Tolerance without Clonal Expansion: Self-Antigen-Expressing B Cells Program Self-Reactive T Cells for Future Deletion[1]

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B cells have been shown in various animal models to induce immunological tolerance leading to reduced immune responses and protection from autoimmunity. We show that interaction of B cells with naive T cells results in T cell triggering accompanied by the expression of negative costimulatory molecules such as PD-1, CTLA-4, B and T lymphocyte attenuator, and CD5. Following interaction with B cells, T cells were not induced to proliferate, in a process that was dependent on their expression of PD-1 and CTLA-4, but not CD5. In contrast, the T cells became sensitive to Ag-induced cell death. Our results demonstrate that B cells participate in the homeostasis of the immune system by ablation of conventional self-reactive T cells. ***The Journal of Immunology,*** **2008, 181: 5748–5759.**

Ample evidence exists to suggest that B lymphocytes have an inhibitory influence on T cells. Fuchs and Matzinger (1) have demonstrated that transfer of male B cells inhibited HY Ag-specific CTL responses. Further, they also demonstrated that activated B cells were potent inhibitory cells of CTL responses. In contrast, naive as well as activated B cells were shown to activate memory T cells rather than tolerizing them (1). Targeting Ags to B cells in vivo also results in tolerance of T cells. For example, injection of mice with rabbit anti-mouse IgD Ab resulted in lower T cell-dependent immune responses to rabbit Ig (2, 3). It was speculated that the anti-IgD Abs reached resting B cells and epitopes of these Abs were presented by B cells leading to tolerance of Ag-specific $CD4^+$ T cells.

B cells were previously shown to exhibit inhibitory function in experimental autoimmune encephalomyelitis (EAE),[3] a T cell-dependent animal model of multiple sclerosis. Hence, rats injected with mouse anti-IgD Abs, coupled to myelin basic protein (MBP) were tolerized to MBP-induced EAE (4, 5). Similar to the experiments of Eynon et al. (2), it was presumed that anti-IgD Abs targeted resting B cells, and due to a lack of costimulatory molecules, these B cells tolerized MBP-specific T cells. Similarly, it was shown that passive transfer of B cells expressing a myelin peptide prevented the induction of EAE (6–8) or even EAE relapses (9). One explanation why B cells induce tolerance of naive but not memory T cells might be the need for expression of costimulatory molecules by the APC to activate naive T cells, specifically B7-1 and/or B7-2, but resting B cells do not express these molecules. On the other hand, memory T cells may not need costimulation and could therefore be activated by B cells. A problem of this hypothesis is that also activated B cells, which normally do express B7 molecules, can induce tolerance of T cells (10). It is therefore not clear whether it is indeed the absence of costimulation what causes B cells to induce tolerance.

To study the role of B cells in tolerance induction we have generated mice that express an MHC class II-restricted immunodominant T cell epitope of myelin oligodendrocyte glycoprotein (MOG) specifically on B cells. These mice were found to be resistant to EAE induction. We could show that, following interaction of naive T cells with B cells presenting their specific Ag, T cells are partially activated, resulting in very marginal proliferation and up-regulation of coinhibitory molecules such as CTLA-4, B and T lymphocyte attenuator (BTLA), PD-1, and CD5. Subsequent in vivo activation of tolerized T cells leads to their deletion. Thus, we assessed that naive B cells induce peripheral tolerance by inducing expression of negative costimulatory molecules by Ag-specific T cells, followed by Ag-induced cell death (AICD) upon the next Ag encounter.

Materials and Methods

Generation of invariant chain (Ii) MOG mice

The targeting vector ROSA26STOP*IiMOG was constructed by introduction into the *Xba*I site of the vector ROSA26–1 (11); a gift from P. Soriano (Fred Hutchinson Cancer Research Center, Seattle, WA) of a fragment comprising (5′ to 3′): adenoviral splice acceptor, *loxP*, 2× SV40 polyadenylation signal, FRT-flanked pGK-*neo*, a STOP cassette (12), *loxP*, mutant invariant chain (termed IiMOG), and bovine poly(A). The mutant invariant chain (IiMOG) was generated by assembly PCR on Ii template cDNA (derived from plasmid pcEX V3 mIi31, carrying the cDNA of the

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Received for publication May 13, 2008. Accepted for publication August 7, 2008.

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[1] This work was funded by the FP6 Marie Curie Research Training Network MRTN-CT-2004-005632, the Deutsche Forschungsgemeinschaft grant SFB548, and by funds from the Böhringer Ingelheim Stiftung (to A.W.).

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[3] Abbreviations used in this paper: EAE, experimental autoimmune encephalomyelitis; MBP, myelin basic protein; MOG, myelin oligodendrocyte glycoprotein; AICD, Ag-induced cell death; Ii, invariant chain; sc, spinal cord; BM, bone marrow; DC, dendritic cell; WT, wild type; Treg, regulatory T cell; c-Flip, cellular FLICE-inhibitory protein; MHCII, MHC class II; BTLA, B and T lymphocyte attenuator.

Ii splice form Ii31 (13), gift from N. Koch(University of Bonn, bonn, Germany)) replacing the coding sequence of CLIP with the one of the MOG peptide 35–55. First, two independent fragments were amplified containing either the 5′ or the 3′ part of Ii (using the external primers LB-Ii (5′-ACATGTAGTACTGGATCCACCATGGATGACCAACGCGACC-3′) and RB-FLAG-Ii (5′-GATATCATTTGTCGTCGTCTTTGTAGTCC AGGGTGACTTGACCCAGTTCCTGCC-3′) and, introduced by the primers ML-Ii and MR-Ii, the sequence of MOGp35–55 (ML-Ii: 5′-CTTG CCATTTCGGTAGAGGTGAACCACTCTTGAGAAGGGAGAACGGT ACCAACCCACCTCCATCTTCATGCGAAGGCTCTCC-3′; ML-Ii/B-FLAG-Ii: 429 base pairs (bp); MR-Ii: 5′-ATGGAGGTGGGTTGGTAC CGTTCTCCCTTCTCAAGAGTGGTTCACCTCTACCGAAATGGCA AGGATAACATGCTCCTTGGGCC-3′; MR-Ii/LB-Ii: 321 bp). The two fragments were assembled by PCR using the external primers LB-Ii and RB-FLAG-Ii thereby generating the IiMOG minigene (688 bp). With the 5′ primer (LB-Ii), a kozak consensus sequence as well as *Sca*I and *Afl*III restriction sites were introduced. Along with the 3′ primer (RB-FLAG-Ii), a FLAG-tag sequence and an *Eco*RV restriction site were added to the 3′ end of the mutant Ii. The two fragments were assembled by PCR using the external primers LB-Ii and RB-FLAG-Ii (688 bp). Eighty micrograms of targeting vector was linearized with *Pvu*I and electroporated into Bruce4 ES cells (14). ES cell culture was performed as described (15). Homologous recombinants were identified by Southern blot analysis using a 700bp genomic *Eco*RI-*Pac*I fragment after *Eco*RI digest (16) (data not shown). Chimeras were generated from two homologous recombinant clones by injection into CB29 blastocysts. Germline transmission was confirmed by Southern blot analysis after *Eco*RI digest using a 1-kb *Sac*II-*Xba*I fragment (probe 1; p1) from pROSA26-1 (17).

Mice

All animal experiments were in accordance with the guidelines of the central animal facility institution (ZVTE, University of Mainz). 2D2 mice were described elsewhere (18). DNA for typing was prepared from tail biopsies. Presence of the different alleles was routinely tested by PCR as published for CD19-Cre (19, 20) and 2D2 mice (18) or using the following primer pairs: for IiMOG/IiMOGΔ (982/456 bp product) 5′-GGC TAC TGC TGA CTC TCA ACA TT −3′ (WSS-F), 5′-ATT TCG GTA GAG GTG AAC CAC TC −3′ (MOG-R), and 5′- CAG GGT TTC CTT GAT GAT GTC A −3′ (Rosa-1). All experiments were performed with 6–12 wk old mice. The experiments were repeated at least once.

Cell preparations and CFSE staining

2D2 $CD4^+$ T cells or B cells were isolated from spleen and lymph node by positive selection using MACS (Miltenyi Biotec) according to the manufacturer's instructions. Purity of the resulting $CD4^+$ T cells or $CD19^+$ B cells was typically >95%. When indicated freshly isolated $CD4^+$ T cells from 2D2 mice were labeled with the vital dye CFSE (Molecular Probes). After washing the cells twice in 10 ml PBS (pH 7.4), they were incubated with 0.5 mM CFSE in 1 ml PBS per 10^7 cells at room temperature for 8 min. To stop the staining reaction, 8 ml RPMI 1640 plus 10% FCS was added. The cells were then washed twice in 10 ml RPMI 1640, resuspended in the appropriate volume of PBS and typically $5–10 \times 10^6$ T cells were injected i.v. into the different mice as indicated.

B and T cell activation

B cells were MACS-purified from B^{MOG} mice using CD19 beads (Miltenyi Biotec). B cells were activated in culture in the presence of anti-CD40 (2.5 μg/ml; FGK45.5) (21), anti-BCR (10 μg/ml; Bethyl Laboratories), and IL-4 (2 μg/ml; R&D Systems) for 4 h at 37°C. B cells were injected i.v. in a concentration of 10×10^6/mouse. To obtain activated 2D2 $CD4^+$ T cells for adoptive transfer, 2D2 mice were immunized as described for active EAE induction, but no pertussis toxin was administered. Ten days after immunization, mice were sacrificed, activated $CD4^+$ T cells from spleen and lymph node were MACS-purified using Va3.2-bio Abs (BD Biosciences), and streptavidin beads (Miltenyi Biotec) and $5–10 \times 10^6$ T cells were i.v. injected into the recipient mice. To generate memory 2D2 $CD4^+$ T cells, activated 2D2 $CD4^+$ T cells were adoptively transferred to MOG-deficient mice (MOGi-Cre homozygous) (22), re-isolated 30 days after transfer using MACS, and the memory phenotype was assessed by FACS analysis ($CD44^+$ $CD62L^{int}$). Three $\times 10^6$ T cells were i.v. injected into the recipient mice.

Flow cytometry

For cytofluorometric analysis, we used Ab conjugates to the following Ags: CD4, CD5, CD25, CD44, CD69, CD103, BTLA, CTLA-4, Fas, Ly6C, PD-1, Tim-3, Thy1.1, Va3.2, and Vb11. Abs were obtained from BD Pharmingen or Natutec (eBiosciences). Intracellular stainings for Foxp3 were performed using the Foxp3 Staining Set (Natutec, eBiosciences) according to the manufacturer's instructions. Anti-Gitr (clone DTA-1) was prepared in our laboratory. Cells from lymphoid organs were stained with the Ab conjugates for flow cytometric analysis on a FACSCalibur or a FACScan (BD Biosciences). Events in a live lymphocyte gate were analyzed with CellQuest (BD Biosciences) software.

Ab treatment

For blockade of CTLA-4 interactions mice were given 0.5 mg of anti-CTLA-4 Ab (23) (4F10; gift from M. van den Broek (University Hospital Zurich, Zurich, Switzerland)) i.p. at the day of T cell transfer and two days later. DCs were activated in vivo by i.p. injection of 50 μg agonistic anti-CD40 Ab (24) (FGK45.5; gift of J. Kirberg (University of Lausanne, Epalinges, Switzerland)) 2 days before T cell transfer. Control mice received PBS.

ELISA

Detection of IFN-γ and IL-17A was performed by ELISA (BD Biosciences) on day 3 supernatants from in vitro restimulated T cell cultures. 2D2 $CD4^+$ T cells were reisolated 5 days after adoptive transfer to wild-type or B^{MOG} mice by MACS using CD90.1-PE Ab (BD Biosciences) and anti-PE beads (Miltenyi Biotec) and cultured with MOG-pulsed wild-type APCs in the absence or presence of MOGp35–55 (10 μg/ml).

RNA analysis

For RNA analysis, total RNA from flow cytometry-sorted cells ($CD4^+Thy1.1^+$) was isolated using the Qiagen mini kit according to the manufacturer's instruction. The expression of mRNA for Caspase-8, FADD, Bim, Bid, Bax, Puma, and c-Flip was analyzed with specific primers from Qiagen as described on their homepage (https://www1.qiagen.com/GeneGlobe/Default.aspx) using the QuantiTect SYBR Green RT-PCR Kit. Expression was normalized to that of the housekeeping gene GAPDH.

Induction and assessment of EAE

$MOG_{35–55}$ peptide (amino acid sequence: MEVGWYRSPFSRVVH-LYRNGK) was obtained from Research Genetics. Active EAE was induced by immunization with 50 μg of $MOG_{35–55}$ peptide emulsified in CFA (Difco Laboratories) supplemented with 8 mg/ml of heat-inactivated *Mycobacterium tuberculosis* H37RA (Difco Laboratories). The emulsion was administered as a 100 μl s.c. injection in the tail base. Mice also received 200 ng of pertussis toxin (Sigma-Aldrich) i.p. on the day of immunization and 2 days later. Passive EAE was induced by injection of MOG-reactive lymphocytes (30×10^6/mouse, generated as described (25). Mice also received 200 ng of pertussis toxin i.p. on the day of immunization and 2 days later. For induction of EAE with spinal cord (sc) homogenate, sc from C57BL/6 mice was isolated and a 1g/1 ml sc in 0.9% (w/v) NaCl homogenate was made by several passages through a syringe with needles of decreasing diameter. The homogenate was emulsified in CFA to a final ratio (v/v) of 1:1. The emulsion was administered as a 100 μl s.c. injection in the tail base. Mice also received 200 ng of pertussis toxin i.p. on the day of immunization and 2 days later. Clinical assessment of EAE was performed daily according to the following criteria: 0, no disease; 1, decreased tail tone; 2, abnormal gait (ataxia) and/or impaired righting reflex (hind limb weakness or partial paralysis); 3, partial hind limb paralysis; 4, complete hind limb paralysis; 5, hind limb paralysis with partial fore limp paralysis; and 6, moribund or dead.

In vivo depletion of $CD25^+$ cells

Endogenous $CD25^+$ cells were depleted from mice 2 days before induction of EAE by i.p. injection with 1 mg of anti-CD25 Ab PC61 (rat IgG1). Control mice received an i.p. injection of 1 mg of MAC49 (rat IgG1, anti-phytochrome). Confirmation of $CD25^+$ cell depletion by PC61 was determined by staining peripheral blood of all mice 2 days after treatment with an Ab that recognizes a different epitope of CD25 (7D4), and it resulted in >90% $CD25^+$ cell depletion (data not shown).

Statistics

Values are presented as mean ± SEM. Statistical significance was assessed using 2-tailed Student's *t* test. p values <0.05 were regarded significant.

Results

A new genetic system to investigate peripheral tolerance

To better understand the in vivo function of naive B cells in the induction of tolerance and regulatory mechanisms, we generated

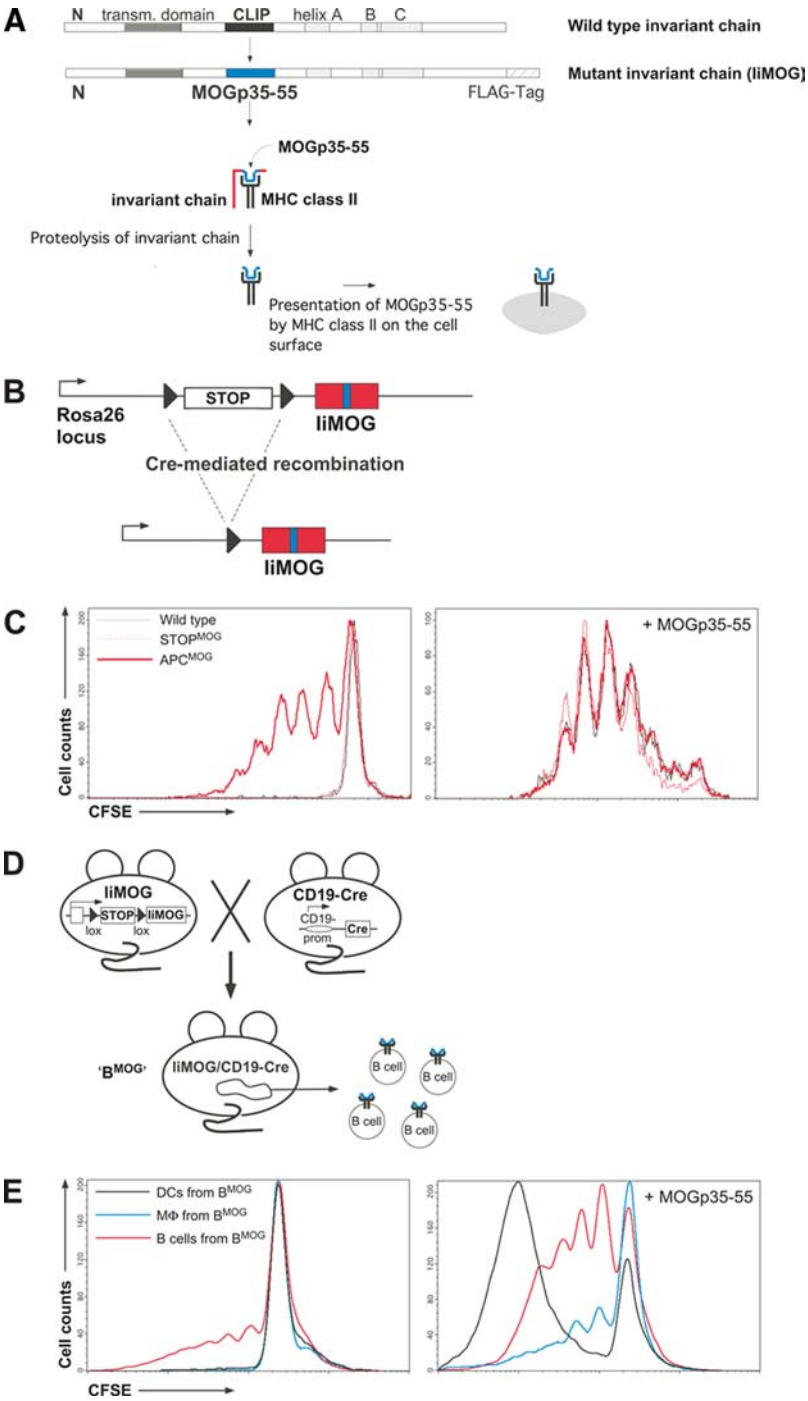

FIGURE 1. *A*, Scheme of the IiMOG system. The CLIP peptide of the invariant chain (Ii) was replaced by the sequence of MOGp35–55. Cleavage of the mutant Ii (IiMOG) leads to the presentation of MOGp35–55 on MHC class II on the cell surface. *B*, General scheme of the IiMOG mouse strain. IiMOG is expressed under the Gt(ROSA)*26Sor*/ROSA26 promoter only upon Cre-mediated removal of a STOP cassette. The STOP cassette, which prohibits IiMOG expression, is removed by crossing the IiMOG strain to a specific Cre-expressing mouse strain. *C*, To prove functionality of the IiMOG system BM-derived DCs from WT, $STOP^{MOG}$, and APC^{MOG} mice were cultured with CFSE-labeled MOG-specific 2D2 $CD4^+$ T cells either with (*right histogram*) or in the absence (*left histogram*) of externally added MOGp35–55. Five days later cells were monitored for proliferation. DCs from the different mice are indicated with different lines in the histogram. *D*, Specific presentation of MOGp35–55 on B cells is achieved by crossing of the IiMOG strain to B cell-specific CD19-Cre mice. *E*, To show specificity of MOGp35–55 presentation by B cells, DCs, Mϕ, and B cells from B^{MOG} mice were cultured with CFSE-labeled 2D2 $CD4^+$ T cells either with (*right histogram*) or in the absence (*left histogram*) of externally added MOGp35–55. The different APCs are indicated with different lines in the histogram. IiMOG, mutant invariant chain (containing MOGp35–55 instead of the CLIP peptide); triangles, *loxP* sites; arrows, transcriptional activity; open ovals, promoter.

a new mouse model that allows the cell-specific presentation of MOGp35–55 on MHC class II. We constructed a mutated invariant chain (Ii) where we replaced the sequence of the CLIP peptide by the peptide sequence of MOGp35–55, resulting in the IiMOG gene (Fig. 1*A*). To express the IiMOG gene in a tissue-specific manner, it was inserted in the ROSA26 locus by homologous recombination as it has previously been shown for the diphtheria toxin receptor gene (26). The IiMOG gene was placed under the control of the ROSA26 promoter following a loxP flanked transcriptional STOP cassette (see schematics in Fig. 1*B*). The STOP cassette prevents expression of the IiMOG gene, as shown previously for the diphtheria toxin receptor gene (26). We crossed the IiMOG knock-in mice to a mouse strain that expresses the Cre-recombinase early in embryogenesis (deleter-Cre) (27), resulting in mice that express the IiMOG gene in all cells including all APCs (mice termed APC^{MOG}). In APC^{MOG} mice, all cells that express MHC class II should present the MOG peptide (see schematics in Fig. 1, *A* and *B*). To determine whether cells isolated from APC^{MOG} mice could present MOGp35–55 after removal of the STOP cassette, we prepared bone marrow (BM)-derived dendritic cells (DCs) from wild type (WT) mice, mice that still contained the STOP cassette in the genome ($STOP^{MOG}$ mice), and from APC^{MOG} mice. These cells were cultured with CFSE-labeled MOG-specific TCR transgenic $CD4^+$ T cells (termed 2D2 $CD4^+$ T cells; Ref. 18). As seen in Fig. 1*C*, only DCs derived from BM of APC^{MOG} mice, but not of WT or of $STOP^{MOG}$ mice could induce proliferation of 2D2 $CD4^+$ T cells. This indicates that the IiMOG-encoded MOG peptide is indeed loaded onto MHC class II molecules and efficiently presented to T cells. In addition, these data show that the STOP cassette

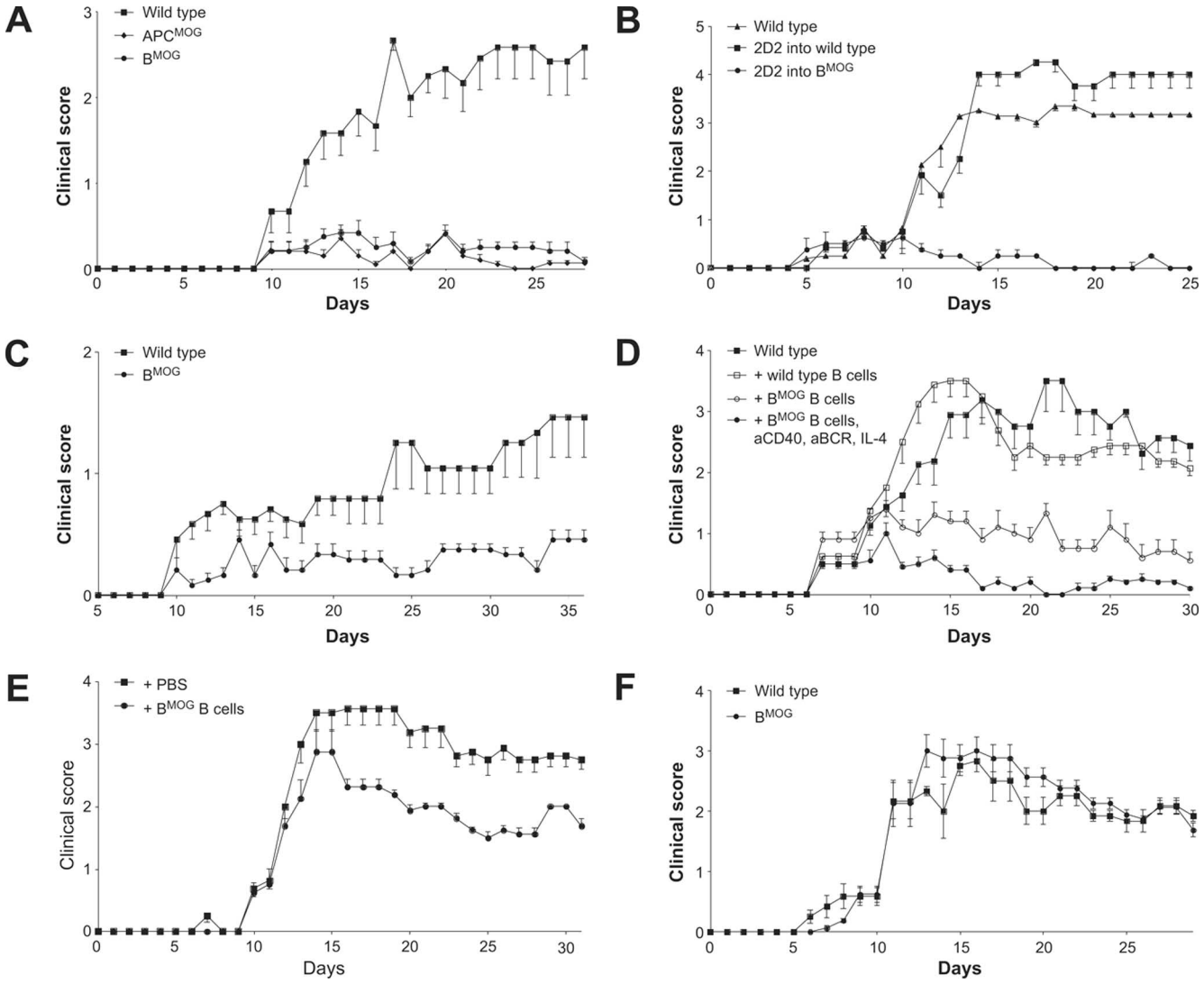

FIGURE 2. Ag presentation by naive B cells induces tolerance to EAE. *A*, EAE was actively induced by immunization of B^{MOG} (●), APC^{MOG} (♦), or WT mice (■) with MOGp35–55. B^{MOG} and APC^{MOG} mice develop significantly less severe EAE compared with WT mice ($p < 0.05$, days 13–28). Shown is one representative of two individual experiments ($n \geq 5$ mice/group). *B*, MOG-specific 2D2 $CD4^+$ T cells were adoptively transferred into B^{MOG} (●) or WT mice (■). After 1 wk, EAE was actively induced by immunization with MOGp35–55 including untreated WT control mice (▲). B^{MOG} mice develop significantly less severe EAE than WT mice ($p < 0.05$). Shown is one representative of two individual experiments ($n = 6$ mice/group). *C*, EAE was induced by adoptive transfer of MOG-reactive lymphocytes into B^{MOG} (●) or WT mice (■). B^{MOG} mice develop significantly less severe EAE than WT mice ($p < 0.05$). Shown is one representative of two individual experiments ($n = 6$ mice/group). *D*, EAE was actively induced in WT mice 2 days after transfer of B cells from WT (□), from B^{MOG} (○), activated B cells from B^{MOG} mice (●), or without transfer of B cells (■). Mice injected with naive or activated B^{MOG} B cells developed significantly less severe EAE compared with untreated WT or WT-B cell-injected mice ($p < 0.05$). *E*, EAE was actively induced in WT mice and 12 days after immunization, B cells from B^{MOG} mice (●) were transferred. Control mice received PBS (■). Mice injected with naive B^{MOG} B cells developed significantly less severe EAE compared with PBS-treated control mice ($p < 0.05$; days 16–32). *F*, EAE was induced in B^{MOG} (●) or WT mice (■) by immunization with spinal cord homogenate. Values are represented as mean ± SEM.

is stringent, not allowing expression of IiMOG before its excision by Cre-recombinase.

Next, the B cell-specific deletion of the STOP cassette was investigated by crossing the IiMOG allele to B lymphocyte-specific CD19-Cre mice (20) (Fig. 1*D*). From double-transgenic mice (termed B^{MOG}), B cells, macrophages, and DCs were isolated and cultured separately with CFSE-labeled 2D2 $CD4^+$ T cells for 5 days. As shown in Fig. 1*E*, only B cells, but not DCs or macrophages from B^{MOG} mice induced proliferation of T cells in vitro. Upon addition of external MOGp35–55, either macrophages or DCs could support T cell proliferation, indicating that these are indeed proficient APCs (Fig. 1*E*).

B cells induce tolerance and protect from the induction of CNS inflammation

Because we could show that the B cells in B^{MOG} mice presented the MOG peptide and could induce moderate proliferation of MOG-specific T cells in culture, it was of interest to investigate whether these mice are susceptible to MOG-induced EAE. As seen in Fig. 2*A*, B^{MOG} mice as well as APC^{MOG} mice are resistant to EAE upon immunization with MOGp35–55 in CFA. We reasoned that B^{MOG} mice are resistant to EAE induction due to the B cell-specific presentation of MOGp35–55 in vivo, which could lead either to central tolerance in the thymus, if indeed B cells can participate in this process, or to peripheral tolerance.

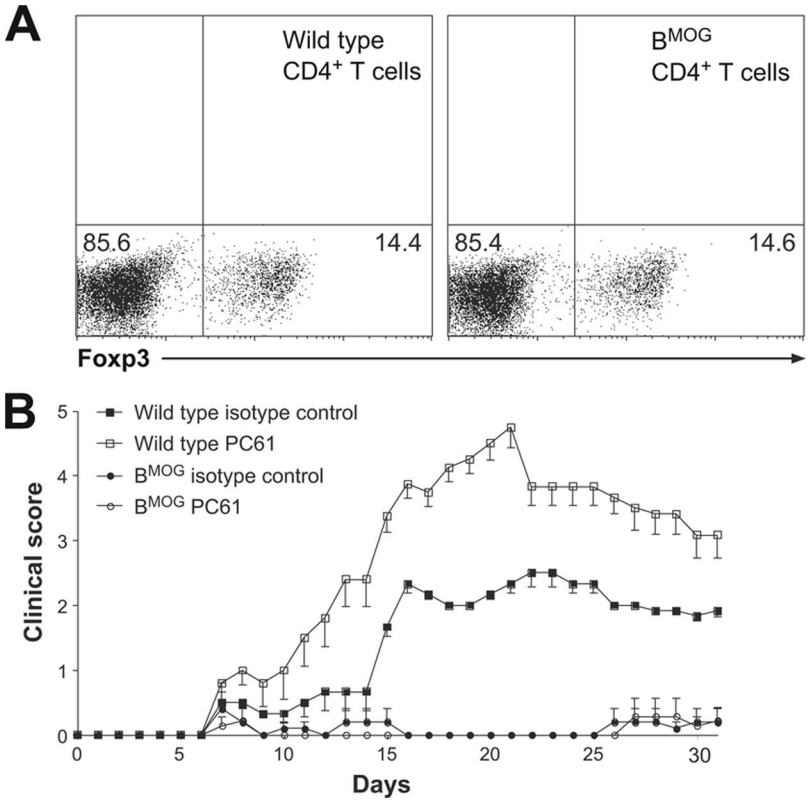

FIGURE 3. Tolerance of B^{MOG} mice is not due to enhanced Treg cell levels. *A*, Intracellular staining for Foxp3 on MACS-purified $CD4^+$ T cells from WT and B^{MOG} mice. *B*, WT (square) and B^{MOG} (circle) mice were injected with 1 mg of PC61 (open symbols) or isotype control Ab (MAC49; filled symbols). EAE was induced by immunization 2 days later. B^{MOG} mice were resistant to EAE, also after PC61 treatment, whereas WT mice developed significantly more severe EAE compared with isotype control injected WT mice ($p < 0.05$, days 13–26).

To investigate whether B cells are involved in the process of peripheral tolerance, resulting in resistance to MOG-induced EAE in B^{MOG} mice, we adoptively transferred 2D2 $CD4^+$ T cells to B^{MOG} mice, and actively induced EAE 1 wk later. As seen in Fig. 2*B*, the transfer of naive MOG-specific T cells did not bypass the resistant phenotype of B^{MOG} mice. In contrast, WT mice, which received 2D2 $CD4^+$ T cells, were as susceptible to EAE as control WT mice that had not received 2D2 $CD4^+$ T cells. Furthermore, we adoptively transferred MOG-reactive lymphocytes from C57BL/6 mice to induce passive EAE in B^{MOG} as well as WT mice. We observed that B^{MOG} mice were also resistant to passive EAE induction (Fig. 2*C*).

We showed that the specific presentation of MOG peptide by B cells renders the B^{MOG} mice resistant to EAE that was actively induced by the presented peptide or by passive transfer of encephalitogenic MOG-reactive T cells. We reasoned that the MOG-specific T cells are able to interact with the MOG-presenting B cells, but as a consequence of this interaction the T cells are anergized and thus unable to induce EAE. To investigate whether tolerance can be induced also by transfer of MOG-presenting B cells, we injected isolated B cells from B^{MOG} to WT C57BL/6 mice and induced active EAE 2 days later. We observed that mice injected with MOG-presenting B cells developed only a mild form of EAE compared with WT or WT-B cell-injected control mice (Fig. 2*D*). Interestingly, mice injected with activated MOG-presenting B cells (before transfer B cells were activated in vitro with anti-CD40, anti-BCR, and IL-4) were also resistant to EAE (Fig. 2*D*). B cell transfer to EAE mice on day 12 after immunization results in significantly less severe EAE compared with PBS-treated control mice (Fig. 2*E*). Thus, we conclude that also transferred MOG-presenting B cells have the capacity to induce tolerance to MOG-induced EAE.

Because the presentation of MOGp35–55 by B cells induced tolerance to EAE, it was of interest to investigate whether B^{MOG} mice develop disease when encountering all immunogenic epitopes of the myelin sheath or whether tolerance induced by MOG-presenting B cells is dominant. To this end, B^{MOG} and WT mice were immunized with C57BL/6 sc homogenate covering all immunogenic epitopes of the myelin sheath. Upon immunization with sc homogenate, B^{MOG} mice developed EAE to the same extent as WT mice (Fig. 2*F*), indicating that tolerance induced by B cells presenting MOGp35–55 is not dominant.

Activation of T cells by B cells does not lead to clonal expansion

Targeting of Ag to immature DCs was previously shown to induce the formation of regulatory T cells (Tregs) in vivo (28). To investigate if presentation of the MOG peptide by B cells will induce conversion of naive T cells to Tregs, we analyzed the population of $Foxp3^+$ T cells in B^{MOG} mice. As demonstrated in Fig. 3*A*, we could not detect an obvious change in the percentage of $Foxp3^+$ T cells among $CD4^+$ T cells in these mice. As it was previously demonstrated that B-T cell interactions can lead to the development of Tregs (29), it is possible that MOG-presenting B cells induce the development of MOG-specific Treg cells which might be responsible for the tolerance we observe. Because the number of specific Tregs is relatively small, it may not be possible to detect this change in the total $Foxp3^+$ population. To investigate whether elevated levels of MOG-specific Tregs contribute to the resistance to EAE in B^{MOG} mice, we depleted endogenous $CD25^+$ cells by injection of anti-CD25 Ab (PC61) before induction of EAE. It was reported that $Foxp3^+CD25^+$ are depleted by this treatment (30). The depletion of $CD25^+$ T cells does not change the resistant phenotype of B^{MOG} compared with isotype-treated B^{MOG} mice (Fig. 3*B*). As expected, WT mice treated with anti-CD25 before EAE induction developed disease of increased severity compared with isotype-treated WT controls (Fig. 3*B*). These results indicate

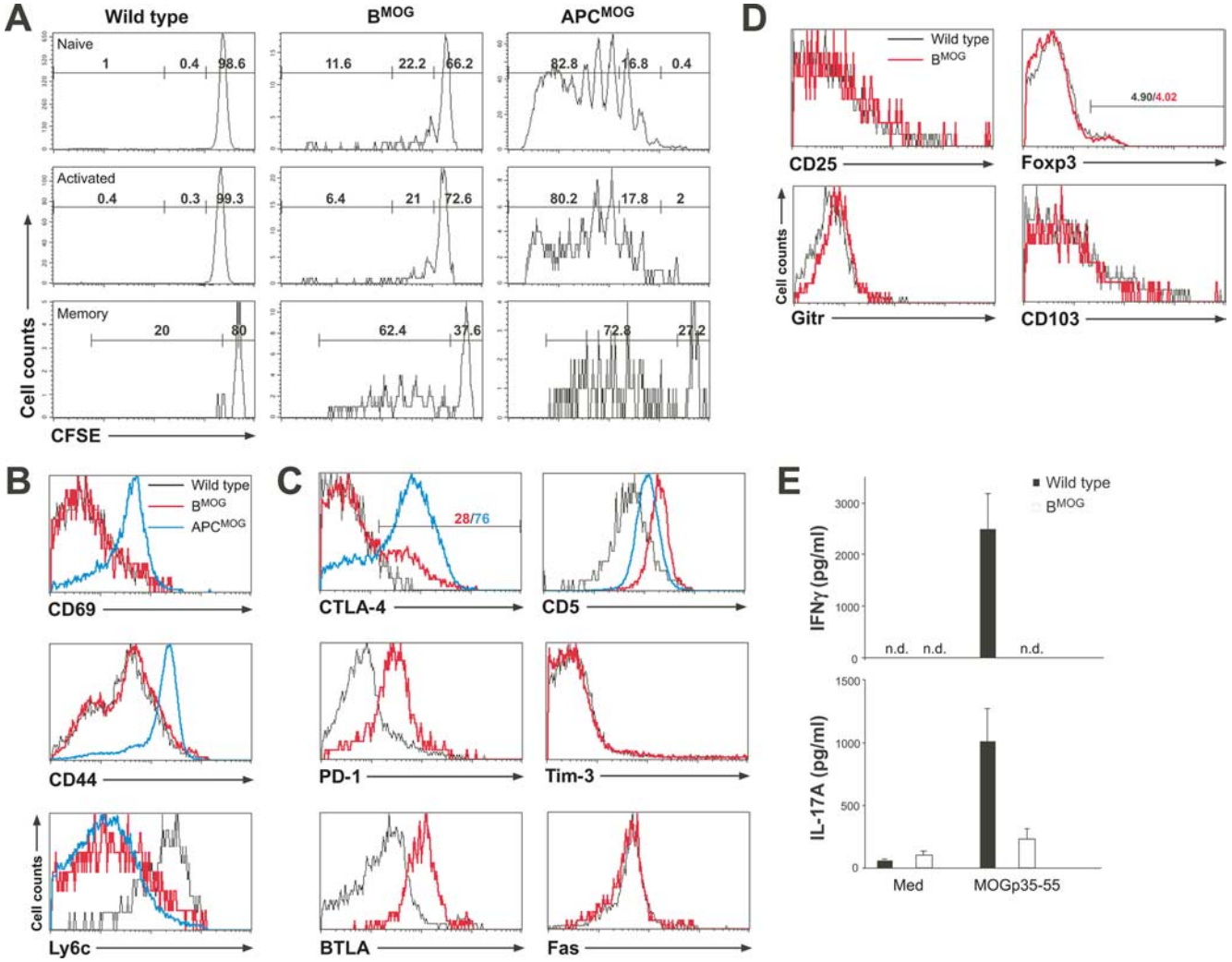

FIGURE 4. MOG-specific T cells show a tolerized phenotype after interaction with MOG-presenting B cells. *A*, Proliferation of naive (*upper row*), activated (*middle row*), or memory (*lower row*) 2D2 CD4$^+$ T cells, 5 days after adoptive transfer to WT (*left panel*), B^{MOG} (*middle panel*), or APC^{MOG} mice (*right panel*). Numbers in histograms indicate percent positive cells in each marker region. Data are representative of three independent experiments. *B–D*, FACS analysis of 2D2 CD4$^+$ T cells 5 days after transfer to WT (black line), B^{MOG} (red line), or APC^{MOG} mice (blue line). Stainings were performed using Abs to CD44, CD69, and Ly6C (*B*) or CTLA-4, PD-1, BTLA, CD4, Tim-3, and Fas (*C*) or CD25, CD103, Gitr, and Foxp3 (*D*). *E*, Secretion of IFN-γ (*upper diagram*) and IL-17A (*lower diagram*) was detected performing ELISA on SN from 2D2 CD4$^+$ T cells transferred to WT (■) or B^{MOG} (□) mice, 72 h after restimulation with MOGp35–55 in vitro. n.d., not detectable.

that CD4$^+$CD25$^+$ T cells are not involved in the resistance to EAE of B^{MOG} mice.

To better follow the effect of B cell-specific presentation of MOGp35–55 on MOG-specific T cells, we labeled 2D2 CD4$^+$ T cells with CFSE and adoptively transferred them to B^{MOG}, APC^{MOG}, and WT mice. Five days after transfer, different lymphoid organs were isolated and proliferation of the transferred T cells was monitored, as depicted by the loss of CFSE labeling. MOG-specific T cells divided extensively after in vivo encounter of MOG-presenting APCs in APC^{MOG} mice (Fig. 4*A*). No division of T cells transferred to WT mice was seen. This lack of division was expected because in the absence of Ag, or space for homeostatic proliferation, T cells do not divide (31, 32). In contrast to APC^{MOG} mice, 2D2 CD4$^+$ T cells transferred to B^{MOG} mice hardly proliferated within the period of 5 days (Fig. 4*A*). Also, activation of the MOG-presenting B cells in B^{MOG} mice with anti-CD40 did not result in enhanced proliferation of transferred MOG-specific T cells (data not shown).

We were interested to assess whether the lack of proliferation of T cells is a consequence of their naive state, and whether activated or memory T cells will proliferate after interaction with B cells. 2D2 TCR transgenic mice were immunized with MOGp35–55 in CFA. Ten days later, CD4$^+$ T cells were purified (activated T cells) and transferred to WT, B^{MOG}, or APC^{MOG} mice. For induction of memory T cells, activated 2D2 CD4$^+$ T cells were transferred and left for 1 mo in MOG-deficient mice (22), assuming that in the period of 30 days in which the cells are not further activated, a large proportion of the transferred T cells differentiated to memory cells. Proliferation of activated and memory 2D2 CD4$^+$ T cells was analyzed 5 days after transfer. As seen in Fig. 4*A*, also activated 2D2 T cells did not proliferate in B^{MOG} mice. In contrast, memory T cells proliferated to a similar extent as in APC^{MOG} mice. We conclude that B cells have the capacity to induce proliferation of Ag-experienced resting T cells, but not of naive or recently activated T cells.

Although MOG-specific T cells hardly proliferated upon interaction with MOG-presenting B cells, we observed one up to two cycles of proliferation and wondered if we could detect expression of activation markers on these cells. As seen in Fig. 4*B*, T cells do

not up-regulate expression levels of CD69 and CD44, after interaction with B cells, but interestingly, they down-regulate the expression of Ly6C. In contrast, following interaction with B cells presenting their cognate Ag, T cells up-regulate the expression levels of the coinhibitory molecules CTLA-4, PD-1, BTLA, and CD5 (Fig. 4*C*). We could not detect differences in the expression levels for Tim-3 and Fas between unactivated T cells and T cells that interacted with B cells (Fig. 4*C*).

Also, expression of receptors and markers normally up-regulated on surface of Treg cells was unaltered (Fig. 4*D*). When activating 2D2 CD4$^+$ T cells with the MOG peptide in culture, T cells that had interacted with their cognate Ag presented in vivo by B cells did not secrete IFN-γ or IL-17A, indicating that these cells are functionally tolerized (Fig. 4*E*).

IL-10 does not play a role in tolerance induced by B cells

How do B cells induce tolerance? Recently, it was shown that IL-10 specifically produced by B cells is active at the remission stage of EAE and allows the mice to recover from disease (33). According to that study, it is essential that B cells produce IL-10, otherwise recovery from EAE cannot occur, similar to B cell-deficient mice (33). It is not clear from that study at which phase of disease B cell-produced IL-10 is functional: is it at the stage of T cell activation or do IL-10-producing B cells indeed invade the brain and directly tolerize encephalitogenic T cells? Because induction of EAE is similar in WT and B cell-IL-10-deficient mice (33), it is also not clear whether IL-10 produced by B cells has a general role in tolerance to EAE or only in causing remission. To investigate whether the tolerization by B cells is associated with their production of IL-10, presentation of MOG by IL-10-deficient B cells was achieved by crossing the B^{MOG} mice to mice that allow the tissue-specific deletion of the *il-10* gene (IL-10^{fl}; (34). The *il-10* gene is efficiently inactivated in IL-10$^{fl/fl}$/CD19-Cre mice (A.R. and W.M., manuscript in preparation). 2D2 CD4$^+$ T cells were transferred to these mice (termed B^{MOG}/IL-10$^{fl/fl}$ mice) and proliferation of the T cells was assessed 5 days after transfer. As seen in Fig. 5*A*, the presentation of MOG by IL-10-deficient B cells did not lead to an enhanced proliferation of MOG-specific T cells, indicating that the production of IL-10 by B cells had no role in tolerance of T cells, when the peptide was presented by B cells. Consistently, B^{MOG}/IL-10$^{fl/fl}$ mice showed similar resistance to MOG-induced EAE as B^{MOG} mice (Fig. 5*B*).

B cells mediate their tolerance effect via CTLA-4 and PD-1

It was previously shown that PD-1 plays a role in the induction of peripheral tolerance of CD8$^+$ T cells by resting DCs (35). In Fig. 4, we demonstrated that MOG-presenting B cells induced an up-regulation of CD5, BTLA, PD-1, and CTLA-4 by MOG-specific T cells. To assess the contribution of CD5, we investigated whether injection of anti-CD5 Ab could prevent induction of T cell tolerance by MOG-presenting B cells. No difference in proliferation ensued when CD5 was blocked (data not shown). To reveal whether PD-1 is required for the induction of CD4$^+$ T cell tolerance in our system, PD-1-deficient mice were crossed to 2D2 mice to obtain MOG-specific T cells that cannot interact through PD-1. Before transfer, PD-1$^{-/-}$ 2D2 T cells were analyzed for the transgenic CD4$^+$ T cell population and displayed no obvious difference compared with WT 2D2 mice (data not shown). To investigate the involvement of CTLA-4 in B cell-induced CD4$^+$ T cell tolerance, recipient mice were treated with a mAb that blocks signaling through CTLA-4 in vivo (23, 35). Either WT or PD-1$^{-/-}$ transgenic 2D2 CD4$^+$ T cells (Thy1.1) were CFSE-labeled and adoptively transferred to B^{MOG} and WT mice (Thy1.2). Recipient mice were additionally treated with anti-CTLA-4 or PBS, and the transferred T cells were monitored for proliferation. The treatment of recipient B^{MOG} mice with anti-CTLA-4 as well as the transfer of PD-1$^{-/-}$ 2D2 T cells led to an increase in proliferation of the transferred T cells up to 71 and 79%, respectively (Fig. 6). The combination of PD-1 deficiency and blockade of CTLA-4 led to a higher increase in proliferation up to 97% of total proliferating T cells, with most cells dividing at least twice (Fig. 6), which indicates that both PD-1 and CTLA-4 play an important role in the induction of peripheral CD4$^+$ T cell tolerance by naive B cells. Further, the importance of PD-1 and CTLA-4 was shown by inducing EAE in anti-CTLA-4-treated PD-1$^{-/-}$ mice upon transfer of B^{MOG} B cells. The percentage of paralysis after transfer of B^{MOG} B cells increased from 0% in WT mice to 33% in anti-CTLA-4-treated PD-1$^{-/-}$ mice, respectively (Fig. S1).[4] These data demonstrate that the tolerance mediated by B cells is partially dependent on PD-1 and CTLA-4.

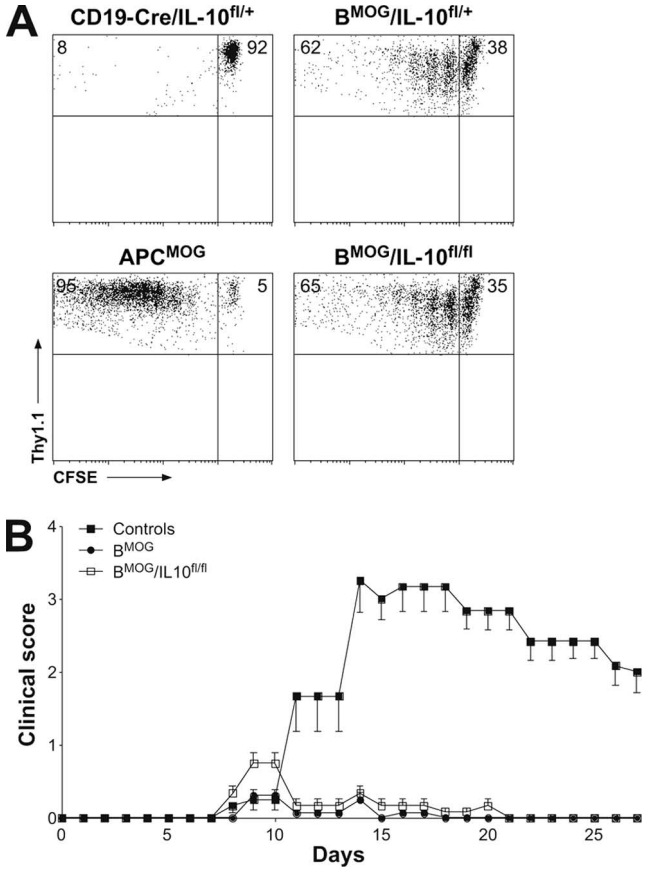

FIGURE 5. The tolerization of MOG-specific T cells by B cells is independent of IL-10. *A*, Proliferation of naive 2D2 CD4$^+$ T cells 5 days after transfer to CD19-Cre/IL-10$^{fl/fl}$, B^{MOG}/IL-10$^{fl/+}$, B^{MOG}/IL-10$^{fl/fl}$, and APC^{MOG} mice. Cells were gated on Thy1.1$^+$ cells. Numbers in quadrants indicate percent positive cells in each. *B*, EAE was actively induced by immunization of control (WT, IL-10$^{fl/+}$ and IL-10$^{fl/fl}$ mice; (■)), B^{MOG} (●), or B^{MOG}/IL-10$^{fl/fl}$ (□) mice with MOGp35–55. B^{MOG} and B^{MOG}/IL-10$^{fl/fl}$ mice develop significantly less severe EAE compared with control mice ($p < 0.05$, days 14–26). Values are represented as mean ± SEM.

B cells induce peripheral tolerance by sensitizing T cells to Ag-induced cell death

To investigate how B cell-anergized T cells react to restimulation in vivo, 2D2 CD4$^+$ T cells (Thy1.1) were adoptively transferred to

[4] The online version of this article contains supplemental material.

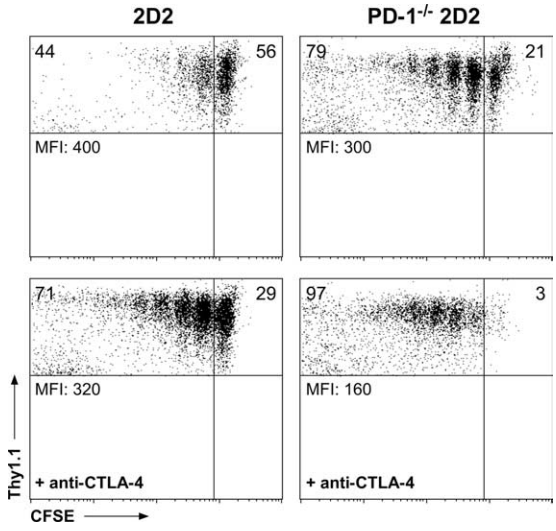

FIGURE 6. PD-1 deficiency and blockade of CTLA-4 partially rescue T cells from tolerance. Proliferation of naive WT 2D2 CD4$^+$ (*left row*) or PD-1$^{-/-}$ 2D2 CD4$^+$ T cells (*right row*) 5 days after adoptive transfer to anti-CTLA-4-treated (*lower row*) or untreated (*upper row*) B^{MOG} mice. Numbers in quadrants indicate percent positive cells in each. MFI, mean fluorescence intensity.

B^{MOG} or WT mice (Thy1.2). Then, total CD4$^+$ T cells were reisolated from the recipients 5 days after transfer, labeled with CFSE and transferred into APC^{MOG} mice (Thy1.2), which were injected with agonistic anti-CD40 to activate DCs in vivo (as depicted in the scheme of Fig. 7*A*). Before transfer, we determined the ratio between Thy1.1$^+$ 2D2 T cells and Thy1.2$^+$ host CD4$^+$ T cells. Because the 2D2 T cells did not proliferate extensively in either of the hosts, as demonstrated in Fig. 4*A*, the ratio was similar, namely 1:12 for Thy1.1$^+$ vs Thy1.2$^+$ cells (Fig. 7*B*). As seen in Fig. 7*C*, 2D2 CD4$^+$ T cells that were transferred to WT mice, reisolated, and transferred to APC^{MOG} mice proliferated extensively, to a similar extent as when directly transferred to APC^{MOG} mice (Fig. 4*A*). The transferred Thy1.2$^+$ CD4$^+$ WT host T cells are CFSE$^+$, but do not proliferate, as they are not MOG-specific, and serve in this study as an internal control for proliferation and ratio changes. Thus, we showed that transfer of 2D2 CD4$^+$ T cells to WT mice does not alter their potential to be activated as seen when reisolated and transferred to APC^{MOG} mice. In contrast, 2D2 CD4$^+$ T cells that first encountered MOG by B cells in B^{MOG} mice and were then transferred to APC^{MOG} mice were dramatically decreased in number. As seen in Fig. 7*C*, only very few Thy1.1$^+$ T cells were left after in vivo restimulation, indicating that T cells initially triggered by B cells are rendered sensitive to deletion upon a second exposure to Ag on activated APC in vivo. The ratio of Thy1.1$^+$ to Thy1.2$^+$ cells among all CD4$^+$ T cells originally reisolated from WT mice was ~1:1 (Fig. 7, *C* and *D*). In contrast, the ratio of Thy1.1$^+$ to Thy1.2$^+$ cells among all CD4$^+$ T cells was ~1:12 when CD4$^+$ T cells were transferred to B^{MOG} before transfer to APC^{MOG} mice, although Thy1.1$^+$ cells appeared to proliferate in APC^{MOG} mice. These results indicate that 2D2 CD4$^+$ T cells that were reisolated from WT mice as well as the ones reisolated from B^{MOG} mice were triggered and proliferated when restimulated in APC^{MOG} mice. In contrast to 2D2 CD4$^+$ T cells that never encountered Ag before, 2D2 CD4$^+$ T cells that encountered MOG on B cells died upon activation, most likely after proliferation was

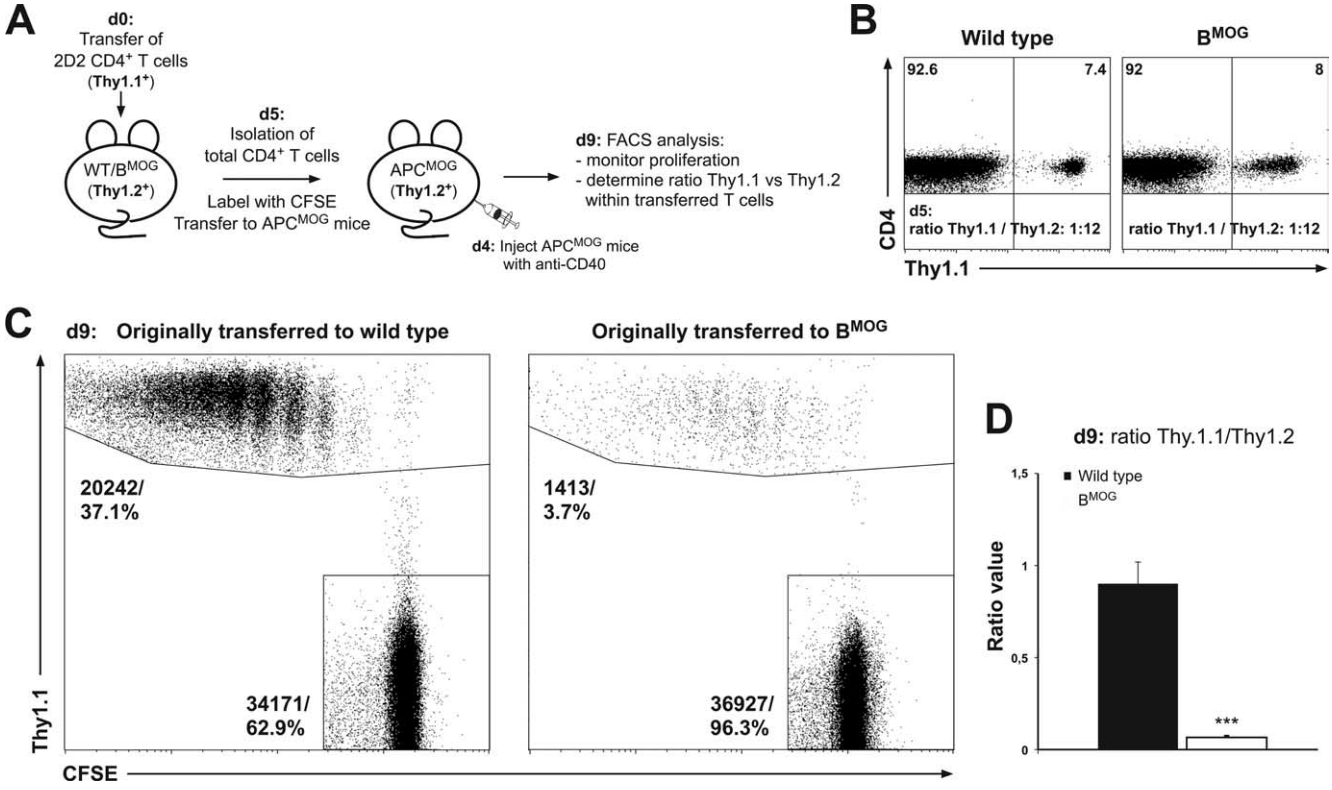

FIGURE 7. B^{MOG}-tolerized T cells are deleted upon restimulation in vivo. *A*, Schematic presentation of the experiment. *B*, Ratio of 2D2 CD4$^+$ Thy1.1$^+$ vs WT Thy1.2$^+$ CD4$^+$ 5 days after transfer to WT or B^{MOG} mice. *C*, Proliferation of 2D2 CD4$^+$Thy1.1$^+$ T cells reisolated from WT or B^{MOG} mice after restimulation for 4 days in anti-CD40 injected APC^{MOG} mice. Cells were gated on Thy1.1$^+$ and CFSE$^+$ cells. Numbers close to gates refer to events and percentage of positive cells in each. *D*, Ratio values of CFSE-labeled 2D2 CD4$^+$ Thy1.1$^+$ vs WT Thy1.2$^+$ CD4$^+$ 4 days after restimulation in anti-CD40-injected APC^{MOG} mice.

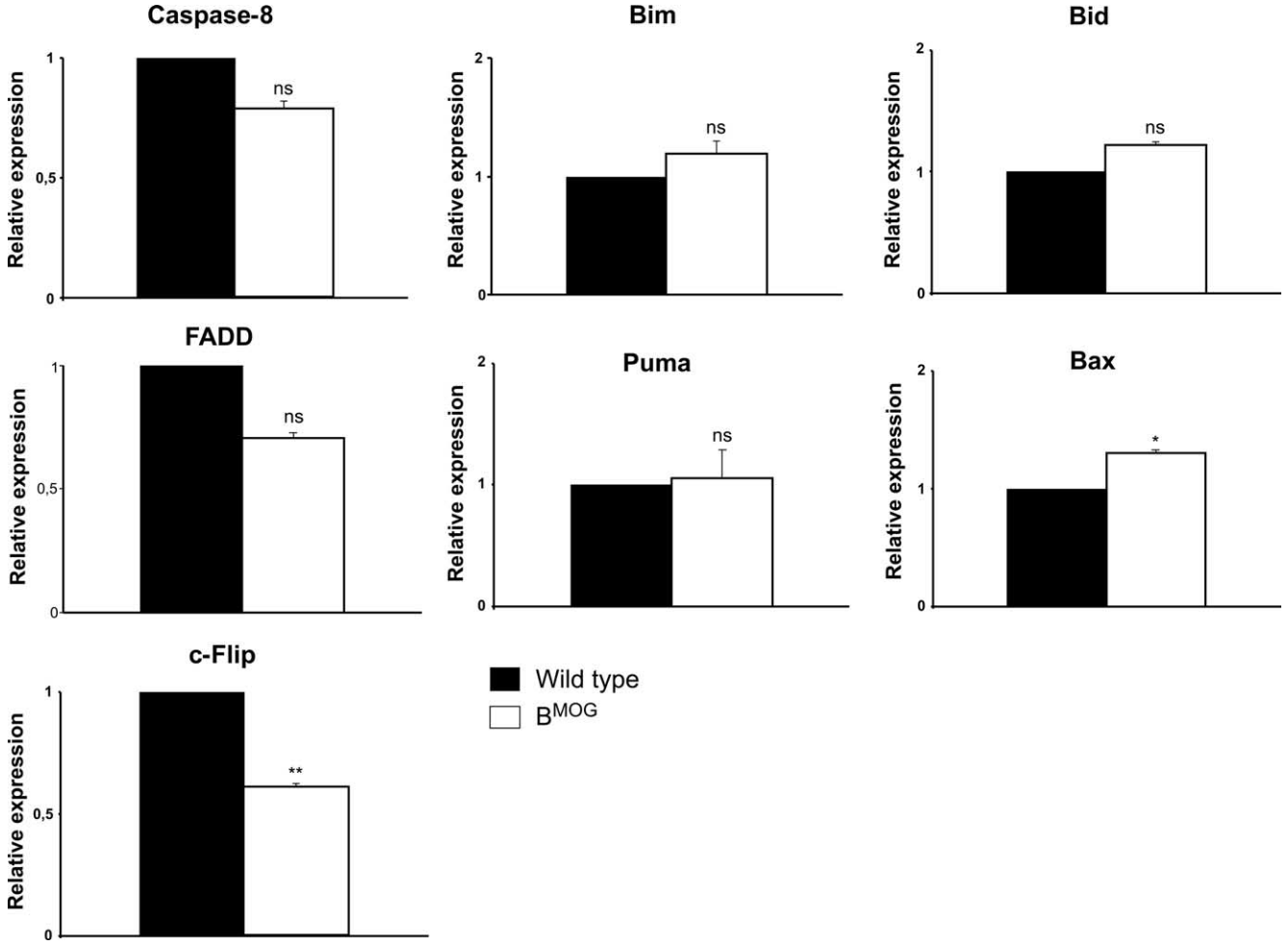

FIGURE 8. MOG presentation by B cells enhances Bax and decreases c-Flip expression by MOG-specific T cells. Real-time PCR for Caspase-8, Bim, Bid, FADD, Puma, Bax, and c-Flip was performed on mRNA from 2D2 CD4$^+$ T cells sorted 5 days after transfer to WT (■) or B^{MOG} (□) mice.

initiated, as no nonproliferating Thy1.1$^+$ T cells could be detected (Fig. 7*C*).

In view of our data, we suggest a model, in which the interaction of T cells with Ag-presenting B cells leads to partial activation of the T cells. This is manifested by their marginal proliferation, by the absence of expression of activation markers, but also by the expression of coinhibitory molecules. T cells that interact with more potent APCs, as in APC^{MOG} mice, will expand and contribute to the pool of effector T cells. Both types of T cell activation, i.e., following interaction with B cells or DCs in vivo, will ultimately lead to AICD once the cells are retriggered by potent APCs (Fig. 7).

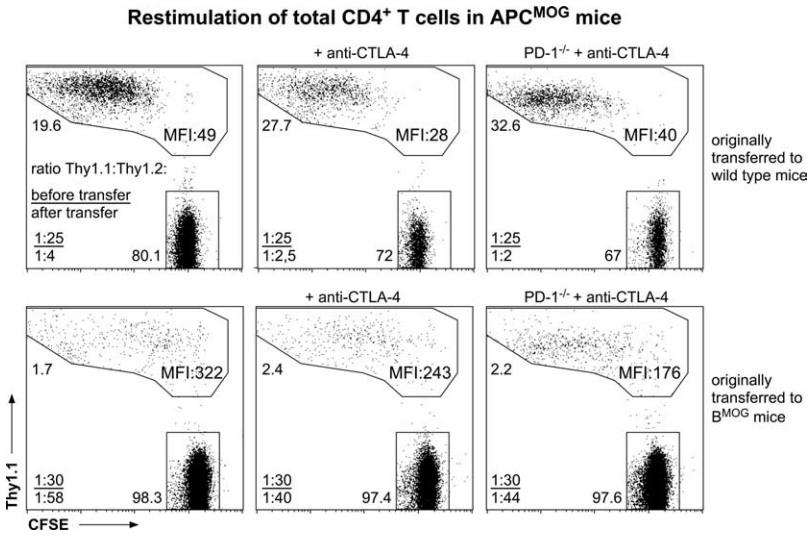

FIGURE 9. PD-1 deficiency and blockade of CTLA-4 do not rescue tolerized T cells from deletion. Proliferation of 2D2 CD4$^+$Thy1.1$^+$ T cells reisolated from WT or B^{MOG} mice after restimulation for 4 days in anti-CD40-injected APC^{MOG} mice. On the day of the second transfer and 2 days later, mice were additionally injected with anti-CTLA-4 in combination with (*right panel*) or without (*middle panel*) PD-1 deficiency. Cells were gated on Thy1.1$^+$ and CFSE$^+$ cells. Numbers close to gates refer to percentage of positive cells in each. MFI, mean fluorescence intensity.

RNA was prepared from sorted 2D2 T cells 5 days after transfer to B^{MOG} or WT mice. The RNA was subjected to real-time RT-PCR using primers specific for the proapoptotic molecules Caspase-8, FADD, Bim, Bid, Bax, Puma, and the anti-apoptotic molecule cellular FLICE-inhibitory protein (c-Flip). A significant difference was only found in the increased expression of Bax and the decreased expression of c-Flip (Fig. 8). The increase of Bax and the reduction in c-Flip expression levels may render the cells sensitive to apoptosis, since c-Flip is able to modulate activation of procaspase-8 and thereby prevents induction of apoptosis mediated by death receptors (36) whereas Bax is required for mitochondrial dysfunction during apoptosis (37).

We could show that the interaction of T cells with B cells induces only modest T cell proliferation, but the expression of negative costimulatory molecules. Further, we noted that this interaction supported T cell proliferation when signaling through PD-1 and CTLA-4 was blocked (Fig. 6). To investigate whether blocking the signaling through these molecules might have an effect on AICD, we performed similar in vivo restimulation experiments as seen in Fig. 7, using PD-$1^{-/-}$ T cells and treatment with anti-CTLA-4. Then, the T cells were CFSE-labeled and transferred to APC^{MOG} mice as already demonstrated in Fig. 7. The tolerized T cells were not rescued from deletion when signaling via CTLA-4 and PD-1 was blocked (Fig. 9).

Discussion

In this study, we show that constitutive Ag presentation by B cells on MHC class II (MHCII) results in profound peripheral tolerance. We have shown that T cells that specifically interact with B cells in vivo are partially activated, but this activation does not lead to their proliferation. Rather, they up-regulate the expression of coinhibitory molecules and become highly sensitive to AICD. The process we propose in this study is a part of general peripheral tolerance: we suggest that in the steady state, $CD4^+$ T cells that recognize their specific Ag presented by B cells undergo a process that eventually results in their death once these T cells recognize the Ag in a more potent antigenic context. As B cells are the most prevalent MHCII-expressing cells in the body, they are the natural candidates to serve as cells that enforce tolerance of autoreactive $CD4^+$ cells, once these cells develop and migrate from the thymus to the periphery. In this study, we demonstrate a central role for B cells in preventing autoimmune responses that are mediated by MHCII-restricted T cells.

Using the Cre/loxP system, we were able to direct the expression of the MOG peptide p35–55 specifically to B cells. This system ensures B cells to be able to interact with Ag-specific T cells in all secondary immune organs. We found that in contrast to the specific presentation of MOG by B cells, its presentation by all $MHCII^+$ cells, including macrophages and different DC populations, enables MOG-specific T cells to proliferate extensively. This finding indicates that the tolerance mediated by B cells is not dominant over other APCs; in a system that allows peptide presentation not only by B cells, but by all $MHCII^+$ cells, the latter can nevertheless induce T cell proliferation.

Tolerance induced by B cells is profoundly different to that induced by DCs or other professional APCs. When DCs present peptide to $CD4^+$ T cells following injection with DEC205/peptide conjugate, the T cells first go through a phase of abortive proliferation (28, 38). Likewise, when mice are rendered tolerant by i.v. injection with peptide, the T cells first go through a few cycles of proliferation before their deletion that results in tolerance to EAE (39, 40). B cells therefore provide a more efficient form of tolerance compared with DCs. This tolerance takes less immunological space, as the Ag-specific T cells to not need to be expanded and then eliminated. In contrast to DCs, B cells provide a more economical tolerance mechanism, by which the T cells do not need to go first through a phase of abortive proliferation, but are rendered sensitive to elimination upon further activation, when it is done in a more physiological context.

B^{MOG} as well as APC^{MOG} mice are resistant to active EAE induction. As a result of IiMOG expression in the thymus, MOG-specific T cells could be eliminated, resulting in mice that lack the MOG-specific T cell repertoire. Although B cells are not normally implicated in the process of negative selection, their presence in the thymus was shown before (41). Therefore it is possible that both MOG-expressing mouse strains are resistant to EAE due to central tolerance. Thus, we have used MOG-specific T cells isolated from IiMOG-negative mice and shown that also these cells, although they were not subjected to central tolerance by MOG, were not able to induce EAE in B^{MOG} mice.

The interaction of B cells with naive as well as activated MOG-specific T cells did not result in massive proliferation of the T cells, which is seen when these cells are activated by DCs and macrophages in APC^{MOG} mice. However, activation of resting Ag-experienced T cells by B cells was indistinguishable from activation of these cells in APC^{MOG} mice. These data indicate that B cells do not support the expansion of naive or activated T cells, but do induce proliferation of memory T cells. These findings also support the dogma, according to which memory T cells require less stimulation for their activation, which could also be supplied by B cells (1). It is possible that naive B cells do not express the costimulatory molecules that are necessary for the activation of naive or activated T cells, but the conditions needed for the activation of memory T cells are lower and therefore they can be activated by B cells. On the other hand, we have performed experiments in which the B cells of B^{MOG} mice were activated in vivo using LPS or anti-CD40, but naive T cells transferred to these mice were not sufficiently stimulated to proliferate. It has previously been shown that activated transferred B cells need to express B7 molecules to induce tolerance (10). It is possible that, in our system, tolerance can also be achieved by costimulatory molecules other than B7. This raises the possibility that B cells have to be activated by cognate Ag in conditions that differ from these given by TLRs or the CD40 receptor to efficiently activate naive T cells.

Studies by several groups demonstrated that B cells do interact with T cells in culture, resulting in proliferation of the T cells (42) or even their conversion into Tregs (29, 43). B cells isolated from B^{MOG} mice are able to induce moderate proliferation of naive T cells in culture without the addition of exogenous Ag. Possibly, when both cell types are present in close proximity in tissue culture conditions, B cells form a stable long term interaction with T cells that could lead to their proliferation and/or differentiation into Tregs (29). Our present work does not contradict such a possible function of B cells. We suggest that in vivo, the main process by which B cells contribute to peripheral tolerance is via sensitization of self-reactive T cells to AICD. These results are also support by the previous work of Townsend and Goodnow (44). The latter work demonstrated that B cells can stimulate rare Ag-specific T cells resulting in abortive proliferation of the T cells (44). It is also possible that in B^{MOG} mice some of the T cells differentiate into Tregs, as has been shown following the interaction of naive T cells with steady-state DCs (28). Although we did not notice a general elevation of $FoxP3^+$ T cells and the deletion of $CD25^+$ Tregs in B^{MOG} mice did not alter their resistant phenotype, we

cannot answer this question with certainty without using reagents that can specifically label MOG-specific T cells in B^{MOG} mice. Previously, it was shown that depletion of Treg cells abrogated tolerance induced by transfer of activated B cells (45). These results do not contradict with our findings since our data do not imply that B cells do not induce the generation of Tregs, but indicate that the B cell-mediated tolerance by directly inhibiting effector T cells is dominant over tolerance mediated by the generation of Tregs, or at least is sufficient for the inhibition of EAE induction.

Deletion of the B cell-tolerized T cells occurs upon in vivo reactivation and is contrary to studies by Seamons et al. (42) showing less proliferation but no deletion of T cells that were restimulated by B cells. The fact that they use bulk cultures and ex vivo stimulation might account for the discrepancy to our data. Our results do not explain how B cells are able to interact with T cells during a germinal center reaction without the tolerization of these T cells. Germinal center B cells possibly behave different from naive or even LPS-stimulated B cells and may down-regulate negative costimulatory receptors that are responsible for the general T cell tolerance observed in B^{MOG} mice.

Previously, Fillatreau and colleagues demonstrated that IL-10 produced by B cells has a suppressive role in EAE, and when B cells were incapable to produce this cytokine, mice could not recover from the disease (33). Moreover, B cell-produced IL-10 has been shown to play an important role in the prevention of arthritis (46) (47). In the present work, we could demonstrate that B cells are able to serve as suppressor cells in the initiation of the disease. Also, transfer of MOG-presenting B cells after disease onset significantly attenuated the course of EAE, demonstrating a suppressive potential of B cells, as it is especially challenging to reverse ongoing EAE. Using mice in which B cells presented the MOG peptide, but were not able to produce IL-10, we could show that the effect of peripheral tolerance that is mediated by B cells is independent from their IL-10 production.

Studies by the groups of Y. Ron and D. Scott (6–9) have previously shown that B cells can efficiently serve as tolerogenic cells that prevent the induction of EAE or even serve as a therapy in a relapsing-remitting model of the disease. We have now extended these studies by using a chronic model of EAE and a mouse strain that allows Ag presentation by B cells that were not manipulated in culture or applied by transfer. Importantly, we could show that the mechanism by which B cells mediate the process of peripheral tolerance involves the coinhibitory receptors PD-1 and CTLA-4. These studies are important in the designation of future cell-based therapies for autoimmune diseases, as B cells of patients are easy to obtain and manipulate.

Acknowledgments

We thank Claudia Uthoff-Hachenberg, Annette Shrestha, and Marina Snetkova for excellent technical assistance.

Disclosures

The authors have no financial conflict of interest.

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