Increased Transcriptional Activity of Milk-Related Genes following the Active Phase of Experimental Autoimmune Encephalomyelitis and Multiple Sclerosis[1]

David Otaegui,[*][§] **Sara Mostafavi,**[†] **Claude C. A. Bernard,**[‡] **Adolfo Lopez de Munain,**[§] **Parvin Mousavi,**[†] **Jorge R. Oksenberg,**[*] **and Sergio E. Baranzini**[2][*]

We analyzed global transcriptional changes in the lymph nodes of mice with experimental autoimmune encephalomyelitis in a longitudinal fashion. Most of the transcriptional activity was observed between 3 and 5 days postimmunization. After that period, gene expression changes decayed sharply back to baseline levels. A comparison of transcriptional profiles between immunized and control mice at the time of peak disease activity revealed 266 transcripts, mostly involved in cell-cell interaction and protein synthesis. When the same comparison was performed at the time of recovery from an attack, increased expression of genes coding for milk components were identified. Specifically, casein α (Csn1s1), β (Csn2), γ (Csn1s2a), and κ (Csn3), in addition to lactoalbumin α and extracellular proteinase were elevated >3-fold in immunized animals compared with CFA-injected controls. We confirmed these findings by quantitative RT-PCR and immunostaining of Csn3. Interestingly, the expression of Csn3 was also found elevated in the blood of multiple sclerosis (MS) patients after a relapse. Altogether, our data suggest that increased production of milk-related transcripts in the lymph nodes and blood succeeds an inflammatory event in experimental autoimmune encephalomyelitis and MS. The potential role of lactogenic hormones in MS is discussed. *The Journal of Immunology,* 2007, 179: 4074–4082.

Multiple sclerosis (MS)[3] is a neurological disease driven by a dysregulation of the immune system leading to an autoimmune response against one or several Ags of the CNS white matter. It is generally acknowledged that a complex interplay between environmental and genetic factors are involved, but the etiology of MS remains unknown. Experimental autoimmune encephalomyelitis (EAE) is an induced, T cell-mediated autoimmune inflammatory disease of the CNS that mimics many of the clinicopathological findings of MS (1). EAE can be induced either by transfer of activated encephalitogenic T cells or by active immunization with myelin Ags and, depending on the strain and antigenic stimulus, different disease courses can be provoked. For example, while relapsing EAE develops in the SJL mouse strain

immunized with spinal cord homogenate or purified myelin Ags, a chronic progressive disease is characteristic of C57BL/6 mice. In addition to a distinct disease course, each model differs in pathological aspects such as the extent of CNS inflammation and demyelination. When the disease is induced with myelin oligodendrocyte glycoprotein (MOG) in the nonobese diabetic (NOD) mouse, a very reproducible course of relapsing remitting disease can be obtained with abundant leukocyte infiltrates and marked demyelination (2).

Although a significant body of knowledge has accumulated on various aspects of EAE, a detailed description of the underlying molecular mechanism is still lacking. Furthermore, the extent and contribution of the different cell types involved and the anatomical organization of the overall neuroinflammatory processes are not well understood. A currently accepted model states that the injected Ag is taken up by APCs (dendritic cells and macrophages) in the peripheral tissues and within a few hours is transported via afferent lymphatic vessels to the lymph nodes (LN) (3). Once there, the Ag is processed and presented to naive T cells in the context of molecules of the MHC. During the next few days, activated T cells are released into the circulation from where they gain access to virtually every organ in the body. Because activated T cells have an increased ability to cross the blood-brain barrier, they will be subject to reactivation by resident CNS APC. This unleashes a cascade of events resulting in myelin damage and axonal pathology, which in turn causes the neurological disability. In the NOD mouse, neurological dysfunction is evident as early as 13 days postimmunization (dpi).

One way of examining the evolution of the disease is to analyze samples obtained at closely spaced time points, since this design allows for correlations of measured variables with the observed clinical phenotype. We recently reported the longitudinal gene expression profiling of spinal cords obtained at 12 different time points after immunization of NOD mice with MOG_{35-55} peptide

[3] Abbreviations used in this paper: MS, multiple sclerosis; EAE, experimental autoimmune encephalomyelitis; MOG, myelin oligodendrocyte glycoprotein; dpi, days postimmunization; BL, baseline; EE, early EAE; PE, peak EAE; ER, early recovery; LR, late recovery; LN, lymph node; EDSS, Expanded Disability Status Scale; qRT-PCR, quantitative PCR; BTN, butyrophilin; SPMS, secondary progressive multiple sclerosis; OPN, osteopontin; Ltf, lactotransferrin; DEG, differentially expressed gene; CC, class comparison; Lalba, lactoalbumin; Expi, extracellular proteinase inhibitor.

Table I. *Experimental design and sample collection scheme*

| | | RNA | | | | | | Histology |
| | | Control (CFA) | | | Immunized (MOG$_{35-55}$) | | | Immunized (MOG$_{35-55}$) |
Time Point	dpi	Grouping	No. of Animals[a]	No. of arrays OK[b]	Grouping	No. of Animals[c]	No. of Arrays OK[d]	No. of Animals[e]
0	0	BL	4[f]	4	BL	4[f]	4	3[g]
1	3	t1–t6	2	2	BE	4	3	3
2	5	t1–t6	2	2	BE	4	2	3
3	7	t1–t6	2	2	BE	4	3	3
4	9	t1–T6	2	2	BE	4	4	3
5	11	t1–t6	2	2	BE	4	4	3
6	12	t1–t6	2	2	BE	4	4	3
7	13	T7–T9	2	1	EE	4	3	3
8	14	t7–t9	2	1	EE	4	4	3
9	15	t7–t9	2	1	EE	4	4	3
10	16	t10	2	2	PE	4	4	3
11	17	t11	2	2	ER	4	4	3
12	18	t12	2	2	LR	4	4	3

[a] A total of 84 animals were immunized with MOG$_{35-55}$ in CFA (indicated in *columns c* plus *e* minus the 7 mice used at BL). Seven animals were sacrificed at BL and at each of 12 subsequent time points, and their LN were removed. The same four animals served as BL for the immunized and control groups (*f*). LN from four of these animals were processed for RNA extraction and posterior microarray analysis, whereas those from the remaining three animals (*g*) were formalin fixed and embedded in paraffin for histological examination. In parallel, 24 mice were injected with CFA only and sacrificed following the same schedule as their immunized littermates (indicated in *column a* minus the four mice used at BL). Two animals were sacrificed at each time point and their LN were processed for RNA and microarray analysis. The number of microarrays that passed our quality control are indicated at each time point (indicated in *columns b* and *d*). t1–t12 are time points used to identify control animals.

(4). In that study, we identified expression signatures that correlated with disease stage and histological profiles. In addition, we found that discrete phases of neuroinflammation were accompanied by distinctive expression signatures with altered immune vs neural gene expression ratios. According to the model of pathogenesis described previously, the immune response unfolds as a wave of events where the activity of molecular and cellular events shifts from the LN, to the blood, to the CNS. To evaluate the molecular activity that precedes the pathogenic cellular infiltration into the brain and spinal cord, we now investigated the transcriptional profiles of LN dissected from MOG-immunized mice.

Several recent reports have attempted to characterize the transcriptional profiles in the CNS or LN of EAE mice, helping to define the molecular fingerprint of the demyelinating process (3, 5–8). However, most of these studies focused on cross-sectional analyses associated with a concrete EAE status. In this article, we report an extensive longitudinal analysis of transcription in the LN of NOD mice after induction of EAE by MOG$_{35-55}$.

Materials and Methods

Mice

Eight- to 13-wk-old NOD mice were kept at the La Trobe University central animal house (Melbourne, Australia). All of the experiments were conducted in accordance with the Australian code of practice for the care and use of animals for scientific purposes (National Health and Medical Research Council, 1997), after approval by the La Trobe University Animal Ethics Committee.

Immunization and clinical scoring

EAE was induced in 84 female NOD mice by s.c. injection into their lower flanks with 200 μl of MOG$_{35-55}$ peptide (MEVGWYRSPFSRVVH-LYRNGK; Auspep) emulsified in CFA containing 4 mg/ml *Mycobacterium tuberculosis* (Difco). Immediately thereafter and 48 h later, an i.v. injection of 350 ng of *Bordetella pertussis* toxin was administered to the animals. The control group consisted of another 26 female mice treated with the same protocol except MOG peptide. We also used seven non-injected mice as baseline (BL) group (time 0). Animals were monitored and scored daily. According to the evolution of these scores, the mice were ascribed to one of the following groups: presymptomatic or before EAE (BE), early EAE (EE), peak EAE (PE), early recovery (ER), and late recovery (LR). The time-matched groups in control animals were: baseline (*t0*), times 1–6 (*t1–6*), times 7–9 (*t7–9*), time 10 (*t10*), time 11 (*t11*), and time 12 (*t12*).

Sample collection

Mice.

At each of the 12 time points (except baseline) 9 animals were sacrificed under the same conditions. At each time point, three animals from the immunized group were immediately perfused with 4% paraformaldehyde in 0.1 M phosphate buffer and their inguinal and axillary LN were removed and processed for histological staining (Table I). LN from the other six animals (four immunized and two controls) were also collected and immersed in RNAlater (Ambion) and frozen at –20°C. The time correlate with dpi is shown in Table I.

Human.

All patients were diagnosed with MS according to the McDonald criteria (42). The Expanded Disability Status Scale (EDSS) score was calculated by a single neurologists using the Kurtzke scale according to clinical history data and patient examination (43). These scores were reconverted using the Multiple Sclerosis Severity Score evolution time value (44). All patients were recruited by Neurology Departments of hospitals from the region of Gipuzkoa (Spain) according to local institutional review board regulations.

Histology

Freshly isolated axillary and inguinal LN were fixed in 4% paraformaldehyde overnight and embedded in paraffin. Eight-micrometer sections were cut, mounted on glass slides, and stained with H&E. Selected sections were stained with an Ab against Csn2 by the avidin-biotin peroxidase complex method according to standard protocols. Briefly, after deparaffinization, sections were treated with 3% H$_2$O$_2$ for 10 min and then processed for Ag retrieval. Sections were incubated with 10% normal serum for 30 min; they were later incubated with the primary Ab (Csn2; Sigma-Aldrich). After washing with TBS, the sections were incubated at room temperature for 30 min with peroxidase-conjugated secondary Abs (DakoCytomation), followed by incubation with the avidin-biotin complex (DakoCytomation) for 30 min. Peroxidase labeling was visualized with 10% 3,3-diaminobenzidine. Sections were counterstained with hematoxylin.

Expression analysis

Mice.

LN from selected animals were removed from RNAlater and homogenized in TRIzol (Invitrogen Life Technologies) using an electric homogenizer. cDNA was synthesized according to previously published protocols (4).

The LN probes were labeled with Cy-3 fluorescent dye (Amersham Biosciences) while a pool of brain and spleen RNA from naive animals was labeled with Cy-5. These probes were hybridized onto glass slides containing 18,144 spotted 60- to 70-mer oligonucleotides (from the Operon mouse set; Operon), followed by washing and scanning (University of California, San Francisco microarray core facility). Ten genes showing differential expression at EAE stages were selected for validation by quantitative RT-PCR (qRT-PCR) using SYBR Green (Applied Biosystems) dye. Reactions were conducted in an Applied Biosystems HT-7900 according to the manufacturer's instructions.

Human.

Blood samples from 105 patients and 21 controls were extracted by the same phlebotomist and approximately at the same time of the day. RNA was purified from PBMC using a Versagene kit (Gentra). The RNA concentration was measured by spectrophotometry (Nanodrop Technologies) and equal quantities of RNA were converted to total cDNA using the RT kit (Applied Biosystems). Csn3 and GAPDH were amplified using specific validated primers (codes QT00010577 and QT00079247, Geneglobe; Qiagen). To determine expression values we used the $\Delta\Delta CT$ method (User Bulletin 2; Applied Biosystems). Samples from selected patients were collected at different times to check the stability of the Csn3 expression pattern.

Statistical analysis

A quality control check was performed for each microarray assay based on the diagnostic plots generated by the marrayTools package from the Bioconductor Suite (www.bioconductor.org). We verified that A) the normalized ratio of intensities (M) and the normalized product of intensities (A) for the controls spots (positive and negative) followed the expected distribution; B) the spatial distribution of normalized M and A values for all of the probes in the array was homogeneous; and C) the mean signal:noise ratio for all probes for each fluorescence exceeded a previously set threshold of 1.4.

Arrays were considered of high quality if no more than one of these tests failed. Arrays raw data were imported into BRB-array Tools (Biometric Research Branch, National Institutes of Health, version 3.3 Beta) and filtered by flagged spots. All class comparison (CC) and gene ontology analyses were performed in BRB-array Tools. The significance level for the CC analysis was set at 0.001 for the univariate tests. Permutation based false discovery rate correction was performed for all CC tests. Clustering analyses were performed using GeneLinker Platinum (Improved Outcomes). Gene oncology relation tree and bar analysis were made using a Gene Ontology Tree Machine (45).

Analysis of neural vs immune genes

To establish the origin and function of each of the genes contained in the array, its Unigene record was examined and the source tissues from where the cDNA libraries derived were recorded. A gene was classified as either neural or immune if at least 20% of the source cDNA libraries contained one or more of a list of key words previously defined (see Ref. 4 for details).

Sequencing

A total of 547 bp upstream of the transcriptional start site of the Csn3 gene was sequenced in addition to the complete transcribed sequences from exons 1 and 2 in those patients that presented elevated Csn3 expression and in three controls. The primers used were 1 forward: 5′-CTT GAA ATC CAG GTT CTT AAA C-3′, 1 reverse: 5′-AAA TTT GAA CAT AAC ACC ATC CAG-3′; 2 forward: 5′-TTC ACA TCG GCT AAA TCT AC-3′ and 2 reverse: 5′-TTG CAT AGT TAA AGC CCT TA-3′.

The changes found in these sequences were compared with DNA samples from all MS patients and controls. The deletion upstream of exon 1 was tested by PCR and agarose electrophoresis at 4% and the single nucleotide polymorphism was tested by PCR-RFLP using the restriction enzyme *Cac*8I (New England Biolabs).

Results

The transcriptional and pathological changes detected in the LN of NOD mice during the first 18 days after the injection of either CFA plus MOG-(immunized group) or CFA only (control group) are presented. To detect changes that correlated with neurological disability, we first divided all of the animals in groups showing a similar disease course at the time of sample collection (Table I). This resulted in the following groups: baseline (BL, *t*0), early EAE

Table II. *EAE scores of mice used for RNA expression analysis*[a]

| Animal	Stage					
	EE			PE	ER	LR
	*t*7	*t*8	*t*9	*t*10	*t*11	*t*12
1	0.5	0.5	0.5	3	1	1
2	0.5	0.5	0.5	3	1	0.5
3	0.5	0.5	0.5	3.5	1.5	1.5
4	0.5	0.5	0.5	3	1	0.5
Average	0.5	0.5	0.5	3.125	1.125	0.875

[a] The EAE score at the moment of sacrifice is indicated for each of the four animals at each time point. Only time points where positive scores were observed are reported.

(EE, *t*7–9), peak EAE (PE, *t*10), early recovery (ER, T11), and late recovery (LR, *t*12). A detailed description of EAE scores of each animal used for RNA expression analysis is provided in Table II. To match controls with immunized animals in all subsequent comparisons, we also arranged them into similar groups, based on time after immunization.

Of the 84 immunized mice, 48 were used for tissue harvesting followed by RNA collection at different time points and 36 for tissue harvesting and fixation for immunostaining. With the addition of 24 control animals, and the 4 naive mice, the total number of arrays performed was 76. Of these, 68 arrays passed our quality control protocol (see *Materials and Methods* and Table I). After filtering out low signal:noise spots, we selected 11,913 genes for further analysis. We first compared the expression profiles from LN and spinal cords across all time points from the same animals from our earlier study (4) and selected the 3,000 genes with highest expression difference across tissues for comparative visualization (Fig. 1*A*). The two profiles are clearly distinguishable with clusters of high expression in both spinal cords (identified by open bars to the *right* of the expression heat map in Fig. 1*A*) and LN (Fig. 1*A*, filled bars). We then classified each of the genes encompassed by the bars as either immune, neural, both, or none (see *Materials and Methods* for details). We found that only 2.7% of the selected 3,000 genes with detectable expression in spinal cords were of immune origin, while >58% of these genes with high expression in LN belonged to this category. Although the percentage of immune-related genes expressed in spinal cords was small in comparison to the total number of transcripts, these were almost exclusively present in immunized animals and their expression was not detected until 7 days after immunization. In contrast, although almost 40% of the genes expressed in spinal cords were exclusively of neural origin, only 2.5% of highly expressed genes in the LN belonged to this category (Fig. 1*B*). The most likely explanation for identifying genes of neural origin in the LN is that these transcripts were originally cloned and annotated from brain-derived cDNA libraries and hence detected by our algorithm, thus suggesting pleiotropy Although these findings are not unexpected, the relative contribution of transcripts from each tissue is in agreement with their anatomical origin, thus validating our experimental approach, data handling, and microarray analysis.

When we compared how changes in the number of differentially expressed genes (DEG) in LN and spinal cords of immunized animals evolved over time, we observed almost opposing profiles (Fig. 2*A*). Although the number of DEG in LN decreased sharply, the number of differentially expressed transcripts identified in spinal cords increased almost exponentially within the same time frame. For the remainder of the analysis, we focused on the expression profiles of the LN.
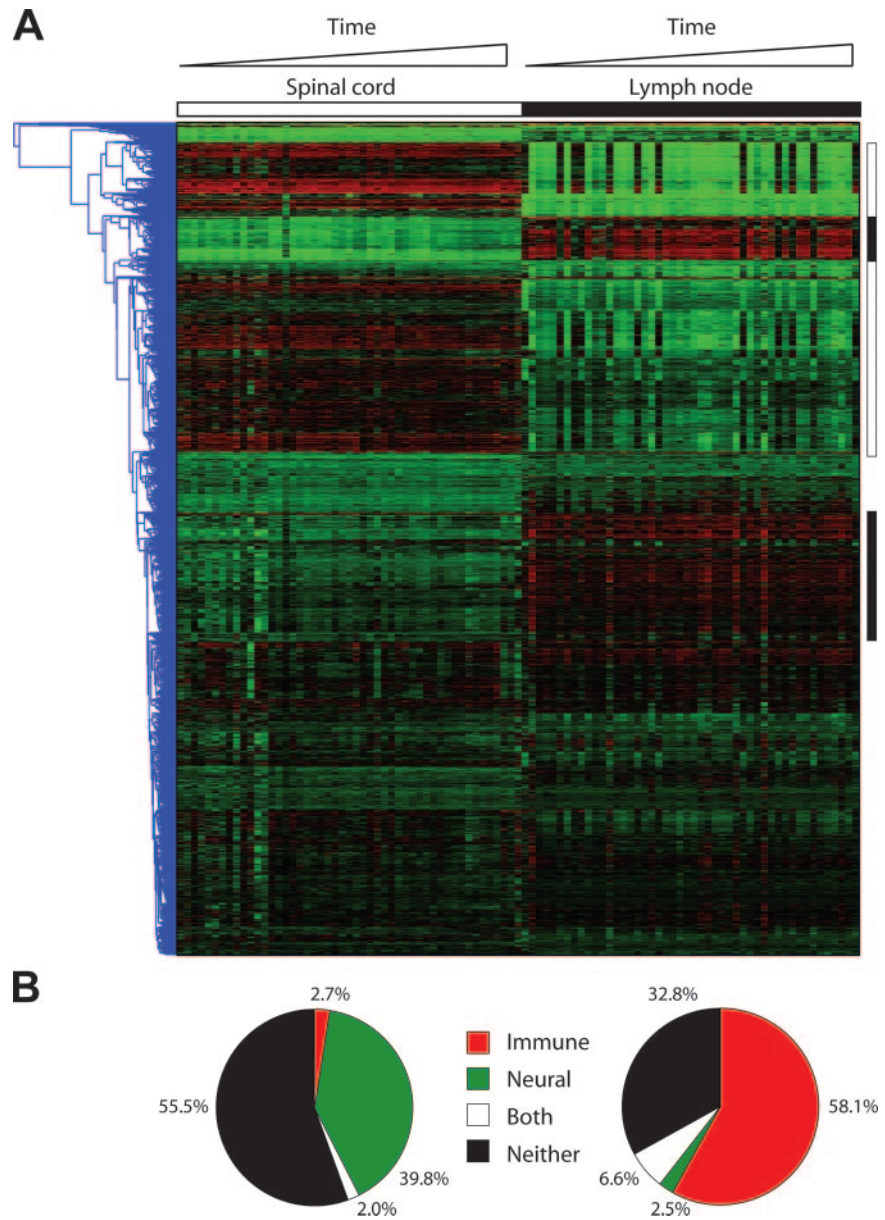
FIGURE 1. Global expression profiles in spinal cords and LN after EAE induction. *A,* Expression of the most variable 3000 genes is shown for both tissues at all time points. Genes were clustered according to their similarity of expression using an Euclidean distance algorithm, but samples were kept in their original positions according to time after immunization. Two clusters of genes with overall elevated expression in one tissue but low expression in the other were identified and identified by vertical bars to the side of the clustergram (open bars, high expression in spinal cords; filled bars, high expression in LN). *B,* The origin of genes in each cluster was analyzed with a custom algorithm (see *Materials and Methods*) that classified transcripts as immune, neural, both, or neither. The proportion of immune and neural genes from the selected clusters, clearly differentiate both tissues.

A longitudinal analysis of global expression differences between immunized and controls revealed 63 DEG. Of interest, many of these genes had been also identified in our previous analysis of the spinal cords such neutrophilic granule protein, myeloperoxidase, and haptoglobin. These genes are commonly expressed by activated neutrophils and are typically associated with acute inflammatory processes (9, 10). We next looked for genes whose expression at each disease stage differed significantly from that observed
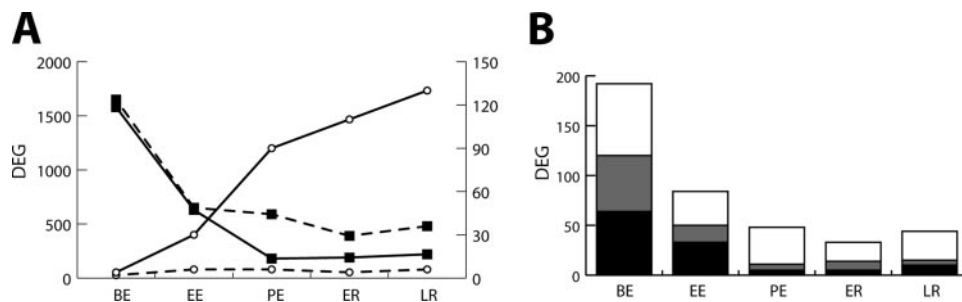
FIGURE 2. Number of DEG in spinal cords and LN after EAE induction. *A,* Although the number of DEG in comparison to baseline increased almost exponentially in spinal cords (●, left axis) of immunized animals (solid lines), transcriptional activity was almost reversed in the LN (■, right axis), with most genes expressed soon after immunization. Note the difference in the magnitude of changes between the two tissues. Adjuvant-injected animals (dashed lines) showed a similar expression to that of immunized animals in the LN only. *B,* A detailed count of DEG in the LN at each EAE stage in response to adjuvant (□), MOG (■) peptide, or both (▦).
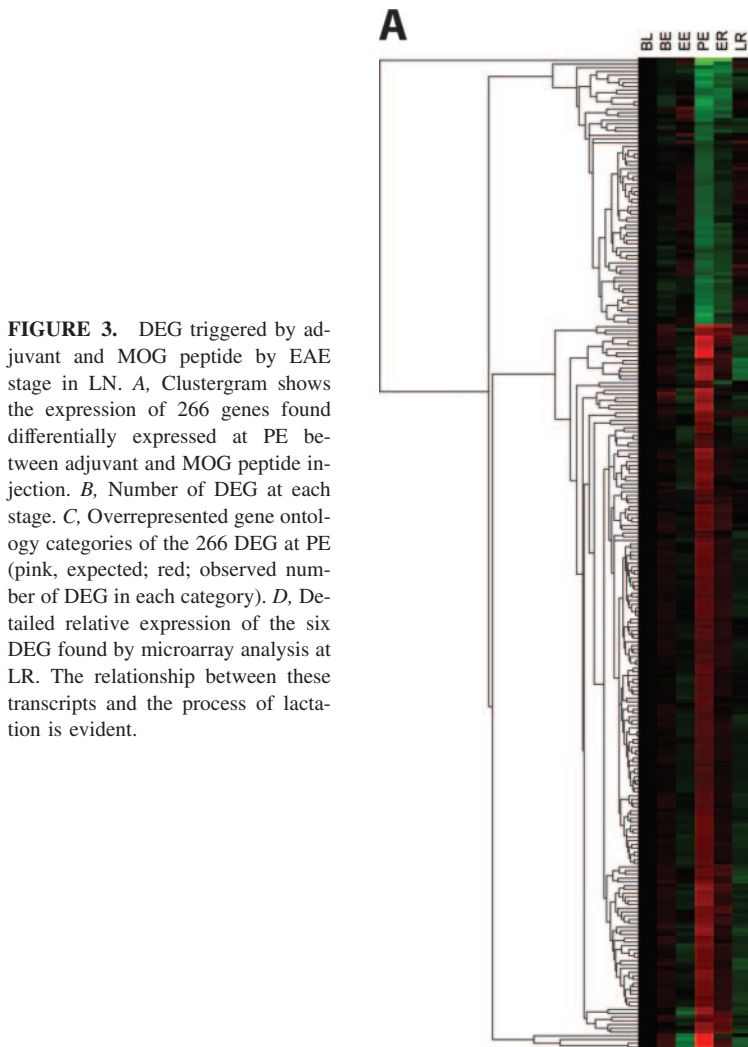
FIGURE 3. DEG triggered by adjuvant and MOG peptide by EAE stage in LN. *A,* Clustergram shows the expression of 266 genes found differentially expressed at PE between adjuvant and MOG peptide injection. *B,* Number of DEG at each stage. *C,* Overrepresented gene ontology categories of the 266 DEG at PE (pink, expected; red; observed number of DEG in each category). *D,* Detailed relative expression of the six DEG found by microarray analysis at LR. The relationship between these transcripts and the process of lactation is evident.

at baseline. We identified 192 such DEG at the BE stage (Fig. 2*B*). Although immunized and control animals shared almost a third of the genes differentially expressed at the BE stage (Fig. 2*B*, ▨), each group also showed a unique set of DEG (controls, □; immunized, ■, Fig. 2*B*). Notably, although the total number of DEG decreased with time, the proportion of those in response to the adjuvant became larger. This observation may reflect the fact that a large proportion of the cells (and their corresponding molecular activity) evidenced in the LN at early stages in immunized mice eventually translocate to the CNS, thus disappearing from these organs. Conversely, that transcriptional activity may persist for a longer time in mice receiving only adjuvant reflecting a transient nonspecific activation of the immune system in those animals.

To identify disease-specific transcriptional signatures in the LN of EAE mice, we conducted cross-sectional comparisons between the immunized and control groups at each disease stage (Fig. 3, *A* and *B*). Interestingly, 279 genes were identified as differentially expressed between PE and *t*10 (controls) stages, but much fewer DEG were identified for any of the other stages (5 for BE, 1 for EE, 1 for ER, and 6 for LR). One possible explanation for this findings is that a common transcriptional response may be triggered by the adjuvant, thus masking any real differences induced by the MOG peptide in LN. In addition, this may be consistent with the fact that, although actively induced EAE is primarily a disease of the CNS, their inflammatory consequences might be reflected in multiple organs, including the LN. A similar study

performed in mice where EAE is induced by adoptive transfer will be needed to firmly establish this hypothesis. Interestingly, a gene ontology analysis of these 279 genes shows transcripts involved in the biosynthesis of macromolecules, cell-cell interaction, and protein synthesis and folding, consistent with the innate immune response triggered by the adjuvant in the LN of control mice.

Although only six DEG were detected between immunized and controls at the LR phase, their characteristics strongly argues against false discoveries. For example, four of these genes belong to the casein family (casein α or Csn1s1, casein β or Csn2, casein κ or Csn3, casein γ or Csn1s2a), while the two remaining genes are lactoalbumin α (Lalba) (another component of milk) and extracellular proteinase inhibitor (Expi), an inducer of mammary involution. Fig. 3*D* shows that the differences in expression between immunized and control mice during recovery are largely significant. Furthermore, real-time PCR confirmed the elevated expression of caseins in LN, whereas no differences were detected in the spleens from the same mice, where the expression was minimal (data not shown). The expression of casein genes was undetectable in spinal cords.

To test whether these findings were also evident at the protein level, we examined the expression of one of the members of the casein family (Csn2) by immunohistochemistry in the LN of mice taken at different times after immunization. A strong staining was
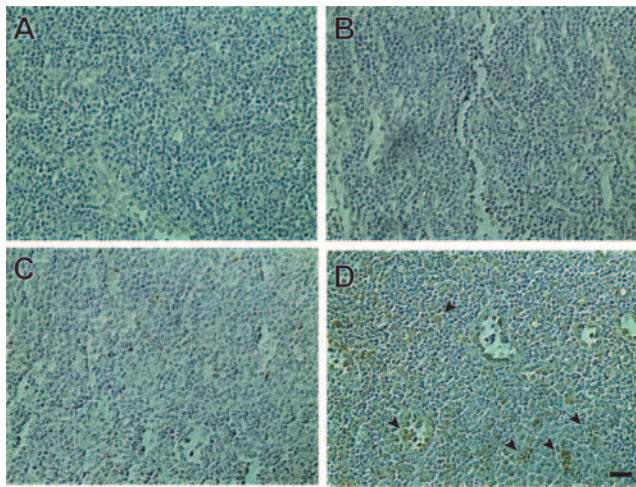
FIGURE 4. Immunohistochemistry of Csn2. The expression of Csn2 was analyzed at BL (*A*), *t*2 (*B*), *t*11 (*C*), and *t*12 (*D*) at ×40 magnification. Visible Csn2 expression was only evident at T12 (arrowheads). Bar, 20 μm.

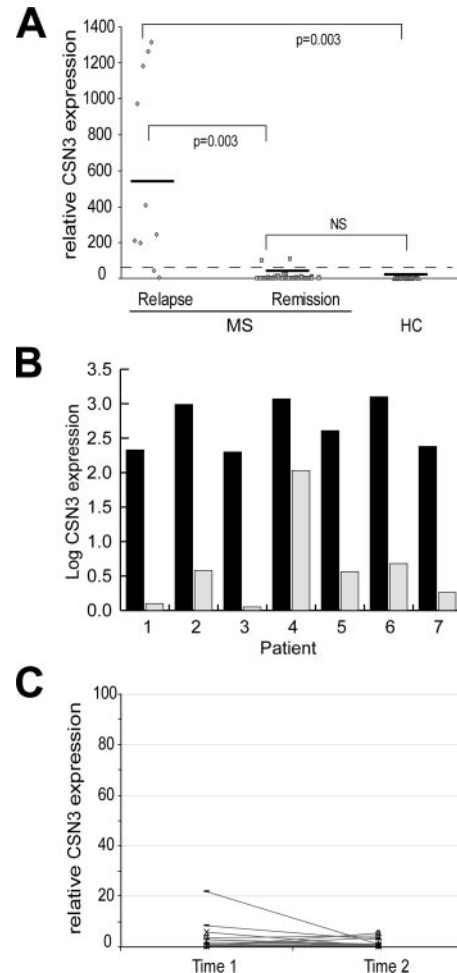observed only at t12, corresponding to the LR phase of the disease (Fig. 4).

The elevated expression of genes coding for caseins prompted us to investigate their expression in humans suffering from MS. If a transcriptional increase of caseins was indeed correlated to an attack, patients with relapsing remitting multiple sclerosis (RRMS) would also be expected to show an elevated expression shortly after a relapse. Although we initially analyzed murine LN, when testing humans we decided to collect and analyze whole blood for practical reasons. We thus analyzed the expression of CSN1S1 and CSN3 in the blood of 112 MS patients (3 secondary progressive MS, secondary progressive multiple sclerosis (SPMS), 109 RRMS) and 21 healthy controls by qRT-PCR (see Table III for clinical details). Although the expression of CSN1S1 was not detectable in any of the control samples, an elevated expression was identified in 10 of 112 MS samples ($p < 0.003$, *t* test; Fig. 5). Interestingly, six of these samples were obtained from RRMS patients shortly after a clinical relapse. Of the remaining four samples with elevated CSN1S1, two were from SPMS patients, and two from RRMS with no apparent clinical exacerbations.

To exclude the possibility that the observed elevated CSN3 expression in some patients was constitutive, we tested its expression at a later time, once the relapse had subsided. In all patients tested, the CSN3 expression levels at the time of the relapse differ markedly from those measured during remission (Fig. 5*B*). In contrast, samples taken from 17 patients at two different times during remission did not differ significantly (Fig. 5*C*). These results suggest that the observed differences in CSN1S1 expression are not constitutive. However, to rule out the existence of DNA polymorphisms near the promoter region that could affect the expression, we sequenced 547 bases up-

FIGURE 5. Expression of CSN3 in humans. The expression of CSN3 was assessed by qRT-PCR in MS patients and healthy controls (HC). *A,* Patients who were experiencing a relapse or worsening of their symptoms had increased expression of CSN3 when compared with patients in remission or healthy controls. Horizontal bars, The mean for each group. *B,* Patients with elevated CSN3 within 72 h of a relapse (■) showed reduced levels when tested at the time of remission (▨) (data was log transformed for visualization). *C,* The expression of CSN3 in patients during remission is stable over time.

stream of the transcriptional start site. We identified a 195-bp deletion at position −309 and a single nucleotide substitution at position +37 relative to the transcriptional start site, neither of which had been previously reported, that appear to be polymorphic in the population studied. Although our sample size clearly did not provide sufficient statistical power, the allelic frequencies of either polymorphism did not differ significantly between MS and controls (Table IV). Altogether, our data suggest that the elevated CSN1S1 expression is due to regulatory rather than constitutive expression.

Table III. *Clinical characteristics of MS patients[a]*

	Relapse	Remission
EDSS	3.21 (1.95)	3.92 (2.60)
MSSS	4.37 (2.84)	4.67 (3.08)
Duration (days)	6.78 (6.40)	11.20 (8.71)
Age (years)	33.33 (6.14)	43.36 (12.35)
Female (%)	66.67	63.3

[a] Average and SD (in parentheses) of each parameter is shown.

Table IV. *Genotyping of single nucleotide polymorphism +37 in human samples[a]*

	G/G	G/A	A/A
MS	1.1	20.2	78.7
Controls	2.2	20.4	77.4

[a] The percent frequency of each genotype is indicated for cases and controls.

Discussion

We have recently reported the transcriptional analysis of early stages of MOG-induced EAE in spinal cords from NOD mice (4). We have now analyzed the RNA profiles of LN collected from the same group of animals. Contrary to what was observed in spinal cords, a relatively limited number of genes in the LN changed their expression pattern over the course of the disease when compared against baseline animals (Fig. 2A). We interpret that the larger number of DEG in the spinal cord was largely an effect of the immune cell infiltration that accompanies EAE progression. In this scenario, transcripts carried inside immune cells across the blood-brain barrier would appear as differentially expressed in the CNS simply because they were not expressed at earlier time points. In contrast, no foreign cells are expected to traverse into the LN during an immune response. Thus, although variability in the composition of the cells inside the LN may occur, detectable differences in gene expression are most likely representative of regulatory processes at play during this period.

During the first days of the immune response, a common pattern of expression was observed in the LN of both immunized and adjuvant-injected (control) mice. Neutrophilic granule protein, Ltf, and myeloperoxidase were among the most prominent transcripts in that pattern showing elevated expression as early as 3 dpi, suggesting the presence of activated neutrophils in both groups of animals. Neutrophils are one of the earliest cells of the innate immune system in arriving at sites of inflammation, playing an important role in migration across the endothelium (11). Interestingly, we found a similar profile in spinal cords (4). We interpret these findings as a shared reaction to the adjuvant, mostly driven by the innate immune response.

When we compared the number of genes differentially expressed at each disease stage with respect to baseline in both immunized and control mice, we observed different profiles. Although the number of DEG waned for both groups shortly after injection, MOG-immunized animals showed a more abrupt decay when compared with controls (Fig. 2B). A third group of genes, those differentially expressed in both immunized and controls, also declined over time, suggesting that most of the transcriptional activity in the LN occurs early after immunization and then quickly returns to baseline levels. Some of these genes include the S100 calcium-binding proteins A8 and A9 (S100 A8 and S100 S9), matrix metalloproteinase 8, and several members of the small inducible cytokine family. Many of these genes have been categorized as immune related in an earlier study based on their expression profiles in various tissues and on the presence of common upstream regulatory elements (12).

Although analyzing the expression profiles of immunized vs control mice at each disease stage, we observed that most of the genes with statistically significant expression differences were detected only at the peak of the disease (PE) (Fig. 3B). Interestingly, several genes with reduced expression in the LN of immunized animals appear highly expressed in their spinal cords (4). Because most of the genes up-regulated in spinal cords were of immune origin, it is likely that these reciprocal profiles reflect migration of lymphocytes and macrophages from the periphery to the CNS. A detailed analysis of these transcripts reveled genes of immune nature (several CD Ags, lymphocyte markers, interleukins, etc.), thus supporting our hypothesis.

The six genes differentially expressed between immunized and control mice at the LR stage represent an interesting group, because all of them are related to milk production or mammary gland development. This is relevant to MS pathogenesis in light of the numerous hypotheses relating milk consumption and susceptibility
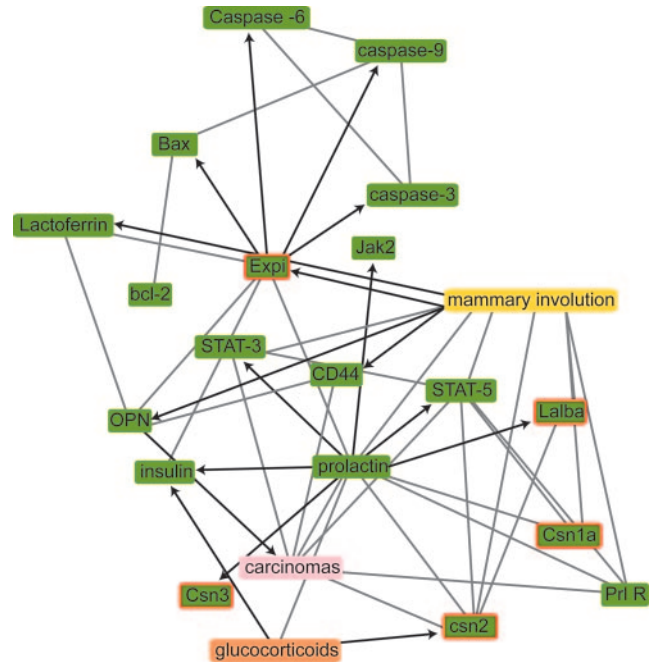
FIGURE 6. Network of genes and processes regulating the synthesis of caseins. During lactation, Prl binds to its receptor in mammary cells and stimulates the expression of Lalba and casein genes through the phosphorylation of STAT 3 and STAT5. After lactation, a number of genes are activated that result in mammary involution. These genes include Expi, OPN, lactoferrin, and several components of the apoptotic cascade of proteases. Genes are indicated in green, drugs in orange, processes in pink, and tissues in yellow. Black arrows indicate known interactions. Gray lines depict co-occurrence of terms in curated PubMed abstracts. The network was created using Ali Baba, an automated scientific text parser tool (46).

to this disease (13–22). Casein α (Csn1s1), casein β (Csn2), casein κ (Csn3), casein γ (Csn1s2a), Lalba, and extracellular proteinase inhibitor (Expi) were strongly up-regulated, particularly at the recovery stage in immunized mice (Fig. 3D). Caseins represent ~80% of total protein in bovine milk, ~30–35% of which is β-casein (23). One variant, A1 β-casein, has been implicated as a potential etiological factor in type 1 diabetes mellitus (24), ischemic heart disease (25), and even autism (26). Casein genes localize to a cluster in chromosome 5 in mice and in chromosome 4 (q21.1) in humans. Interestingly, osteopontin (OPN or SPP1), an inflammatory cytokine previously implicated in the pathogenesis and susceptibility to EAE and MS, also localizes to this cluster (27–29). The four caseins are evolutionary conserved and like OPN belong to the group of proteins known as "disordered," a term referring to their undefined folding pattern which usually correlates with multifunctionality (30, 31). Moreover, Csn2 and Csn3 share a common regulatory element that binds STAT5 and are activated by prolactin through JAK2 signaling, NF-κB, and TNF-α. This pathway has been found to be critical in the activation of CNS microglia by IL-3 (32).

It has been shown that caseins or its derivatives have a strong immunosuppressive effect both in vivo and in vitro (33–35). Although these experiments suggest that caseins may suppress activation of immune cells, the exact mechanism of action of this process is currently unknown. In contrast, caseins were also found to be potent immunoenhancers in vitro (36) and that T cell responses in casein-stimulated PBMC from MS and diabetes patients were higher than in healthy controls (15). These apparently conflicting roles of caseins, as inhibitors and activators or the immune

response, are intriguing and warrant further investigation. However, it is possible that, like many other molecules involved in immune responses and development, they exhibit both effects depending on the context and/or their concentration.

To better understand a pathological process that results from the interaction of multiple genes and proteins through many levels of organization, it might be useful to attempt a description through their integration into a wider network of related molecules and processes (Fig. 6). After completion of lactation, mammary glands undergo involution during the weaning period, regressing to a state resembling that of a virgin animal. This phase is characterized by dramatic epithelial cell death and tissue remodeling, a process inhibited by epidermal growth factor (37). During this stage of involution, high expression levels of Ltf and Expi have been reported in mammary-derived HC11 cells (38). It has been also shown that elevated levels of Expi trigger apoptosis through a BAFF-mediated mechanism (39). This could also be the mechanism by which Expi induces mammary involution and, in addition, to inhibiting the action of proteases also inhibits tumor metastasis (39). The proinflammatory molecule OPN and its receptor (CD44) are also highly expressed during mammary involution and are inhibited during lactation. Thus, the expression of Expi during EAE recovery may represent the net result of the interaction of a network of molecules that also includes Prl, caseins, Ltf, and epidermal growth factor, acting in autocrine and paracrine fashion in response to the inflammatory process. Remission of MS during pregnancy led to the hypothesis that remyelination might be enhanced in the maternal brain. In an experiment that supports this hypothesis, an increase in the number of oligodendrocytes and of myelinated axons has been described in pregnant mice (40). Furthermore, the authors found that the hormone Prl regulates oligodendrocyte precursor proliferation, thus mimicking the regenerative effects of pregnancy. This suggests that the maternal CNS has the ability to repair demyelination, at least in part, through the local action of Prl, a main regulator of caseins.

In another set of experiments, it has been recently shown that butyrophilin (BTN), another milk protein, is capable of inducing EAE by virtue of its structural similarity ("molecular mimicry") with MOG (13, 41). Interestingly, BTN elicits both T and B cell responses, but pretreatment of experimental animals with BTN can also ameliorate or even suppress EAE in a mechanism mediated by anergic and regulatory T cells (16). Although the influence of milk-derived proteins like caseins and BTN on EAE is intriguing, additional experiments are needed to firmly establish their role in the disease.

The analysis of CSN3 expression in the blood of MS patients showed elevated levels during resolution of a relapse. Because elevated expression of caseins was also consistently observed shortly after the peak of EAE, where residual disease was still evident (Table II), we hypothesize that they could play a protective role in the disease. These elevated levels of CSN3 could not be explained by DNA polymorphisms within the gene. In two patients, the expression of CSN3 was slightly elevated without a relapse being reported. Interestingly, one of these individuals had SPMS with an aggressive evolution (Expanded Disability Status Scale of 8); therefore, we reasoned that a relapse could have been masked by the patient's clinical condition. Although the other individual with elevated CSN3 had RRMS, he/she did not report any exacerbation, it is possible that this is due to subclinical disease activity. In contrast, in two individuals reporting a relapse, CSN3 levels were normal. Interestingly, both of these patients experienced a sensorial relapse, suggesting that these type of relapses may involve a different mechanism. Although due to the limited size of our dataset we cannot draw any definite conclusions on the

role of caseins in MS, the present results warrant further investigation.

In summary, the longitudinal transcriptional analysis of LN during EAE allowed us to characterize the molecular response underlying the pathology. We uncovered the molecular signature of the innate immune response during the first days of the disease and identified several genes involved in milk production as potentially important players in the development and regulation of autoimmunity. Our data could open important venues to firmly establish the role of these genes in EAE and MS.

Acknowledgments

Disclosures

The authors have no financial conflict of interest.

References

1. Steinman, L., and S. S. Zamvil. 2006. How to successfully apply animal studies in experimental allergic encephalomyelitis to research on multiple sclerosis. *Ann. Neurol.* 60: 12–21.
2. Bernard, C. C., T. G. Johns, A. Slavin, M. Ichikawa, C. Ewing, J. Liu, and J. Bettadapura. 1997. Myelin oligodendrocyte glycoprotein: a novel candidate autoantigen in multiple sclerosis. *J. Mol. Med.* 75: 77–88.
3. Hugues, S., L. Fetler, L. Bonifaz, J. Helft, F. Amblard, and S. Amigorena. 2004. Distinct T cell dynamics in lymph nodes during the induction of tolerance and immunity. *Nat. Immunol.* 5: 1235–1242.
4. Baranzini, S. E., C. C. Bernard, and J. R. Oksenberg. 2005. Modular transcriptional activity characterizes the initiation and progression of autoimmune encephalomyelitis. *J. Immunol.* 174: 7412–7422.
5. Mix, E., S. Ibrahim, J. Pahnke, D. Koczan, C. Sina, T. Bottcher, H. J. Thiesen, and A. Rolfs. 2004. Gene-expression profiling of the early stages of MOG-induced EAE proves EAE-resistance as an active process. *J. Neuroimmunol.* 151: 158–170.
6. Matejuk, A., C. Hopke, J. Dwyer, S. Subramanian, R. E. Jones, D. N. Bourdette, A. A. Vandenbark, and H. Offner. 2003. CNS gene expression pattern associated with spontaneous experimental autoimmune encephalomyelitis. *J. Neurosci. Res.* 73: 667–678.
7. Whitney, L. W., S. K. Ludwin, H. F. McFarland, and W. E. Biddison. 2001. Microarray analysis of gene expression in multiple sclerosis and EAE identifies 5-lipoxygenase as a component of inflammatory lesions. *J. Neuroimmunol.* 121: 40–48.
8. MacKenzie-Graham, A. J., T. M. Pribyl, S. Kim, V. R. Porter, A. T. Campagnoni, and R. R. Voskuhl. 1997. Myelin protein expression is increased in lymph nodes of mice with relapsing experimental autoimmune encephalomyelitis. *J. Immunol.* 159: 4602–4610.
9. Theilgaard-Monch, K., L. C. Jacobsen, M. J. Nielsen, T. Rasmussen, L. Udby, M. Gharib, P. D. Arkwright, A. F. Gombart, J. Calafat, S. K. Moestrup, B. T. Porse, and N. Borregaard. 2006. Haptoglobin is synthesized during granulocyte differentiation, stored in specific granules, and released by neutrophils in response to activation. *Blood* 108: 353–361.
10. Hansson, M., I. Olsson, and W. M. Nauseef. 2006. Biosynthesis, processing, and sorting of human myeloperoxidase. *Arch. Biochem. Biophys.* 445: 214–224.
11. Borregaard, N., and J. B. Cowland. 1997. Granules of the human neutrophilic polymorphonuclear leukocyte. *Blood* 89: 3503–3521.
12. Hutton, J. J., A. G. Jegga, S. Kong, A. Gupta, C. Ebert, S. Williams, J. D. Katz, and B. J. Aronow. 2004. Microarray and comparative genomics-based identification of genes and gene regulatory regions of the mouse immune system. *BMC Genomics* 5: 82.
13. Guggenmos, J., A. S. Schubart, S. Ogg, M. Andersson, T. Olsson, I. H. Mather, and C. Linington. 2004. Antibody cross-reactivity between myelin oligodendrocyte glycoprotein and the milk protein butyrophilin in multiple sclerosis. *J. Immunol.* 172: 661–668.
14. Monetini, L., M. G. Cavallo, S. Manfrini, L. Stefanini, A. Picarelli, M. Di Tola, A. Petrone, M. Bianchi, M. La Presa, C. Di Giulio, et al. 2002. Antibodies to bovine β-casein in diabetes and other autoimmune diseases. *Horm. Metab. Res.* 34: 455–459.
15. Winer, S., I. Astsaturov, R. K. Cheung, K. Schrade, L. Gunaratnam, D. D. Wood, M. A. Moscarello, P. O'Connor, C. McKerlie, D. J. Becker, and H. M. Dosch. 2001. T cells of multiple sclerosis patients target a common environmental peptide that causes encephalitis in mice. *J. Immunol.* 166: 4751–4756.
16. Stefferl, A., A. Schubart, M. Storch, A. Amini, I. Mather, H. Lassmann, and C. Linington. 2000. Butyrophilin, a milk protein, modulates the encephalitogenic T cell response to myelin oligodendrocyte glycoprotein in experimental autoimmune encephalomyelitis. *J. Immunol.* 165: 2859–2865.
17. Malosse, D., H. Perron, A. Sasco, and J. M. Seigneurin. 1992. Correlation between milk and dairy product consumption and multiple sclerosis prevalence: a worldwide study. *Neuroepidemiology* 11: 304–312.
18. Butcher, P. J. 1986. Milk consumption and multiple sclerosis–an etiological hypothesis. *Med. Hypotheses* 19: 169–178.

19. Verdy, M. 1977. Multiple sclerosis and milk. *Can. Med. Assoc. J.* 117: 27.

20. Butcher, J. 1976. The distribution of multiple sclerosis in relation to the dairy industry and milk consumption. *N. Z. Med. J.* 83: 427–430.

21. Christensen, J. C. 1975. Multiple sclerosis: some epidemiological clues to etiology. *Acta Neurol. Latinoam.* 21: 66–85.

22. Agranoff, B. W., and D. Goldberg. 1974. Diet and the geographical distribution of multiple sclerosis. *Lancet* 2: 1061–1066.

23. Cavallo, M. G., D. Fava, L. Monetini, F. Barone, and P. Pozzilli. 1996. Cell-mediated immune response to β casein in recent-onset insulin-dependent diabetes: implications for disease pathogenesis. *Lancet* 348: 926–928.

24. Pozzilli, P. 1999. β-casein in cow's milk: a major antigenic determinant for type 1 diabetes? *J. Endocrinol. Invest.* 22: 562–567.

25. Tailford, K. A., C. L. Berry, A. C. Thomas, and J. H. Campbell. 2003. A casein variant in cow's milk is atherogenic. *Atherosclerosis* 170: 13–19.

26. Knivsberg, A. M., K. L. Reichelt, T. Hoien, and M. Nodland. 2002. A randomised, controlled study of dietary intervention in autistic syndromes. *Nutr. Neurosci.* 5: 251–261.

27. Chabas, D., S. E. Baranzini, D. Mitchell, C. C. Bernard, S. R. Rittling, D. T. Denhardt, R. A. Sobel, C. Lock, M. Karpuj, R. Pedotti, et al. 2001. The influence of the proinflammatory cytokine, osteopontin, on autoimmune demyelinating disease. *Science* 294: 1731–1735.

28. Sinclair, C., M. Mirakhur, J. Kirk, M. Farrell, and S. McQuaid. 2005. Up-regulation of osteopontin and αβ-crystallin in the normal-appearing white matter of multiple sclerosis: an immunohistochemical study utilizing tissue microarrays. *Neuropathol. Appl. Neurobiol.* 31: 292–303.

29. Chiocchetti, A., C. Comi, M. Indelicato, L. Castelli, R. Mesturini, T. Bensi, M. C. Mazzarino, M. Giordano, S. D'Alfonso, P. Momigliano-Richiardi, et al. 2005. Osteopontin gene haplotypes correlate with multiple sclerosis development and progression. *J. Neuroimmunol.* 163: 172–178.

30. Huq, N. L., K. J. Cross, M. Ung, and E. C. Reynolds. 2005. A review of protein structure and gene organisation for proteins associated with mineralised tissue and calcium phosphate stabilisation encoded on human chromosome 4. *Arch. Oral. Biol.* 50: 599–609.

31. Gerencser, A., E. Barta, S. Boa, P. Kastanis, Z. Bosze, and C. B. Whitelaw. 2002. Comparative analysis on the structural features of the 5′ flanking region of κ-casein genes from six different species. *Genet. Sel. Evol.* 34: 117–128.

32. Natarajan, C., S. Sriram, G. Muthian, and J. J. Bright. 2004. Signaling through JAK2-STAT5 pathway is essential for IL-3-induced activation of microglia. *Glia* 45: 188–196.

33. Daddaoua, A., V. Puerta, A. Zarzuelo, M. D. Suarez, F. Sanchez de Medina, and O. Martinez-Augustin. 2005. Bovine glycomacropeptide is anti-inflammatory in rats with hapten-induced colitis. *J. Nutr.* 135: 1164–1170.

34. Otani, H., T. Watanabe, and Y. Tashiro. 2001. Effects of bovine β-casein (1–28) and its chemically synthesized partial fragments on proliferative responses and immunoglobulin production in mouse spleen cell cultures. *Biosci. Biotechnol. Biochem.* 65: 2489–2495.

35. Monnai, M., and H. Otani. 1997. Effect of bovine κ-caseinoglycopeptide on secretion of interleukin-1 family cytokines by P3888D1 cells, a line derived from mouse monocyte/macrophage. *Milchwissenschaft* 52: 192–196.

36. Li, E. W., and Y. Mine. 2004. Immunoenhancing effects of bovine glycomacropeptide and its derivatives on the proliferative response and phagocytic activities of human macrophagelike cells, U937. *J. Agric. Food Chem.* 52: 2704–2708.

37. Lee, M., I. Hwang, Y. Choi, S. Paik, H. B. Lee, and M. Baik. 1997. EGF inhibits expression of WDNM1 and sulfated glycoprotein-2 genes in mammary epithelial cells. *Biochem. Biophys. Res. Commun.* 240: 104–107.

38. Baik, M. G., M. J. Lee, and Y. J. Choi. 1998. Gene expression during involution of mammary gland (Review). *Int. J. Mol. Med.* 2: 39–44.

39. Jung, D. J., J. J. Bong, and M. Baik. 2004. Extracellular proteinase inhibitor-accelerated apoptosis is associated with B cell activating factor in mammary epithelial cells. *Exp. Cell Res.* 292: 115–122.

40. Gregg, C., V. Shikar, P. Larsen, G. Mak, A. Chojnacki, V. W. Yong, and S. Weiss. 2007. White matter plasticity and enhanced remyelination in the maternal CNS. *J. Neurosci.* 27: 1812–1823.

41. Mana, P., M. Goodyear, C. Bernard, R. Tomioka, M. Freire-Garabal, and D. Linares. 2004. Tolerance induction by molecular mimicry: prevention and suppression of experimental autoimmune encephalomyelitis with the milk protein butyrophilin. *Int. Immunol.* 16: 489–499.

42. McDonald, W. I., A. Compston, G. Edan, D. Goodkin, H. P. Hartung, F. D. Lublin, H. F. McFarland, D. W. Paty, C. H. Polman, S. C. Reingold, et al. 2001. Recommended diagnostic criteria for multiple sclerosis: guidelines from the International Panel on the diagnosis of multiple sclerosis. *Ann. Neurol.* 50: 121–127.

43. Kurtzke, J. F. 1983. Rating neurologic impairment in multiple sclerosis: an expanded disability status scale (EDSS). *Neurology* 33: 1444–1452.

44. Roxburgh, R. H., S. R. Seaman, T. Masterman, A. E. Hensiek, S. J. Sawcer, S. Vukusic, I. Achiti, C. Confavreux, M. Coustans, E. le Page, et al. 2005. Multiple Sclerosis Severity Score: using disability and disease duration to rate disease severity. *Neurology* 64: 1144–1151.

45. Zhang, B., D. Schmoyer, S. Kirov, and J. Snoddy. 2004. GOTree Machine (GOTM): a web-based platform for interpreting sets of interesting genes using gene ontology hierarchies. *BMC Bioinformatics* 5: 16.

46. Plake, C., T. Schiemann, M. Pankalla, J. Hakenberg, and U. Leser. 2006. AliBaba: PubMed as a graph. *Bioinformatics* 22: 2444–2445.