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Involvement of Twisted Gastrulation in T Cell-Independent Plasma Cell Production

Sotiris Tsalavos,* Katerina Segklia,* Ourania Passa,* Anna Petryk,†‡ Michael B. O’Connor,‡ and Daniel Graf†

Bone morphogenetic protein (BMP) signaling is increasingly implicated in immune cell differentiation and function; however, direct in vivo evidence for such a role is still missing. In this article, we report that Twisted gastrulation (TWSG1), an extracellular regulator of BMP signaling, is expressed in activated B cells and regulates T-independent B cell responses in the mouse. TWSG1-deficient B cells mount stronger T-independent type 2 responses reflected as increased IgM levels and numbers of Ag-specific IgM-secreting cells. BCR stimulation of TWSG1-deficient B cells results in hyperproliferation, hyperresponsiveness, and decreased apoptosis, whereas TLR stimulation results in hyperpolarization and increased IgG3 production. These changes are reflected on the molecular level by increased transcription of Bcl-6, Pax5, and the BMP-responsive gene Id-2. The TWSG1 effects on B cells appear to be cell intrinsic, suggesting that TWSG1 expression in B cells serves to interpret BMP signals on a per-cell basis. In summary, our observations on the role of TWSG1 in B cell function is opening new paths toward the exploration of the role of BMP signaling in immunological processes. The Journal of Immunology, 2011, 186: 6860–6870.
or negative manner (31–34). Several in vitro studies point toward an important role of TWSG1 in the regulation of the immune system. TWSG1 expressed by developing thymocytes in a TCR-dependent manner synergizes with Chordin to block BMP2/4 that negatively regulate thymocyte proliferation and differentiation (17). TWSG1 expressed in mature T cells in a Tб-dependent manner has been shown to inhibit proliferation and cytokine production of alloreactive CD4+ T cells (35). The in vivo requirements for Twsgl are background dependent. Whereas it has been reported that Twsgl-deficient mice are viable but show impaired lymphocyte development in some mice (36), Twsgl null mutants on the C57Bl6 background die in utero and display craniofacial malformations of variable severity (37) (D. Graf and O. Passa, unpublished observations).

We have found that Twsgl is also expressed in activated B cells. Using a conditional gene ablation strategy, we investigated its function specifically in B cells in vivo. We found that B cell-derived TWSG1 is not required for B cell development per se, but rather that it is involved in the regulation of TI responses in vivo. Twsgl−/− B cells show enhanced proliferation, activation, and IgG3 production after stimulation in vitro. This indicates a role for TWSG1 in regulating plasma cell production and implies for the first time, to our knowledge, the presence of a BMP signaling network to regulate B cell function.

**Materials and Methods**

**Mice**

Heterozygous Twsgl−/lacZ reporter mice (Twsgl+/lacZ) have been described elsewhere (38). Mice carrying a conditional allele (Twsglf/f) (37) were crossed with FcγCre mice to remove the neo cassette and were subsequently backcrossed into C57BL/6 mice for at least six and up to eight generations. The F1 progeny homozygous for the floxed allele (Twsglf/f) was crossed with CD19 cre mice (39) to generate mice with a conditional loss of Twsg1 in the B cell compartment [Twsglf/f(CD19 cre/f)] as designated by Twsg1 conditional knockout (kO) in this article (Supplemental Fig. 2A). Mice homozygous for the floxed allele (Twsglf/f) were used as control animals, because they showed no statistical differences in activation assays to Twsglf/f(CD19 cre/f) mice. Similar (Twsglf/f) mice were crossed with VavCre mice (40) to generate mice with a conditional loss of Twsgl in the complete hematopoietic compartment. As a control, we also deleted Twsgl in the germline, which was perinatally lethal. Mice were maintained at the Biomedical Sciences Research Center Alexander Fleming’s Animal Research and Ethics Committee for compliance with the Federation of European Laboratory Animal Science Associations’ regulations.

**Abs and flow cytometry**

Erythocyte-depleted single-cell suspensions from bone marrow (BM), spleen, lymph nodes, and peritoneal cavity were stained with the following Abs: FITC and biotin anti-IgM F(ab’2) (Southern Biotechnology), LPS (Sigma-Aldrich), 100 ng/ml recombinant BMP2 (Bmp2; PeproTech), recombinant Twsgl (Twsgl1), and 1 µg/ml recombinant Chordin (rChordin; R&D Systems). For measuring proliferation, 2 × 105 purified B cells from RPMI were plated in 96-well, flat-bottom plates, and proliferation was measured after 72 h by pulsetting the cells with 0.5 µCi [3H]thymidine 16 h before harvesting. For B cell activation, cells were stimulated with 5 µg/ml anti-IgM F(ab’2) or 1 µg/ml LPS. For plasma cell induction, B cells were incubated with 1 µg/ml LPS. Secreted Ig in the supernatant were measured by ELISA as described after 7d of culture.

**Immunohistochemistry and lacZ staining**

OCT (BDH)-embedded tissues were “snap frozen” in liquid nitrogen steam. Five- to 6-µm cryostat sections were placed in gelatin-coated slides, air-dried, fixed in ice-cold acetone for 10 min, and rehydrated in PBS.

Sections were incubated overnight with Abs against rabbit anti-Bmp2 (PeproTech) and detected with AP-labeled anti-rabbit IgG (Southern Biotechnology) followed by staining with naphthol-AS-MX-phosphate/ fast blue BB. Subsequent staining was performed with bovine labeled F4/80 (BM8; BioLegend), detected with AP-labeled anti-rabbit IgG (Southern Biotechnology) and rat anti-macrophages/microphages (MOMA-2; Serotec), detected with biotinylated anti-rat IgG (BioLegend) and streptavidin-HRP followed by staining with 3,3'-diaminobenzidine tetrahydrochloride. For lacZ staining, sections were fixed in cold glutaraldehyde/formaldehyde and incubated with 2 mg/ml 5-bromo-4-choro-3-indolyl-β-D-galactopyranoside (BT Bio-technology, Cambridge, UK) at 37°C overnight. Subsequently, the sections were incubated with bovine B220 (RA3-6B2) followed by streptavidin-HRP. Sections were visualized on a Nikon Eclipse microscope.

**Western blotting**

A total of 5 × 105 purified B cells suspended in RPMI/1% FCS were serum starved for 1 h before stimulation with 1 µg/ml LPS + 100 ng/ml Bmp2. Cells were lysed at the various time points with radioimmunoprecipitation
assay cell extraction buffer (50 mM Tris, 150 mM NaCl, 1% NP-40, 0.1% SDS, 1 mM Na3VO4, 1 mM PMSF, 1 × protease inhibitor mixture; Sigma-Aldrich). Ten micrograms cell lysate was loaded on acrylamide gel for SDS-PAGE electrophoresis. Western blotting carried out on nitrocellulose membrane (Whatman) blocked with PBS/0.1% Tween 20/5% BSA and probed with goat anti-tsg (R&D Systems) overnight in PBS/0.1% Tween 20/1% BSA. Membranes were washed 3 × 10 min with PBS/T and incubated with anti-goat IgG-HRP (Southern Biotechnology). Membranes were visualized using ECL plus detection kit (Amersham) on Biomax film (Kodak). Membranes were stripped and reprobed with β-actin (Sigma-Aldrich), to assess equal protein loading.

Results

Twsg1 is preferentially expressed in LPS-activated B cells

Twsg1 is a bona fide BMP modifier, and its expression correlates with sites of BMP activity (31, 41). Twsg1 is expressed in several adult tissues including primary and secondary lymphoid organs (17, 36, 42, data not shown). Because BMP signals have been implied in the regulation B cell function (20, 21, 43), we hypothesized that Twsg1 was also differentially expressed in B cells. Using a Twsg1-lacZ reporter mouse (Twsg1+HlacZ) that allows visualization of Twsg1 gene expression in situ, we observed lacZ expression in the lymphoid follicles of the spleen and mesenteric lymph nodes (MLNs) (Supplemental Fig. 1). Staining was observed in some B220+ cells, suggesting a restricted or dynamic expression in B cells. Testing for Twsg1 expression in purified B cells ex vivo, we found that Twsg1 was not expressed in resting B cells but was induced by LPS (Fig. 1A, 1B). No expression of other BMP antagonists was observed (Fig. 1C, Table I). BMP receptor expression was also induced by LPS similar to Twsg1. Hardly any receptor expression was seen in resting B cells, but several BMP type I receptors (Alk1, Alk2, Alk3, Alk6) and BMP type II receptors (BMPR-II, ActR-II, ActR-Ib) were induced in LPS-activated B cells (Fig. 1D, Table I). This suggests that activated B cells are the predominant BMP targets, though the weak expression of Alk-6 and BMPR-II in unstimulated B cells suggests that at least some resting B cells might also be receptive to BMP signals.

In thymocytes, pre-TCR–induced TWSG1 antagonizes BMP activity in an autocrine fashion (17). The activation-induced expression of Twsg1 suggested a similar function in B cells. To address this, we crossed a conditional Twsg1 allele to CD19Cre to delete Twsg1 specifically in B cells (Supplemental Fig. 2A). Successful deletion was confirmed in LPS-activated B cells by RT-PCR and Western blot (Fig. 2A, 2B, Table I). Twsg1-cKO mice did not exhibit any gross abnormalities in the lymphoid organs. Total cell counts of BM, spleen, and lymph nodes were comparable with littermate controls (Fig. 2C). The relative frequencies of B and T cells in spleen and peripheral blood were also not altered (Fig. 2D). This was in contrast with the phenotype observed in mice with global deficiency of TWSG1 (36). Vav-Cre–mediated deletion of Twsg1 in all hematopoietic cells also resulted in normal cellularity of the lymphoid organs (Supplemental Fig. 2B), suggesting that it is not the loss of hematopoietic cell-derived Twsg1, but rather of BM stroma-derived Twsg1 that is responsible for the block in lymphoid development observed in global TWSG1 deficiency (36, 40).

B cell-specific deletion of Twsg1 does not affect B cell development

We next investigated in more detail the effects of TWSG1 deficiency on the development of B cell subcompartments in BM and spleen. In the BM, similar percentages of pro-B (CD19+CD20−IgM+IgD−c-Kit+CD25−) and pre-B (CD19+B220−IgM−IgD−c-Kit−CD25−) cells in both control and Twsg1-cKO mice were observed. Similarly, the immature (B220+CD19+IgM−IgD−/+) and mature/recirculating (B220+CD19+IgM+IgD−/−) B cell subsets showed no significant changes, suggesting normal B cell development in Twsg1-cKO mice (Fig. 3A). In Twsg1-cKO spleens, follicular B cell (CD23+CD21+) numbers were similar to the wild-type (wt) control, but the numbers of MZB (CD23−CD21−), as well as the newly formed/transitional B cells (CD23−CD21low), were reduced (Fig. 3B, top panel). This was also reflected in a small decline in the fraction III population (IgD−IgM−) that

![FIGURE 1.](http://www.jimmunol.org/Downloadedfrom.jpg) Twsg1 is expressed in activated B cells. A and B, Twsg1 promoter activity measured by FACS in FDG-stained purified splenic B cells of wt (Twsg1++) and Twsg1−/lacZ mice stimulated with 1 μg/ml LPS at several time points. B, Mean fluorescence intensity (MFI) of the FDG intensity of the Twsg1−/lacZ and Twsg1++ cells. C and D, RT-PCR of (C) BMP antagonists and (D) BMP receptors expressed in unstimulated and LPS-stimulated purified splenic B cells.
contains MZB and transitional stage 1 (T1)/newly formed cells (Fig. 3B, middle panel). The percentages of the B1 cell compartment (B220+CD23−IgM+CD43+CD5+) were unchanged (Fig. 3B, bottom panel).

To gauge whether B cell-specific ablation of Twsg1 affects Ig production, we assessed the baseline Ig titers. No differences were observed in blood serum levels for IgM, IgG and its subtypes, and IgA in the peritoneal cavity (Fig. 3C). These results are in line with the observation of normal development of spontaneous germinal centers (GCs) in Peyer’s patches, MLNs, and spleen (not shown). Overall, no significant alterations toward B cell development and homeostatic Ig production were observed in Twsg1-cKO mice, suggesting that TWSG1 is not critically involved in these processes.

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**Table I. RT-PCR primer sequences**

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<tr>
<th>Gene</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
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<tr>
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<td>5’-AGGGGGCGTGGACATTG-3’</td>
<td>5’-CTGTGCTCCTCCGGCTGTCCG-3’</td>
</tr>
<tr>
<td>Noggin</td>
<td>5’-CTCTACGCCCTGGTGGTG-3’</td>
<td>5’-AAGCCCGGGTGCAATGCTG-3’</td>
</tr>
<tr>
<td>Chordin</td>
<td>5’-CAAGCGAAACGCAACGAG-3’</td>
<td>5’-CAACAGCTGAGAGCCGACG-3’</td>
</tr>
<tr>
<td>Gremlin</td>
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<td>5’-CAAGCCCAGCCAATAGAACT-3’</td>
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<tr>
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<td>5’-TTCTGGGGAGGTGACAACTGG-3’</td>
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<tr>
<td>Gapdh</td>
<td>5’-TTCTCTGTGCTCAATCTGACC-3’</td>
<td>5’-ACTCTACGGACATACCTGACC-3’</td>
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**FIGURE 2.** Ablation of Twsg1 from B cells does not affect lymphocyte development. A, Semiquantitative RT-PCR for Twsg1 from 72-h LPS-stimulated B cells from control and Twsg1-cKO mice. B, Western blot for Twsg1 in control and Twsg1-cKO LPS-activated B cells. β-actin was used to assess equal protein loading. C and D, Assessment of effect of twsg1 B cell deletion in control and Twsg1-cKO mice (C) total cell counts from spleen, MLN, and BM (n = 5 mice/group), and (D) frequencies of B (B220+) and T (CD5+) cells in spleen and peripheral blood analyzed by FACS (means and SE of n = 3 mice/group). Representatives from three independent experiments are shown.

**Increased proliferation and activation in Twsg1-deficient B cells**

The Twsg1 expression in LPS-activated B cells indicated a potential involvement of TWSG1 in B cell activation-dependent processes. We therefore measured B cell proliferative responses to mitogenic stimuli. Twsg1-cKO purified B cells were hyperproliferative compared with control cells over a range of anti-IgM and LPS doses (Fig. 4A). In contrast, proliferation induced by anti-CD40 was not altered, and CD40 stimulation did not reverse anti-IgM– or LPS-induced hyperproliferation (Fig. 4B). Next, we wanted to test whether the hyperproliferation was due to increased activation or increased survival. Three days after stimulation, control and Twsg1-cKO B cells increased similarly in size and granularity, as is typical of “blasting” cells (Fig. 4C). Expression
of several activation markers was assessed. CD25 and CD69 levels were increased on Twsg1-cKO cells relative to littermate controls after 24 h in response to anti-IgM and lesser to LPS (Fig. 4D, left panels, 4F). To exclude that the increased activation was not due to haploinsufficiency for CD19 because of the use of CD19Cox, we compared the activation profiles between Twsg1f/f and Twsg1f/+ Cd19Cox. No significant difference between the controls was observed (Supplemental Fig. 3). No alterations in expression kinetics were observed for MHC-II and CD86 (Supplemental Fig. 4). Observation of expression kinetics was done for IgM, and peritoneal cavity IgA (left panel), and IgG subtypes (right panel) from control (open square) and Twsg1-cKO mice (filled triangle), n = 5 mice/group. F-I, fraction I; F-II, fraction II; F-III, fraction III.

**Enhanced TI Ig production of Twsg1-cKO mice**

The hyperresponsiveness of Twsg1-deficient B cells prompted us to investigate the in vivo responses of Twsg1-cKO mice. Because the hyperresponsiveness was restricted to IgM and LPS stimulation, and did not appear to involve CD40 signals, we focused on TI-specific Ab responses. Fourteen days after immunization with the TI-1 Ag DNP-Ficoll (DNP-Ficoll), Twsg1-cKO mice showed significantly higher Ag-specific IgM responses (Fig. 5A). The IgG3 titers were lower at day 7 but recovered by day 14. Immunization with the TI-2 Ag DNP-Ficoll resulted in significantly higher IgM and IgG3 levels by day 14 (Fig. 5B). To assess whether the increased Ig levels corresponded to more Ag-specific B cells, we performed an ELISPOT assay on in vitro restimulated B cells 4 d after DNP-Ficoll immunization. As shown in Fig. 5C, Twsg1-cKO mice had more DNP-specific ASCs. These data establish that TWSG1 functions as a negative regulator of TI type 2 responses in vivo.

Increased Pax5, Bcl-6, and Id2 expression and IgG3 production in Twsg1-cKO B cells

The increased number of ASCs prompted us to investigate whether TWSG1 directly affects plasma cell production. For this, purified B cells were stimulated with LPS for 4 d in vitro. No significant differences in the percentage of plasma cells (B220lowCD138+) and expression of several transcription factors controlling plasma cell generation and fate (Blimp1, Irf4, Xbp-1, and Mitf) also remained unaltered (Fig. 6B, Table I). However, we detected enhanced expression of Pax-5 and Bcl-6 (Fig. 6B). LPS stimulation induces IgM, IgG2b, and IgG3 secretion. We thus investigated whether the levels of these Ig subtypes were altered. IgM and IgG2b titers remained unaltered, but we observed increased IgG3 levels in the culture supernatant from Twsg1-deficient B cells 7 d after LPS stimulation (Fig. 6C).

To gauge whether these differences were the result of altered BMP signaling in Twsg1-cKO B cells, we probed for changes in the expression of BMP target genes. We focused on the Id gene family (Id1-4) because these molecules are downstream targets of BMP signaling and have, at least in part, been implicated in B cell function and IgG production (25, 27, 44, 45). Semiquantitative
RT-PCR analysis showed increased expression of \( \text{Id}2 \) in LPS-activated Twsg1-cKO B cells compared with control cells. Levels of \( \text{Id}1 \) and \( \text{Id}3 \) were unaltered, and \( \text{Id}4 \) remained, as expected, undetected. These findings indicate that TWSG1 regulates B cell responsiveness to polyclonal activators, possibly by antagonizing BMP signals as reflected by altered expression levels of \( \text{Id}2 \).

**Presence of BMP signaling network components in B cells**

As TWSG1 appeared to modulate BMP signals, it raised the question whether BMPs are expressed in B cells or within their local environment in secondary lymphoid organs. Several BMPs are expressed in primary and secondary lymphoid organs, but no expression was observed either in resting or in LPS-activated B cells.
B cells in vitro (data not shown). We thus investigated whether BMPs were present in B cell areas of the lymphoid follicles. We focused first on BMP2, which is clearly expressed in the spleen (data not shown). Using double immunohistochemistry, we identified BMP2 in the marginal zone of lymphoid follicles in the spleen (Fig. 7A), which colocalized, at least in part, with F4/80 and Moma-II+ marginal zone macrophages. We thus tested whether BMP2 could affect plasma cell differentiation directly. For this, we stimulated control and Twsg1-cKO B cells with LPS in the presence of BMP2. We did not observe any effects on plasma cell differentiation (Fig. 7B). Similarly, Ab secretion remained unaltered and, not surprisingly, was also not affected by the addition of Chordin (Fig. 7C). Furthermore, BMP2 had no direct effect on B cell activation and proliferation (data not shown). Thus, whereas the molecular data indicate that B cells are capable of receiving BMP signals, we failed to detect any effect in vitro.

**B cell intrinsic effect of TWSG1**

TWSG1 is predominantly produced in activated B cells rather than resting B cells. Because we failed to detect any direct effect of BMPs on B cell activation, proliferation, and Ig production, we wondered whether the TWSG1 was responsible for the effects on B cell activation, or whether any other mechanism was in operation. To address this, we tested the effect of rTwsg1 in a range of doses on anti-IgM–stimulated B cells. As readout we used CD25 expression levels 24 h after stimulation because we had established that CD25 levels were increased on activated Twsg1-cKO B cells. rTwsg1 by itself had no effect on B cell activation (data not shown). Anti-IgM–induced upregulation of CD25 was not affected by any dose of rTwsg1 tested, neither in Twsg1-cKO nor control B cells (Fig. 7D). Because exogenous Twsg1 did not acutely interfere with BCR signaling, we tested whether Twsg1-deficient B cells were already altered in situ and the increased BCR activation observed in vitro was a reflection of this. To address this, we mixed control or Twsg1-cKO B cells (CD45.2) with competitor wt (CD45.1) B cells at a 1:1 ratio and stimulated them with anti-IgM. If the Twsg1 effect was mediated by secreted TWSG1, then the competitor CD45.1 B cells should “rescue” the Twsg1–cKO–B cell effect, resulting in decreased CD25 levels in cKO B cells. If the TWSG1 effect was cell intrinsic, then introduction of competitor B cells should have no effect on the activation phenotype (increased CD25 levels) observed in Twsg1-cKO B cells. If the TWSG1 effect was cell intrinsic, then introduction of competitor B cells should have no effect on the activation phenotype (increased CD25 levels) observed in Twsg1-cKO B cells. As shown in Fig. 7E and 7F, 24 h after stimulation, both control and Twsg1-cKO B cells displayed their respective CD25 signature independent of the presence of competitor B cells. CD25 levels neither decreased in Twsg1-cKO B cells nor increased in CD45.1 wt B cells. The observations that rTwsg1 did not affect B cell activation and that wt or Twsg1-cKO B cells did not alter their activation profile even if cocultured suggests that the TWSG1 effect on B cell activation is cell intrinsic and predetermined in vivo.
In summary, in this report, we show that B cells express the BMP modifier TWSG1 in an activation-dependent manner. B cell-specific TWSG1 deficiency does not affect B cell development, but it does alter B cell responses. Twsg1-deficient B cells show increased TI-2 responses, which is reflected in vitro by increased activation, proliferation, and Ig secretion (IgM and IgG3). These
effects could not be modulated in vitro, either through addition of recombinant BMP, BMP antagonists, or rTwsg1 itself. Competitor experiments established that the Twsg1 effect on B cell activation is B cell intrinsic, indicating a role for TWSG1 in a preceding B cell maturation/selection step.

Discussion

In this report, we have demonstrated that TWSG1, a bona fide BMP modulator, regulates B cell activation, proliferation, TI plasma cell production, and Ab secretion. We provide evidence that TWSG1 is not acting autonomously on B cells, but it might be part of an extracellular BMP signaling network that regulates B cell function in the lymphoid follicles of spleen and lymph node. To our knowledge, this is the first report to show an in vivo function of a member of the BMP signaling machinery in secondary lymphoid organs.

To date, there is no direct proof that BMP signals regulate lymphocyte development and function in vivo. In vitro studies have implied BMP2 and BMP6 as negative regulators of B cell proliferation (19, 21). Furthermore, Bmp6 is part of the gene signature that defines diffuse large B cell lymphoma with predicted poor outcome (46). TWSG1 is a modifier of BMP signaling, and has been shown to both positively and negatively affect BMP signaling (31–34). In developing and mature T lymphocytes, TWSG1 has been identified as a BMP antagonist. In the thymus, pre-TCR signaling induced TWSG1 interacts with stroma-derived Chordin to attenuate BMP2/4 binding, which inhibits thymocyte proliferation and differentiation (17). In mature T cells, TWSG1 regulates cytokine production in CD4⁺/alloreactive T cells in vitro (35). Interestingly, both reports suggest that TWSG1 acts in an autocrine manner.

In mice, TWSG1 deficiency affects lymphocyte development (36). This phenotype is not fully penetrant and is not observed on all genetic backgrounds. Whereas it is seen on a mixed C57Bl6:129 background (O. Passa and D. Graf, unpublished observations), TWSG1 deficiency on the C57Bl6 background is perinatally lethal and accompanied by a series of severe craniofacial malformations (37, 47) (O. Passa and D. Graf, unpublished observations). Because of this background dependence, we have chosen to perform our studies on mice backcrossed at least six generations to C57Bl6 background.

After B cell-specific deletion of Twsg1, primary or secondary lymphoid organs had normal B cell counts, in contrast with a global TWSG1 deficiency in mice. Similarly, conditional deletion in all hematopoietic cells using the Vav-Cre (40) resulted in normal cellularity of the lymphoid organs. This indicates that BM stroma-derived TWSG1 rather than hematopoietic cell-derived TWSG1 might be responsible for the lymphoid defects observed in Twsg1 null mice.

More detailed analysis of Twsg1⁻/⁻KO spleens revealed a 30% reduction of MZB. This reduction was also reflected in the IgD⁺low IgM⁺high fraction, which contains newly formed/T1 and MZB cells. Interestingly, these B cell populations reside in the outer ring of the marginal zone, the very region where functional defects are being observed (increased TI responses) (48, 49). The transition of newly formed immature B cells to follicular cells and MZB is regulated by multiple signaling pathways. Of interest are Id2-deficient mice, in which newly formed/T1 and MZB populations are also impaired with a shift toward follicular cells (27). Id2 is a BMP target gene; thus, it is conceivable that a BMP signal regulates this aspect of B cell differentiation. In line with this, reduced percentages in newly formed/T1 and MZB populations are also observed in Twsg1⁻/⁻KO mice.

In vitro stimulation of purified B cells with anti-IgM or LPS revealed increased proliferation and reduced apoptosis in Twsg1⁻/⁻KO cells. In contrast, proliferation induced by anti-CD40 was not altered, and CD40 stimulation did not reverse anti-IgM- or LPS-induced hyperproliferation. The hyperproliferation was accompanied by increased expression levels of CD25 and CD69, whereas other activation/differentiation markers such as CD86 and MHC-II were not altered. It is not clear whether the increased CD25 and CD69 levels are a direct effect of TWSG1 deficiency or whether they are an epiphenomenon associated with the increased proliferation. We used the altered CD25 expression levels to address whether the observed hyperproliferation was the result of activation-induced TWSG1 acting on the B cells in an autocrine/paracrine fashion, or whether Twsg1-deficient cells were already altered in situ and the increased proliferation was only a reflection of this. Because CD45.1 (competitor)-derived Twsg1 could not affect the hyperactivation of Twsg1⁻/⁻KO B cells in coculture experiments, we conclude that the propensity for hyperproliferation is B cell intrinsic. Therefore, it was not surprising that addition of exogenous BMP pathway molecules (rTwsg1, rBmp2, rChordin) had no effect on in vitro activated B cells.

The in vivo and in vitro differences pointed toward a predominant role of TWSG1 in TI rather than TD responses. In line with this, immunization with both TI-1 and TI-2 resulted in increased secretion of IgM and, in some subclasses, of IgG, which as shown for DNP-Ficoll was accompanied by more Ag-specific ASCs. In contrast, no significant differences to IgM or IgG production were observed in TD responses, and the size of GCs or the percentage of GC B cells were comparable (data not shown). TI responses are thought to be initiated by MZB cells, because they have the ability to respond very rapidly to TI Ags because of their low activation threshold allowing them to cope with low Ag concentrations, thus enhancing cellular responses (50). Although spleens of Twsg1⁻/⁻KO mice contain lower numbers of MZB, our data suggest that their ability to respond to TI-1 and TI-2 Ags is enhanced.

LPS stimulation of Twsg1⁻/⁻KO B cells in vitro resulted in increased production of IgG3, indicating a possible role of TWSG1 in the class switch to IgG3. These higher IgG3 levels did not correspond to a global increase in plasma cells or in the altered expression of the main transcription factors that drive plasma cell differentiation. We did observe, however, an increase in the expression of the Bcl-6, Pax5, and Id2. Bcl-6 and Pax5 are known for their key roles in B cell proliferation, maturation, and plasma cell differentiation (51–54). Gonda et al. (28) have proposed that the modulation of the relative abundance of Pax5, E2A, and Id proteins provides a mechanism for subtle regulation of the effects of Pax5 and E2A in B cell activation, and these differential activities determine the activation of the master switch regulator, AID. Based on our data, we propose that the enhanced expression levels of Pax5 and Id2, regulated by TWSG1 activity, are shifting the balance toward the germline transcription of the Cγ3 locus, thus enhancing IgG3 production in activated B cells under specific conditions.

TWSG1 is a bona fide modulator of BMP activity, and no function for TWSG1 without BMP interaction has been reported. In the search of a BMP signaling network that could be regulated by TWSG1 in situ, we identified BMP2 in the vicinity of the splenic marginal zone ring. The question is whether TWSG1 modulates BMP2 activity in vivo. The differences observed in Twsg1⁻/⁻KO B cells on IgM and Ig subtype kinetics suggest that such a BMP signal could affect B cell activation and subsequent Ig responses based on the fact that a common integrator of BCR and BMP signaling are the Id proteins (25). Interestingly, Twsg1-deficient B cells have increased expression levels of Id2.
In vitro experiments failed to reveal any direct effect of exogenous BMP2 on B cell activation (data not shown), proliferation, and Ig production in either control or Twsg1−cKO cells. Ig production was also not affected by Chordin, a known interaction partner of TWSG1 (33). Because BMP receptors are hardly expressed on resting B cells but are strongly upregulated after activation in vitro, this could provide a likely explanation for the earlier observations. Although we failed to identify the mechanism and exact circumstances by which BMP signals affect B cells, we cannot exclude that the in vitro hyperresponsiveness observed in the Twsg1−/− deficient B cells is the result of an endogenous BMP signal “reprogramming” the B cell for an altered response in vitro.

By dissecting the role of TWSG1 in B cell activation and TI responses, the results show for the first time, to our knowledge, a potential in vivo role of BMP signaling in B cell differentiation. B cells deficient in the BMP modulator TWSG1 are hyperresponsive to BCR and TLR4 stimulation, resulting in increased IgG3 production and enhanced TI-2 responses. The observed phenotypes in Twsg1−cKO mice do not show an absolute requirement for TWSG1 in these processes, rather pointing to a second level of control toward fine-tuning the B cell’s response to Ag encounter. Unraveling the mechanisms that regulate BMP signaling and how the cells respond to such signals will be another step toward understanding the regulatory complexity behind B cell differentiation and Ab production.

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Disclosures
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References
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**Supplementary figure 1**  Spleen and Mesenteric Lymph Node cryostat sections from Twsg1^{+/lacZ} mice stained with X-gal (blue) and B220 (brown).

**Supplementary figure 2**  A) conditional deletion strategy B) total cell counts from spleen, mesenteric lymph node and bone marrow in control Twsg1-Vav^{Cre} mice (n = 5)

**Supplementary figure 3**  No differences in B cell activation between Twsg1^{V+/Cd19^{cre}} and Twsg1^{V/+Cd19^{cre}} mice in contrast to Twsg1-cKO mice (n = 3). Representative from 3 independent experiments.

**Supplementary figure 4**  Additional activation markers (MHC-II^{I-A-b} and CD86) in unstimulated and 24hour LPS (top) or anti-IgM (bottom) stimulated B cells from control and Twsg1-cKO mice (n = 3). Representative from 3 independent experiments

**Supplementary figure 5**  Increased cell division in Twsg1-cKO B-cells. B cells from Twsg1^{V+Cd19^{cre}} and Twsg1^{V+Cd19^{cre}} mice were labeled with CFSE and cultured for 72hours without stimulation or with LPS (1µg/mL) or anti-IgM (5µg/mL). Numbers in gates denote means and standard error of n=3 mice per group. Representative of two independent experiments.
A

5'UTR  1  2  loxP  3  loxP  4  5  3'UTR
twsg1^flx allele

1  2  frt  4  5
twsg1^flx CD19^{cre/+} allele

+ CD19 Cre

B

![Cell counts graph](image)

- controls
- Twsg1-Vav^{cre}

B

- spleen
- MLN
- BM