Tolerance without clonal expansion: Self-antigen-expressing B cells program self-reactive T cells for future deletion

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

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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 naïve 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.
were performed using the Foxp3 Staining Set (Naturac, ebioscience) 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 FACS caliber 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) (2F10; 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 BMOG 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 instructions. The expression of mRNA for Caspase-8, FADD, Bim, Bid, Bax, Puma, and e-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**

MOG35–55 peptide (amino acid sequence: MEVGWyRSPSRVHLYRNGK) was obtained from Research Genetics. Active EAE was induced by immunization with 50 μg of MOG35–55 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 × 10⁶/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 1/10 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 forelimp paralysis; and 6, moribund or dead.

**In vivo depletion of CD25+ cells**

Endogenous CD25+ T cells were depleted from mice 2 days before induction of EAE by i.p. injection with 1 mg of anti-CD25 Ab (eBioscience). Control mice received an i.p. injection of 1 mg of MAC49 (rat IgG1, Biochemicals). For induction 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...
a new mouse model that allows the cell-specific presentation of MOGp35–55 on MHC class II. We constructed a mutated invariant chain (IiMOG) where we replaced the sequence of the CLIP peptide by the peptide sequence of MOGp35–55, resulting in the IiMOG gene (Fig. 1A). To prove functionality of the IiMOG system BM-derived DCs from WT, STOPMOG, and APCMOG 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 BMOG 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.

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, STOPMOG, and APCMOG 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 BMOG 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.
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. 1D). From double-transgenic mice (termed BMOG), B cells, macrophages, and DCs were isolated and cultured separately with CFSE-labeled 2D2 CD4+ T cells for 5 days. As shown in Fig. 1E, only B cells, but not DCs or macrophages from BMOG 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. 1E).

**FIGURE 2.** Ag presentation by naive B cells induces tolerance to EAE. A, EAE was actively induced by immunization of BMOG (●), APCMOG (♦), or WT mice (■) with MOGp35–55. BMOG and APCMOG 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 = 5 mice/group). B, MOG-specific 2D2 CD4+ T cells were adoptively transferred into BMOG (●) or WT mice (■). After 1 wk, EAE was actively induced by immunization with MOGp35–55 including untreated WT control mice (▲). BMOG 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 BMOG (●) or WT mice (■). BMOG 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 BMOG (○), activated B cells from BMOG mice (●), or without transfer of B cells (■). Mice injected with naive or activated BMOG B cells developed significantly less severe EAE compared with untreated WT or WT-B cell-injected mice (p < 0.05). C, EAE was actively induced in WT mice and 12 days after immunization, B cells from BMOG mice (●) were transferred. Control mice received PBS (■). Mice injected with naive BMOG B cells developed significantly less severe EAE compared with PBS-treated control mice (p < 0.05; days 16–32). F, EAE was induced in BMOG (●) or WT mice (■) by immunization with spinal cord homogenate. Values are represented as mean ± SEM.

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

Because we could show that the B cells in BMOG 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. 2A, BMOG mice as well as APCMOG mice are resistant to EAE upon immunization with MOGp35–55 in CFA. We reasoned that BMOG 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.
To investigate whether B cells are involved in the process of peripheral tolerance, resulting in resistance to MOG-induced EAE in BMOG mice, we adoptively transferred 2D2 CD4\(^{+}\)/H11001 T cells to BMOG mice, and actively induced EAE 1 wk later. As seen in Fig. 2B, the transfer of naive MOG-specific T cells did not bypass the resistant phenotype of BMOG 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 BMOG as well as WT mice. We observed that BMOG mice were also resistant to passive EAE induction (Fig. 2C).

We showed that the specific presentation of MOG peptide by B cells renders the BMOG 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 BMOG 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 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 BMOG as well as WT mice. We observed that BMOG mice were also resistant to passive EAE induction (Fig. 2C).

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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\(^{+}\)/H11001 cells in BMOG mice. As demonstrated in Fig. 3A, we could not detect an obvious change in the percentage of Foxp3\(^{+}\)/H11001 cells among CD4\(^{+}\)/H11001 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\(^{+}\)/H11001 population. To investigate whether elevated levels of MOG-specific Tregs contribute to the resistance to EAE in BMOG 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 BMOG compared with isotype-treated BMOG mice (Fig. 3B). As expected, WT mice treated with anti-CD25 before EAE induction developed disease of increased severity compared with isotype-treated WT controls (Fig. 3B). These results indicate
that CD4+ CD25+ T cells are not involved in the resistance to EAE of BMOG 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 BMOG, APCMOG, 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 APCMOG mice (Fig. 4A). 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 APCMOG mice, 2D2 CD4+ T cells transferred to BMOG mice hardly proliferated within the period of 5 days (Fig. 4A). Also, activation of the MOG-presenting B cells in BMOG 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, BMOG, or APCMOG 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. 4A, also activated 2D2 T cells did not proliferate in BMOG mice. In contrast, memory T cells proliferated to a similar extent as in APCMOG 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. 4B, 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. 4C). 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. 4C).

Also, expression of receptors and markers normally up-regulated on surface of Treg cells was unaltered (Fig. 4D). 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. 4E).

**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, presentation of MOG by IL-10-deficient B cells did not lead to an enhanced proliferation of MOG-specific T cells. 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 IL-10, presentation of IL-10-deficient B cells was achieved by crossing the BMOG mice to mice that allow their production of IL-10, presentation of MOG by IL-10-deficient B cells. The percentage of paralysis after transfer of BMOG B cells was 71% and 79% for transfer of IL-10-deficient B cells and WT B cells, respectively (Fig. S1). These data demonstrate that the tolerance mediated by B cells is partially dependent on PD-1 and CTLA-4.

**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 BMOG 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 BMOG 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 BMOG B cells. The percentage of paralysis after transfer of BMOG B cells increased from 0% in WT mice to 33% in anti-CTLA-4-treated PD-1−/− mice, respectively (Fig. S1). These data demonstrate that the tolerance mediated by B cells is partially dependent on PD-1 and CTLA-4.

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

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

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**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-10fl/fl, BMOG/IL-10fl/fl, BMOG/IL-10fl/fl, and APCMOG 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-10fl/fl and IL-10fl/fl mice (●)), BMOG (○), or BMOG/IL-10fl/fl (□) mice with MOGp35-55. BMOG and BMOG/IL-10fl/fl mice develop significantly less severe EAE compared with control mice (p < 0.05, days 14–26). Values are represented as mean ± SEM.
BMOG 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 APCMOG mice (Thy1.2), which were injected with agonistic anti-CD40 to activate DCs in vivo (as depicted in the scheme of Fig. 7A). 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. 4A, the ratio was similar, namely 1:12 for Thy1.1 vs Thy1.2 cells (Fig. 7B). As seen in Fig. 7C, 2D2 CD4 T cells that were transferred to WT mice, reisolated, and transferred to APCMOG mice proliferated extensively, to a similar extent as when directly transferred to APCMOG mice (Fig. 4A). 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 APCMOG mice. In contrast, 2D2 CD4 T cells that first encountered MOG by B cells in BMOG mice and were then transferred to APCMOG mice were dramatically decreased in number. As seen in Fig. 7C, 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. 7C 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 BMOG before transfer to APCMOG mice, although Thy1.1 cells appeared to proliferate in APCMOG mice. These results indicate that 2D2 CD4 T cells that were reisolated from WT mice as well as the ones reisolated from BMOG mice were triggered and proliferated when restimulated in APCMOG 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 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) BMOG mice. Numbers in quadrants indicate percent positive cells in each. MFI, mean fluorescence intensity.

**FIGURE 7.** BMOG-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 BMOG mice. C. Proliferation of 2D2 CD4+ Thy1.1+ T cells reisolated from WT or BMOG mice after restimulation for 4 days in anti-CD40 injected APCMOG 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 APCMOG mice.
initiated, as no nonproliferating Thy1.1+ T cells could be detected (Fig. 7C).

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 APCMOG 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 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 BMOG (□) mice.

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 BMOG mice after restimulation for 4 days in anti-CD40-injected APCMOG 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 BMOG 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 APCMOG 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 (MHCIId) 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 co-inhibitory 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 MHCIId-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 MHCIId-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 MHCIId+ 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 MHCIId+ 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.

BMOG as well as APCMOG 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 BMOG 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 APCMOG mice. However, activation of resting Ag-experienced T cells by B cells was indistinguishable from activation of these cells in APCMOG 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 BMOG 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 BMOG 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 BMOG 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 BMOG 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\(^{\text{MOG}}\) 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\(^{\text{MOG}}\) 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 co-inhibitory 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.

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References


