B Lymphocytes Producing Demyelinating Autoantibodies: Development and Function in Gene-targeted Transgenic Mice

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Summary

We studied the cellular basis of self tolerance of B cells specific for brain autoantigens using transgenic mice engineered to produce high titers of autoantibodies against the myelin oligodendrocyte glycoprotein (MOG), a surface component of central nervous system myelin. We generated "knock-in" mice by replacing the germline J_H locus with the rearranged immunoglobulin (Ig) H chain variable (V) gene of a pathogenic MOG-specific monoclonal antibody. In the transgenic mice, conventional B cells reach normal numbers in bone marrow and periphery and express exclusively transgenic H chains, resulting in high titers of MOG-specific serum Igs. Additionally, about one third of transgenic B cells bind MOG, thus demonstrating the absence of active tolerization. Furthermore, peritoneal B-1 lymphocytes are strongly depleted. Upon immunization with MOG, the mature transgenic B cell population undergoes normal differentiation to plasma cells secreting MOG-specific IgG antibodies, during which both Ig isotype switching and somatic mutation occur. In naive transgenic mice, the presence of this substantial autoreactive B cell population is benign, and the mice fail to develop either spontaneous neurological disease or pathological evidence of demyelination. However, the presence of the transgene both accelerates and exacerbates experimental autoimmune encephalitis, irrespective of the identity of the initial autoimmune insult.

Key words: B cell tolerance • gene targeting • autoantibodies • experimental autoimmune encephalomyelitis • myelin oligodendrocyte glycoprotein

Self tolerance within the B cell compartment is organized in a highly complex fashion. On the one hand, it is known that the normal B cell repertoire includes a large number of self-reactive clones, most of which are $CD5^+$ polyreactive B cells of the B-1 compartment (1, 2). In contrast, in the conventional B-2 B cell subset, autoreactive clones capable of initiating pathological Ab responses are either removed via deletion (3–5), silenced by mechanisms of anergy (6, 7), or defused by receptor editing (8–10).

How can the immune system discriminate between harmless self-reactive B cell clones and those with a pathogenic potential? This question is of paramount importance, as several human diseases are caused by pathogenic B cell responses that bypass these tolerogenic mechanisms. Examples include autoimmune hemolytic anemias (11), Graves' disease (12), skin-blistering diseases (13), myasthenia gravis (14), and Rasmussen's encephalitis (15). Autoantibody responses are also implicated in the pathogenesis of Guillain-Barré syndrome, motor neuropathies, and multiple sclerosis (16).

In this study, we have used MOG[1] as a model central nervous system (CNS) autoantigen to investigate the mechanisms that normally confer B cell tolerance to antigens sequestered within the CNS compartment. MOG is a minor component of CNS myelin that is expressed on the surface of myelin exclusively in the CNS (17). We have used a knock-in mutant mouse model to study the development and control of conditionally pathogenic B lymphocytes in vivo. We replaced in embryonic stem (ES) cells the genomic region containing the J_H segments and the DQ52 el-

[1] *Abbreviations used in this paper:* AP, alkaline phosphatase; BBB, blood–brain barrier; CNS, central nervous system; EAE, experimental autoimmune encephalomyelitis; ES, embryonic stem; MOG, myelin oligodendrocyte glycoprotein; PLP, proteolipid protein; r, recombinant; R/S ratio, ratio of replacement to silent mutations.

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ement with the rearranged VDJ gene of the H chain from the MOG-specific hybridoma 8.18-C5 (18). This strategy, rather than the construction of conventional transgenic mice, was chosen to allow study of all aspects of B cell differentiation, in particular H chain isotype switch as well as tolerizing events within the natural context of the Ig H genes, such as V gene editing (19, 20) and the different tolerogenic potential of different Ig isotypes (21).

This mouse model offers several advantageous features. In these gene-targeted mice, almost all B cells express transgenic Ig H chains exclusively, as identified using both allotypic and idiotypic markers. The transgenic H chains associate with endogenous Ig L chains to generate large populations of MOG-reactive B cells in both the bone marrow and periphery, accounting for ~30% of total B cells. As a consequence, high titers of MOG-specific Igs are found in the serum of naive knock-in mice but not in naive nontransgenic littermates. Finally, the transgenic B cells are conditionally pathogenic: the mutant mice do not spontaneously develop any neurological deficits. However, when challenged with encephalitogenic antigens or T cells, the autoimmune potential of B cells in the transgenic mice unfolds to increase incidence, severity, and accelerated disease onset of experimental autoimmune encephalomyelitis (EAE).

Materials and Methods

Construction of the Targeting Vector. A 1.7-kb genomic region containing a rearranged V_HDJ_H gene (designated Th gene) was subcloned from hybridoma clone 8.18-C5 (18) and ligated to a PGK-neo gene in opposite transcriptional orientation. The Th gene spanning from an EcoRI site 1.1 kb upstream of the ATG codon to a BamHI site 160 bp downstream of J_H2 had been tested previously for functional expression by transfection into the myeloma line X63-Ag8.6.5.3 (not shown). 5′ and 3′ homologous sequences were derived from a cosmid clone isolated from an ES cell genomic library (a generous gift of S. Mudgett and R. Jaenisch, Whitehead Institute for Bio medical Research, Department of Biology, Cambridge, MA). The cosmid was reduced in size so that the J_H cluster was flanked by a 12-kb region upstream and a 1-kb sequence downstream of J_{H4} including the H chain enhancer. The $DQ52/J_{H1-4}$ region was then deleted and replaced by the neo-Th gene cassette. The final targeting construct (see Fig. 1) was linearized at a unique NotI site and used for transfection.

Homologous Recombination in ES Cells. ES cells of the R1 line (22) were transfected with the NotI-linearized targeting vector and selected with G418 as described (23). Southern blot analysis was performed with SacI-digested ES cell DNA and hybridized to a 0.8-kb ^{32}P-labeled external probe (see Fig. 1, probe $0.8E_H$). We obtained 4 correctly targeted clones out of 320 G418-resistant ES cell clones and confirmed the mutation by sequencing of PCR-amplified DNA. Two of these positive clones were injected into C57Bl/6 blastocysts and reimplanted into pseudopregnant hosts. Both clones yielded germline-transmitting chimeras. These chimeric mice were crossed with C57Bl/6, and agouti offspring were screened for the Th mutation by Southern blot as above.

Flow Cytometry of Lymphocytes. Single cell suspensions were prepared from spleen, LNs, peritoneal cells, and bone marrow from 6–8-wk-old mice and processed. Red blood cells were lysed by incubation in 0.165 M NH_4Cl for 10 min. Cells were washed with PBS/1% FCS and stained with the following Ab conjugates: anti–IgM^a-FITC (clone DS-1; PharMingen, San Diego, CA); anti–IgM^b-BIOTIN (clone AF6-78; PharMingen); anti–CD43-FITC (clone S7; PharMingen); anti–B220-PE (PharMingen); anti–CD5-FITC (clone 53-7.3; PharMingen); anti-IgD (Nordic Immunology Labs, Tilburg, The Netherlands); and 8.18-C5 idiotype–specific Ab (24). Biotinamidocaproate-*N*-hydroxysuccinimide ester (Sigma Chemical Co., Munich, Germany) was used for biotin labeling of recombinant truncated rat MOG (25), which was applied in a 1:500 dilution for staining. Biotin conjugates were developed with streptavidin-PE (Becton Dickinson, San Jose, CA), and the 8.18-C5 idiotype–specific Ab was detected with Cy3-labeled anti–rabbit Ig Ab (Jackson ImmunoResearch Labs, West Grove, PA). After excluding dead cells by staining with propidium iodide, cells were analyzed with a FACScan® (Becton Dickinson).

Serum ELISA and Western Blot Analysis. Peripheral blood of mice was taken by tail bleeding, and after coagulation at 4°C, serum was obtained by centrifugation. For ELISA, 96-well vinyl assay plates (Costar Corp., Cambridge, MA) were coated with one of the following reagents at a concentration of 10 μg/ml in PBS/0.02% NaN_3: goat anti–mouse IgM (Southern Biotechnology Associates, Inc., Birmingham, AL); recombinant MOG (rMOG); and synthetic peptides spanning the extracellular domain of MOG. The expression and purification of rat rMOG were described previously as well as the composition of the synthetic MOG-peptides (25, 26). After blocking with 1% BSA, the assay plates were incubated with serial dilutions of mouse serum. Specific binding was detected with alkaline phosphatase (AP)-conjugated goat anti–mouse IgG (Southern Biotechnology Associates, Inc.), biotinylated anti-IgM^a and anti-IgM^b (PharMingen), and streptavidin-coupled AP (Amersham International, Buckinghamshire, UK) as a secondary reagent. *p*-Nitrophenyl phosphate (Sigma Chemical Co.) was used as a substrate for the AP-catalyzed reaction. The absorbance at 405 nm was read on an ELISA reader (MR-4000; Dynex Technologies, Denkendorf, Germany). For Western blot analysis, 20 μg of mouse brain–extracted proteins and rMOG were run on a 12.5% polyacrylamide gel and transferred to a nitrocellulose membrane (Hybond-ECL; Amersham International). The blot was probed with 1:500 diluted mouse serum or with the 8.18-C5 mAb in a concentration of 4 μg/ml. Specific binding was detected with peroxidase-conjugated goat anti–mouse Ig (1:4,000; Amersham International) applying the ECL system (Amersham International).

Cloning and Sequence Analysis of H Chain Sequences. Spleens were taken from naive and rMOG-immunized mice and immediately frozen in liquid nitrogen. Total RNA was extracted with Trissolv reagent (GIBCO BRL, Eggenstein, Germany) according to the manufacturer's instructions. cDNAs were synthesized from 10 μg freshly dissolved total RNA per cDNA reaction using Superscript reverse transcriptase (GIBCO BRL) with oligo(dT) as a primer. The resulting cDNA was diluted to 100 μl, and 2 μl of each sample (corresponding to 200 ng of total RNA) was used for PCR amplification with the following primers: 5′ primer, 8.18FR1: CGGGATCCACTCCCAGGTTCAGCTGC, and a 3′ located Cγ primer (27). PCR reactions were carried out using the following conditions: 94°C for 5 min (1 cycle); 94°C for 60 s, 60°C for 60 s, 72°C for 120 s (30 cycles), followed by a final step of 10 min at 72°C. The PCR products were purified from an ethidium bromide–stained agarose gel and cloned into pBluescript (Stratagene Inc., Heidelberg, Germany) using the restriction endonuclease BamHI. Several transformed bacterial clones were analyzed by DNA sequencing; the sequencing reaction was performed by MediGene (Martinsried, Germany).

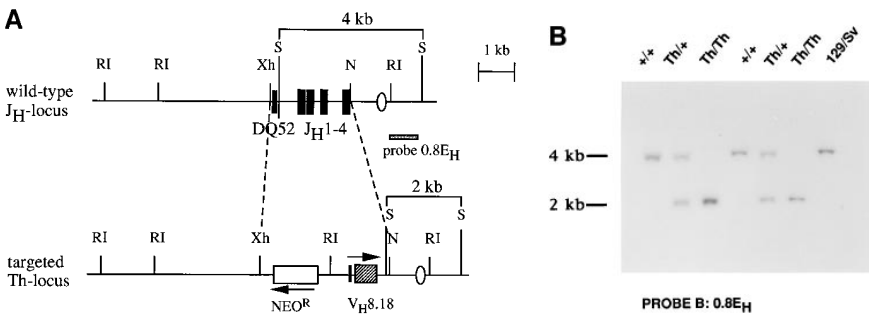

Figure 1. Site-directed replacement of the J_H locus with the Th^{mog} gene. (*A*) Structure and partial restriction map of the wild-type IgH locus and the targeted insertion. *Filled boxes*, the DQ52 and J_H elements; *open oval*, the 3′ enhancer region. *Arrows*, The transcriptional orientation of the Th (VDJ 8.18; *hatched box*) and the neo^r gene. Diagnostic restriction fragments and location of the probe (0.8 E_H) used for Southern blot analysis are shown. *N*, NaeI; *RI*, EcoRI; *S*, SacI; *Xh*, XhoI. (*B*) Southern blot analysis of ES cell–derived offspring. SacI-digested tail DNA from offspring of heterozygous mutant mice was hybridized to the probe 0.8 E_H. Homozygous mutant animals (Th/Th) show only a 2-kb hybridizing fragment, whereas heterozygous mutants (Th/+) show both a 4- and a 2-kb fragment.

Immunization of Mice and Evaluation of EAE. C57Bl/6 mice were obtained from the animal facility at the Max-Planck-Institut für Biochemie, and SJL mice were purchased from Charles River WIGA GmbH (Sulzfeld, Germany). Transgenic F1 (129/Sv × Bl/6) progeny of chimeric mice were bred onto the C57Bl/6 and the SJL strain to perform EAE experiments. Mice of the fourth backcross to SJL were injected with an emulsion of 200 μg proteolipid protein (PLP) 139–154 (28) in CFA (GIBCO BRL) supplemented with 4 mg/ml inactivated *Mycobacterium tuberculosis* (H37 RA; Difco Laboratories, Inc., Detroit, MI) in the flanks on both sides and the tail base. The animals received an additional intraperitoneal injection of 200 ng pertussis toxin (List Biological Labs, Inc., Campbell, CA) in 0.1 ml PBS on the day of immunization and again 48 h later. Mice bred onto the C57Bl/6 genetic background were immunized with 50 μg rMOG in the same way as described above, but without the use of pertussis toxin. Animals were monitored daily for clinical symptoms and weight.

For the clinical evaluation of EAE, the following scale was used: 0, no clinical disease; 1, tail weakness; 2, paraparesis (incomplete paralysis of one or two hindlimbs); 3, paraplegia (complete paralysis of one or two hindlimbs); 4, paraplegia with forelimb weakness or paralysis; and 5, moribund or dead animals.

To analyze the neuropathology, mice were perfused with 4% paraformaldehyde. Brain and spinal cord were removed, postfixed for another 24 h, and routinely embedded in paraffin. The extent of inflammation and demyelination was evaluated on 3-μm spinal cord cross sections stained with hematoxylin/eosin and Klüver Barrera myelin stain.

T Cell Lines. SJL mice were immunized with 100 μg of peptides PLP 139–154 and PLP 130–151 (28) for establishment of T cell lines GK and IH, respectively. 10 d later, cells isolated from draining LNs were cultured at 5×10^6 cells/ml in the presence of 10 μg/ml PLP peptide for 3 d. After growing cells for 10–14 d in IL-2–containing DMEM (GIBCO BRL), the cells were restimulated with antigen and irradiated (4,000 rads) syngeneic spleen cells; the specificity was assessed by [^{3}H]thymidine (Amersham International) incorporation for the last 16–18 h of a 3-d culture period. Activated T cells were harvested on Lymphoprep™ (Nycomed, Oslo, Norway), washed once, and injected via the tail vein into mice. Animals were scored daily for clinical signs of EAE as described above.

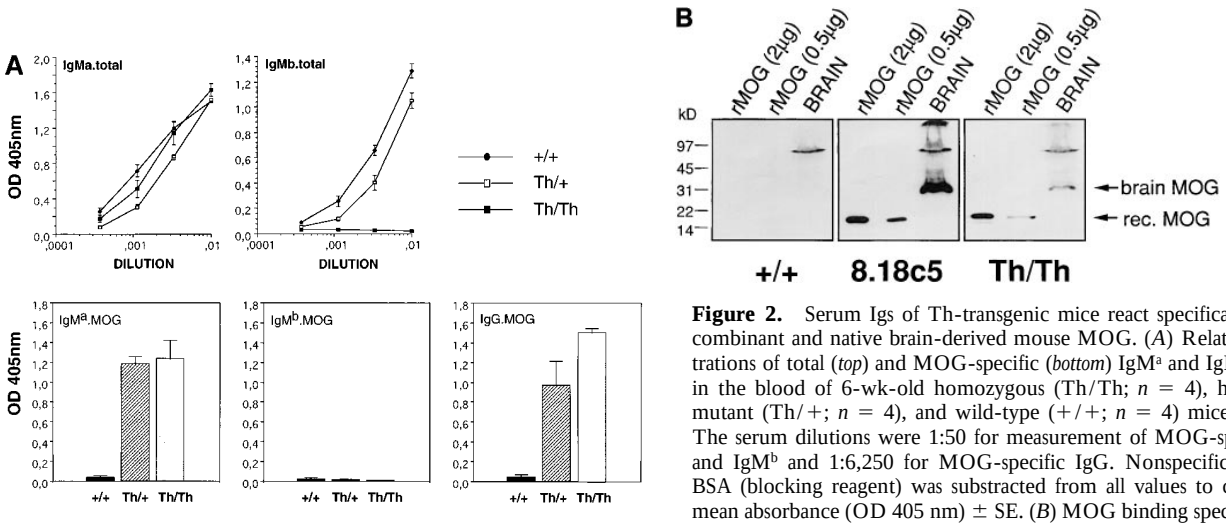

Figure 2. Serum Igs of Th-transgenic mice react specifically with recombinant and native brain-derived mouse MOG. (*A*) Relative concentrations of total (*top*) and MOG-specific (*bottom*) IgM^a and IgM^b allotypes in the blood of 6-wk-old homozygous (Th/Th; $n = 4$), heterozygous mutant (Th/+; $n = 4$), and wild-type (+/+; $n = 4$) mice by ELISA. The serum dilutions were 1:50 for measurement of MOG-specific IgM^a and IgM^b and 1:6,250 for MOG-specific IgG. Nonspecific binding to BSA (blocking reagent) was substracted from all values to calculate the mean absorbance (OD 405 nm) ± SE. (*B*) MOG binding specificity of serum Igs derived from homozygous mutant (Th/Th) and wild-type (+/+) mice, analyzed by Western blot. Mouse brain–extracted proteins and rMOG separated by SDS-PAGE and blotted onto nitrocellulose membrane were incubated with wild-type serum (*left*), the 8.18-C5 mAb (*middle*), and serum from homozygous mutant mice (Th/Th; *right*). The sizes of the molecular weight standard are indicated (*left*), and the position of rMOG- and brain-derived MOG are marked (*arrows*, *right*).

Results

Generation of MOG-specific Ig Gene–targeted Mice. The entire J_H cluster of the Ig H chain gene locus was replaced by the rearranged VDJ gene segment of MOG-specific mAb 8.18-C5 (Fig. 1 *A*). Germline chimeric mice generated by introducing gene-targeted ES cells into blastocysts gave rise to mutant progeny at normal efficiency (Fig. 1 *B*). The transgenic mice were backcrossed onto both C57Bl/6 and SJL/J genetic backgrounds. Endogenous and transgenic Ig H chains can be readily identified serologically by their allotypes. C57Bl/6 and SJL/J mice produce Ig of allotype b (*Igh*^{b}), whereas the targeted Ig H gene locus is derived from the strain 129/Sv, with allotype a (*Igh*^{a}).

The total level of serum IgM of the transgenic (a) allotype in hetero- or homozygous knock-in mice (Th/+ and Th/Th, respectively) is comparable to the serum levels in F1 mice heterozygous for the IgM^a and IgM^b alleles (Fig. 2 *A*). In contrast to Th/+ heterozygous knock-in mice, where serum IgM^b levels are slightly reduced, no IgM^b can be detected in Th/Th mice (Fig. 2 *A*). MOG-binding IgG and IgM^a are found exclusively in naive Th/Th and Th/+ mice but not in wild-type nontransgenic F1 mice (Fig. 2 *A*).

The antigen specificity of the serum Igs derived from transgenic mice was verified by Western blotting. Sera from knock-in but not from nontransgenic littermates bound to rat rMOG, as well as to native mouse brain MOG (Fig. 2 *B*). The transgenic Abs showed a binding pattern identical to the MOG-specific "donor" mAb 8.18-C5.

Distribution and Development of B Cells in Th Mice. Flow cytometric analysis of B lymphocytes obtained from knock-in animals confirmed that autoreactive MOG specificity is not confined to a small subpopulation. Abs specific for transgenic IgM (IgM^a) bound to the vast majority of resting B lymphocytes in the spleen of Th/+ mice, documenting the predominant use of the transgenic Ig H chain. Only a minority of B cells (1–4%) express endogenous Ig H chains (Fig. 3 *A*, *top*). Staining of these few B cells is not due to an artifact, but seems to represent a minor IgM^b-labeled subpopulation specific for Th/+ mice, since such cells are completely absent in Th/Th mice (Fig. 3 *C*). Thus, allelic exclusion leads to exclusive expression of the mutant Ig H allele.

Interestingly, in Th/+, up to 30% and in Th/Th, up to 50% of IgM^a-positive B cells bound biotin-tagged, rMOG protein (Fig. 3, *A* and *C*). Using rabbit antiidiotypic Abs recognizing specifically the V region of the H chain of 8.18-C5 (24), we confirmed that the overwhelming majority of transgenic B cells indeed express the 8.18-C5 V_H idiotype (Fig. 3 *A*). This excludes the possibility that editing of the original gene-targeted V region has occurred within the IgM^a-positive B cell population that is unable to bind MOG, and suggests that many but not all endogenous L chains can associate with the transgenic H chain to generate a functional MOG-specific Ab.

The total number of B and T cells and the distribution of T cell subsets in the spleen and LNs of both Th/+ heterozygous and Th/Th homozygous animals were indistinguishable from wild-type control organs (not shown), indicating normal differentiation of transgenic B lymphocytes. Analysis of early B cell differentiation in the bone marrow of adult Th/+ mice confirmed this assumption. The number and distribution of B cell precursors in the bone marrow of these mice were identical to that seen in wild-type littermates as assessed by the surface expression of the differentiation markers B220, CD43, and IgM (Fig. 3 *B*). In addition, MOG-specific B cells appear in the bone marrow at a frequency similar to that found in the periphery (Fig. 3, *A* and *B*).

The analysis of the peritoneal B cell compartment, the main milieu of B-1 lymphocytes, revealed that due to allelic exclusion, the transgenic IgM^a H chain was also dominant in the peritoneal B cell population (Fig. 4). The pro-

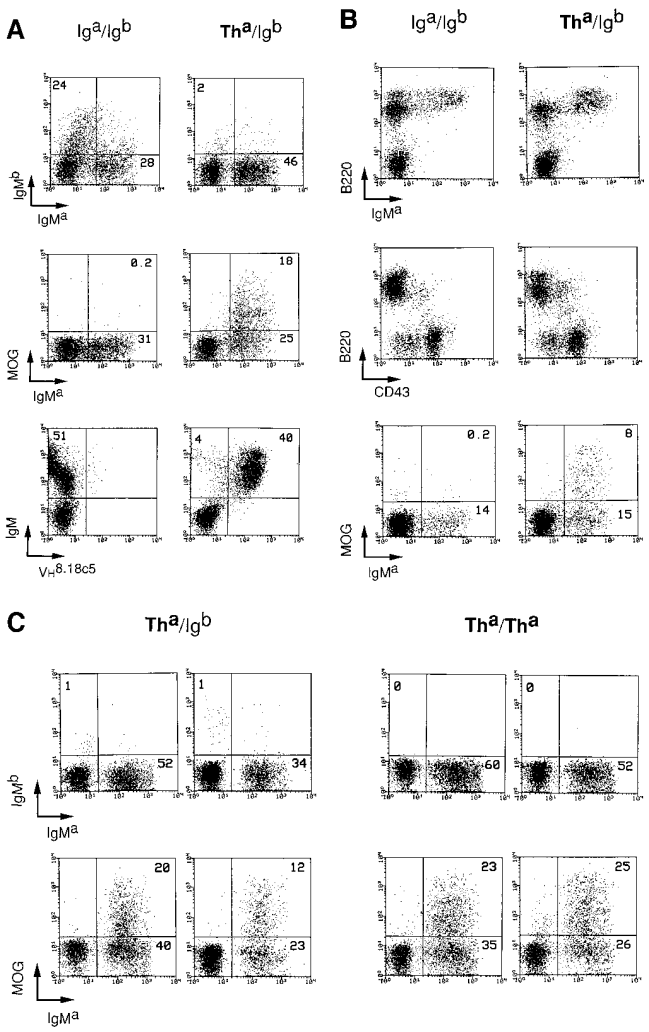

Figure 3. Surface phenotype of lymphocytes isolated from spleen (*A*), bone marrow (*B*), and peripheral blood (*C*) of wild-type (+^{a}/+^{b}), heterozygous (Th^a/+^{b}), and homozygous (Th^a/Th^a) mutant 8-wk-old mice. Cells were stained with anti-IgM^a mAb, anti-IgM^b Mab, biotinylated rMOG, anti-B220, anti-CD43, and an idiotype-specific Ab ($V_H^{8.18\text{-}C5}$) as indicated. Numbers in quadrants refer to the percentage of cells in the lymphocyte gate as defined by forward and side scatter.

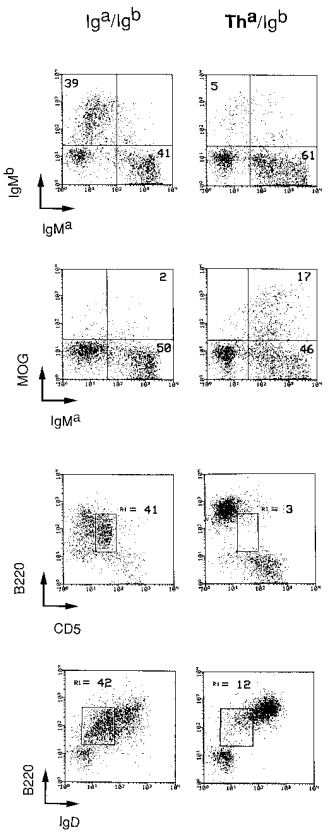

Figure 4. Flow cytometry of peritoneal B cells from wild-type ($+^a/+^b$) and heterozygous mutant ($Th^a/+^b$) 8-wk-old mice. Cells were stained with anti-IgM^a and -IgM^b allotype–specific Abs and MOG as shown previously in Fig. 3. Double stainings of either CD5 or IgD combined with anti-B220 identify CD5-positive B-1 cells, characterized by low expression of B220 and IgD. *Boxes*, B220^{dull}CD5$^+$ cells and B220^{dull}IgD^{dull} cells; percentages in a given box are shown for each plot.

portion of MOG-binding IgM^a B cells was comparable to that seen in other lymphoid organs (Fig. 4). However, the proportion of CD5$^+$ B-1 subset B cells, characterized by low levels of IgD and B220, was severely reduced in the peritoneum of the mutant mice (Fig. 4).

These results demonstrate that association of the targeted Ig H chain with endogenous L chains generate autoreactive MOG-specific B cells which differentiate without restriction in the bone marrow and subsequently colonize the immune organs of Th mice.

Response of Transgenic B Cells to Immunization against MOG. We have shown that in naive transgenic mice, neither are autoreactive MOG-specific B cells deleted, nor are their Ig receptors edited through secondary rearrangements of the transgenic Ig H chain. Also, the density of surface IgM receptors on the populations of B cells that do and do not bind MOG is identical in transgenic animals and similar to that seen in nontransgenic littermates. This argues against anergy of autoreactive B cells (Fig. 3). More cogent evidence for the functional state of autoreactive transgenic B cells comes from immunization of Th/+ mice with recombinant MOG in CFA. This treatment induces a MOG-specific Ab response, with the synthesis of both IgG1 and IgG2a Igs, which are indicative of normal isotype switching (Fig. 5 *A*).

Our knock-in Th mice are "single-transgenics," with only the Ig H chain modified by insertion of a MOG-specific VDJ region. Therefore, L chains used by transgenic B lymphocytes are from endogenous origin, and upon immunization with MOG, this diverse L chain use could result in novel epitope specificities differing from that of the original 8.18-C5 hybridoma. The mAb 8.18-C5 recognizes a nonlinear, conformational but SDS-resistant epitope within the extracellular Ig-like domain of MOG (our unpublished observations). This original specificity pattern was maintained in MOG-immunized Th/+ mice, where no response was detected to a panel of peptides covering the entire rMOG protein (Fig. 5 *B*). In contrast, the Ab response in immunized wild-type littermates recognizes multiple linear MOG peptide epitopes, mainly located in the amino acid sequences 1–25 and 50–71 (Fig. 5 *B*). The absence of these MOG peptide–specific responses in Th mice strongly suggests that the immune response against MOG in knock-in mice is dominated by transgenic B cells of the same specificity as in the original hybridoma 8.18-C5.

Somatic hypermutation of Ig V regions is a hallmark of the B cell immune response. To document somatic mutations within the targeted VDJ gene, we amplified the gene-targeted VDJ region linked to constant regions of γ isotypes via reverse transcription PCR from mRNA from the

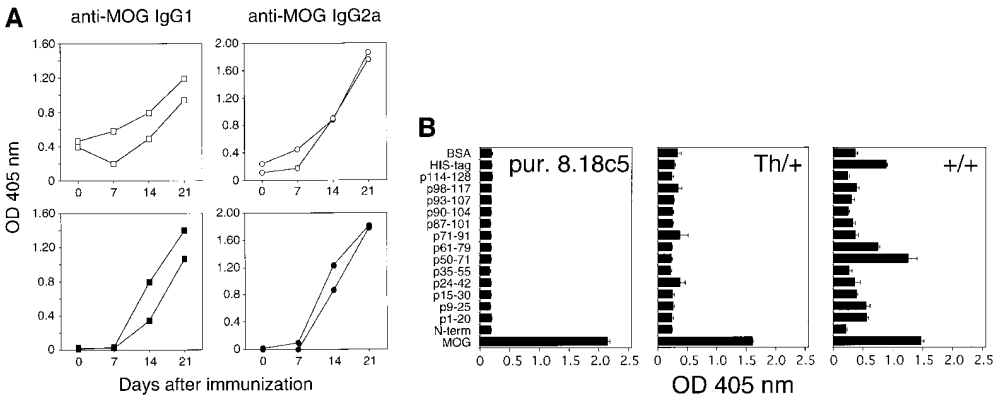

Figure 5. Ab response of Th-transgenic mice immunized with rMOG in CFA. (*A*) MOG-specific IgG1 and IgG2a Abs were measured in the serum (diluted 1:360) of homozygous mutant mice (Th/Th; *top*) and wild-type controls (+/+; *bottom*) by ELISA (see Fig. 2). Samples were taken before immunization and 7, 14, and 21 d after antigenic challenge. Two mice of each group are shown. (*B*) The epitope specificity of serum Igs was analyzed in heterozygous mutant (Th/+) and wild-type (+/+) mice 14 d after MOG immunization. A panel of overlapping peptides spanning the extracellular region of MOG including NH_2-terminal (*N-term*) and COOH-terminal (*HIS-tag*) peptides of rMOG was used to measure specific reactivity by ELISA (OD 405 nm). Purified 8.18-C5 mAb (*pur. 8.18c5*) was used at a concentration of 2 μg/ml, and the serum was diluted 1:100 and developed with goat anti–mouse Ig.

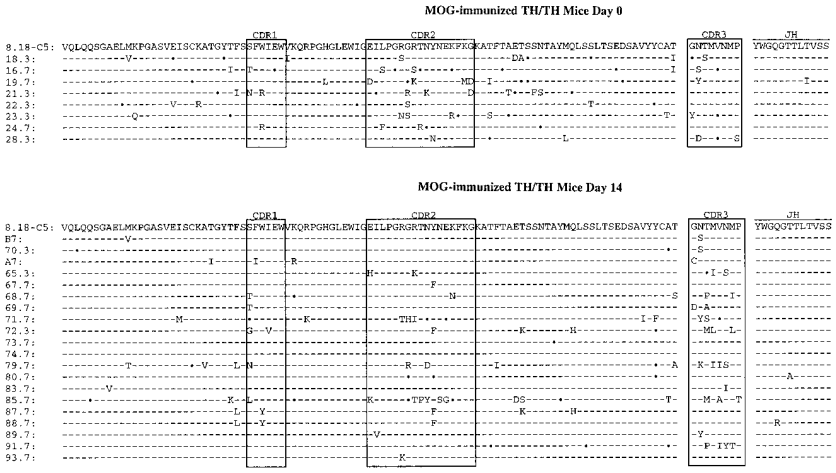

Figure 6. Somatic hypermutation of the transgenic Th gene sequence obtained from homozygous mutant (Th/Th) mice before (day 0) and 14 d after priming with rMOG. Deduced amino acid sequences are compared with that of the 8.18-C5 gene. *Dashes*, identity; amino acid substitutions are shown; *dots*, silent mutations. The sequence data are available from GenBank under the accession number AF042086.

spleens of MOG-immunized homozygous Th/Th mice, and determined individual sequences after cloning. Replacement mutations are already detectable in the transgenic V region before immunization (Fig. 6). These mutations are scattered throughout the entire V sequence without marked accumulation in the CDR3 region (0.06 mutations/amino acid in the framework regions versus 0.1 in the CDR regions). However, at day 14 after immunization, the total replacement to substitution (R/S) ratio (R/S: 3.4) was three times higher than in preimmune sequences (R/S: 1.4), and for the CDR3 region, this increase was tenfold (R/S: 1.5 at day 0 versus R/S: 14.5 at day 14 after immunization; Fig. 6). Although care must be taken correlating sequence data with antigen specificity, the enhanced frequency of replacement mutations in the CDR3 region of the gene-targeted V_H gene is indicative of an antigen-driven process.

We conclude that the MOG-specific transgenic B cells in Th mice arise and develop normally, are not anergized in peripheral immune organs, and are functionally competent and fully able to sustain mature humoral immune responses on immunization with the cognate antigen.

MOG-specific B Cells Are Conditionally Pathogenic. We have demonstrated the persistence of functionally reactive autoreactive MOG-specific B cells in the Th mice, and showed high titers of MOG-specific serum autoantibody. Yet the mutant mice developed neither neurological deficits nor CNS pathology during a period of over one year. The intact endothelial blood–brain barrier (BBB) and intrinsic protective mechanisms in the CNS (29) seem to be sufficient to protect the mice against the potentially pathogenic B cells and their autoantibody products. However, the pathogenic potential of this MOG-specific population of B cells was revealed in the context of a CNS-specific inflammatory response.

Th mice were backcrossed onto SJL and C57Bl/6 strains, which differ markedly in their susceptibility to

Table 1. *Active EAE in 8–12-wk-old Th^{mog} Mice (Th/+) Compared with Littermate Controls (+/+)*

Strain backcross level	Genotype	Antigen	Disease incidence (percent)	Mean maximal disease score ± SE	Mean day of onset (range)
C57B6-n1	Th/+	rMOG	8/14 (57)	2.9 ± 0.44	15.5 (13–18)
	+/+	rMOG	1/12 (8)	2.0	21.0
C57B6-n2	Th/+	rMOG	5/6 (83)	2.9 ± 0.58	14.6 (13–15)
	+/+	rMOG	3/7 (43)	2.3 ± 0.33	22.0 (17–28)
C57B6-n3	Th/+	rMOG	9/13 (69)	2.4 ± 0.28	21.3 (14–32)
	+/+	rMOG	6/16 (38)	3.6 ± 0.20	21.7 (20–26)
SJL-n4	Th/+	PLP 139–154	13/14 (93)	4.89 ± 0.08	8.6 (7–17)
	+/+	PLP 139–154	11/12 (92)	2.14 ± 0.32	12.2 (8–25)

Mice of three distinct generations (*n*1–3) bred into the C57B6 strain and animals of the fourth backcross to SJL (*SJL-n4*) were challenged with rMOG and PLP 139–154, respectively (reference 21). The disease incidence, the mean maximal disease score, and the average onset of clinical EAE were calculated for each group of immunized mice.

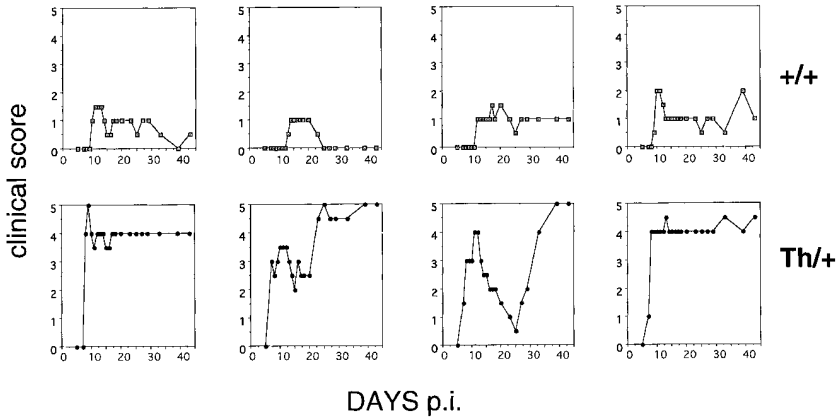

Figure 7. Development of clinical EAE in four wild type (+/+; *top*) and four heterozygous mutant (Th/+; *bottom*) mice after immunization with peptide PLP 139–154 in CFA. The clinical score (*y-axis*) and weight (not shown) were monitored daily over a period of 40 d (*x-axis*) after injection. The shown data are representative of a larger group of immunized animals summarized in Table 1.

EAE. Wild-type SJL mice are highly responsive to immunization with either PLP peptide 139–154 or rMOG protein (reference 28, and our unpublished observations). In SJL mice actively immunized with the PLP peptide, the presence of the transgene accelerated disease onset and aggravated the neurological deficit to a mean maximal disease score of 4.9, as opposed to 2.1 in the littermate controls (Table 1, and Fig. 7). Histopathological analysis revealed that accelerated disease development was associated with widespread CNS inflammation and demyelination by day 9 after immunization in the mutant Th mice, at which time wild-type controls showed no CNS lesions (Fig. 8). As opposed to the relatively mild, relapsing course of disease seen in nontransgenic littermate mice, EAE actively induced in transgenic mice with PLP peptide was severe; remission was observed in only one Th animal, and this was followed by a fatal relapse (Fig. 7, and Table 1).

In contrast to the high susceptibility of SJL mice, C57Bl/6 mice are PLP resistant and only partially susceptible to EAE induced with rMOG protein (reference 30, and our unpublished observations). In transgenic C57Bl/6 mice, the incidence of MOG-induced disease was 70 ± 13%, whereas in wild-type littermates, the incidence was only 29 ± 19% (Table 1).

In transgenic SJL mice, disease onset was also accelerated upon adoptive transfer of PLP peptide–specific SJL T cell lines. These T cell lines induce severe and ultimately lethal EAE in normal SJL mice, but disease onset consistently occurred 3–4 d earlier in the SJL-backcrossed Th/+ animals compared with their littermates, irrespective of the dose of T cells transferred (Table 2). As the T cell lines are PLP-, not MOG-specific, we can exclude the possibility of antigen-dependent cooperation with the transgenic MOG-specific B cell population that could enhance T cell proliferation or conversely activate resident B cells. Therefore, the accelerated onset of disease seen in the Th/+ mice probably represents the pathologic effects of MOG-specific Abs that rapidly amplify the inflammatory/demyelinating response in the CNS (31).

Treatments that do not interfere with BBB permeability, such as pertussis toxin alone, immunization with foreign antigens such as KLH, or the passive transfer of KLH-specific T cells, failed to provoke CNS disease in transgenic mice (data not shown).

Discussion

Numerous studies have used transgenic mice to investigate the mechanisms involved in immunological self tolerance of B lymphocytes. For example, in conventional transgenic mice harboring rearranged Ig genes specific for a natural self protein (the MHC class I molecule H-2K^k), or a transgenically expressed foreign protein (hen egg lysozyme), physical deletion was observed when the autoantigen was expressed as membrane component, whereas expression of the autoantigen as soluble protein led to the functional inactivation, or anergy, of autoreactive B cells (32). Studies of transgenic mice with Abs against MHC determinants or against DNA led later to the discovery of B cell receptor editing, the selective inactivation of the autoreactive Ig receptor by the reactivation of endogenous Ig gene rearrangement, which results in expression of nonautoreactive endogenous L chains (20, 33).

The picture was sharpened and to some degree modified with the advent of gene-replacement mutants. In studies using knock-in mice where some of the germline Ig genes were replaced with rearranged autoantibody genes (20, 33), B cell receptor editing, which was localized in the pre–B cell compartment, proved to be of more substantial importance than anticipated (20, 34). Further, immunization of knock-in mice allows mature self-reactive B cells to be studied in terms of Ig switching and affinity maturation via somatic mutation.

To date, no transgenic models have been described involving Ig gene replacement with autoantibodies known to mediate defined tissue-specific autoimmune disease. We have generated a knock-in mutant in which the VDJ region of the MOG-specific H chain from the hybridoma 8.18-C5 was inserted into the natural location of rearranged V genes in the H gene locus. mAb 8.18-C5 mediates demyelination both in vitro and in vivo (35, 36) and exacerbates clinical disease in EAE (31, 37). Apart from dealing with a proven pathogenic autoantibody, our model combines several features advantageous for studying devel-

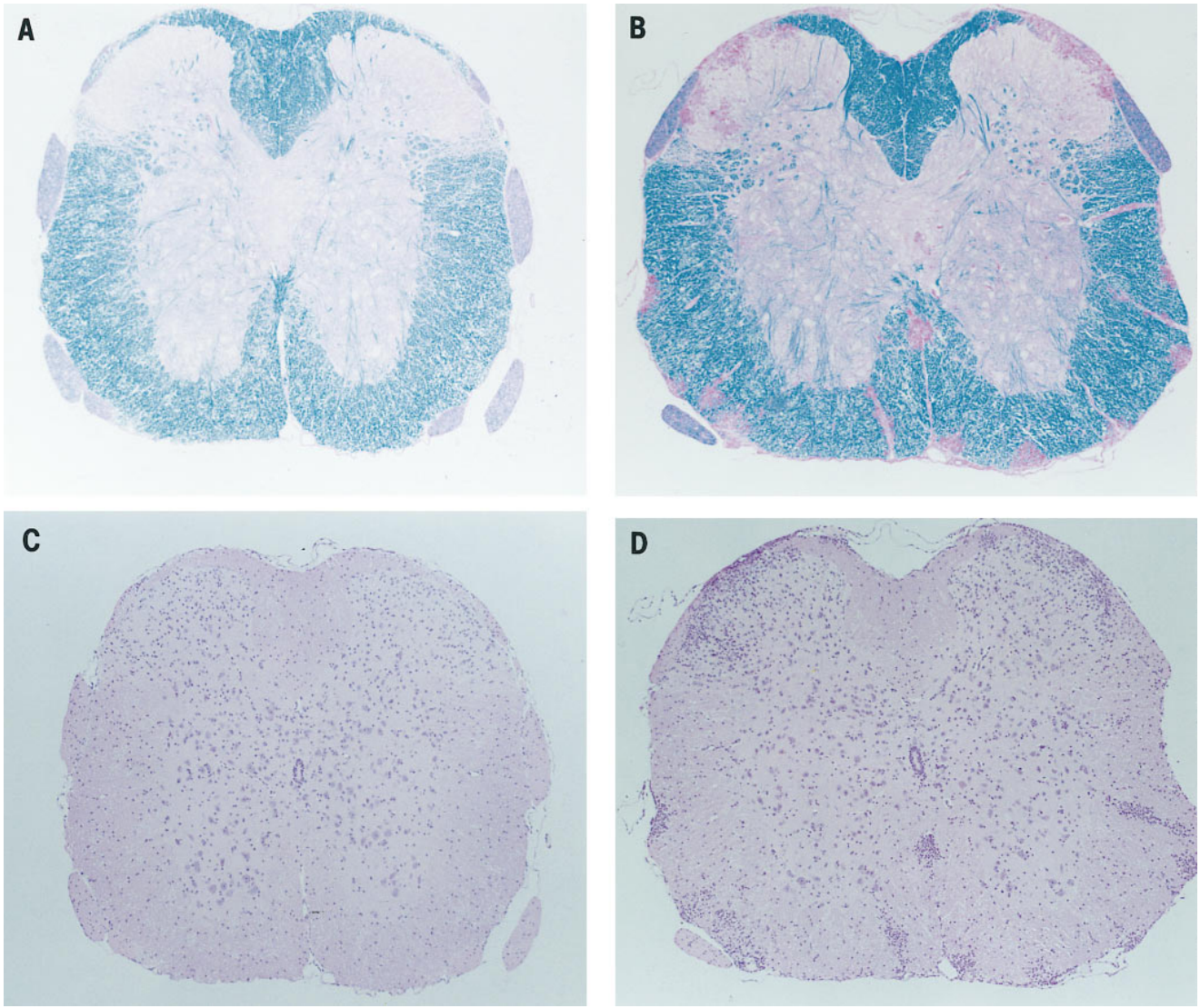

Figure 8. Histological staining on paraffin-embedded spinal cord sections of Th transgenic and wild-type mice after induction of EAE with PLP 139–154 peptide. On day 9 after immunization, early demyelination is found in Th knock-in animals (*B*) but not in littermate control mice (*A*). Large cellular infiltrates are detectable within the areas of demyelination after hematoxylin/eosin staining of sections of Th mice (*D*) but not in sections of control animals (*C*).

opment and maintenance of B cell self tolerance. The presence of defined allotypic and idiotypic markers allows the transgenic H chain to be detected in vivo. Furthermore, the combination of the gene-targeted H chain with endogenous L chains generates MOG specificity in a fraction but not in all transgenic B lymphocytes (Fig. 3). This allowed us to study the maturation of autoreactive B cells under conditions where they have to compete with a large pool of nonautoreactive B cells for appropriate immunological niches (38), in both naive and challenged animals. Finally, the restricted localization of MOG to the CNS allowed us to study the mechanistic basis of tolerance for a sequestered antigen and the role of autoreactive B cell clones in disease development.

The analysis of transgenic B cells in mature immune organs did not provide any evidence of active tolerance induction. Mature mutant mice express the transgenic Ig H chain in almost all B cells and constitutively synthesize high levels of MOG-specific Ig. The peripheral immune organs of adult hetero- and homozygous knock-in Th mice contain B cells indistinguishable from wild-type mice in number and morphology, ruling out substantial B cell deletion. Likewise, anergy is also excluded by the normal density of surface IgM on the transgenic B cells (Fig. 3) and by their full response to immunization against MOG (Figs. 5 and 6), suggesting that circulating amounts of soluble MOG, if present at all, must be negligible (39). Furthermore, using a V_H chain–specific idiotypic marker Ab, we found no evidence of Ig H chain editing (Fig. 3).

The number and composition of immature B lymphocyte subsets developing in the transgenic bone marrow

Table 2. *Passive EAE in Heterozygous Mutant Mice (Th/+) and Littermate Controls (+/+) Induced by Adoptive Transfer of Encephalitogenic T Cells*

Strain-backcross level	Genotype	No. of cells transferred ($\times 10^6$)	Line (restimulation cycles)	EAE incidence	Average day of onset (range)
SJL-n2	Th/+	6	GK (1)	10/10	4.5 (4–5)
	+/+	6	GK (1)	10/10	7.2 (6–9)
SJL-n2	Th/+	5	IH (2)	3/3	4.6 (4–5)
	+/+	5	IH (2)	2/3	9.0 (6–12)
SJL-n3	Th/+	1	GK (1)	7/7	5.1 (4–7)
	+/+	1	GK (1)	5/5	7.8 (6–10)
SJL-n3	Th/+	0.1	GK (1)	3/4	6.3 (6.7)
	+/+	0.1	GK (1)	2/3	10

PLP-specific T cell lines were established from SJL mice as described in Materials and Methods. After one or two cycles of restimulation with antigen, equal numbers of activated T cells were transferred intravenously in Th/+ and +/+ mice of the second and third backcross to SJL.

were also completely regular. In particular, pro–B cells ($CD43^+$, $B220^{low}$, IgM^-), pre–B cells ($CD43^-$, $B220^{low}$, IgM^-), and immature B cells ($B220^{low}$, IgM^+) did not differ from the corresponding subsets in the bone marrow of normal littermate mice (Fig. 3). Further, early B lymphocytes bound MOG at high rates similar to those of mature peripheral B cells, indicating that the peripheral level of MOG specificity is the result of random association of the knock-in Ig H chain with endogenous L chains during B cell development.

The only B cell compartment showing drastic differences between transgenic and wild-type mice was the peritoneal space, the habitat of $CD5^+$ B-1 lymphocytes (1). In our transgenic mice, the peritoneal B-1 compartment was severely depleted of $CD5^+$ B-1 cells (Fig. 4). This is opposite to one case of conventional transgenic mice which expressed the gene for a hemolytic autoantibody predominantly in the B-1 cell subset, in the absence of B cells of the B-2 compartment (40). Yet it is doubtful that depletion of B-1 cells in our transgenic mice is related to B cell self reactivity. In fact, the observation of a similar change in another knock-in model expressing transgenic Ig specific for foreign antigen (27) would rather argue against an autoimmune basis of B-1 cell deficiency.

Immunization of our transgenic mice with rMOG protein resulted in enhanced Ig levels, isotype switching, and the accumulation of somatic mutations in the CDR3 sequence, changes similar to those seen during the conventional antigen-driven selection process (41). The transgenic V_H region found in IgG-expressing B cells of nonimmunized controls contains exchange mutations scattered throughout the entire VDJ gene segment. Since we analyzed homozygous Th/Th mice in these experiments, these mutations most likely reflect the need to select clones recognizing specificities other than MOG (42).

Remarkably, even after immunization, the Ab response is dominated by the original epitope specificity of the hybridoma 8.18-C5, despite the pathogenicity of this response (Fig. 5 *B*). We conclude from these observations that MOG-specific autoreactive B cells in Th knock-in mice mature uncensored through all stages of B cell differentiation, resulting in a large pool of potentially pathogenic B cells that produces high titers of autoreactive serum Igs.

The normal distribution and responsiveness of the MOG-specific B cells in Th mice could be explained by clonal ignorance, with the target autoantigen inaccessible during B cell development and maturation. Indeed, MOG is exclusively produced by oligodendrocytes of the CNS and is therefore sequestered from the peripheral circulation by the endothelial BBB. Although there is protein exchange between the cerebrospinal fluid and lymph (43), in the case of MOG, if this occurs at all, the levels of antigen reaching the periphery are clearly insufficient to induce tolerance. However, mature B cells may be able to cross the BBB. It has already been established that the BBB is permeable for activated T cells but not for resting T lymphocytes (44). The same rule may also hold for B cells, as suggested by the behavior of activated B cell lymphomas, which have a marked propensity to cross the BBB (45).

These observations are clinically relevant, as the MOG-specific transgenic B cells produce conditionally pathogenic autoantibodies. These B cells fail to cause spontaneous autoimmune disease in the healthy organism, but exhibit their pathogenic potential in combination with an encephalitogenic T cell response. Activated myelin-specific T lymphocytes cross the BBB shortly after intravenous transfer. The subsequent interaction with glial cells within the CNS triggers an inflammatory cascade and then results in the opening of the BBB. This leads to edema formation and the influx of inflammatory cells and serum proteins into the CNS (44). Permeating anti-MOG Abs bind to their target epitopes on the myelin surface and initiate widespread complement- and antibody-dependent cell-mediated cytotoxicity (ADCC)-mediated demyelination. In addition,

the autoreactive B cells may also function as highly efficient APCs, enhancing the selective action of MOG-specific T cells (46). Deciphering the precise roles played by B cells in MOG-induced autoimmune disease is of particular interest, as recent evidence stresses the importance of MOG as a prominent candidate autoantigen in multiple sclerosis and the implication of B cells in this disease.

In general terms, our results show that autoreactive B lymphocytes with pathogenic potential can develop and respond to activation in a healthy organism. Comparable autoreactive B cell clones with specificities for other tissue-specific autoantigens (e.g., thyroid, pancreatic islet, epithelial) are also likely to be present in the healthy immune repertoire (47).

From this point of view, B cell tolerance resembles its counterpart on the T cell level, where potentially autoreactive T cell clones are known to be normal components of the intact immune repertoire (48, 49). In this case, the induction of autoimmune disease requires that autoreactive T cells must be activated, for example, through microbial agents (50). Similarly, in the case of autoreactive B cells specific for CNS antigens, their presence in the immune system is in itself insufficient to cause tissue damage. This requires additional inflammatory factors, such as a concomitant encephalitogenic T cell response or CNS inflammation. Alternatively, it could be argued that the high levels of allelic exclusion reached in our Th mice could have led to the depletion of the natural Ab repertoire, which has been suggested to play a role in protecting against autoimmune diseases (for a review, see reference 51). However, an enhanced susceptibility to autoimmunity resulting from repertoire shift rather than from the MOG specificity of the targeted H chain seems less likely, since all EAE studies (Table 1, and Fig. 7) were performed in heterozygous Th/+ mice, which contain almost normal serum titers of nontransgenic Abs (allotype b), including natural Abs, compared with normal littermate animals (see Fig. 2 *A*).

In summary, this paper demonstrates that autoreactive B cells specific for antigens sequestered within the CNS are not actively tolerized, but persist in the immune repertoire due to clonal ignorance. The presence of this potentially pathogenic B cell population is irrelevant in healthy individuals, but modifies the pathophysiology and clinical course of inflammatory CNS disease. Therefore, strategies for immune therapies should take these observations into account and be designed to ablate the B cell as well as T cell arm of the autoimmune response in diseases such as multiple sclerosis.

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