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Gene Therapy Delivery of Myelin Oligodendrocyte Glycoprotein (MOG) via Hematopoietic Stem Cell Transfer Induces MOG-Specific B Cell Deletion

Jie-Yu Chung,* William Figgett,* Kirsten Fairfax,* Claude Bernard, † James Chan, ‡ Ban-Hock Toh, ‡ Fabienne Mackay,* and Frank Alderuccio*

The various mechanisms that have been described for immune tolerance govern our ability to control self-reactivity and minimize autoimmunity. However, the capacity to genetically manipulate the immune system provides a powerful avenue to supplement this natural tolerance in an Ag-specific manner. We have previously shown in the mouse model of experimental autoimmune encephalomyelitis that transfer of bone marrow (BM) transduced with retrovirus encoding myelin oligodendrocyte glycoprotein (MOG) promotes disease resistance and CD4+ T cell deletion within the thymus. However, the consequence of this strategy on B cell tolerance is not known. Using BM from IgHMOG mice that develop MOG-specific B cell receptors, we generated mixed chimeras together with BM-encoding MOG. In these animals, the development of MOG-specific B cells was abrogated, resulting in a lack of MOG-specific B cells in all B cell compartments examined. This finding adds a further dimension to our understanding of the mechanisms of tolerance that are associated with this gene therapy approach to treating autoimmunity and may have important implications for Ab-mediated autoimmune disorders. The Journal of Immunology, 2014, 192: 000–000.

The challenge of treating autoimmune disease has initiated a range of new and novel approaches such as autologous hematopoietic stem cell transplantation (HSCT), which is currently being trialled in a range of disorders including multiple sclerosis (MS) (1–3). The approach is based on the isolation and subsequent transfer of a patient’s stem cells following a preconditioning regimen designed to clear existing pathogenic clones. Although this approach appears to have beneficial outcomes for many patients, some patients display relapse and disease progression (3–6), suggesting room for improvement. In our murine studies of experimental autoimmune encephalomyelitis (EAE), we also observe that treatment of established EAE with HSCT can ameliorate disease. However, upon rechallenge, mice relapse, suggesting that tolerance has not been established (7). We have shown that by adapting this strategy to introduce ectopic expression of autoantigen, we can promote robust immune tolerance that will maintain mice in remission following treatment of established disease (7). By understanding and harnessing the mechanisms of protection associated with this strategy, potential for translation to humans may lead to improvement in the current protocols.

The adaptive arm of the immune system protects the body against foreign pathogens in an Ag-specific manner through the generation of lymphocytes expressing randomly generated TCRs and BCRs (8). Although this process allows for almost unlimited receptor specificities, there is a risk that lymphocytes will recognize self-Ags, with potential activation leading to immune responses against self and subsequent autoimmune pathology. To counter this, developing T and B lymphocytes are exposed to a wide range of self-Ags so that self-reactive cells can be negatively selected and removed from the immune repertoire (9). Within the B cell compartment, tolerance can also extend to include the functional silencing of anergy or receptor editing to salvage nonfunctional or self-reactive clones (10–12). The importance of self-Ag exposure to immune regulation is highlighted in animals and humans deficient in the transcription factor autoimmune regulator element (AIRE), which drives promiscuous gene expression in medullary thymic epithelial cells (13). The lack of AIRE and associated self-Ag expression results in multiorgan autoimmune disease induction in both mice and humans (14, 15). However, even individuals with functional AIRE are still susceptible to autoimmunity, suggesting this system is not perfect and that self-reactive T cell clones can escape negative selection if the interaction with their cognate self-Ag is untested or not sufficient to promote deletion (16).

Artificial introduction of self-Ags into the immune environment raises the possibility of influencing the development of self-reactive immune cells associated with autoimmune disease. To support this view, direct injection of Ags into thymi of mice has been shown to confer transient tolerance (17) and builds upon the very early studies of Waksman and colleagues (18, 19), commenced in the 1960s, demonstrating that thymic exposure to both neoantigen and spinal cord homogenate could influence the development of thymocytes. Furthermore, constitutive Ag exposure in transgenic animals ectopically expressing self-Ag under the MHC class II...
promoter provides long-term tolerance, as demonstrated in mouse models of experimental autoimmune gastritis (20) and type 1 diabetes (21). Importantly, tolerance in these models could be transferred by transplanting bone marrow (BM), thus highlighting the clinical potential (22, 23).

Our approach to the treatment of autoimmunity with HSCT is augmentation by introducing self-Ag exposure through retroviral transduction of BM cells, thus introducing a tolerance-promoting step to the standard HSCT treatment (24). Using this strategy, mice given BM transduced with retrovirus encoding the self-Ag myelin oligodendrocyte glycoprotein (MOG) are protected against induction of EAE (25). This strategy has also been shown with BM-encoding proteolipid protein and the prevention of proteolipid protein–induced EAE (25), transfer of BM-encoding proinsulin II, and reduction in insulin in type 1 diabetes–prone NOD mice (26).

This highlights the possibility and effectiveness of introducing Ags into the system through the BM compartment as a therapeutic procedure for treating autoimmune diseases (27). In mice, this protocol has been shown to enhance negative selection of developing self-reactive MOG-specific CD4+ T cells in the thymus (7). Combined with a course of prednisolone to initially promote remission (28), the transfer of transduced BM can also be used to cure existing EAE and prevent relapses, even following remission (28), the transfer of transduced BM can also be used (7). Combined with a course of prednisolone to initially promote remission (28), the transfer of transduced BM can also be used to cure existing EAE and prevent relapses, even following remission (28), the transfer of transduced BM can also be used (7). Combined with a course of prednisolone to initially promote remission (28), the transfer of transduced BM can also be used (7).

Materials and Methods

Mice

Female C57BL6 (Ly5.1 or Ly5.2), IgH MOG (Ly5.2), and SW HEL (Ly5.2) mice were housed in specific pathogen-free environments at Precinct Animal Centre, Alfred Medical Research & Education Precinct. All experiments were conducted in accordance with local ethics guidelines. The generation and characterization of IgHMOG mice has previously been described (37). SWHEL mice on a C57BL6/6 background have been previously described (38) and are homozygous for the Vh10 anti–hen egg lysozyme (HEL) H chain V region coding exon, as well as the VH10-8 anti–HEL L chain transgene.

Generating MOG and HEL retroviral vectors

pMYs–MOG-IG retroviral vector encoding MOG was generated as previously described (7). Membrane-bound HEL from pIRES-mHELWT (39) was excised and inserted into pMYs-IG using NotI and BamHI to create pMYs-mHEL-IG. Retroviral vectors also encoded GFP driven by an internal ribosome entry site to enable tracking and identification of property cells. Retrovirus was generated by transfecting viral producer cell line BOSCe23 and collecting supernatant containing retroviral particles at 48, 72, and 96 h posttransfection. Supernatants were filtered through a 22-μm filter, snap frozen on dry ice, and stored at −80°C. Thawed retroviral supernatant was titrated by transducing NIH3T3 fibroblast cell lines using serial one in five dilutions and analyzing at 48 h by flow cytometer for the percentage of cells expressing GFP. Retroviral titers were routinely ≥1×10^5 infectious units/ml, and supernatants were used neat for transduction of BM cells.

Donor BM collection and transduction

Prior to BM harvest, C57BL6 Ly5.1 BM donor mice were injected i.p. with 150 mg/kg 5-fluorouracil (Hospira). Four days later, mice were killed and BM flushed from femur and tibia bones with ice-cold PBS. RBCs were lysed using RBC lysis buffer (Invitrogen) and BM cells cultured in complete DMEM (Life Technologies) with 50 ng/ml stem cell factor (R&D Systems) and 10 ng/ml IL-6 (R&D Systems) and 10% FCS and analyzed on an LSRII flow cytometer (BD Biosciences).

To identify B cells expressing MOG-specific Ig receptors on the cell surface, recombinant MOG (MOG) protein was generated as previously described (40) and biotinylated using the Fluoreporter mini-biotin-xx protein labeling kit (Invitrogen) as per the manufacturer’s instructions. Flow cytometry was used to analyze cell populations expressing MOG-binding Ig. One million cells were stained with biotinylated MOG were incubated with streptavidin-allophycocyanin-Cy7 in 50 μl PBS containing 1% FCS for 30 min. Stained cells were washed three times with 200 μl PBS/1% FCS and analyzed on an LSRII flow cytometer (BD Biosciences).

Flow cytometric analysis of rMOG-binding B cells

Single-cell suspensions of lymphoid organs (spleen, BM, and inguinal LNs) were prepared by using a cell scraper at a density of 2.5×10^7 cells/ml in 24-well plates at 37°C, 5% CO2. BM cells were stained with infected NIH3T3 fibroblast cell lines, at 4°C for 30 min. To detect biotinylated MOG, cells were incubated with streptavidin-allophycocyanin-Cy7 in 50 μl PBS with 1% FCS for a further 30 min. Stained cells were washed three times with 200 μl PBS/1% FCS and analyzed on an LSRII flow cytometer (BD Biosciences).

SWHEL B cell transfer

Single-cell suspensions of splenocytes from SWHEL were prepared, and B cells were enriched and negatively isolated using the Dynabeads CD43 B cell isolation kit (>95% purity; Invitrogen) as per the manufacturer’s instructions. A total of 1.9×10^6 SWHEL Tg B cells in PBS was then transferred via tail vein i.v. injection into nBM, i.MOOG, or rHEL mice. Five days postinjection, recipient mice were killed and spleens recovered.

Flow cytometric analysis of recombinant HEL binding B cells

Single-cell suspensions of splenocytes from SWHEL were prepared, and 5 million cells were incubated with 37 ng/ml recombinant HEL (RHEL; Sigma-Aldrich) for 30 min at 4°C. Cells were then washed three times with PBS containing 1%
FCS. HEL-binding cells were detected by staining cells with HyHEL9 anti-HEL mAb conjugated to Alexa Fluor 647 using an Ab Labeling Kit (Molecular Probes) as per the manufacturer’s instructions. Viable transferred SWHEL B cells were identified by staining cells with fluorescent-labeled mAbs against IgM (eBioscience), B220/CD45R, Ly5.1, Ly5.2 (BD Biosciences), and LIVE/DEAD Fixable Violet stain (Molecular Probes) in 50 μl PBS plus 1% FCS for 30 min at 4˚C. Stained cells were washed three times with 200 μl PBS/1% FCS and analyzed on an LSRII flow cytometer (BD Biosciences).

Statistical analysis
All data are presented are means ± SEM. The Mann–Whitney test or two-way ANOVA was used to analyze statistical significance, with p ≤ 0.05 considered statistically significant.

Results
Generating mixed BM chimeras
It is well established that the BM compartment is associated with promoting immunological tolerance (41, 42). Using this in the context of autoimmunity, we have adopted the strategy that promoting ectopic expression of autoantigen via BM manipulation can be used to induce specific tolerance (24). The strategy we have used to promote protection from EAE using BM retrovirally transduced with the self-Ag MOG is shown in Fig. 1A (dotted box). This protective effect has been shown to involve negative selection of developing T cells in the thymus, as determined by analyzing mixed chimeras generated from MOG-transduced BM and BM from 2D2 (MOG35–55-specific) TCR-transgenic mice (7). To extend our understanding of the tolerance induced by the ectopic expression of MOG and if it also includes the B cell compartment, we have used BM from IgH MOG+ -transgenic mice expressing an IgH specific to MOG to track developing MOG-reactive B cells. Using a similar strategy to our 2D2 study, IgHMOG BM was mixed with BM transduced with tMOG, tHEL, or nBM and transferred to C57BL6 recipient mice (Fig. 1A). After 8 wk, splenocytes were analyzed by flow cytometry for immune reconstitution of T and B cells using the congenic Ly5.1 marker and GFP expression for progeny of transduced BM stem cells (Fig. 1B, 1C). Recipient mice were reconstituted at the expected level of ~20% Ly5.1+ cells in both the CD4+ and CD8+ T cell compartments across the various recipient groups (tMOG: 19.2 ± 1.6%; tHEL: 16.0 ± 4.1%; and nBM: 16.0 ± 6.2%) (Fig. 1B). In contrast, reconstitution of Ly5.1+ B cells varied greatly across the recipient groups, with 94% of all B cells in tMOG recipients displaying the Ly5.1 marker compared with 31.2% in tHEL and 48.5% in nBM mice (Fig. 1B). This demonstrates a survival advantage of Ly5.1+B cells in tMOG-recipient mice (Fig. 1B). Analysis of GFP expression associated with the development of cells from transduced BM also confirmed chimerism in lymphocyte populations in tMOG and tHEL mice with none, as expected, in mice that received normal BM or in normal Ly5.2 C57BL/6 mice (Fig. 1C). Analysis of T and B cell development derived from the BM of Ly5.2 IgHMOG mice indicated an absence of B cells in tMOG mice that was not observed in tHEL and normal BM transfer (Fig. 1D).

Abrogation of MOG-specific immature B cell development and recirculating B cells in the BM
To assess what influence ectopic expression of MOG might have on B cell development within the BM, BM cells from reconstituted mixed BM chimeric mice were stained for B cell markers B220, IgM, congenic marker Ly5.2, and biotinylated rMOG to track B cells derived from IgHMOG BM (Fig. 2A). Based on B220 and IgM expression, B220IgM B cell progenitors (PRO), B220IgM+IgM+ immature B cells (IMM), and B220+ IgM+ mature recirculating B cells (RC) (Fig. 2A) were analyzed for binding biotinylated rMOG as indication of successful MOG-specific Ig

![FIGURE 1.](http://www.jimmunol.org/) Generation of mixed BM chimeric mice. (A) BM from Ly5.1+ donor mice is transduced with MOG (tMOG) or HEL (tHEL) retrovirus or nontransduced (nBM) and transferred with Ly5.2 IgHMOG BM into irradiated syngeneic recipient mice to generate mixed BM chimeras. Mixed BM chimeras were killed, and splenic CD4+ and CD8+ T cells and B220+ B cells were analyzed for Ly5.1 chimerism (B) or GFP chimerism (C). Similarly, the proportion of CD4+ and CD8+ T cells and B cells derived from transferred Ly5.2 IgHMOG BM were also analyzed (D). Nonmanipulated mice (Nor) were used as negative controls. n = 6.
generation (Fig. 2B). B220^lo/IgM^- B cell progenitor cells are in the process of rearranging and expressing functional Ig receptors and, as expected, displayed minimal rMOG binding in all recipient mixed chimeric groups (Fig. 2C, Table I). In contrast, B220^lo/+IgM+/hi IMM have successfully rearranged and express their Ig receptors on the cell surface, allowing for detection of MOG specificity through rMOG binding (Fig. 2B).

In this population, we noted that the number of immature B cells capable of binding MOG remained low in tMOG mice and was significantly reduced compared with mixed chimeras generated with control HEL Ag or normal BM (Fig. 2B, 2C, Table I). The level of MOG-reactive immature B cells in tMOG mice was similar to that observed in nonmanipulated normal C57BL/6 mice, which did not receive any IgHMOG BM (Table I).

**Lack of MOG-specific B cells in peripheral lymphoid organs**

To examine the effect on the various B cell populations within the spleen, flow cytometry was used as outlined in Fig. 3A. Developing transitional IMM (B220^+CD93^+) entering the spleen from the BM can be divided into T1 (IgM^-CD23^2), T2 (IgM^-CD23^+), and T3 (IgM^-CD23^-) subsets (Fig. 3A). The degree of rMOG-specific B cells in the T1, T2, and T3 subsets of tMOG mice cotransferred with IgHMOG BM was slightly elevated compared with that observed in unmanipulated Nor (Fig. 3B, 3C, Table I). In contrast, rMOG binding in T1, T2, and T3 cells in mice transferred with normal BM or BM encoding the irrelevant Ag HEL was significantly higher and similar across all populations (Fig. 3B, 3C, Table I).

Also within the spleen, we could analyze mature follicular (FO) CD93^-CD23^-CD21^- and marginal zone (MZ) CD93^-CD23^-CD21^- B cells (Fig. 3A). Mature FO B cells are able to recirculate and populate the periphery, whereas MZ B cells remain in the spleen MZ microenvironment. Both FO and MZ subsets showed a significant decrease in rMOG binding in tMOG mice compared with tHEL and nBM, respectively (Fig. 3B, 3C, Table I) and similar to that observed in normal mice (Fig. 3C, Table I).

The final populations of B cells to be assessed were mature B220^+ MOG-specific B cells in the peripheral blood and LNs (Fig. 4A). rMOG-specific B cells were detected in the LNs and peripheral blood of tHEL and nBM mice cotransferred with IgHMOG BM (Fig. 4B, 4C, Table I). However, the proportion of rMOG-binding B cells was much reduced in tMOG mice and similar to that detected in Nor (Fig. 4B, 4C, Table I).

Overall, these results indicate that IgHMOG B cells, with specificity for the MOG Ag, are developmentally blocked from the IMM stage in the BM onwards, resulting in the loss of mature MOG-specific B cells in the periphery of chimeric mice that have been transferred in tMOG compared with control groups.

**The effect on mature Ag-specific B cells**

The data above have focused on the development of Ag-specific B cells associated with this model and do not specifically address what may be the effect on mature B cells in this chimeric environment that may survive the preconditioning procedure. To address this, we have used the SWHEL B cell transgenic mice with BCR specificity for HEL (43). Mature HEL-specific B cells were detected in the LNs and peripheral blood of tHEL and nBM mice cotransferred with IgHMOG BM (Fig. 4B, 4C, Table I). However, the proportion of rMOG-binding B cells was much reduced in tMOG mice and similar to that detected in Nor (Fig. 4B, 4C, Table I).

In contrast, tMOG binding to RC is further reduced in tMOG mice cotransferred with IgHMOG BM compared with the control groups tHEL and nBM cotransferred with IgHMOG BM (Fig. 2B, 2C, Table I). Again, the degree of rMOG binding was similar to that observed in normal mice (Table I). From these data, we conclude that MOG-specific B cells developing within the BM are capable of being deleted due to ectopic expression of MOG following the transfer of MOG-encoding BM.

**FIGURE 2.** IgHMOG MOG-specific B cells in the BM. Mixed BM chimeras were generated using MOG (tMOG), HEL (tHEL), or nontransduced Ly5.1^+ BM (nBM) plus Ly5.2^+IgHMOG BM. At 8 wk posttransfer, chimeras were killed, and Ly5.2^+ B cell populations in the BM compartment were analyzed for rMOG binding. (A) Gating strategy used to identify PRO (B220^-IgM^-), IMM (B220^-IgM^-), and RC (B220^+IgM^-). rMOG binding for B cell subsets was analyzed by flow cytometry (B) and quantitated for the different cohorts of mice (C). Nonmanipulated mice without BM transfer (Nor) were used as negative controls. n = 6. ***p < 0.001. FSC, Forward light scatter; SSC, side scatter.
analyzed by flow cytometry for rHEL binding (Fig. 5A). Within these B cells, we saw a population of strong HEL binding cells in mice transplanted with normal or MOG-encoding BM (Fig. 5A). In contrast, mice receiving HEL-encoding BM showed a marked reduction in B cell rHEL binding profile (Fig 5A). The significant reduction in rHEL binding (Fig. 5B) was not associated with a reduction in cell numbers, and there was no difference in cell numbers of rHEL binding or non-rHEL binding adoptively transferred cells across the three experimental groups (Fig. 5C). Phenotypic examination of rHEL-binding B cells in mice showed that B cells transferred to mice transplanted with HEL-encoding BM had reduced expression of B220 and IgM and were larger in size compared with rHEL-binding B cells recovered from MOG- or nBM-transplanted mice (Fig 5D).

Table I. The percentage of MOG-specific B cell populations found in the lymphoid compartments of mixed chimeric mice ectopically expressing MOG following the transfer of BM transduced with retrovirus-encoding MOG

<table>
<thead>
<tr>
<th>Lymphoid Compartment</th>
<th>Cell Population</th>
<th>tMOG</th>
<th>tHEL</th>
<th>nBM</th>
<th>Nor</th>
</tr>
</thead>
<tbody>
<tr>
<td>BM</td>
<td>PRO: B220 IgM&lt;sup&gt;+&lt;/sup&gt;IgM&lt;sup&gt;+&lt;/sup&gt;</td>
<td>1.8 ± 0.3</td>
<td>5.6 ± 1.2</td>
<td>3.9 ± 0.2</td>
<td>1.4 ± 0.1</td>
</tr>
<tr>
<td>IMM: B220&lt;sup&gt;+&lt;/sup&gt;IgM&lt;sup&gt;+&lt;/sup&gt;</td>
<td>13.2 ± 1.1</td>
<td>47.3 ± 1.1</td>
<td>35.8 ± 6.1</td>
<td>11.3 ± 0.6</td>
<td></td>
</tr>
<tr>
<td>RC: B220&lt;sup&gt;+&lt;/sup&gt;IgM&lt;sup&gt;+&lt;/sup&gt;</td>
<td>4.9 ± 0.3</td>
<td>62.2 ± 0.5</td>
<td>51.6 ± 1.5</td>
<td>7.5 ± 0.2</td>
<td></td>
</tr>
<tr>
<td>Spleen</td>
<td>T1: IgM&lt;sup&gt;+&lt;/sup&gt;CD23&lt;sup&gt;+&lt;/sup&gt;</td>
<td>10.4 ± 2.7</td>
<td>60.3 ± 4.9</td>
<td>58.2 ± 6.1</td>
<td>7.9 ± 0.5</td>
</tr>
<tr>
<td>T2: IgM&lt;sup&gt;+&lt;/sup&gt;CD23&lt;sup&gt;+&lt;/sup&gt;</td>
<td>18.0 ± 0.3</td>
<td>65.6 ± 2.3</td>
<td>62.5 ± 11.9</td>
<td>11.9 ± 0.6</td>
<td></td>
</tr>
<tr>
<td>T3: IgM&lt;sup&gt;+&lt;/sup&gt;CD23&lt;sup&gt;+&lt;/sup&gt;</td>
<td>15.1 ± 2.3</td>
<td>60.8 ± 0.4</td>
<td>58.7 ± 4.6</td>
<td>8.5 ± 0.5</td>
<td></td>
</tr>
<tr>
<td>FO: CD23&lt;sup&gt;+&lt;/sup&gt;CD21&lt;sup&gt;+&lt;/sup&gt;</td>
<td>1.1 ± 0.1</td>
<td>50.0 ± 0.8</td>
<td>44.1 ± 5.3</td>
<td>2.5 ± 0.4</td>
<td></td>
</tr>
<tr>
<td>MZ: CD23&lt;sup&gt;+&lt;/sup&gt;CD21&lt;sup&gt;+&lt;/sup&gt;</td>
<td>2.1 ± 0.3</td>
<td>64.0 ± 5.7</td>
<td>56.6 ± 13.2</td>
<td>3.1 ± 0.04</td>
<td></td>
</tr>
<tr>
<td>LN</td>
<td>B220&lt;sup&gt;+&lt;/sup&gt;</td>
<td>2.1 ± 0.2</td>
<td>57.1 ± 1.0</td>
<td>52.1 ± 0.5</td>
<td>4.6 ± 0.3</td>
</tr>
<tr>
<td>Peripheral blood</td>
<td>B220&lt;sup&gt;+&lt;/sup&gt;</td>
<td>4.4 ± 1.4</td>
<td>55.4 ± 3.9</td>
<td>57.4 ± 2.0</td>
<td>7.2 ± 0.9</td>
</tr>
</tbody>
</table>

nBM, Mixed BM chimeras receiving nontransduced BM plus BM from Ig<sup>H<sub>MOG</sub></sup>-transgenic mice; Nor, nonmanipulated C57BL/6 mice; tHEL, mixed BM chimeras receiving transduced BM-encoding HEL plus BM from Ig<sup>H<sub>MOG</sub></sup>-transgenic mice; tMOG, mixed BM chimeras receiving transduced BM-encoding MOG plus BM from Ig<sup>H<sub>MOG</sub></sup>-transgenic mice.

**FIGURE 3.** Ig<sup>H<sub>MOG</sub></sup> MOG-specific B cells in the spleen. Mixed BM chimeras were generated using MOG (tMOG), HEL (tHEL), or nontransduced Ly<sup>5.1</sup> BM (nBM) plus Ly<sup>5.2</sup> Ig<sup>H<sub>MOG</sub></sup> BM. Chimeras were killed after 8 wk, and Ly<sup>5.2</sup>B220<sup>+</sup> B cell populations in the spleen were analyzed for rMOG binding. (A) Gating strategy shown for CD93<sup>+</sup>FO (CD23<sup>+</sup>CD21<sup>+</sup>), MZ cells (CD23<sup>+</sup>CD21<sup>+</sup>), and CD93<sup>−</sup> transitional T1 (IgM<sup>+</sup>CD23<sup>+</sup>), T2 (IgM<sup>+</sup>CD23<sup>+</sup>), and T3 (IgM<sup>+</sup>CD23<sup>+</sup>) B cells. rMOG binding for the above B cell subsets is shown in (B) and quantitated for each population across the different cohorts (C). Nonmanipulated mice without BM transfer (Nor) were used as negative controls. n = 6. ***p < 0.001. FSC, Forward light scatter; SSC, side scatter.
Discussion

Meeting the challenge of treating autoimmune disease has taken many approaches, with the use of autologous HSCT being trialed in a range of diseases (44). Although this process can provide benefit to some individuals, the threat of relapse is also present and suggestive that the procedure is not establishing immunological tolerance and long-term protection. Indeed, in EAE, we have shown that in mice with established EAE, treatment with total-body irradiation and syngeneic BM transplantation can promote remission; however, upon rechallenge with Ag, there is rapid relapse (7, 45).

We have found the transfer of genetically modified BM encoding autoantigen has proven to be an effective improvement over the standard BM transplantation therapy. Not only are naive animals protected against induction of EAE following MOG BM transfer, but also animals with pre-existing EAE disease are effectively cured upon MOG BM transfer (7). Our analysis of this model to date attributes this protection and tolerance to the observed thymic deletion of developing MOG-specific thymocytes (7). This is in accordance with the earlier findings of Ally et al. (46), which demonstrated deletion of lymphocytic choriomeningitis virus–specific T cells in mice transplanted with BM encoding lymphocytic choriomeningitis virus. However, B cells also undergo Ag-mediated negative selection, and therefore, the question arises whether the B cell compartment is also affected by the introduction and ectopic expression of autoantigen.

With the availability of IgH^{MOG}-transgenic mice with B cell specificity for MOG, we directly addressed the possibility of using this strategy to examine the induction of B cell deletion in our MOG model. Using BM from IgH^{MOG} mice transgenically expressing IgH receptor specific to MOG and nontransgenic BM transduced with retrovirus encoding MOG, we were able to show in mixed chimeras that IgH^{MOG} (MOG-specific) B cells were being deleted from the IMM stage in the BM compartment, resulting in a complete loss of mature MOG-specific B cells in the periphery. This result is in concordance with a number of earlier studies using neoantigens that demonstrated Ag exposure could promote B cell deletion (47, 48). However, this is the first example, to our knowledge, that has demonstrated the profound effect on B cell deletion that is associated with the procedure of transfer of BM cells transduced with a bona fide autoantigen.

In the context of using this strategy in an established disease setting, the potential fate of any mature B cells that may survive the preconditioning regimen was examined by transferring mature HEL-specific B cells into BM chimeric mice encoding HEL. We found that in an environment of HEL expression, SW_{HEL} B cells were not deleted nor did they expand in numbers. However, within rHEL chimeric mice, there was a marked reduction in B cell binding of HEL accompanied by a reduced expression of surface B220 and IgM expression and an increase in cell size. Although a reduction in IgM expression is associated with anergy (49), reduced B220 and BCR expression and increased cell size are seen when naive cells become plasmablasts (50). The significance of this observation is not clear. The lack of expansion in this population suggests these cells have seen Ag but have not been fully activated. B cells require costimulation for Ab production, and in the absence of T cell help, which is lacking in this model, we believe these cells are likely to represent a transient state that may lead to cell death due to persistent Ag exposure in the absence of T cell help (51) rather than mature plasma cell formation and Ab production. From our published studies with an established EAE model, we do not see MOG Ab production in animals that have received MOG-encoding BM, even after rechallenge with MOG (7), suggesting that if any MOG-specific B cells were still present in the animals, they were not capable of being activated to generate autoantibody. It has been suggested that anergy may exist in range of states for different B cells and may be influenced by Ag structure and presentation but also BCR affinity as illustrated by BCR-transgenic studies comparing neoantigen (HEL)-specific or autoantigen (dsDNA)-specific driven B cell tolerance (52, 53). We may be observing a similar B cell phenotype as that observed by Roark et al. (52), who also reported a nonresponsive B cell population with reduced B220 expression and surface IgM. However, further studies will need to be performed to fully elucidate this. To promote clearance of pre-existing self-reactive B cells, it may be prudent to include B cell–depleting agents such as rituximab that are currently used in a range of human conditions, including the treatment of human autoimmune disease to target CD20^+ B cells (54). In particular,
rituximab has been included in the preconditioning regimen for patients with refractory rheumatoid arthritis undergoing autologous hematopoietic stem cell transfer as a treatment for their disease (55). Thus, the inclusion of B cell depletion in our gene therapy model as part of the preconditioning regimen has feasibility for reducing the possibility of activating chemotherapy resistant B cells. Mature plasma B cells, however, no longer require Ag stimulation for survival or Ab production and as a result downregulate CD20, which leads to resistance to rituximab treatment (56). Mature plasma B cells are highly dependent on their microenvironment for survival factors, and in the BM, this microenvironment is made up primarily of stromal cells and eosinophils, among other immune cells that themselves are susceptible to chemotherapy (57, 58). Therefore, although plasma cells themselves can be difficult to target, the BM lymphopenia associated with chemotherapy preconditioning may itself be enough to ablate the survival niches that plasma cells rely on. However, we are yet to specifically test this and the effectiveness of our strategy on depleting long-lived plasma cells, but this is notionally supported by a previous observation in which MOG-specific autoantibody reactivity is diminished in mice with established EAE, which are subsequently preconditioned and transplanted with genetically manipulated BM (7).

In summary, these results add to our understanding of the effectiveness of deletion that is generated by using the BM compartment. With deletion directed to both T and B cell populations, it provides further impetus in understanding and developing this strategy for treating autoimmunity. In addition, studies such as these indicate that the natural process of immune deletion associated with self-tolerance is not fixed and that it can be manipulated. This provides avenues for exploitation, with the potential of enhancing and correcting failed tolerance associated with many

**FIGURE 5.** Mature SW HEL B cell adoptive transfer. Total of $1.9 \times 10^6$ mature SW HEL Ly5.2$^+$ B cells were adoptively transferred into Ly5.1$^+$ nBM, tMOG, or tHEL mice. Five days post-adoptive transfer, recipients were killed and spleens recovered. (A) Gating strategy to identify rHEL-binding B220$^+$ IgM$^+$Ly5.2$^+$ SW HEL B cells. (B) Mean fluorescence intensity (MFI) of rHEL-binding SW HEL B cells recovered from tHEL mice compared with nBM and tMOG mice. (C) The absolute number of recovered rHEL-binding and nonbinding SW HEL B cells in the spleens of recipient mice. (D) Downregulation of B220 and IgM and increase in cell size in rHEL-binding SW HEL B cells. Solid line: tHEL; dashed line: tMOG; shaded: nBM. $n = 3$. ***$p < 0.001$. FSC, Forward light scatter.
autoimmune disorders. However, a number of key questions remain to be answered, such as whether defining the relevant or key autoantigens within individuals is critical in light of epitope spreading. Although we have demonstrated the ability to promote specific T and B cell deletion, it is not clear if other mechanisms of tolerance are also generated, such as regulatory cells, which may have important implications for controlling immune response to multiple Ags. Also of interest is understanding which of the BM-derived cells, which will be ectopically expressing autoantigen, are responsible for the tolerance we observe. It may be that multiple mechanisms of tolerance are being exploited and explain why the tolerance is so profound. Another important aspect of BM transplants in this setting is the degree of toxicity and mortality associated with the preconditioning regimes (4). Positive inroads are being made on this front, and we have recently shown in our studies that a nonmyeloablative preconditioning regimen can be used to reproduce tolerance to MOG in our EAE model (45). Underpinning this is the observation that only low-level microchimerism is required to promote robust tolerance (45, 59). Taken together, the data that are gradually accumulating surrounding this strategy of using a gene therapy approach to tackling autoimmunity will bolster its significance as a feasible and future option for potential clinical translation.

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References


