Published in final edited form as:

Eur J Immunol. 2013 July ; 43(7): . doi:10.1002/eji.201242784.

Peripheral CD4⁺ T-cell tolerance is induced in vivo by rare antigen-bearing B cells in follicular, marginal zone, and B-1 subsets

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Summary

B cells are efficient APCs when they internalize antigen via BCR-mediated uptake. Adoptively transferred antigen-presenting B cells can induce T-cell tolerance to foreign and self antigens; however, it is unknown whether endogenous B cells presenting self-peptides interact with naïve T cells and contribute to peripheral T-cell self-tolerance. Moreover, the relative abilities of mature B-cell subsets to induce T-cell tolerance have not been examined. To address these questions, we created a new mouse model wherein a very small fraction of B cells expresses an antigen transgene that cannot be transferred to other APCs. We limited antigen expression to follicular, marginal zone, or B-1 B-cell subsets and found that small numbers of each subset interacted with naïve antigen-specific T cells. Although antigen expressed by B-1 B cells induced the most T-cell division, divided T cells subsequently disappeared from secondary lymphoid tissues. Independent of which B-cell subset presented antigen, the remaining T cells were rendered hyporesponsive, and this effect was not associated with Foxp3 expression. Our data show that physiologically relevant proportions of B cells can mediate peripheral T-cell tolerance, and suggest that the mechanisms of tolerance induction might differ among follicular, marginal zone, and B-1 B-cell subsets.

Keywords

B-cell subsets; CD4⁺ T cells; tolerance

Introduction

Negative selection in the BM and thymus eliminates many newborn, self-reactive lymphocytes, but this process is not complete, and self-tolerance relies on additional peripheral mechanisms to limit immune responses by autoreactive T cells and T cells specific for commensal microbiota and environmental antigens. These mechanisms include death, anergy, and suppression by regulatory lymphocytes. APCs play a central role in providing signals that determine tolerance versus immunity in T cells. Therefore, it is essential to identify the APCs that induce and maintain peripheral tolerance. DCs are well-studied as effective APCs for both priming and peripheral tolerance [1], but B cells can also present antigen efficiently. Though poorly phagocytic, B cells can concentrate antigen for

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Conflict of interest

The authors declare no financial or commercial conflict of interest.

presentation to T cells up to 10,000 fold via BCR-mediated endocytosis, even in the absence of BCR cross-linking [2, 3], and may be essential APCs for immunity or tolerance to some low concentration antigens [4–7]. Thus, while B cells of any given specificity constitute a small fraction of total B cells, they may represent a physiologically relevant tolerizing APC. Indeed, very small numbers of short-lived, anergic self-antigen specific B cells can induce tolerance that is partially dependent on the ability of the B cells to directly present antigen on MHC class II [8].

Besides short-lived anergic B cells, two subsets of self-renewing B cells, B-1 and marginal zone (MZ) B cells, possess an increased proportion of self-reactive and poly-reactive specificities. B-1 B cells express a restricted set of unmutated Ig V genes lacking N region additions that react with self and foreign carbohydrate and phospholipid antigens and are protective against certain encapsulated bacteria [9]. They reside primarily in the peritoneal and pleural cavities, although they also represent ~2% of splenic B cells [10, 11]. MZ B cells are more heterogeneous, but a portion has a restricted set of rearranged V region genes, and weakly polyreactive or autoreactive BCRs can select B cells into the MZ subset [12, 13]. Both MZ and B-1 B cells are larger than resting follicular (Fo) B cells and resemble partially activated Fo B cells in some of their properties and surface markers [14–16], although molecules involved in antigen presentation have not been directly compared among all three subsets. MZ B cells are superior to Fo B cells as APCs for T cells in vitro [14, 17], but virtually nothing is known about the ability of different B-cell subsets to induce T-cell tolerance in vivo.

Many experiments have shown that transferred unfractionated B cells pulsed with or expressing antigen can tolerize or delete naïve T cells [18–22]. Likewise, antigen targeted to or expressed by a majority of endogenous B cells can induce antigen-specific T-cell tolerance to foreign or self-antigens [18, 23, 24]. In most studies of endogenous B cells as tolerizing APCs, a large portion of the entire B-cell repertoire presents antigen [18, 23], which does not reflect the ability of a limited repertoire of BCR specific for self-antigen to tolerize antigen-specific T cells. In adoptive transfer studies where more physiologic proportions of antigen-presenting B cells can be achieved, antigen can be transferred to other APCs in vivo [19, 25, 26], so it is not clear whether B cells are directly presenting antigen. Recently, Wysocki and colleagues [8] showed that anergic self-reactive Ars/A1 B cells depend partially, but not entirely, on MHC class II to induce tolerance. Likewise, Townsend and Goodnow [19] showed that antigen-pulsed B cells transferred antigen to host APCs in sufficient amounts to activate antigen-specific T cells. However, if host APCs did not bear the restricting MHC, the transferred B cells were also capable of inducing T-cell division. These studies showed that transferred B cells can interact with naïve antigen-specific T cells, but did not address whether this occurs in the absence of acute BCR signals. They also did not examine potential differences between mature B-cell subsets in their ability to interact with and tolerize naïve antigen-specific T cells.

Our central questions here are 1) whether low precursor frequencies of antigen-presenting polyclonal B cells that are activated only by endogenous self-antigen can interact with antigen-specific T cells to induce tolerance, and 2) whether Fo, MZ, and B-1 B-cell subsets differ in this capacity. We created a new mouse model whereby, in the context of an otherwise normal immune system, a very small proportion of B cells (<0.5%) bears a peptide antigen that is covalently linked to MHC class II and cannot be transferred to other APCs [27]. We then devised methods to largely limit this antigen-bearing B-cell population to Fo, MZ, or B-1 B-cell subsets in order to compare the relative capacities of these subsets to tolerize naïve antigen-specific T cells. We found that naïve antigen-specific T cells could interact with antigen-presenting B cells of all subsets, but that T-cell proliferation was most robust in response to antigen presented by B-1 cells. Nonetheless, all three subsets induced

substantial tolerance to subsequent T-cell stimulation that was not accompanied by differentiation to Foxp3⁺ Treg cells. Mechanistically, we propose that the *in vivo* interactions with B cells led to T-cell anergy and/or death, depending on the interacting B-cell subset.

Results

MZ and B-1 B cells express increased levels of costimulatory and adhesion molecules

MZ and B-1 B cells have been reported to possess markers associated with a semi-activated state [14–16], which suggests they may form more successful B-T conjugates for T-cell activation or tolerance. We directly compared B-1, MZ, and Fo B cells (Fig. 1A) from spleen and peritoneal cavity in terms of their MHC class II, adhesion, and costimulatory molecule expression levels. MHC class II levels were not different between the subsets, but all other markers varied distinctly among B-cell subsets (Fig. 1B). MZ and B-1 B cells expressed more CD86, ICAM-1, and PD-L1 than did Fo B cells. In addition, B-1, but not MZ B cells, expressed more CD80 than Fo B cells. B-1 and MZ B cells expressed similarly elevated levels of CD86 and PD-L1, but MZ B cells expressed a level of ICAM-1 intermediate between B-1 and Fo B cells. The increased levels of activation markers on B-1 B cells was not a universal feature of B cells from the peritoneal cavity because B-2 B cells from the peritoneal cavity were indistinguishable from Fo B cells from the spleen using these markers, with the exception of CD86, which was slightly increased compared with Fo B cells (Fig. 1C).

Repopulation of BM chimeric mice with low frequencies of antigen-transgenic B-1, MZ, and Fo B cells

Given the increased level of costimulatory and adhesion molecules on B-1 and MZ B cells, we expected these subsets to more efficiently interact with antigen-specific naïve T cells *in vivo*. To test the intrinsic T-cell stimulatory capacity of these B-cell subsets *in vivo* we developed a system in which antigenic peptide could not be transferred from traceable antigen-expressing B cells to other APCs, such as DCs. We used B cells from antigen transgenic mice that express a peptide covalently linked to MHC class II I-E^k under the control of a class II promoter (Ag-tg) [27]. Aside from transgene expression, B cells from these mice are normal. They are polyclonal and can bind and take up antigen, including self-antigen, for which their BCR is specific. Intracellular signal transduction associated with BCR cross-linking (or lack thereof) reflects the constitutive signals normally received by polyclonal B cells of the different subsets. While the endogenous C57BL/6 class II (IA^b) is highly and equivalently expressed on WT and Ag-tg B cells [27], the IE^k-peptide transgene is expressed at a low level only on Ag-tg B-cell subsets (Fig. 2A). This expression level is ~1.5 logs less than IE^k expression on mouse strains that endogenously express IE^k, such as B10.BR [27].

To create mice whose repertoires contain a physiologically low frequency of antigen-bearing B cells of each subset, we exploited the self-renewing potential of MZ and B-1 B cells. We made BM chimeras in which we co-injected lethally irradiated recipients with B6.Thy1.1 BM along with graded numbers of highly pure FACS-sorted CD45.1 congenically marked Ag-tg MZ or B-1 B cells (Fig. 2C). PerC B-1 B cells were sorted as CD19⁺CD11c⁻CD11b⁺B220^{lo} (Fig. 2B), and were further assessed for CD5 expression, which was enriched after sorting to the same degree as seen in gated B-1 B cells pre-sort (Supporting Information Fig. 1). MZ B cells were sorted as CD19⁺CD93⁻CD21^{hi}CD23^{lo} (Fig. 2B), and were further assessed for IgM and CD1d expression, which was enriched after sorting to the same degree as seen in gated MZ B cells pre-sort (Supporting Information Fig. 1). Because these B-cell subsets normally undergo homeostatic proliferation as they self-

renew, we thought that proliferation in the irradiated hosts would not compromise their identity and phenotype, which indeed was the case.

In contrast, preliminary experiments showed that when Fo B cells were co-injected with BM they lost their follicular phenotype during expansion in the lymphopenic host and acquired markers and localization properties associated with the self-renewing MZ and B-1 B-cell subsets by the time the BM had reconstituted the recipients (Supporting Information Fig. 2 and [28]). Thus, for the Fo B-cell group, we transferred congenically marked Ag-tg FACS-sorted Fo B cells (CD19⁺CD93⁻CD23^{hi}CD21^{lo}) 2 months after BM reconstitution and 7 days before introduction of antigen-specific T cells to avoid acute activation associated with the transfer (Fig. 2B and C). In the B-1 and MZ B-cell groups, we reliably detected congenically marked Ag-tg B cells 2 months after combined B-cell and BM transfer (Fig. 3A). Despite the presence of allo-MHC class II, the transferred Fo B cells were also accepted by the immunocompetent hosts (Fig. 3A). This is not surprising, since accessory cell-depleted B cells can effectively induce transplantation tolerance across other MHC class II barriers [29], the transgenic antigen is expressed at low levels, and most of the transgenic MHC molecules are loaded with the same peptide [30, 31]. By adjusting the number of B cells injected, we achieved equivalent ranges of Ag-tg B-1, MZ, and Fo B-cell percentages in chimeras, from ~0.03% to 0.3% of total spleen (Fig. 3A, 4B and C).

B-1, MZ, and Fo B cells retain their phenotypic identity in chimeras

We next assessed the phenotype of Ag-tg B-1, MZ, and Fo B cells from chimeric mice. Ag-tg Fo B cells remained mostly CD21^{lo}CD23^{hi}IgM^{lo}IgD^{hi} and were enriched for Fo phenotype compared with endogenous B cells (Fig. 3A and Supporting Information Fig. 3). The Ag-tg self-renewing MZ and B-1 B-cell subsets also retained expression of subset-specific markers, with over half showing CD21^{hi}CD23^{lo}IgM^{hi}IgD^{lo} and CD21^{lo}CD23^{lo}IgM^{hi}IgD^{lo} phenotypes respectively (Fig. 3A and Supporting Information Fig. 3). B-1 B cells normally occupy 1–2% of the spleen, but also home to the peritoneal cavity. Importantly, we found Ag-tg B-1 B cells in the peritoneal cavity, which further confirms they maintain stable B-1 identity after transfer (Fig. 3B). Although we initially sorted on total Ag-tg B-1 B cells (comprising B-1a and B-1b), those that repopulated the peritoneal cavity adopted a B-1a phenotype as assessed by expression of CD11b, B220, and CD5 (Fig. 3B). The Ag-tg B-1 cells occupy a large portion of peritoneal B cells and express higher levels of CD5 than the BM-derived B-1 cells because only B-1b cells, which are CD5^{neg}renew from BM [32]. In some B-1 and MZ B-cell chimeric mice we observed a small proportion of CD45.1⁺CD19⁻ cells, which theoretically could have derived from contaminating Ag-tg macrophages or DCs in the FACS sort. However, these cells expressed neither CD11b nor CD11c, but instead expressed kappa light chain and varying levels of CD138 (Fig. 3C). This strongly suggests that they are B cells in the process of differentiating to plasmablasts.

Small numbers of Ag-tg B-1, MZ, and Fo B cells interact with naïve T cells in vivo

To determine the ability of these very small numbers of antigen-bearing B cells to interact with antigen-specific T cells, we transferred syngeneic AND TCR transgenic T cells, which are positively selected by I-A^b in the thymus but recognize only I-E^k-PCC in the periphery [33], into chimeras created as described in Fig. 2C. In our chimeras, the only antigen available for AND T cells to recognize is the antigen transgene expressed on small numbers of B-cell subsets. Importantly, the polyclonal Ag-tg B cells will receive (or not) signals through their endogenously rearranged BCRs via any self-antigens for which they are specific. We assessed T-cell activation in vivo by CFSE dilution, and found that small numbers of all Ag-tg B-cell subsets interacted with AND T cells in an antigen-specific manner. However, Ag-tg Fo B cells induced far less proliferation than MZ or B-1 B cells,

with >60% of T cells remaining undivided at all concentrations of Fo B cells (Fig. 4A and B). In the B-1 group, some AND T cells appear to have diluted out CFSE sufficiently to begin merging into the endogenous population of V β 3⁺V β 11⁺ T cells. These are mostly radioresistant Thy1.2⁺ host-derived T cells that happened to rearrange V β 3 and V β 11 (Supporting Information Fig. 4A). While this could theoretically cause us to underestimate the number of AND T cells, we do not believe this is the case, because if it were, one would expect 1) an increase in total V β 3⁺V β 11⁺CFSE⁻ cells, and/or 2) an increase in the CFSE mean fluorescence intensity of the CFSE negative gate. Neither of these differences was observed (Supporting Information Fig. 4B and C).

We assessed T-cell proliferation as a function of percent of Ag-tg B cells within the spleen. There was a very high degree of correlation between the proportion of T cells that remained undivided and the number of Ag-tg B cells of each subset present (Fig. 4B). When comparing the regression curves between B-cell subsets, it is clear that at any given proportion of Ag-tg B cells, B-1 B cells induce the most proliferation, followed by MZ B cells and then Fo B cells. It should be noted that even in the B-1 group, the level of AND T-cell proliferation is far less than maximal, as demonstrated previously by injecting AND T cells into Ag-tg hosts in which all class II⁺ cells express the antigen [27]. Interestingly, the overall recovery of AND T cells in the spleen correlated poorly with the Ag-tg B-cell proportion (Fig. 4C), and the population of T cells that proliferated the most—those that encountered the highest numbers of Ag-tg B-1 B cells—accumulated the least. In fact, within the B-1 group, AND T-cell division negatively correlated with AND T-cell accumulation (Fig. 4D). Numerous preliminary experiments showed that total spleen cell numbers did not vary across experimental conditions (Supporting Information Fig. 5), so AND T-cell and Ag-tg B-cell percentages accurately reflect total AND and Ag-tg cell numbers. The loss of AND T cells was not due to trafficking to the peritoneal cavity, as AND T cells were absent from this location in all experimental groups. These data suggest that a likely result of activation by B-1 B cells is T-cell death, although we cannot exclude trafficking to other tissues.

Small numbers of all B-cell subsets induce clonal T-cell hyporesponsiveness but not Foxp3 expression

Given the absence of any infection, adjuvant, or other “danger” signal, we thought the outcome of any T-cell activation in our chimeras might be immune tolerance. However, the low level of T-cell proliferation in response to antigen-bearing Fo B cells suggested that most T cells in this group had not encountered antigen, and thus would remain responsive. To test these hypotheses, one week after transfer of CFSE labeled AND T cells into chimeras, we enriched those T cells from individual spleens and restimulated equal numbers of AND T cells in vitro with Ag-tg APCs. Independent of the B-cell subset that presented antigen to T cells in vivo, AND T cells thus recovered were hyporesponsive as compared to AND T cells recovered one week after transfer into control BM chimeric mice lacking any Ag-tg APCs (Fig. 5A and Supporting Information Fig. 6). In the absence of antigen, the latter remain naïve, and thus show maximal responsiveness.

Under certain conditions, B cells presenting antigen have been shown to induce or expand Treg cells in vivo [24, 34], so we considered that the naïve AND T cells might be differentiating into Treg cells upon antigen encounter on some Ag-tg B-cell subsets in vivo. In particular, B-1 B cells, which express high levels of PD-L1 might induce Treg-cell differentiation from naïve T cells [35, 36]. To test this possibility we assessed AND T cells for expression of Foxp3 after transfer into chimeric mice bearing Ag-tg B cells of each subset. The transferred T cells were uniformly negative for Foxp3 (Fig. 5B) as well as CD25 (data not shown). We conclude that in vivo, the primary effect of cognate interactions

between naïve T cells and rare antigen-bearing B cells is T-cell hyporesponsiveness and, in the case of B-1 B cells, T-cell death.

Discussion

In addition to producing antibodies, it is now clear that B cells can either enhance or inhibit T-cell immunity, including autoimmune responses [37]. When regulating T-cell immunity, B cells may employ several different mechanisms. IL-10-producing regulatory B cells (also called B10) restrain autoimmunity in many models [37, 38]. B cells also may be important in Treg-cell-mediated suppression of responses since B cells can expand or induce Treg cells in vivo [24, 34], although in other models B-cell-mediated T-cell tolerance is independent of Treg cells [23, 39]. We and others have shown that B cells can act as tolerizing APCs in vivo. In models in which most endogenous B cells present the same self-antigen, naïve T cells can be rendered anergic and subsequently deleted [18, 23]. Similarly, several studies have shown that when antigen-pulsed or antigen-expressing B cells are acutely transferred, they can induce T-cell tolerance to the antigen they present [18–22].

An important unanswered question was whether rare self-reactive endogenous B cells have the potential to present antigen in a tolerizing fashion to naïve T cells in the steady state. It has been assumed that for both T-cell immunity and tolerance, DCs are the APCs that interact with naïve T cells, and that only once T cells have been primed do antigen-specific B and T cells collaborate [40–43]. Recently, Cahalan and colleagues [44] showed that naïve antigen-expressing B cells and antigen-specific T cells formed stable conjugates as early as 72 hours after adoptive transfer, but this time point does not preclude the possibility that T cells had already been primed on B-cell-derived antigen presented by DCs. Wysocki and colleagues [8] showed that self-antigen specific anergic B cells can induce tolerance, and that this was partially dependent on direct presentation of antigen the B cells had internalized via the BCR. Here, we used Ag-tg B cells in which peptide transfer to other APCs is prohibited to test whether small precursor numbers of antigen-bearing Fo, MZ, and B-1 B cells can interact with naïve antigen-specific T cells, and if so, what the functional outcome of such an interaction might be. These subsets differ in the repertoire of antigens, including self-antigens, they bind. In our system the B cells are polyclonal and selected normally, and are not acutely activated through a fixed antigen receptor or TLR stimulation. Instead, B cells from each subset receive only tonic signals and BCR signals provided by autoantigens that they normally would encounter. Another important component of our system is that antigen-bearing B cells occupy only ~0.05–0.5% of the total B-cell pool in the context of an otherwise WT immune system. This proportion agrees with the range of estimates for B-cell precursor frequency for self-antigens [45, 46].

Due to the covalent linkage of transgenic I-E^k to peptide and the MHC restriction of AND T cells, we eliminated the possibility of peptide transfer to other APCs. Although we cannot rule out a role for trogocytosis (it is possible that myeloid APCs can capture membrane patches encompassing entire I-E^k-peptide molecules from B cells), we do not favor this mechanism. Trogocytosis is primarily thought to occur between cells that interact via specific receptor-ligand interaction — most notably transfer of MHC from APCs to T cells and NK cells and transfer of antibody-bound surface proteins from opsonized cells to FcR-expressing myeloid cells [47, 48]. Thus, it seems highly unlikely that MHC would be transferred from B cells to myeloid APCs.

Using this new system, we found that small numbers of B cells of all subsets interacted with naïve T cells as assessed by T-cell division in vivo. B-1 B cells induced more T-cell proliferation than MZ or Fo B cells, but despite the increased proliferation, T cells did not accumulate in the spleen or peritoneal cavity, and in fact decreased significantly upon

increasing rounds of division. This suggests that an outcome of this interaction is T-cell death, although we cannot rule out trafficking to peripheral tissues. The low level of T-cell proliferation induced by Fo B cells suggested that these cells might not be interacting efficiently with naïve T cells. This turned out not to be the case as all three subsets of B cells strongly induced T-cell hyporesponsiveness to subsequent antigen encounter. Although T-cell anergy is usually preceded by cell division, this is not universally true [23, 49], and antigen expressed on B cells in particular has been shown to induce tolerance in the absence of T-cell division [23]. We think it is likely that all B-cell subsets are capable of inducing T-cell anergy, though we cannot rule out hyporesponsiveness due to cell death upon secondary challenge.

Given the distinct T-cell proliferative profiles induced by different B-cell subsets, it is tempting to speculate that the mechanism of tolerance induction might differ. B-1 B cells, which induced the most T-cell proliferation, also express the highest level of costimulatory molecules among B-cell subsets. On B-1 cells, the high levels of ICAM-1 and B7 (which would favor more stable contacts and ligation of CD28) may lead to T-cell proliferation followed by death in the absence of signal 3. Conversely, low levels of ICAM-1 and B7 on Fo B cells might favor more ligation of CTLA-4, which has a higher affinity for B7 than does CD28 [50], and which contributes to B-cell-mediated T-cell tolerance [23]. The high expression level of the inhibitory molecules PD-L1 (Fig. 1) and PD-L2 [51] on B-1 B cells may also play a role in T-cell tolerance in response to that B-cell subset, but not to others [35, 36]. Indeed, we hypothesized that the high levels of PD-L1 on B-1 B cells may bias tolerance towards a Treg cell phenotype, but we found no evidence of conversion of antigen-specific naïve T cells to Treg cells in any of the groups in our system. When physiologic proportions of B cells present antigen to naïve T cells, Treg cell conversion may preferentially occur in mucosal environments [34, 52] that provide additional Treg cell-inducing factors such as TGF- β , retinoic acid, and IL-10. Regardless of the mechanisms, future studies should determine whether there are qualitative differences in T-cell hyporesponsiveness induced by different B-cell subsets—T-cell lifespan, trafficking patterns, or ability to acquire helper functions upon inflammatory stimuli. Such differences may aid design of immunotherapies that employ clonally expanded or genetically modified B cells [53–55] to regulate autoimmune or allergic responses.

B-cell deficient animals and humans do not suffer from generalized T-cell-mediated autoimmunity, so B cells clearly are not necessary for T-cell tolerance to all self-antigens. However, in many models B-cell deficient animals experience exacerbated autoimmunity upon antigen challenge [39, 56, 57]. We propose that B-cell-mediated tolerance is important specifically for those self-antigens likely to be presented much more efficiently by B cells than by other APCs, namely low concentration soluble self-antigens, which could be concentrated and presented by resting Fo B cells without cross-linking the BCR [2], and self antigens for which B1 and MZ B-cell subsets have clonally enriched BCR specificities. Antigen presentation by B cells may deepen naïve T-cell tolerance to these self-antigens to prevent T helper cell activation and autoantibody formation in the event that that self-antigen becomes more widely available in an inflammatory context.

Materials and Methods

Animals

Mice were housed under specific pathogen free conditions at the Oregon Health & Science University animal facility. All procedures were approved by the Institutional Animal Care and Use Committee of Oregon Health & Science University in Portland, Oregon. B6.Thy1.1 and B6.CD45.1 mice were from The Jackson Laboratory. AND/Rag- α mice and antigen-transgenic mice expressing I-E^k and covalently linked PCC with the amino acid T102S

substitution (Ag-tg) were both maintained on a C57BL/6 background and were described previously [27]. Congenically marked Ag-tg mice were made by crossing Ag-tg mice to B6.CD45.1 mice.

Antibodies

Fluorescently conjugated antibodies used for flow cytometry were B220 (RA3-6B2), CD1d (1B1), CD4 (RM4-5), CD5 (53-7.3), CD11b (M1/70), CD11c (N418), CD19 (1D3), CD21 (4E3), CD23 (B3B4), CD25 (PC61.5), CD45.1 (A20), CD54/ICAM-1 (3E2), CD80 (16-10A1), CD86 (GL1), CD90.1 (HIS51), CD93 (AA4.1), CD138 (281-2), CD274/PD-L1 (MIH5), Foxp3 (FJK-16s), I-A^b (25-9-17), I-E^k (17-3-3), Ig kappa light chain (187.1), IgM (R6-60.2), IgD (11-26c.2a), V 11 (RR8-1), and V 3 (KJ25). All antibodies were from BD Bioscience, except CD1d, CD4, CD19, CD21, CD25, Foxp3 (eBioscience) and IgD (Biolegend).

Flow cytometry and cell sorting

Spleen cell suspensions were stained directly *ex vivo* for surface antigens as described [27] or for surface antigens followed by intracellular staining for Foxp3, performed per manufacturer's instructions (Biolegend Foxp3 Fix/Perm Buffer kit). Cells were analyzed on a FACSCalibur or LSR II flow cytometer (BD Biosciences) and analyzed using FlowJo (Tree Star). To sort B-cell subsets for transfer, cells were dissociated from LN, spleens, and peritoneal cavity washes of Ag-tg mice. Spleens and LN were pooled, magnetically enriched for B cells via negative selection (EasySep mouse B cell enrichment kit, Stem Cell Technologies), and stained with antibodies to CD19, CD93, CD21, and CD23. Fo B cells were sorted as CD19⁺CD93⁻CD21^{low}CD23^{hi}; MZ B cells were sorted as CD19⁺CD93⁻CD21^{hi}CD23^{low}. B-1 B cells were sorted from the peritoneal cavity as CD19⁺CD11c⁻CD11b⁺B220^{low}.

Adoptive transfers

For T-cell transfers, single cell suspensions were prepared from spleen and LN of AND/Rag^{-/-} mice, depleted of erythrocytes by hypotonic lysis, and labeled with CFSE as described [58]. The percentage of TCR transgenic cells was assessed (typically ~70%) and total leukocytes containing 1×10⁶ TCR transgenic T cells were transferred intravenously. For Fo B-cell transfers, Ag-tg Fo B cells were sorted as described in *Flow cytometry and cell sorting* and 2–20×10⁶ were injected intravenously 2 months post-chimerism and one week before T-cell transfer.

BM chimeras

BM was harvested from femurs and tibiae of B6.Thy1.1 mice, and 1×10⁶ nucleated BM cells were injected alone or mixed with varying numbers (0.2–1×10⁶) of sorted Ag-tg MZ or B-1 B cells into lethally irradiated C57BL/6 recipients.

In vitro T-cell stimulation and ³H-thymidine incorporation

Transferred AND/Rag^{-/-} CD4⁺ T cells were magnetically enriched from individual spleens of recipient mice per manufacturer's instructions for CD4 T-cell purification (EasySep mouse CD4⁺ T-cell enrichment kit, Stem Cell Technologies) with addition of biotinylated anti-Thy1.1 antibody to the negative selection antibody cocktail. This allowed greater enrichment of AND/Rag^{-/-} T cells by depleting a proportion of the recipient CD4⁺ T cells. Without this necessary step, the proportion of AND/Rag^{-/-} T cells among recipient splenocytes was too low (0.05–0.2%) to measure antigen-specific ³H-thymidine incorporation above background. After enrichment, populations were 1–4% AND/Rag^{-/-} T cells. Each enriched population from individual recipient spleens was assessed for percent

AND/Rag^{-/-} T cells by flow cytometry. The number of total cells added to each well was adjusted such that 8000 AND/Rag^{-/-} T cells were added to each well of a 96 well round-bottom plate. For controls that did not receive AND/Rag^{-/-} T cells, a number of total magnetically purified T cells was added equaling the average number of total T cells plated in experimental groups. T cells were stimulated with 300,000 irradiated (1000 rads) splenocyte APCs obtained from unmanipulated Ag-tg or C57BL/6 control mice. T cells were stimulated for 6 days, and 1 µCi of ³H-thymidine was added per well for the last 18–20 hours. Cell associated ³H-thymidine was counted on a Packard TopCount-NXT microplate scintillation counter (Perkin Elmer). Stimulation indices were calculated for T cells from each individual recipient as (mean cpm of wells with Ag-tg APCs)/(mean cpm of wells with WT APCs).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank M. Boyd and P. Canaday at the OHSU Flow Cytometry Core Facility for cell sorting. This work was supported by National Institutes of Health grant AI070934 (to D.C.P.) and Medical Research Foundation of Oregon (to D.C.P.).

Abbreviations

Ag-tg	antigen transgenic
Fo	follicular
MZ	marginal zone

References

1. Steinman RM, Hawiger D, Nussenzweig MC. Tolerogenic dendritic cells. *Annu. Rev. Immunol.* 2003; 21:685–711. [PubMed: 12615891]
2. Tony H-P, Phillips NE, Parker DC. Role of membrane immunoglobulin (Ig) crosslinking in membrane Ig-mediated, major histocompatibility-restricted T cell-B cell cooperation. *J. Exp. Med.* 1985; 162:1695–1708. [PubMed: 3877141]
3. Lanzavecchia A. Receptor-mediated antigen uptake and its effect on antigen presentation to class II-restricted T lymphocytes. *Annu. Rev. Immunol.* 1990; 8:773–793. [PubMed: 2188679]
4. Crawford A, Macleod M, Schumacher T, Corlett L, Gray D. Primary T cell expansion and differentiation in vivo requires antigen presentation by B cells. *J. Immunol.* 2006; 176(6):3498–3506. [PubMed: 16517718]
5. Tsitoura DC, Yeung VP, DeKruyff RH, Umetsu DT. Critical role of B cells in the development of T cell tolerance to aeroallergens. *Int. Immunol.* 2002; 14(6):659–667. [PubMed: 12039917]
6. Valujskikh A, VanBuskirk AM, Orosz CG, Heeger PS. A role for TGFβ and B cells in immunologic tolerance after intravenous injection of soluble antigen. *Transplantation.* 2001; 72(4): 685–693. [PubMed: 11544432]
7. Ashour HM, Niederkorn JY. Peripheral tolerance via the anterior chamber of the eye: role of B cells in MHC class I and II antigen presentation. *J. Immunol.* 2006; 176(10):5950–5957. [PubMed: 16670303]
8. Aviszus K, Macleod MK, Kirchenbaum GA, Detanico TO, Heiser RA, St Clair JB, Guo W, Wysocki LJ. Antigen-specific suppression of humoral immunity by anergic arsa1 B cells. *J. Immunol.* 2012; 189(9):4275–4283. [PubMed: 23008448]
9. Baumgarth N. The double life of a B-1 cell: self-reactivity selects for protective effector functions. *Nat. Rev. Immunol.* 2011; 11(1):34–46. [PubMed: 21151033]

10. Ding C, Liu Y, Wang Y, Park BK, Wang CY, Zheng P, Liu Y. Siglecg limits the size of B1a B cell lineage by down-regulating NFkappaB activation. *PLoS ONE*. 2007; 2(10):e997. [PubMed: 17912374]
11. Herzenberg LA, Stall AM, Lalor PA, Sidman C, Moore WA, Parks DR, Herzenberg LA. The Ly-1 B cell lineage. *Immunol. Rev.* 1986; 93:81–102. [PubMed: 3096879]
12. Chen X, Martin F, Forbush KA, Perlmutter RM, Kearney JF. Evidence for selection of a population of multi-reactive B cells into the splenic marginal zone. *Int. Immunol.* 1997; 9:27–41. [PubMed: 9043945]
13. Wen L, Brill-Dashoff J, Shinton SA, Asano M, Hardy RR, Hayakawa K. Evidence of marginal-zone B cell-positive selection in spleen. *Immunity*. 2005; 23(3):297–308. [PubMed: 16169502]
14. Oliver AM, Martin F, Kearney JF. IgM^{high} CD21^{high} lymphocytes enriched in the splenic marginal zone generate effector cells more rapidly than the bulk of follicular B cells. *J. Immunol.* 1999; 162:7198–7207. [PubMed: 10358166]
15. Martin F, Kearney JF. CD21^{high} IgM^{high} splenic B cells enriched in the marginal zone: distinct phenotypes and functions. *Curr. Top. Microbiol. Immunol.* 1999; 246:45–50. discussion 51–2. [PubMed: 10396038]
16. Hastings WD, Tumang JR, Behrens TW, Rothstein TL. Peritoneal B-2 cells comprise a distinct B-2 cell population with B-1b-like characteristics. *Eur. J. Immunol.* 2006; 36(5):1114–1123. [PubMed: 16609926]
17. Attanavanich K, Kearney JF. Marginal zone, but not follicular B cells, are potent activators of naive CD4 T cells. *J. Immunol.* 2004; 172(2):803–811. [PubMed: 14707050]
18. Eynon EE, Parker DC. Small B cells as antigen-presenting cells in the induction of tolerance to soluble protein antigens. *J. Exp. Med.* 1992; 175:131–138. [PubMed: 1730913]
19. Townsend SE, Goodnow CC. Abortive proliferation of rare T cells induced by direct or indirect antigen presentation by rare B cells in vivo. *J. Exp. Med.* 1998; 187:1611–1621. [PubMed: 9584139]
20. Parker DC, Greiner DL, Phillips NE, Appel MC, Steele AW, Durie FH, Noelle RJ, et al. Survival of mouse pancreatic islet allografts in recipients treated with allogeneic small lymphocytes and antibody to CD40 ligand. *Proc. Natl. Acad. Sci.* 1995; 92:9560–9564. [PubMed: 7568172]
21. Fuchs EJ, Matzinger P. B cells turn off virgin but not memory T cells. *Science*. 1992; 258:1156–1159. [PubMed: 1439825]
22. Su Y, Zhang AH, Li X, Owusu-Boaitey N, Skupsky J, Scott DW. B cells "transduced" with TAT-fusion proteins can induce tolerance and protect mice from diabetes and EAE. *Clin. Immunol.* 2011; 140(3):260–267. [PubMed: 21546313]
23. Frommer F, Heinen TJ, Wunderlich FT, Yogev N, Buch T, Roers A, Bettelli E, et al. Tolerance without clonal expansion: self-antigen-expressing B cells program self-reactive T cells for future deletion. *J. Immunol.* 2008; 181(8):5748–5759. [PubMed: 18832734]
24. Morlacchi S, Soldani C, Viola A, Sarukhan A. Self-antigen presentation by mouse B cells results in regulatory T-cell induction rather than anergy or clonal deletion. *Blood*. 2011; 118(4):984–991. [PubMed: 21652680]
25. Valdez Y, Mah W, Winslow MM, Xu L, Ling P, Townsend SE. Major histocompatibility complex class II presentation of cell-associated antigen is mediated by CD8alpha⁺ dendritic cells in vivo. *J. Exp. Med.* 2002; 195:683–694. [PubMed: 11901195]
26. Whipple EC, Shanahan RS, Ditto AH, Taylor RP, Lindorfer MA. Analyses of the in vivo trafficking of stoichiometric doses of an anti-complement receptor 1/2 monoclonal antibody infused intravenously in mice. *J. Immunol.* 2004; 173(4):2297–2306. [PubMed: 15294942]
27. Lathrop SK, Huddleston CA, Dullforce PA, Montfort MJ, Weinberg AD, Parker DC. A signal through OX40 (CD134) allows anergic, autoreactive T cells to acquire effector cell functions. *J. Immunol.* 2004; 172:6735–6743. [PubMed: 15153490]
28. Agenes F, Freitas AA. Transfer of small resting B cells into immunodeficient hosts results in the selection of a self-renewing activated B cell population. *J. Exp. Med.* 1999; 189:319–330. [PubMed: 9892614]
29. Hori S, Sato S, Kitigawa S, Azuma T, Kokudo S, Hamaoka T, Fujiwara H. Tolerance induction of allo-class II H-2 antigen-reactive L3T4⁺ helper T cells and prolonged survival of the

- corresponding class II H-2-disparate skin graft. *J. Immunol.* 1989; 143:1447–1452. [PubMed: 2527264]
30. Ignatowicz L, Kappler J, Marrack P. The repertoire of T cells shaped by a single MHC/peptide ligand. *Cell.* 1996; 84:521–529. [PubMed: 8598039]
 31. Liu C-P, Parker DC, Kappler J, Marrack P. Selection of antigen-specific T cells by a single IEK peptide combination. *J. Exp. Med.* 1997; 186:1441–1450. [PubMed: 9348301]
 32. Herzenberg LA. B-1 cells: the lineage question revisited. *Immunol. Rev.* 2000; 175:9–22. [PubMed: 10933587]
 33. Kaye J, Vasquez NJ, Hedrick SM. Involvement of the same region of the T cell antigen receptor in thymic selection and foreign peptide recognition. *J. Immunol.* 1992; 148:3342–3353. [PubMed: 1316916]
 34. Sun JB, Flach CF, Czerkinsky C, Holmgren J. B lymphocytes promote expansion of regulatory T cells in oral tolerance: powerful induction by antigen coupled to cholera toxin B subunit. *J. Immunol.* 2008; 181(12):8278–8287. [PubMed: 19050244]
 35. Francisco LM, Salinas VH, Brown KE, Vanguri VK, Freeman GJ, Kuchroo VK, Sharpe AH. PD-L1 regulates the development, maintenance, and function of induced regulatory T cells. *J. Exp. Med.* 2009; 206(13):3015–3029. [PubMed: 20008522]
 36. Wang L, Pino-Lagos K, de Vries VC, Guleria I, Sayegh MH, Noelle RJ. Programmed death 1 ligand signaling regulates the generation of adaptive Foxp3+CD4+ regulatory T cells. *Proc. Natl. Acad. Sci. U S A.* 2008; 105(27):9331–9336. [PubMed: 18599457]
 37. Lund FE, Randall TD. Effector and regulatory B cells: modulators of CD4(+) T cell immunity. *Nat. Rev. Immunol.* 2010; 10(4):236–247. [PubMed: 20224569]
 38. Bouaziz JD, Yanaba K, Tedder TF. Regulatory B cells as inhibitors of immune responses and inflammation. *Immunol. Rev.* 2008; 224:201–214. [PubMed: 18759928]
 39. Hoehlig K, Shen P, Lampropoulou V, Roch T, Malissen B, O'Connor R, Ries S, et al. Activation of CD4(+) Foxp3(+) regulatory T cells proceeds normally in the absence of B cells during EAE. *Eur. J. Immunol.* 2012; 42(5):1164–1173. [PubMed: 22539290]
 40. Banchereau J, Steinman RM. Dendritic cells and the control of immunity. *Nature.* 1998; 392:245–252. [PubMed: 9521319]
 41. Mempel TR, Henrickson SE, Von Andrian UH. T-cell priming by dendritic cells in lymph nodes occurs in three distinct phases. *Nature.* 2004; 427(6970):154–159. [PubMed: 14712275]
 42. Guery JC, Ria F, Adorini L. Dendritic cells but not B cells present antigenic complexes to class II-restricted T cells after administration of protein in adjuvant. *J. Exp. Med.* 1996; 183(3):751–757. [PubMed: 8642279]
 43. MacLennan IC, Gulbranson-Judge A, Toellner KM, Casamayor-Palleja M, Chan E, Sze DM, Luther SA, Orbea HA. The changing preference of T and B cells for partners as T-dependent antibody responses develop. *Immunol. Rev.* 1997; 156:53–66. [PubMed: 9176699]
 44. Matheu MP, Su Y, Greenberg ML, Blanc CA, Parker I, Scott DW, Cahalan MD. Toll-like receptor 4-activated B cells out-compete Toll-like receptor 9-activated B cells to establish peripheral immunological tolerance. *Proc. Natl. Acad. Sci. U S A.* 2012; 109(20):E1258–E1266. [PubMed: 22511718]
 45. Portnoï D, Freitas A, Holmberg D, Bandeira A, Coutinho A. Immunocompetent autoreactive B lymphocytes are activated cycling cells in normal mice. *J. Exp. Med.* 1986; 164(1):25. [PubMed: 3487614]
 46. Souroujon M, White-Scharf ME, Andreschwartz J, Geftner ML, Schwartz RS. Preferential autoantibody reactivity of the preimmune B cell repertoire in normal mice. *J. Immunol.* 1988; 140(12):4173. [PubMed: 2453571]
 47. Wetzel SA, Parker DC. MHC transfer from APC to T cells following antigen recognition. *Crit. Rev. Immunol.* 2006; 26(1):1–21. [PubMed: 16472066]
 48. Ahmed KA, Munegowda MA, Xie Y, Xiang J. Intercellular trogocytosis plays an important role in modulation of immune responses. *Cell. Mol. Immunol.* 2008; 5(4):261–269. [PubMed: 18761813]
 49. Skokos D, Shakhar G, Varma R, Waite JC, Cameron TO, Lindquist RL, Schwickert T, et al. Peptide-MHC potency governs dynamic interactions between T cells and dendritic cells in lymph nodes. *Nat. Immunol.* 2007; 8(8):835–844. [PubMed: 17632517]

50. Linsley PS, Brady W, Urnes M, Grosmaire LS, Damle NK, Ledbetter JA. CTLA-4 is a second receptor for the B cell activation antigen B7. *J. Exp. Med.* 1991; 174:561–569. [PubMed: 1714933]
51. Zhong X, Tumang JR, Gao W, Bai C, Rothstein TL. PD-L2 expression extends beyond dendritic cells/macrophages to B1 cells enriched for V(H)11/V(H)12 and phosphatidylcholine binding. *Eur. J. Immunol.* 2007; 37(9):2405–2410. [PubMed: 17683117]
52. Singh A, Carson WFT, Secor ERJ, Guernsey LA, Flavell RA, Clark RB, Thrall RS, Schramm CM. Regulatory role of B cells in a murine model of allergic airway disease. *J. Immunol.* 2008; 180(11):7318–7326. [PubMed: 18490731]
53. Calderon-Gomez E, Lampropoulou V, Shen P, Neves P, Roch T, Stervbo U, Rutz S, et al. Reprogrammed quiescent B cells provide an effective cellular therapy against chronic experimental autoimmune encephalomyelitis. *Eur. J Immunol.* 2011; 41(6):1696–1708. [PubMed: 21469107]
54. Chen CC, Rivera A, Dougherty JP, Ron Y. Complete protection from relapsing experimental autoimmune encephalomyelitis induced by syngeneic B cells expressing the autoantigen. *Blood.* 2004; 103(12):4616–4618. [PubMed: 14988158]
55. Skupsky J, Su Y, Lei TC, Scott DW. Tolerance induction by gene transfer to lymphocytes. *Curr. Gene Ther.* 2007; 7(5):369–380. [PubMed: 17979683]
56. Fillatreau S, Sweeney CH, McGeachy MJ, Gray D, Anderton SM. B cells regulate autoimmunity by provision of IL-10. *Nat. Immunol.* 2002; 3(10):944–950. [PubMed: 12244307]
57. Matsushita T, Yanaba K, Bouaziz JD, Fujimoto M, Tedder TF. Regulatory B cells inhibit EAE initiation in mice while other B cells promote disease progression. *J. Clin. Invest.* 2008; 118(10):3420–3430. [PubMed: 18802481]
58. Huddleston CA, Weinberg AD, Parker DC. OX40 (CD134) engagement drives differentiation of CD4+ T cells to effector cells. *Eur. J. Immunol.* 2006; 36(5):1093–1103. [PubMed: 16541471]

Figure 1.

Costimulatory and adhesion molecule expression by Fo, MZ, and B-1 B cells. Spleen and peritoneal cavity cells were stained with the indicated antibodies. (A) Gating of Fo, MZ, B-1 and B-2 subsets in the spleen and peritoneal cavity. Plots are gated on CD19⁺CD93⁻ cells (spleen) or CD19⁺ cells (PerC). (B and C) Surface marker expression on B-cell subsets gated as in (A). Results are representative of 3–4 experiments, n=3 mice per experiment.

Figure 2.

Creation of BM chimeric mice with small proportions of Ag-tg B-cell subsets. (A) Dot plots are gated on CD19⁺CD93⁻ cells (spleen) or CD19⁺ cells (PerC) and show gating strategy for Fo, MZ, and B-1 B cells. Histograms illustrate equivalent levels of I-E^k staining (which detects the antigen transgene) on each subset compared with background levels on WT B6 cells. (B) Pre-sort and post-sort purity of B-1, MZ and Fo B-cell subsets transferred into chimeric mice. (C) Experimental design showing construction of BM chimeras and transfer and assessment of AND T cells. Results are representative of two (A) or five (B) experiments, n=3 mice per group per experiment (A); n=12–17 pooled mice per experiment for sorting (B).

Figure 3.

Phenotype of Ag-tg B-cell subsets in chimeric mice 2 months post-chimerism. (A) Proportion and phenotype of Ag-tg Fo, MZ and B-1 B-cell subsets in spleen. Numbers represent percent of total splenocytes (left) or percent of CD19⁺CD45.1⁺ gated cells (middle, right). (B) Proportion and phenotype of Ag-tg B-1 B cells in peritoneal cavity. Numbers represent percent of gated cells or cells in each quadrant. (C) CD45.1⁺CD19⁻ cells in chimeric mice do not express DC or macrophage markers, and instead have a phenotype consistent with plasmablasts. Numbers represent percent of gated cells; far left dot plot shows total nucleated splenocytes. All other dot plots show cells gated as in far left dot plot.

CD45.1⁻CD19⁻ and CD45.1⁻CD19⁺ populations illustrate appropriate gating. Results are representative of three experiments, n=2–4 mice per group per experiment.

Figure 4.

Small numbers of Ag-tg B cells interact with T cells. CFSE-labeled AND T cells were transferred to chimeric mice, and splenic T and B cells were assessed 3 days later. (A) Identification of AND T cells. Numbers on dot plots represent percent of total splenocytes (left) or percent of gated cells (middle, right). V 3 and V 11 identify the AND TCR. Numbers on histograms represent percent of gated cells that remain undivided (no CFSE dilution). (B) Regression curve showing the effect of increasing numbers of Ag-tg B cells on AND T-cell division. Numbers on the x-axis are percent Ag-tg (CD45.1⁺) B cells within total splenocytes after subtracting background CD45.1 staining in mice that received B6 BM only. Numbers on the y-axis are percent of AND T cells that remain undivided as depicted

in histograms in (A). Each symbol represents an individual mouse. $p < 0.05$ for all R^2 values, t-test for Pearson's correlation coefficient. (C) Regression curve showing no effect of increasing numbers of Ag-tg B cells on AND T-cell accumulation. Numbers on the x-axis as in (B); numbers on the y-axis are percent of AND T cells ($CD4^+V\beta 3^+V\beta 11^+CFSE^+$) within total splenocytes. p is not significant for all R^2 values. (D) Regression curve showing negative correlation of AND T-cell division and T-cell accumulation after encountering antigen presented by B-1 B cells. Numbers on the x-axis are percent of AND T cells within total splenocytes as in (C); numbers on the y-axis are percent of AND T cells that remain undivided as in (A) and (B). $p < 0.05$ for R^2 value. Results are representative of three experiments, $n = 4-9$ mice per group per experiment.

Figure 5.

All B-cell subsets induce T-cell hypo-responsiveness without Treg-cell induction. (A) AND T-cells were enriched from individual recipient spleens 7 days after transfer into BM chimeric mice that harbored no Ag-tg cells (B6 BM only) or that harbored small numbers of Ag-tg B-1, MZ, or Fo B cells. AND T cells were restimulated in vitro for 6 days with irradiated splenocytes from unmanipulated Ag-tg mice in which all MHC class II⁺ cells express the antigen transgene or with irradiated splenocytes from control C57BL/6 mice. Proliferation was assessed by ³H-thymidine incorporation. Stimulation indices were calculated as (average cpm of 3–5 sample replicates stimulated with Ag-tg APCs)/ (average cpm of 3–5 sample replicates stimulated with B6 APCs). Data show mean stimulation indices ± SEM (n=3–18 mice per group). Both experiments performed are shown. *p<0.05, two-tailed t-test. (B) Foxp3 expression by transferred AND T cells directly ex vivo 7 days

after transfer into chimeric mice. AND T-cell plots are gated on Thy1.1⁻CD4⁺V β 3⁺V β 11⁺; endogenous V β 3⁺ T cells (Thy1.1⁺CD4⁺V β 3⁺V β 11⁻) are shown in the lower plots to indicate appropriate gating. Results are representative of two experiments, n=4–9 mice per group per experiment.