Development of Myelin Oligodendrocyte Glycoprotein Autoreactive Transgenic B Lymphocytes: Receptor Editing In Vivo After Encounter of a Self-Antigen Distinct from Myelin Oligodendrocyte Glycoprotein

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Development of Myelin Oligodendrocyte Glycoprotein Autoreactive Transgenic B Lymphocytes: Receptor Editing In Vivo After Encounter of a Self-Antigen Distinct from Myelin Oligodendrocyte Glycoprotein

Tobias Litzenburger,* Horst Blüthmann,† Patricia Morales,‡ Danielle Pham-Dinh,§ André Dautigny,§ Hartmut Wekerle,* and Antonio Iglesias†*

We explored mechanisms involved in B cell self-tolerance against brain autoantigens in a double-transgenic mouse model carrying the Ig H-chain (introduced by gene replacement) and/or the L-chain κ (conventional transgenic) of the mAb 8.18C5, specific for the myelin oligodendrocyte glycoprotein (MOG). Previously, we demonstrated that B cells expressing solely the MOG-specific Ig H-chain differentiate without tolerogenic censure. We show now that double-transgenic (THκMOG) B cells expressing transgenic Ig H- and L-chains are subjected to receptor editing. We show that in adult mice carrying both MOG-specific Ig H- and L-chains, the frequency of MOG-binding B cells is not higher than in mice expressing solely the transgenic Ig H-chain. In fact, in THκMOG double-transgenic mice, the transgenic κMOG L-chain was commonly replaced by endogenous L-chains, i.e., by receptor editing. In rearrangement-deficient RAG-2−/− mice, differentiation of THκMOG B cells is blocked at an immature stage (defined by the B220lowIgM−/−IgD− phenotype), reflecting interaction of the autoreactive B cells with a local self-determinant. The tolerogenic structure in the bone marrow is not classical MOG, because back-crossing THκMOG mice into a MOG-deficient genetic background does not lead to an increase in the proportion of MOG-binding B cells. We propose that an as yet undefined self-Ag distinct from MOG cross-reacts with the THκMOG B cell receptor and induces editing of the transgenic κMOG L-chain in early immature B cells without affecting the pathogenic potential of the remaining MOG-specific B cells. This phenomenon represents a particular form of chain-specific split tolerance. The Journal of Immunology, 2000, 165: 5360–5366.

Myelin oligodendrocyte glycoprotein (MOG)2 is a brain autoantigen with intriguing properties. First, increasing evidence suggests that MOG-specific T and B cells play a crucial role in inflammatory demyelinating disease of the CNS in both experimental and clinical situations (1–4). Second, MOG has several properties setting it apart from other myelin proteins. It is exclusively localized on the outer surface of the myelin sheath, where it can be readily attacked by specific Ab mediating demyelination (5, 6). In addition, the expression of MOG is restricted to the CNS, and it appears late during development of myelin (7–11).

Several mechanisms of B cell tolerance have been identified in Ig-transgenic animal models (12–17). Clonal deletion and receptor editing occur at distinct developmental stages during B cell development in the bone marrow (18). On contact with self-Ag, immature B cells are preferentially induced to undergo receptor editing, i.e., secondary rearrangements of the Ig genes to alter their self-reactive specificity (16, 17). Cells that fail to edit their autoreactive Ag receptors are prone to apoptotic deletion even before entering the mature B cell pool.

Would any of these mechanisms act during the development of MOG-specific B cells? Alternatively, considering that recirculating MOG-specific Abs remain innocuous as long as the tight endothelial blood-brain barrier prevents them from reaching their target structure on the myelin surface, would MOG-autoreactive B cells be generated in bone marrow and spleen without any self-tolerogenic control?

We have recently generated an Ig H-chain “knock-in” transgenic mouse (denominated TH) to retrace the function and fate of MOG-reactive B cells in vivo (4). The Ig H-chain V region was derived from the MOG-specific mAb 8.18C5, an Ab, which induces strong demyelination and clinical exacerbation in experimental autoimmune encephalomyelitis experiments (1, 6). The TH mice showed a diverse B cell repertoire with about one-third of the B cells binding MOG. Thus, in this transgenic setup, the MOG-specific B lymphocytes were not tolerized and secreted MOG-binding Ab, which, on opening the endothelial blood-brain barrier, caused substantial myelin destruction. The absence of B cell tolerance in this case is possibly due to clonal ignorance, because under normal circumstances MOG is sequestered behind the blood-brain barrier and does not interact with peripheral immune cells.

In this study, we show that transgenic B cells expressing both the Ig H- and Ig L-chain of the 8.18C5 mAb are eliminated from the mature B cell repertoire. In Ig H + L-chain-transgenic mice, the transgenic κMOG L-chain is silenced by editing in immature B cells of the B220−/−IgM−/−IgD− phenotype. In addition, we show that elimination of this particular specificity is not mediated by MOG itself but rather by another cross-reacting self-Ag(s).
Materials and Methods

Transgenic mice

The variable region of the L-chain gene was cloned from the MOG-specific hybridoma clone 8.18C5 (7) and inserted in an expression vector including the Cκ region, the κ intron enhancer, and the κ 3′-enhancer. A 1.7-kb SacI fragment of genomic DNA containing the rearranged Vκmog gene was subcloned from a larger clone isolated from a genomic λ phage library of the hybridoma 8.18C5. This DNA fragment including 1 kb 5′-regulatory and untranslated sequences, the two exons encoding the leader sequence, and the entire Vκmog rearranged gene and the intron between them was then inserted 5′ of the Cκ intron sequence in transcriptional orientation, followed by a 1.1-kb DNA fragment containing the κ 3′-enhancer. The entire sequence of the Vκmog gene, including coding exons and the intron between leader sequences and Vκ, as well as 125 bp of the region 5′ of the AUG codon including promoter sequences was determined. The obtained sequence corresponded with the germ-line Vκ8 gene joined to Jκ5. On comparison with known Ig genes, the Vκmog gene was found to be identical with the κ chain gene of the phosphocholine-specific hybridoma 253.15D10 (19), except for a single silent point mutation at aa 110 (CTG/Leu→TTG/Leu) in the Jκ5 region of our Vκmog gene. This point mutation served for unequivocal identification of the transgenic sequence, where the expression of κ genes was studied in immune organs.

For generation of transgenic mice, a 8.5-kb EcoRI fragment of genomic DNA was separated from vector sequences and injected into fertilized oocytes of (C57BL/6 × CBA) F1 mice. Three lines of Vκmog-transgenic mice were obtained, bearing ~2, 8, and 20 copies of the κ transgene. The two κ-transgenic lines with the higher copy numbers of the transgene were crossed back into C57BL/6 and with the TH knock-in mice. The TH knock-in mice have been described elsewhere (20). RAG-2-deficient mice were kindly provided by Dr. H. Mossmann (Max-Planck-Institut für Immunobiologie, Freiburg, Germany). The generation and phenotype of MOG knockout mice will be described elsewhere (D. Pham-Dinh, P. Daubas, C. Vizler, C. Delarasse, D. Dimitri, J. Bauer, A. Dietrich, M. LeMeur, G. Rousset, J.-L. Nassbaum, R. Liblau, and A. Dautigny, manuscript in preparation).

Flow cytometric analysis

Single-cell suspensions were prepared from spleen and bone marrow of adult (6- to 8-week-old) mice or from spleen and liver of first day newborn mice. RBC were lysed by incubation in 0.165 M NH4Cl for 10 min. Cells were washed with PBS/1% FCS and stained with the following Ab conjugates: anti-IgM-FITC (clone DS-1; PharMingen, San Diego, CA); anti-IgM-biotin (Southern Biotechnology Associates, Birmingham, AL); anti-B220-FITC (PharMingen), and dead cells were excluded by labeling with propidium iodide. Samples were analyzed by a FACSscan (Becton Dickinson) and evaluated with the Lysis II program.

Cell sorting and RT-PCR

This experiment was performed as follows. First, individual newborn livers from appropriate crosses were analyzed in FACS for the presence of the MOG mRNA. RBC were lysed by incubation in 0.165 M NH4Cl for 10 min. Cells were washed with PBS/1% FCS and stained with the following Ab conjugates: anti-IgMa-FITC (clone DS-1; PharMingen, San Diego, CA); anti-IgMb-FITC (clone AF6-78, PharMingen); goat anti-mouse IgM-biotin (Southern Biotechnology Associates, Birmingham, AL); anti-B220-FITC (PharMingen); anti-IgD-FITC (PharMingen); and biotin-labeled combinator MOG. The biotinylated mAb 493 (20) was supplied by Ton Rolink (Southern Biotechnology Associates, Birmingham, AL); anti-B220-FITC (PharMingen), and dead cells were excluded by labeling with propidium iodide. Samples were analyzed by a FACSscan (Becton Dickinson) and evaluated with the Lysis II program.

For primer 1 (5′-TGCAGAGCCACGGCAGTAATC-3′) and primer 2 (5′-TCAACTAGCTGCTGTTGGCC-3′), RNA extracted from mouse bone marrow was used as positive control for the amplification of RAG-1 and RAG-2. In all cases, the conditions for PCR amplification were 30 cycles at 1 min at 94°C, 1 min at 60°C, and 1 min at 72°C for each cycle. PCR products were identified on ethidium bromide-stained agarose gels. Primers 5′κ (5′-CAGATCTGCGTGTCACAGACAAACC-3′) and 5′κ (5′-CGGATCTACTGATGGTGGGAAGTGG-3′) were used for amplification of Ig κ-chain sequences which were subsequently cloned into the vector pCR2.1 (Invitrogen, Groningen, The Netherlands). Individual clones were analyzed by Southern blot hybridization using digoxin-labeled primers. First, the identity of the κ clones was determined with an internal Cκ primer (5Cκ: 5′-GCTGATGTGCGTCCACACTGF-3′), and then transgenic κmog sequences were identified with a Vκ-Jκ junctional primer (CSV3xκi: 5′-GGACGTACGGGTAACTACTGT-3′) specific for the κmog transgene. Finally, the sequence of selected transgenic and nontransgenic κ clones was established by TopLab (Martinsried, Germany).

Western blot analysis

Protein extracts of mouse brain and rMOG were run on a 12.5% polyacrylamide gel and blotted onto a nitrocellulose membrane (Hybond ECL, Amersham International, Arlington Heights, IL). The blot was probed with biotinylated 8.18C5 mAb (1:1000) or anti-actin mouse mAb (1:1000, Amersham) and detected with streptavidin-peroxidase (PharMingen) and peroxidase-conjugated goat anti-mouse Ig (Jackson ImmunoResearch), respectively. The signals were developed with the ECL system according to the instruction of the manufacturer (Amersham).

Results

Generation of THκmog-transgenic mice

We generated Ig-transgenic mice the B cells of which share their specificity with the MOG reactive mAb 8.18C5, an Ig with a highly demyelinating potential (23). We cloned the Ig H- and L-chain genes from the 8.18C5 hybridoma, and the rearranged VH region was used for the generation of the VH knock-in (TH) mice, as described previously (4). Genomic DNA including the rearranged Vκmog gene as well as intron and Cκ 3′-enhancer was injected into oocytes to produce conventional IgL-transgenic mice (designated κmog).

Both TH knock-in and κmog-transgenic mice were crossed into the C57BL/6 strain. To generate double-transgenic animals (THκmog), TH and κmog-transgenic mice of the fourth backcross into C57BL/6 were bred. The functional expression of the transgenic κmog gene was demonstrated both by the presence of Vκmog in-frame transcripts in the spleens of transgenic mice and by the association with the transgenic Ig H-chain on the surface of immature B cells (see below).

B cell phenotype of adult THκmog mice

In κmog single-transgenic mice, the κmog L-chain does not contribute to MOG specificity. There are neither MOG-specific B cells nor Abs detectable in these animals (not shown). In striking contrast, we had shown before (4) that in knock-in single-transgenic TH mice ~30% of their B cells bind MOG (Fig. 1), although the B cell repertoire is diverse. The transgenic Ig H-chain combines with some, but not all, endogenous L-chains to reconstitute MOG specificity. The MOG-binding pattern of specific B cells is heterogeneous, ranging from very low to high density by identical surface IgM (Fig. 1), but MOG-reactive B cells are not demonstrably tolerant and seem to undergo normal development (4).

Unexpectedly, B cells isolated from spleen or bone marrow of double-transgenic THκmog mice showed a frequency of MOG-binding cells indistinguishable from that of single-transgenic TH mice (Fig. 1). Furthermore, the titer of MOG-specific Ab in the serum of THκmog mice was not higher than in TH mice. The absence of an increased frequency of MOG-reactive B cells cannot be explained by clonal deletion (12, 13, 24), because the total number of (B220+IgM+) B cells in the spleen and bone marrow of
**THk^mog** mice were not reduced, as compared with TH mice or wild-type controls (Fig. 1). We also exclude anergy, given that the concentrations of surface IgM as compared with surface IgD are normal in these mice (Fig. 1). We also exclude anergy, given that the concentrations of surface IgM as compared with surface IgD are normal in these mice (Fig. 1). 

Expression of transgenic k^mog transcripts was demonstrated by RT-PCR analyses of total mRNA from spleen of single-transgenic adult k^mog mice (not shown) and neonatal liver of double-transgenic THk^mog mice (see below). Functional expression of transgenic k^mog L-chains was also demonstrated on the surface of B cells in the liver of neonatal THk^mog and in the bone marrow and the spleen of adult THk^mog/RAG-deficient mice (see below).

**Transgenic Igk^mog expression in immature B cells**

To track the regulation of the k^mog transgene at early stages of differentiation, we analyzed immature B cells in immune organs from neonatal mice. Like other Ig-transgenic mice, our transgenics showed a global acceleration of B cell development, best explained by the presence of preformed Ig (H or L) genes. The ratio of B220^+IgM^+ to immature B220^+IgM^- pre-B cells was 2-fold higher in the neonatal liver of single-transgenic TH mice than in wild-type littermates (1 vs 0.66, Table I), and this ratio was further elevated in double-transgenic THk^mog neonates (2 vs 1, Table I).

More specifically, the proportion of MOG-binding IgM^+ B cells in the liver of double-transgenic THk^mog neonates is higher than in single-transgenic TH littermates (Table II and Fig. 2). In fact, double staining with IgM^+ allotype-specific Ab (which detect the TH chain) and biotylated MOG reveals a tight correlation between IgM surface density and MOG binding intensity in THk^mog neonates (Fig. 2, top). This particular staining pattern, which was found in THk^mog, but much less in TH pups, indicates a B cell population expressing both transgenic Ig H- and L-chains exclusively.

**Table I. Ratio of B220^+IgM^+ to B220^+IgM^- cells in neonatal immune organs (mean ± SE)**

<table>
<thead>
<tr>
<th></th>
<th>Liver</th>
<th>Spleen</th>
</tr>
</thead>
<tbody>
<tr>
<td>THk (n = 5)</td>
<td>2.02 ± 0.39</td>
<td>6.79 ± 0.46</td>
</tr>
<tr>
<td>TH (n = 5)</td>
<td>1.02 ± 0.07</td>
<td>5.28 ± 0.30</td>
</tr>
<tr>
<td>wt (n = 1)</td>
<td>0.66</td>
<td>2.27</td>
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</tbody>
</table>

* wt, wild type.

**Table II. Ratio of IgM^+MOG^+/IgM^−MOG^- MOG-binding to non-binding cells in neonatal immune organs (mean ± SE)**

<table>
<thead>
<tr>
<th></th>
<th>Liver Day 1</th>
<th>Spleen Day 1</th>
<th>Spleen Day 6</th>
</tr>
</thead>
<tbody>
<tr>
<td>THk (n = 5)</td>
<td>0.89 ± 0.14</td>
<td>0.78 ± 0.05</td>
<td>0.33 (n = 2)</td>
</tr>
<tr>
<td>TH (n = 5)</td>
<td>0.31 ± 0.01</td>
<td>0.39 ± 0.02</td>
<td>0.32 (n = 2)</td>
</tr>
</tbody>
</table>

The proportion of MOG-binding B cells within this IgM^+MOG^+ population is higher in the liver than in the spleen of neonatal THk^mog mice (Fig. 2, top, and Table II). This distinctive B cell phenotype prevailed in perinatal THk^mog mice, whereas it decreased in the spleen within the first 6 days after birth. By then, the MOG-binding pattern of splenic B cells is indistinguishable in THk^mog and TH mice and comparable to the phenotype of B cells in adult transgenic mice (Table II).

To identify the transgenic L-chains during perinatal development, we separated neonatal liver cells into MOG-binding and -nonbinding B cell fractions and analyzed their L gene transcripts by RT-PCR. As shown in Table III, in MOG-binding B cell populations the transgenic k^mog sequence was predominantly expressed (representing 10 of 12 analyzed Ig k sequences), whereas nonbinding B cells preferentially used endogenous L genes (only 4 k^mog sequences found in 36 Ig k sequences analyzed). The identity of the k^mog transcripts was confirmed by sequencing (not shown). These data, together with the Ig k sequences found in fetal liver B cells of TH mice (Table III), demonstrate that predominant expression of k^mog in the MOG^+ fraction of THk^mog fetal liver is not due to an unspecific preference of similar k sequences within this B cell compartment. We conclude that in neonatal THk^mog mice, the transgenic k^mog L-chain actively suppress endogenous L gene expression by allelic exclusion. During further development, however, the transgenic k^mog L-chain is replaced by endogenous L-chains, presumably through receptor editing.

**RAG expression in IgM^+ B cells from neonatal double-transgenic THk^mog liver**

The concept of receptor editing implies that encounter of immature IgM-positive B cells with a self-Ag induces secondary Ig gene rearrangements through reactivation of Ig-recombining genes like...
RAG-1 and RAG-2 (25–27). To learn whether receptor editing was involved in replacing κ<sup>mog</sup> by endogenous L-chains, we analyzed sorted, IgM<sup>+</sup> MOG-binding B cells from neonatal liver of TH<sup>k</sup>mog and TH mice for expression of RAG genes (Fig. 3). As in normal B cell differentiation, where the appearance of surface IgM coincides with a shutdown of the rearrangement machinery, we detected neither RAG-1 nor RAG-2 in the IgM<sup>+</sup> MOG-binding cells from single-transgenic TH neonatal liver (Fig. 3). In contrast, both RAG-1 and RAG-2 transcripts were detected in the MOG-binding cell fraction from neonatal double-transgenic TH<sup>k</sup>mog liver (Fig. 3). Transcription of RAG-1 and RAG-2 was also detected in the excluded fractions from both TH<sup>k</sup>mog and TH mice, given that this fraction includes surface Ig-negative pre-B cells, actively rearranging endogenous L-chains. Persistent or reactivated Ig rearrangement in IgM-positive B cells of neonatal TH<sup>k</sup>mog mice explains the observed expression of endogenous L genes. Receptor editing in such early TH<sup>k</sup>mog-transgenic B cells leads to silencing of the transgenic κ<sup>mog</sup> gene and expression of endogenous L genes. However, like in single-transgenic TH mice, this process does not abrogate MOG specificity totally, because about one-third of endogenous L-chains generate MOG binding on pairing with the TH chain (Fig. 1 and Table II).

**Transgenic TH<sup>k</sup>mog B cells on a RAG-2-deficient background**

What would be the destiny of self-reactive transgenic B cells without rescue by receptor editing? We crossed the RAG-2 knockout mutation into the TH<sup>k</sup>mog-transgenic mice and followed the appearance of B lymphocytes in these animals. As expected, IgM<sup>+</sup> B cells are completely absent both in the adult parental RAG<sup>2</sup>−/− (28) and in TH/RAG<sup>2</sup>−/− mice (Fig. 4). In contrast, in TH<sup>k</sup>mog/RAG<sup>2</sup>−/− mice, a small but significant population (bone marrow) of B220<sup>+</sup>IgM<sup>+</sup> B cells is reproducibly observed in both bone marrow and spleen (Fig. 4, marked region of upper panels). Interestingly, all of these B220<sup>+</sup>IgM<sup>+</sup>IgD<sup>−/low</sup> B cells bind MOG in the “monospecific” staining pattern also seen in neonatal liver cells of TH<sup>k</sup>mog mice (Figs. 2 and 4, middle panels). Judging from their binding of the recently described mAb 493, a further marker of immature B lymphocytes (20) (Fig. 4), excluding the possibility that these cells represent a special form of mature, functionally inactivated, anergic B cells (29). As shown in Fig. 5, IgM<sup>+</sup>493<sup>+</sup> mature B cells are not present in the spleen of TH<sup>k</sup>mog/RAG<sup>2</sup>−/− mice and the small population of IgM<sup>493</sup> B cells is absent in the RAG<sup>2</sup>−/− nontransgenic littersmates.

In TH<sup>k</sup>mog/RAG<sup>2</sup>−/− mice, in which rearrangement of endogenous Ig genes is blocked, the B cells rely on the transgenic Ig chains to sustain development. In this situation, the transgenic B cells are clearly unable to proceed to full maturation and are blocked at the immature stage defined by the B220<sup>+</sup>IgM<sup>low</sup>IgD<sup>−/low</sup> phenotype. These immature B cells in the bone marrow...
FIGURE 5. Flow cytometry analysis of bone marrow (top) and spleen cells (bottom) with the mAb 493. RAG-positive THs\textsuperscript{mo\textsubscript{g}}-transgenic mice are compared with RAG2-deficient THs\textsuperscript{mo\textsubscript{g}}-transgenic and -nontransgenic (non-tg.) mice. Double staining with anti-IgM (a or b allotypes) and 493 mAb demonstrates the presence of IgM\textsuperscript{493} immature B cells but the absence of IgM\textsuperscript{493} mature B cells in THs\textsuperscript{mo\textsubscript{g}} RAG2\textsuperscript{+/-} mice. Both subpopulations are found in THs\textsuperscript{mo\textsubscript{g}} RAG2\textsuperscript{+/-} with predominance of IgM\textsuperscript{493} B cells in the spleen. Nontransgenic RAG-2-deficient mice do not contain IgM-positive cells.

and the spleen of THs\textsuperscript{mo\textsubscript{g}}/RAG-2\textsuperscript{-/-} mice cannot proceed in further differentiation (Figs. 4 and 5). In RAG-2-sufficient THs\textsuperscript{mo\textsubscript{g}} mice, in contrast, expression of the transgenic $\kappa$\textsuperscript{mo\textsubscript{g}} gene is abolished by editing, and development can reach the fully mature stage of B220$^+$ IgM$^+$ IgD$^+$ cells found in peripheral immune organs (Figs. 1B and 5).

MOG gene expression does not affect differentiation of MOG-specific transgenic B cells

The present results suggest that the transgenic, MOG specific B cells are affected by self-tolerizing processes that result in receptor editing. However, they do not reveal the nature of the censoring self-determinant involved in tolerization. To explore the possible participation of the MOG gene products, we crossed TH and THs\textsuperscript{mo\textsubscript{g}} mice into a MOG knockout mutant, i.e., completely devoid of MOG protein (Fig. 6A). In the absence of MOG, the phenotype of MOG binding B cells in TH and THs\textsuperscript{mo\textsubscript{g}} mice is identical with that observed in MOG-sufficient animals (Fig. 6B, top). This experiment also excludes a possible editing of the TH gene, as the number of Id-positive B cells in the MOG$^{-/-}$ backcross is comparable with that found in MOG-expressing mice (Fig. 6B, bottom). Thus, MOG per se cannot be responsible for the tolerization of the transgenic specificity in THs\textsuperscript{mo\textsubscript{g}} mice.

Discussion

We have conducted experiments designed to explore whether (and which) mechanisms of self-tolerance could affect the development of B lymphocytes specific for a brain myelin autoantigen, MOG. The paradigm used was a transgenic gene replacement mouse carrying in its genome the rearranged Ig genes of the MOG-reactive hybridoma 8.18C5. Previous work had demonstrated that in single-transgenic mice the 8.18C5-derived Ig H-chain is expressed along with endogenous L-chains without any apparent impairment and that this IgH chain alone confers MOG specificity in about one-third of the B cells (4). We now report that, in striking contrast, in double-transgenic mice carrying both the Ig H and L genes of 8.18C5 (THs\textsuperscript{mo\textsubscript{g}}), the expression of the transgenic $\kappa$\textsuperscript{mo\textsubscript{g}} gene is not permitted but is replaced by endogenous L-chains.

Ig receptor editing on contact with self-determinants is one of the cardinal mechanisms to generate and maintain immunological self-tolerance on the B cell level. To date, it has been mainly been studied in immature B cells arising in the bone marrow of adult mice, leaving neonatal B cell tolerance less well characterized (30). In our system, in neonatal liver and spleen of double-transgenic THs\textsuperscript{mo\textsubscript{g}} mice, a high proportion of B cells expressed the transgenic B cell receptor, as indicated by the IgM/MOG double staining pattern characteristic of monospecific B lymphocytes. As may be expected, the MOG-binding B cells almost exclusively expressed $\kappa$\textsuperscript{mo\textsubscript{g}} gene transcripts, showing that at this developmental stage the cells are not yet completely tolerized. However, the expression of RAG-1 and RAG-2 genes in this cell subset points to an ongoing process of receptor editing. In contrast, neonatal B cells, which did not bind MOG, predominantly expressed endogenous L-chains, indicating replacement of the transgenic $\kappa$\textsuperscript{mo\textsubscript{g}} chain as a result of editing.

The molecular nature of the mechanism(s) responsible for obliteration of the transgenic $\kappa$\textsuperscript{mo\textsubscript{g}} chain in THs\textsuperscript{mo\textsubscript{g}} mice remains speculative. In an anti-DNA Ig transgenic model, Gay et al. (16) proposed that editing would occur at the level of Ig H- and L-chain pairing. Our system seems to rely primarily on transcriptional down-regulation, as indicated by the predominance of endogenous Igc transcripts over transgenic $\kappa$\textsuperscript{mo\textsubscript{g}} transcripts in edited THs\textsuperscript{mo\textsubscript{g}}-transgenic B cells. Editing of the Ig receptor in THs\textsuperscript{mo\textsubscript{g}} mice affects only the $\kappa$\textsuperscript{mo\textsubscript{g}} L-chain, although replacement inactivation of the knocked-in TH gene would also alter the transgenic specificity, and can be demonstrated upon immunization of TH mice (31). Yet the Id encoded by the TH transgene is maintained in double-transgenic THs\textsuperscript{mo\textsubscript{g}} B cells in a proportion similar to that in single-transgenic TH B cells (Fig. 6 and Ref. 4). This type of secondary rearrangement has been described also in other IgH knock-in mice.

FIGURE 6. The frequency of MOG binding THs\textsuperscript{mo\textsubscript{g}}-transgenic B cells is not changed in the MOG-deficient genetic background. A, Western blot analysis of purified, recombinant (rec.) MOG protein (2 $\mu$g) and brain protein extracts (30 $\mu$g) of wild-type (WT) and MOG knockout (KO) mice, probed with anti-MOG mAb 8.18C5. Detection of actin was used as loading control. Numbers on the left indicate relative migration of m.w. standards. B, Flow cytometry of splenocytes derived from MOG-expressing (THs MOG$^+$) and MOG-deficient THs\textsuperscript{mo\textsubscript{g}}-transgenic (THs MOG$^{-}$) mice shows a similar proportion of MOG-binding IgM-positive B cells (top). Double staining with anti-IgM and Id-specific Ab VH8.18 (bottom) identifies the transgenic variable region of the heavy chain.
The lack of an enhanced MOG-binding fraction of B cells in THk\textsuperscript{mog} double-transgenic mice, as compared with TH single-transgenic animals, could alternatively be interpreted to indicate imperfect fit of the transgenic k\textsuperscript{mog} L-chain with the knock-in TH chain. This could result in insufficient allelic exclusion of endogenous L-chains and/or lack of positive selection of the transgenic Ig pair. Our experimental observations, however, clearly contradict this interpretation. First, in the MOG\textsuperscript{+} fraction of fetal liver and in the RAG-deficient background, the transgenic k\textsuperscript{mog} is able to form disulfide bonds with the TH chain and be efficiently anchored onto the surface of IgM\textsuperscript{+} B cells (Figs. 2 and 4). The concentrations of surface IgM on these cells is comparable with that of normal cells at the same developmental stage (Figs. 2 and 4). Second, we show that early B cells in fetal liver of double-transgenic mice include a large fraction (the MOG-binding one) in which the transgenic k\textsuperscript{mog} chain is expressed almost exclusively. As shown in Table III, \(>80\%\) of expressed L-chains correspond to the transgenic k\textsuperscript{mog} chain. This percentage is reduced to \(11\%\) in the MOG-nonbinding fraction, as a result of the action of receptor editing on the MOG-binding fraction. Taken together, 14 of 48, or \(\sim30\%\), of all k chains expressed in THk\textsuperscript{mog} fetal liver are of transgenic origin. Interestingly, in adult spleens of k\textsuperscript{mog} single-transgenic mice, we find a similar proportion of k\textsuperscript{mog} transcripts (32\%), as revealed in similar RT-PCR analysis of L-chain usage, whereas in THk\textsuperscript{mog} adult spleens this proportion is further reduced to 7\% (data not shown). These data reinforce the interpretation that the combination of TH and k\textsuperscript{mog} chains generates a nonpermitted autospecificity, without excluding the possibility that the k\textsuperscript{mog} chain by itself is autoreactive.

Third, during B cell development, two obligatory checkpoints have been defined, at which B cells are positively selected based on structural requirements. In early, so-called pre-B II cells, newly formed \(\mu\) H-chains are tested for appropriate binding to surrogate L-chains and formation of the pre-B receptor complex (pre-BCR). Successful pre-BCR formation is followed by inhibition of H gene rearrangement, extensive proliferation, and, once proliferation ceases, by rearrangement of L-chain genes (see Ref. 36 for review). Now the surrogate L-chains are substituted by newly generated L-chains, and B cells must pass the second check point for selection, as H + L chain (IgM) complexes are checked for adequate binding and surface deposition. Only B cells are now selected that express a good fitting pair of Ig's composed of \(\mu\) and a L-chain and able to form membrane-bound IgM. B cells unable to express a BCR on their surface undergo rapid apoptosis (Ref. 36 and references therein). Such “immature,” IgM\textsuperscript{+}IgD\textsuperscript{−} B cells can now leave the bone marrow and colonize the spleen, where they develop into IgM\textsuperscript{+}IgD\textsuperscript{−} mature B cells, provided that they do not react against self-components. This last transition is thus governed by negative rather than positive selection principles. On the basis of this model, we interpret the appearance of IgM\textsuperscript{+}IgD\textsuperscript{−}\textsuperscript{−}MOG\textsuperscript{+} B cells in the spleen of THk\textsuperscript{mog}-RAG\textsuperscript{−/−} mice as demonstration of successful positive selection of the transgenic BCR and its subsequent negative selection at the stage of “transitional” B cells. The principle governing the transition from IgM\textsuperscript{+}IgD\textsuperscript{−} into IgM\textsuperscript{+}IgD\textsuperscript{−} B cells is not yet understood. Thus far, no experimental evidence exists to invoke a positive principle as being active at this stage.

Recently, Melamed et al. (18), studying B cell development in cultures of adult bone marrow, have identified the B220\textsuperscript{low} IgM\textsuperscript{low}IgD\textsuperscript{−} subset of immature B cells, where receptor editing occurs during B cell differentiation. According to this model, immature B220\textsuperscript{low}IgM\textsuperscript{low}IgD\textsuperscript{−} B cells that fail to alter their self-reactive specificity by editing during a limited time window will be eliminated by apoptotic deletion at the following B220\textsuperscript{high}IgM\textsuperscript{high}IgD\textsuperscript{+} stage. Because B cells in THk\textsuperscript{mog} mice develop into sIgM\textsuperscript{+}IgD\textsuperscript{low} B lymphocytes and because we observe editing of the k\textsuperscript{mog} chain at this stage in fetal liver, we conclude that receptor editing abolishes a self-reactive specificity distinct from MOG, as given by the combination of the two transgenic Ig chains.

Why are MOG-binding B cells that simultaneously express both transgenic specific Ig H- and L-chains censored while at the same time MOG-binding B cells using the same Ig H-chain but different L-chains develop normally? It appears that an unknown self-structure present in the B cell compartments controls initiation of editing. MOG is not expressed outside the CNS (9). Although we assume that in wild-type as well as in double transgenic THk\textsuperscript{mog} mice, the formation of the MOG-specific repertoire occurs in the absence of the nominal Ag, this is definitely the case in MOG knockout mice (Fig. 6). There is no difference in MOG-specific B cell development between MOG-expressing and MOG-deficient mice. Indeed, all our data indicate that the MOG-specific B cell receptor in THk\textsuperscript{mog} double-transgenic B cells is confronted with a self-Ag distinct of MOG and that this encounter is responsible for the induction of tolerance. The nature of the self-Ag(s) cross-reacting with MOG is unknown. Preliminary attempts to detect putative autoantigens in tissue preparations using the 8.18C5 Ab failed. However, it is known that B cell receptor editing can be induced by very low affinity interactions that may be difficult to measure (37, 38). We are currently testing butyrophilins, a large protein family that, apart from MOG, contains numerous milk proteins and costimulatory molecules of the B7 series (39–41).

Considering the highly efficient tolerization of double-transgenic THk\textsuperscript{mog} B lymphocytes in vivo, the derivation of the original hybridoma 8.18C5 appears enigmatic. At least in theory, the hybridoma precursor B cell should have been edited in the donor mouse, just as the double-transgenic B cells in our experiments are edited. Alternatively, the original B cell may have acquired its MOG/self specificity at a mature stage, after immunization. However, as stated before, the V region of the k\textsuperscript{mog} transgene represents an unmutated V\(\kappa\)-8 gene, identical with a V\(\kappa\) gene previously found in a phosphorylcholine-specific hybridoma (19). In addition, the only bona fide somatic mutation found in the V region of the TH gene corresponds to a silent point mutation (ACT-Thr\rightarrow ACG-Thr) within the rearranged DSP2.7 element (4). Therefore, extensive somatic mutation seems not to explain the heteroclitic specificity associated with the nominal MOG specificity of 8.18C5.

Alternatively, the autoreactive B cell clone could have been derived from the B-1 subset of lymphocytes, a population of mainly peritoneal B cells enriched for self-reactivity. Our finding that TH (and THk\textsuperscript{mog}) mice are depleted of peritoneal B-1 cells and that the few B-1 cells left in these mice express preferentially endogenous Ig's may argue against this possibility (Ref. 4 and data not shown). Finally, some B cells expressing autoreactive IgG but not IgM are viable and provoke autoimmunity in transgenic mice (42, 43). There is the possibility that under the conditions of repeated immunization, rare, autoreactive, MOG-specific B cell clones, like 8.18C5, sneaking through tolerogenesis in bone marrow were recruited to undergo isotype switch and escape tolerance.

Our data show that the developing immune system is able to distinguish between self-specificity defined by the TH chain alone (MOG), which is not recognized and censored outside the CNS, and a second self-specificity given by the combination of the TH chain with this particular k\textsuperscript{mog} L-chain. We propose that through this second specificity THk\textsuperscript{mog} B cells bind to an autoantigen cross-reactive with MOG and become efficiently tolerized via editing of their transgenic L-chain without eliminating the MOG.
binding potential. This particular form of chain-specific split tolerance within B cells results in divergent tolerance and persistence of an organ-specific, potentially pathogenic autoreactive clone.

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References