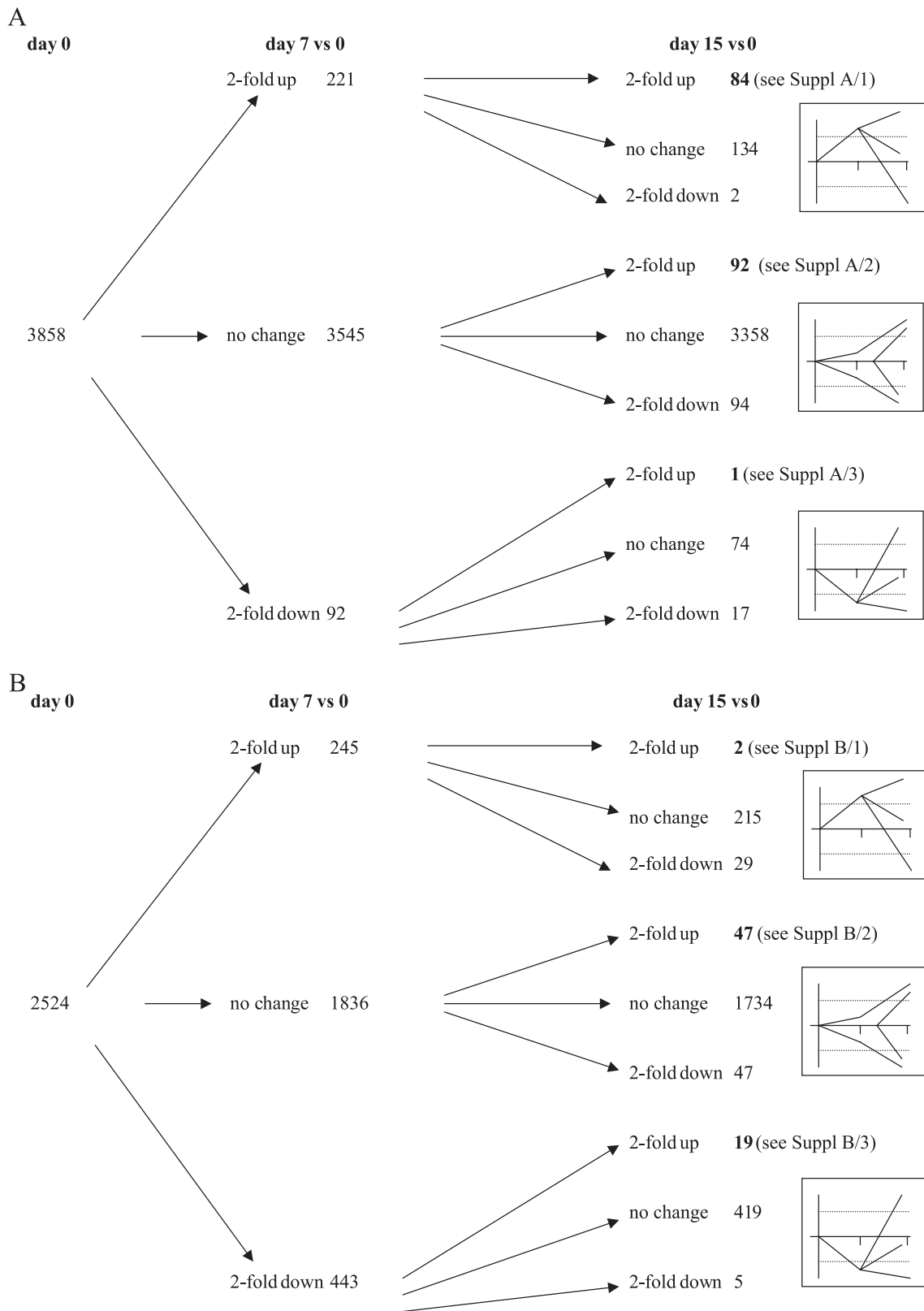


Fig. 3. Changes of gene expressions in lymph nodes of EAE-resistant (B10) (A) and EAE-susceptible (B6) mice (B), respectively, as assessed by DNA microarray technique. The numbers given correspond to genes with a present call expression level according to Affymetrix software at all three time points investigated (see Fig. 1) and the indicated pattern of changes during the initial phase after immunization (days 7 and 15 p.i., respectively). Numbers of genes of special interest, i.e. those that are up-regulated at day 15 in comparison to day 0 are highlighted.

Table 2
Mapping of differentially expressed genes to EAE loci^a

EAE locus	Chr	NCBI	Abbreviation	Full name	Gene locus (cM)	EAE QTLs (cM)
1/5	17	AF049850	C4	Complement component 4 (within H-2S)	18.8	20/21.1
1/5	17	AF049850	Cyp21a1	Cytochrome P450, 21, steroid 21 hydroxylase	18.77	20/21.1
1/5	17	AF049850	D17H6S45	DNA segment, Chr 17, human D6S45	18.84	20/21.1
1/5	17	AF049850	Slp	Sex-limited protein	18.83	20/21.1
1/5	17	AF109906	Bat8	HLA-B-associated transcript 8	18.87	20/21.1
1/5	17	AF109906	C2	Complement component 2 (within H-2S)	18.86	20/21.1
1/5	17	AF109906	D17Ert710e	DNA segment, Chr 17, ERATO Doi 710, expressed	18	20/21.1
1/5	17	AF109906	D17H6S45	DNA segment, Chr 17, human D6S45	18.84	20/21.1
1/5	17	AF109906	D17H6S56E-2	DNA segment, Chr 17, human D6S56E2	18.95	20/21.1
1/5	17	AF109906	G7e-pending	G7e protein	19	20/21.1
1/5	17	AF109906	H2-Bf	Histocompatibility 2, complement component factor B	18.85	20/21.1
1/5	17	AF109906	Hsp70-1	Heat shock protein, 70 kDa 1	18.94	20/21.1
1/5	17	AF109906	Hspa11	Heat shock 70 kDa protein-like1	18.955	20/21.1
1/5	17	AF109906	Neu1	Neuraminidase 1	18.93	20/21.1
1/5	17	AF109906	Vars2	Valyl-tRNA synthetase 2	19.02	20/21.1
4	7	AF053974	Swap70	Ig switch regulator complex protein, 70 kDa	50	50
7/22	11	U09504	Thra	Thyroid hormone receptor alpha (c-ERB-Aalpha2)	57	48/61
9	9	U73478	Anp32	Acidic nuclear phosphoprotein 32	36	35
12	7	M62362	Cebpa	CCAAT/enhancer binding protein (C/EBP), alpha	12	16
15	10	AB015598	Tim	Timeless homolog (Drosophila)	18	16
16	12	U73445	Dld	Dihydroipoamide dehydrogenase	15.1	6
17	10	D87896	Gpx4	Glutathione peroxidase 4	43	36
20	3	X94127	Sox2	SRY-box containing gene 2	15	13.8
21	2	D50430	Gdm1	Glycerol phosphate dehydrogenase 1, mitochondrial	33	37
22	11	U32446	Brcal	Breast cancer tumor suppressor 1	60.5	61
23	11	U14390	Aldh3a2	Aldehyde dehydrogenase family 3, subfamily A2	34.3	38

^a Summary of differentially expressed genes mapping to chromosomal regions containing EAE linked susceptibility loci. Gene expression profiling was carried out using the Mu74A Affymetrix oligonucleotide microarrays. Only genes designated as present by the Affymetrix software in at least one time point of investigation were included in the analysis. The chromosomal positions were obtained from the Jackson Laboratory (<http://www.informatics.jax.org>). EAE QTL were defined as intervals of ± 10 cM of the genetic marker showing highest lod score or as the interval described in references. Genes up-regulated in resistant mice and not changed in susceptible mice are printed in bold.

differentially expressed genes with known chromosomal locations in relation to known QTLs linked to susceptibility to EAE (Table 2). Twenty-six genes mapped to 12 of the 26 known QTLs. As expected the majority (15 genes) mapped to the QTLs EAE1 and EAE5 at the MHC locus. Surprisingly, most of these genes were up-regulated in the EAE resistant strain. Up-regulation of MHC locus genes suggest the intriguing possibility that the resistance to EAE of B10 mice might be more an active than a passive process.

3.4. Confirmation of the expression of resistance-linked genes

As a second and independent validation of the expression data, we analyzed the mRNA by quantitative RT-PCR (LightCyclerTM) of five EAE QTL-linked genes that were up-regulated in EAE resistant mice at least at one time point of investigation, i.e. the genes of thyroid

hormone receptor- α (Thra), CCAAT/enhancer binding protein- α (Cebpa), Timeless (Tim), breast cancer tumor suppressor-1 (Brcal) and immunoglobulin switch regulator of 70 kDa (Swap70) (Table 2). The quantitative RT-PCR confirmed the microarray results with the exception of Swap70. Thra and Cebpa were found to be up-regulated by microarray analysis at day 7, but not on day 15 (Fig. 4A). Accordingly, they revealed lower cross-points (higher LC-changes) corresponding to higher mRNA levels at day 7 than at day 15 in the Light-CyclerTM analysis (Fig. 4B). On the other hand, Tim, Brcal and Swap70 were up-regulated at day 15, but not at day 7 according to the microarray analysis (Fig. 4A). Correspondingly, Tim showed higher LC-changes at day 15 than on day 7. Brcal revealed high LC-changes at both time points confirming the microarray results partly (day 15). The only clear discrepancy between microarray and LightCyclerTM results was noticed for Swap70, which was up-regulated at day 15 according to the microarray

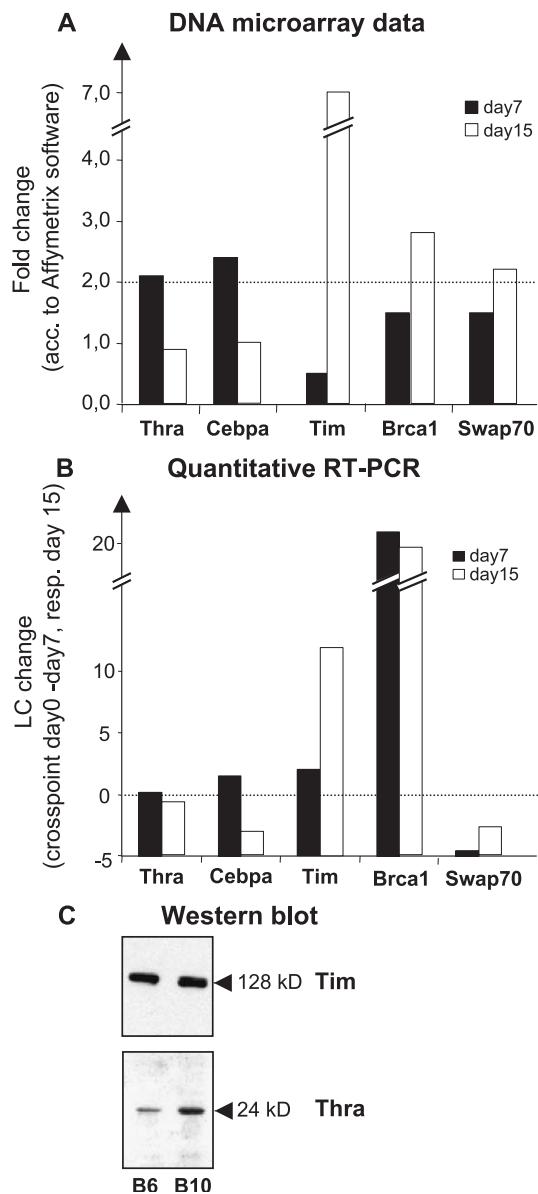


Fig. 4. Verification of differential expression of candidate genes for EAE resistance Thra, Cebpa, Tim, Brca1 and Swap70 in lymph nodes of B10 mice at days 7 and 15 p.i. Results of gene expression are given as assessed by DNA microarray technique (A) and quantitative RT-PCR by LightCycler™ technology (B). Presented data are fold changes for microarray results and LC-changes for LightCycler™ results, respectively, as explained in Materials and methods. (C) Protein expression of the genes Thra and Tim in B6 and B10 mice at day 7 as assessed by Western blot.

results, but down-regulated according to the LightCycler results at both time points of investigation.

In order to control for the protein expression of the putative EAE resistance-linked genes, we analyzed the expression of Thra and Tim in lymph nodes, which had previously shown consistent gene expression in microarray and LightCycler™ assays. Western blot analysis of Thra and Tim at day 7 revealed higher Thra expression in B10 mice than in B6 mice, whereas a high Tim expression was seen in both mice strains (Fig. 4C).

4. Discussion

In this study, we determined the gene expression profile during the initiation phase of MOG-EAE in an EAE-resistant strain, C57Bl/10.S (B10) and a related, but EAE-susceptible strain C57Bl/6 (B6). Our aim was to identify genes responsible for susceptibility, respectively resistance, to the disease expressed in the peripheral lymphatic system. The study complements our earlier findings (Ibrahim et al., 2001) where we determined the expression profile of later stages of the disease in the target tissue, i.e. inflamed spinal cord. Here, the expression profiles of more than 12,000 genes in lymph nodes of B10 and B6 mice were assessed by microarray technology at two time points after disease induction: at the time of established anti-MOG immune response (day 7 p.i.) and at the onset of clinical signs (day 15 p.i.). At both time points, the EAE resistant mice developed a systemic immune response similar to the EAE susceptible mice as evidenced by proliferation assays and IFN- γ release in response to the immunizing antigen MOG peptide 35–55. Correspondingly, the number of up-regulated immune related genes was similar albeit low in both mouse strains, i.e. 13 in B6 and 11 in B10 mice at onset of clinical signs.

The gene expression profiling was recorded on the basis of pooled RNA from the lymph nodes of different regions of three animals in each group. It is to be expected that gene expression patterns of individual lymph nodes draining the regions of injection sides differ from those of distant lymph node regions. However, since it is our aim in the long range to determine genes, which might be suitable as marker genes in a situation mimicking the clinical situation and to use genes as surrogate marker in plasma or peripheral leucocytes (Bomprezzi et al., 2003; Koike et al., 2003; Stürzebecher et al., 2003), we needed to determine common regulation patterns. A widely used method suggests to pool RNA from tissues or parts thereof for this purpose (Miller et al., 2001; Agrawal et al., 2002; Pletcher et al., 2002) and this method has been evaluated as being statistically valid and efficient (Kendzierski et al., 2003; Peng et al., 2003).

A striking finding of gene expression analysis of the pooled peripheral lymph nodes is the selective and persistent (on days 7 and 15 p.i.) up-regulation of 84 genes in B10 mice, whereas only two genes showed the same behavior in B6 mice. However, more interesting than the higher number may be the relation of the selectively up-regulated genes to functional groups. More than 50% belong to one of the following five groups (Suppl. A/1): (1) cell growth and apoptosis (e.g. caspase 3), (2) protein processing (e.g. hsp40 and proteasome- β 6), (3) cell metabolism (e.g. glutathione S-transferase and aldehyde dehydrogenase), (4) transcription (e.g. zinc finger protein OZF), and (5) transport and secretion (e.g. trans-golgi network protein 1 and importin- α 1). This argues for a higher metabolic activity and cell turnover of lymph node cells in EAE-resistant B10 mice as compared

to EAE-susceptible B6 mice during the initiation phase of the disease. The finding is corroborated by a dominance of the gene expression for cell metabolism, mitochondrial function and protein transport and secretion in lymph nodes of B10 mice on day 15 only (Suppl. A/2). We conclude that active mechanisms of resistance are initiated in parallel to the induction of the autoimmune reaction, i.e. before the autoreactive lymphocytes reach the central nervous system (CNS). On the other hand, activated CNS-reactive lymphocytes may have left the lymph nodes and reached the CNS in the susceptible B6 mice earlier and/or to a higher extent than in B10 mice. The latter may, therefore, harbor more activated lymphocytes in the peripheral lymph nodes. This would point to enhanced lymphocyte migration and adhesion to the CNS in B6 mice. In contrast, active processes may inhibit a CNS homing in B10 mice.

Because of the strong peripheral anti-MOG response in B10 mice deletional central tolerance (anergy) is unlikely to be responsible for their EAE resistance. However, peripheral tolerance can be envisaged, e.g. on the basis of active processes inhibiting CD8⁺ T cell-mediated effector activity. In addition, there is still the possibility of resistance to EAE in B10 mice and susceptibility to EAE in B6 mice being determined within the nervous system through the interaction between antigen-specific immune cells and CNS parenchymal cells. At the blood–brain barrier, CD95L (Fas-ligand) may be up-regulated or MHC-molecules on target cells may be down-regulated. Many examples of strong systemic immune response to immunizing antigen without active disease have been documented. One published example confers to Fas mutant mice (B6-lpr/lpr) (Waldner et al., 1997). These mice mount a strong immune response to immunizing antigen reflected by autoantibodies, and higher levels of Th1 cytokines; however, they are almost completely resistant to EAE. The mechanism that prevents the disease in B10 mice remains unresolved.

Regarding the disease promotion in B6 mice, only two genes were consistently up-regulated in these mice at day 7 and day 15, cathepsin C prepropein (Ctsc) and β -enolase repressor factor-1 (Berf-1). Cathepsins are cysteine endoproteases, some of which have been shown to be essential for antigen presentation to autoreactive T cells. This is in agreement with susceptibility to autoimmunity (Saeguse et al., 2002). Berf-1 codes for a Krüppel-like zinc finger protein (Zfp148), which (1) is expressed in several cell types including T cell lineages, (2) suppresses basal and induced expression several genes including stomelysin, gastrin and vimentin, and (3) plays an important role for cell-cycle regulation via activation of p53 (Takeuchi et al., 2003). It may have a counter-regulating function in activated lymph node cells of immunized B6 mice preventing unlimited expansion.

Another important evaluation of the expression data is the mapping of the chromosomal localization of putative resistance-genes. Twenty-six genes mapped to 13 of the known 26 QTLs. As expected, the majority (15 genes)

mapped to the QTLs EAE1 and EAE5 at the MHC locus, which reflects a lower percentage as compared to our earlier study of gene expression mapping in the nervous system of B6 EAE mice (Ibrahim et al., 2001). This fact points to a role of non-MHC-linked genes in the periphery contributing to the disease control. Surprisingly, a few genes that are up-regulated systemically in B10 mice, but not in B6 mice neither at day 7 nor day 15, map to EAE linked QTLs. These are the genes for (1) thyroid hormone receptor- α (Thra), (2) CCAAT/enhancer binding protein c/EBP- α (Cebpa), (3) circadian clock related factor Timeless (Tim), (4) breast cancer susceptibility factor Brca1 and (5) B cell-specific protein Swap70. The up-regulation has been confirmed by real-time quantitative RT-PCR except for Swap70, which was, however, higher expressed at day 15 than at day 7, in both tests (see Fig. 4A and B). Thra, also known as c-erbA α , belongs to the nuclear hormone receptor family and acts, together with retinoid receptors, via ligand and DNA binding sites as transcriptional repressor (Yoh and Privalsky, 2001). Its expression is altered in breast cancer (Silva et al., 2002), but a linkage to autoimmune processes has not been described so far. The transcription factor Cebpa is crucial for neutrophilic differentiation. Mutations in this gene are recognized to cause myeloid leukemia (Snaddon et al., 2003). Involvement in autoimmunity is yet unknown. The DNA-binding protein Tim interacts with the period protein (Per) as a negative regulator of rhythmic processes, e.g. generated in the superchiasmatic nucleus (Barnes et al., 2003). Relations of Tim to autoimmune processes have not been reported so far. The zinc-finger domain containing Brca1 protein is a tumor suppressor, which is frequently mutated in breast and ovarian cancer (King et al., 2003). However, a function in autoimmunity is not known. The 70 kDa protein Swap70 is an immunoglobulin switch recombinase. In addition, it is involved in B cell activation (Masat et al., 2000). Therefore, a role of Swap70 in humoral autoimmune responses is conceivable. All five described genes, which map near EAE QTLs, have not been previously related to autoimmune processes. More importantly, they seem to be up-regulated in parallel with pro-inflammatory genes in the peripheral immune system. An involvement in the suppression of the disease in resistant animals despite full-blown autoimmune response is suggestive. Therefore, they may represent new candidate genes counteracting the autoimmune process in EAE. However, conclusions concerning functional roles of newly detected candidate genes for EAE modification are still speculative and await loss of function and gain of function experiments. In addition, mechanisms of EAE-resistance may not be universal, but rather be restricted to the respective autoantigen involved in the pathogenic process, e.g. myelin basic protein (MBP), MOG, PLP, MOBP peptides. The restriction may be determined by MHC genes (Skundric et al., 2003), by non-MHC genes (Jagodic et al., 2001; Weissert et al., 2001; Becanovic et al., 2003) and by environmental factors (Costa et al., 2003).

So far, EAE is the most widely used animal model for studying pathogenetic aspects of MS on the one hand and to validate new targets for MS therapy on the other hand (Carmody et al., 2002; Lock et al., 2002; Matejuk et al., 2002; Nicot et al., 2003; Pedotti et al., 2003; Steinman and Zamvil, 2003). However, the disease processes are different and materials investigated in recent studies are different, e.g. lymph nodes (Carmody et al., 2002) and spleen (Matejuk et al., 2002) in EAE, and mononuclear blood cells (Bomprezzi et al., 2003; Koike et al., 2003; Stürzebecher et al., 2003) and brain lesions (Whitney et al., 2001; Lock et al., 2002; Mycko et al., 2003; Pedotti et al., 2003) in MS. This makes it difficult to compare results of studies in EAE and MS. Therefore, it is not surprising that analysis of MS plaques (Whitney et al., 1999, 2001; Lock et al., 2002; Mycko et al., 2003) revealed only a few genes, which were also differentially regulated in EAE lymph nodes in our study. Special reasons for these differences may be (1) different time kinetics of gene expression in the peripheral immune system as compared to the target tissue of EAE and MS, and (2) a higher degree of heterogeneity of MS lesions as compared to lymphoid tissue of immunized mice (Whitney et al., 1999; Chabas et al., 2001; Ramanathan et al., 2001; Whitney et al., 2001; Lock et al., 2002; Bomprezzi et al., 2003; Mycko et al., 2003). As to the first possibility, only five up-regulated genes in spinal cord of B6 mice of our previous study (Ibrahim et al., 2001) were also up-regulated in lymph nodes of B6 or B10 mice in the present study. These genes are coding for the following proteins: haematopoietic-specific protein-1 and cystein-rich intestinal protein (both found to up-regulated in B6 mice), interferon-induced 15 kDa protein, skeletal α -actin and adipose differentiation-related protein (all were found to be up-regulated in B10 mice). Homologous genes found to be up-regulated in chronic or acute MS lesions by Lock et al. (2002) and in EAE lymph nodes in our study are the following: connexin 43, pro- α 1(III) collagen, calcium-binding protein CAPL, microsomal aldehyde dehydrogenase ALDH10, stromal interaction molecule-1 GOK and intracellular serine proteinase inhibitor 98 kDa. We conclude that genes concomitantly up-regulated in peripheral lymphoid organs and in CNS of EAE mice as well as MS patients are rare, heterogeneous and ubiquitously expressed rather than typically expressed in inflamed lymph nodes or nervous tissue.

Previous studies have also shown that there is only minor overlapping of gene expression in between lymphoid tissue and CNS in MOG-EAE mice (Carmody et al., 2002). In the CNS of MBP-EAE rats, gene expression related to nerve conduction was mainly decreased (Nicot et al., 2003). On the other hand, Matejuk et al. (2003) found in the CNS of transgenic mice with MBP-Ac1-11 peptide specific T cell receptor and Rag-1 mutation, which develop spontaneous progressive EAE, strong up-regulation of immune related as well as cell cycle related genes. Considering the heterogeneity of morphological changes in the parenchyma and the variety of the corresponding clinical symptoms of MS it is

highly unlikely that the gene expression profile of a few MS plaques of individual patients (Lock et al., 2002) will be representative for larger populations of patients. In contrast, systematic use of DNA microarray analysis of mononuclear blood cells in larger cohorts of patients with similar clinical and radiological (magnetic resonance imaging) characteristics will give more insights in the dynamics and phenotypic heterogeneity of the disease process, in particular with regard to disease promoting and inhibiting factors that can be monitored in longitudinal studies analogously to reproducible animal models. For this purpose, our present results of EAE lymph node gene expression profiling may provide cues for further patient trials, since it may reflect changes in the systemic immune response over time just as the profiling in mononuclear blood cells. In MS patients such profiling has been first described by Ramanathan et al. (2001) and Wandinger et al. (2001) and more recently by Bomprezzi et al. (2003), Koike et al. (2003) and Stürzebecher et al. (2003). An additional aspect of our study is the possibility to test effects of therapeutic strategies in MS and EAE. Application of interferon- β (Wandinger et al., 2001; Koike et al., 2003; Stürzebecher et al., 2003), 17 β -estradiol (Matejuk et al., 2002), histamine receptor type 1 antagonists (Pedotti et al., 2003) or atorvastatin (Youssef et al., 2002) differentially regulates genes, which may support the discrimination of responders from non-responders to the respective therapies by the help of expression analysis.

In conclusion, selectively up-regulated genes in EAE resistant mice may serve as predictors of the clinical course and therapy response in EAE and MS. Finally, genes down-regulated in EAE-resistant animals and up-regulated in EAE-susceptible animals may represent new targets of MS therapy (Steinman and Zamvil, 2003; Zamvil and Steinman, 2003).

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