Bone morphogenetic proteins inhibit CD40L/IL-21-induced Ig production in human B cells: Differential effects of BMP-6 and BMP-7

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Bone morphogenetic proteins (BMPs) are members of the TGF-β superfamily. TGF-β can affect class switch recombination in human B cells, but whether BMPs also play a role have not been tested. We investigated the functional effects of exogenously added BMPs on CD27-/naive and CD27+ memory B cells from healthy donors. BMP-2, -4, -6 and -7 inhibited CD40L/IL-21-induced production of IgM, IgG and IgA. BMP-6 reduced Ig production by 70% in memory B cells and more than 55% in naive B cells, whereas the other BMPs were slightly less potent. We observed a striking difference in functional effects between the structurally similar BMP-6 and BMP-7, as BMP-6 mainly inhibited plasmablast differentiation, and BMP-7 mainly induced apoptosis. In memory B cells, BMP-6 upregulated expression of DNA-binding protein inhibitor genes, but potently inhibited CD40L/IL-21-induced upregulation of the transcription factor XBP1, necessary for the late stages of plasmacytic differentiation. Expression of transcription factors regulating earlier stages (IRF4, PRDM1) was not affected by BMP-6. Taken together, these results show that BMPs are potent suppressors of naive and memory B cells.

Key words: B cells · Bone morphogenetic proteins · Maturation · Plasmablasts

Introduction

When B cells are activated by T-cell-dependent antigens, they start proliferating and can form germinal centers (GCs) where affinity maturation and class switch recombination (CSR) of the immunoglobulin (Ig) take place. Secreted and membrane-bound molecules made by T cells are important for the GC reaction, and CD40L is one of the essential molecules [1]. GC B cells can differentiate to Ig-producing plasma cells, and cytokines like IL-4, IL-6, IL-10 and TGF-β direct which Ig isotype is produced [2–4]. IL-21 has emerged as a strong inducer of B-cell differentiation and Ig production in vitro, and the strength of IL-21 exceeds other positive regulators like IL-2, IL-4 and IL-10 [5–8]. The combination of CD40L and IL-21 can induce CSR to IgA and IgG [7].

The different stages of plasma cell development are regulated by a web of interacting transcription factors. Pax5 and BCL6 are
highly expressed in GC B cells, but they are not expressed in plasma cells where B-lymphocyte-induced maturation protein 1 (Blimp-1) and X-box binding protein 1 (XBP-1) are highly expressed [9]. BCL6 is required for GC formation [9] and Pax5 upregulates the enzyme activation-induced cytidine deaminase (AID) which is necessary for CSR [10, 11]. Another primary function of BCL6 and Pax5 is to repress Blimp-1 and XBP-1 respectively, which are both necessary for plasma cell differentiation [12, 13]. To allow terminal B-cell differentiation, Pax5 and BCL6 must be repressed by Blimp-1 [14, 15] and the mutual repression of Blimp-1 and BCL6 forms a feedback loop enforcing irreversible plasmacytic differentiation. Blimp-1 induces plasma cell differentiation by repressing genes involved in proliferation and GC functions [15], and indirectly induces XBP-1 expression by downregulating Pax5 [16]. The role of XBP-1 is to enhance the secretory capacity of plasma cells [17]. The transcription factor interferon regulatory factor 4 (IRF-4), functioning upstream of XBP-1, is also required for plasma cell differentiation and an important role for IRF-4 is to repress BCL6 [18, 19].

Bone morphogenetic proteins (BMPs) are members of the TGF-β superfamily, and mediate their effects by binding to a hetero-oligomeric complex of type I and type II serine-threonine kinase receptors. In humans, three BMP type I receptors and three BMP type II receptors have been identified [20]. When BMPs bind to the receptors, the type II receptor phosphorylates the type I receptor, which subsequently phosphorylates the receptor-regulated Smads: Smad1, Smad5 and Smad8. Together with Smad4, Smad1/5/8 form a complex which translocates to the nucleus and induces transcription of BMP target genes including the DNA-binding protein inhibitors (IDs) ID-1, ID-2 and ID-3 [20, 21]. ID proteins are helix–loop–helix proteins which heterodimerize with basic helix–loop–helix proteins and inhibit their binding to DNA. ID proteins are generally known to inhibit differentiation and induce proliferation, and have been shown to mediate many of the BMP effects in various cell systems [21]. BMPs play crucial roles during embryonic development, and they regulate cell growth, differentiation and apoptosis of various types of cells, including osteoblasts, neural cells and epithelial cells [22]. BMP-4 acts as a survival factor for hematopoietic stem cells from both adult and neonatal sources [23], whereas BMP-2, -4, -6 and -7 inhibit proliferation and induce cell death in myeloma cells [24–27]. The growth of human peripheral blood B cells is also inhibited by BMP-6 [28].

The effect of BMPs on the differentiation of various cell types, especially their known effect on the proliferation and apoptosis of both healthy B cells and myeloma cells, encouraged us to study the effect of BMPs on the in vitro differentiation of healthy human B lymphocytes. Several in vitro models of B-cell differentiation have been described [6, 7, 29–32] and based on these prior data, we used the combination of CD40L and IL-21 to induce differentiation from peripheral blood naive and memory B cells. CD40L/IL-21 efficiently induced differentiation to the plasmablast maturation stage. The presence of BMP-2, -4, -6 or -7 greatly suppressed CD40L/IL-21-induced differentiation, and this was further investigated in terms of how the various BMPs affected proliferation, viability, Ig production and differentiation, as well as target gene transcription.

**Results**

**BMPs inhibit Ig production in naive and memory B cells**

TGF-β is known to induce IgA CSR [33], but reduce the production of other Ig isotypes [34]. We therefore hypothesized that also BMPs could affect B-cell differentiation. Purified CD19+ B cells from peripheral blood were FACS-sorted into CD19+CD27+ naive B cells and CD19+CD27+ memory B cells. Stimulation with CD40L did not induce Ig production above the level for unstimulated cells, but a combination of CD40L and IL-21 potently induced Ig production (Supporting Information Fig. 1). Co-culturing with BMPs inhibited the CD40L/IL-21-induced production of IgM, IgG and IgA in naive and memory B cells (Fig. 1). BMP-6 inhibited Ig production with an average reduction in Ig concentrations of more than 55 and 70% in supernatants from naive and memory B cells respectively. BMP-2, -4 and -7 were slightly less potent as BMP-2 and -4 reduced the Ig levels by at least 35% and BMP-7 by at least 14% (Fig. 1).

To verify that the BMP-mediated suppressive effects on Ig production were specific and not due to non-specific toxic effects, we used the soluble BMP antagonist Noggin which has been shown to bind BMP-2, -4 and -7, and thereby prevent them from binding to receptors [35]. When the BMPs were pre-incubated with Noggin for 1 h prior to stimulation with CD40L/IL-21, the inhibitory effect of BMP-2, -4 and -7 were counteracted (Supporting Information Fig. 2A), indicating that the BMP-effects were specific. In the absence of exogenously added BMPs, Noggin slightly, but significantly, enhanced CD40L/IL-21-induced Ig production (Supporting Information Fig. 2A, p<0.05). Noggin had no or limited effect on BMP-6-induced suppression of Ig production (Supporting Information Fig. 2A), probably because Noggin binds BMP-6 with low affinity [36]. However, using an anti-BMP-6 neutralizing mAb, the inhibitory effects of BMP-6 was partially counteracted (Supporting Information Fig. 2B). Overall, BMPs inhibited CD40L/IL-21-induced production of IgM, IgA and IgG in naive and memory B cells.

**BMPs inhibit cell growth in memory and naive B cells**

The observed inhibition of CD40L/IL-21-induced Ig production by BMPs could be due to suppression of cell division, induction of cell death and/or inhibition of plasma cell differentiation. To investigate whether cell division and cell death was affected by BMPs, DNA synthesis was measured in CD40L/IL-21-stimulated naive and memory B cells. IL-21 did not induce DNA synthesis, and CD40L alone showed limited induction of DNA synthesis compared to the combined effects of CD40L and IL-21 (Supporting Information Fig. 3). In naive B cells, DNA synthesis was further investigated in terms of how the various BMPs...
was increased 30-fold and only BMP-7 significantly inhibited CD40L/IL-21-induced cell growth, with 44% inhibition of DNA synthesis and 3-fold increase in cell death (Fig. 2A, Table 1). In memory B cells, DNA synthesis was increased 9-fold and BMP-7 had the most suppressive effect with 40% inhibition of DNA synthesis and 3-fold increase in cell death (Fig. 2A, Table 1). Detection of apoptotic cells using the TdT-mediated dUTP-X nick end labeling (TUNEL) assay, confirmed that BMP-7 had prominent apoptosis-inducing effects and largely counteracted the viability-promoting effects of CD40L in naive as well as in memory B cells (Fig. 2B). This was in contrast to BMP-6 which had no significant apoptosis-inducing effect. Altogether, BMP-7 showed a potent apoptosis-inducing effect, whereas BMP-2, -4 and -6 had no or limited effects on DNA synthesis and cell viability.

BMP-6 and BMP-7 have differential effects on CD40L/IL-21-induced development of CD27+CD38+ plasmablasts

To investigate whether plasma cell differentiation was affected by BMPs, we analyzed CD40L/IL-21-induced differentiation to CD27+CD38+ plasmablasts by flow cytometry. Stimulation with CD40L/IL-21 for 5 days induced on the average 3 and 44% CD27+CD38+ plasmablasts from naive and memory B cells respectively (Fig. 3A and B). BMP-6 mediated a strong suppressive effect on CD40L/IL-21-mediated plasmablast differentiation from naive and memory B cells, with a 7.1-fold and 4.6-fold decrease in percent plasmablasts respectively (Fig. 3B). Furthermore, the CD27+CD38lo cells remained CD20hi whereas CD27+CD38hi plasmablasts displayed lower levels of CD20 after CD40L/IL-21 stimulation (data not shown). In contrast to the prominent apoptosis-inducing effects of BMP-7 (Fig. 2B), this BMP had the smallest inhibitory effect on CD40L/IL-21-induced plasmablast differentiation in naive B cells and no significant effect in memory B cells (Fig. 3A and B). Plasma cell differentiation requires several rounds of cell division, in particular when plasma cell differentiation is induced from naive B cells [7, 37]. To distinguish whether BMPs inhibited differentiation or if the reduced percentages of plasmablasts mainly were a result of reduced proliferation, naive and memory B cells were labeled with CFSE prior to culturing in the presence of CD40L/IL-21 with or without various BMPs. This experimental design made it possible to follow differentiation per cell division. Of the memory B cells that had divided four times or more, only 6.5% of the BMP-6-treated cells compared with 21% of the BMP-7 treated cells had differentiated to CD38hi cells (Fig. 3C). This shows that BMP-6, more potently than BMP-7, inhibits plasma cell differentiation. These findings were further confirmed by division slicing [37] and subsequent calculations of percentage of cells in each cell division that had differentiated to CD27+CD38hi plasmablasts. This approach identified BMP-6 as the most potent suppressor of CD40L/IL-21-induced plasma cell differentiation.

**Figure 1.** BMPs inhibit Ig production in naive and memory B cells. CD19+CD27− naive (top) and CD19+CD27+ memory (bottom) B cells from peripheral blood were stimulated with or without CD40L/IL-21 in the presence or absence of BMPs as indicated and cultured for 7 days. Supernatants were collected and analyzed by ELISA for Ig production. Shown are mean values of six independent experiments + SEM. *p<0.05 versus CD40L/IL-21-stimulated control, Wilcoxon test for paired samples.
whereas BMP-2 and -4 had intermediate suppressive effects and BMP-7 had limited effects (Fig. 3D). CFSE tracking of cell division further showed that the BMPs inhibited cell cycle progression as the percentage of cells that had divided four times or more was reduced in the BMP-treated cells (Fig. 3C and not shown). Taken together, the data shown suggest that BMP-6 inhibits Ig production mainly by inhibiting plasma cell differentiation, but also via suppression of proliferation.

To further investigate plasma cell differentiation, we sorted memory B cells by FACS and cultured them with CD40L/IL-21 in the presence or absence of BMP-6 and BMP-7 for 5 days and then analyzed the acquisition of the plasma cell markers IRF-4, CD138 and XBP-1 by immunocytochemistry. Freshly purified memory B cells had no or low expression of IRF-4 and no detectable level of XBP-1 (Fig. 4). After 5 days of culture in the presence of CD40L/IL-21, 84% of cells expressed IRF-4 and 50% of them co-expressed XBP-1 (Fig. 4 and Supporting Information Fig. 4). CD138 was not detected (not shown), indicating that the differentiated cells were plasmablasts, and not fully mature plasma cells. In contrast, fewer cells were present when they had been cultured in the presence of BMP-6 or BMP-7 (compare Hoechst staining across the different culture conditions), and 44 and 36% of them expressed IRF-4 in the presence of BMP-6 and BMP-7 respectively. These data further support the finding that BMP-6 and BMP-7 block CD40L/IL-21-induced differentiation to plasmablasts (Fig. 4).

Naive and memory B cells express BMP type I and type II receptors

We have previously shown that BMP receptors can be detected by flow cytometry [38]. To characterize BMP receptor expression in naive and memory B cells, CD19" cells from peripheral blood

Table 1. Analysis of BMP-induced cell death in naive and memory B cells

|                      | PI<sup>+</sup> cells (%)<sup>a,b</sup> | CD40L/IL-21 |
|----------------------|--------------------------------------|-------------|
|                      | Unstimulated                         | BMP-2       |
| Naive B cells        | 65 ± 9                               | 10 ± 1      |
| Memory B cells       | 47 ± 3                               | 11 ± 3      |
|                      | BMP-4                                | 10 ± 1      |
|                      | BMP-6                                | 13 ± 1      |
|                      | BMP-7                                | 16 ± 3*     |
|                      |                                      | 33 ± 6*     |
|                      | <sup>a</sup>p<sub>0.05</sub> by paired t-test. |
|                      | <sup>b</sup>Cells were cultured for 3 days before PI-positive cells were detected by FACS analysis. |
|                      | <sup>c</sup>Values represent mean ± SEM. |

Figure 2. BMP-7 inhibits DNA synthesis and counteracts the viability-promoting effects of CD40L. CD19<sup>+</sup>CD27<sup>+</sup> naive and CD19<sup>+</sup>CD27<sup>+</sup> memory B cells from peripheral blood were cultured with or without CD40L or CD40L/IL-21 in the absence or presence of various BMPs for 3 days. (A) <sup>3</sup>H-thymidine incorporation was measured. Shown are mean values of five independent experiments ± SEM. *p<0.05, Wilcoxon test for paired samples. (B) The percentage of apoptotic cells was determined using the TUNEL assay and shown are mean values of four independent experiments ± SEM. *p<0.05, two-tailed, paired t-test.
were stained with anti-BMP receptor Abs combined with detection of CD27 and CD20. A distinct fraction of CD27− naive B cells as well as CD27+ memory B cells had detectable expression of the BMP type II receptor activin receptor type IIB (ACTR-IIB), with 15.1 and 18.5% positive cells respectively (Fig. 5A and B). Furthermore, 23.3% of the memory B cells expressed the type I receptor activin receptor-like kinase (Alk) 2. In naive B cells, none of the three type I receptors were detected. Since a hetero-oligomeric receptor complex consisting of type I and type II receptors are needed to bind BMP and induce signaling, the functional effects observed in naive B cells were surprising, unless the stimulation conditions used (CD40L/IL-21) could upregulate BMP receptor expression. To test this hypothesis, we cultured mononuclear cells from peripheral blood in the presence of CD40L/IL-21 for 24 h and then stained with anti-BMP receptor Abs, anti-CD19 or anti-CD20 Abs. Interestingly, stimulation with CD40L/IL-21 doubled the MFI values of Alk-2 expression in CD19+ B cells, whereas only minor differences were seen for the other receptors (Fig. 5C). Specific analysis of naive and memory B cells by anti-CD27 Ab was not possible in stimulated mononuclear cells as CD40L/IL-21-induced down-regulation of CD27 (Supporting Information Fig. 5) as shown previously [39]. Stimulation of FACS-sorted naive B cells for 48 h confirmed that Alk-2 expression could be induced in naive B cells (Fig. 5D). Taken together, naive and memory B cells expressed the type II receptor ACTR-IIB and the BMP type I receptor Alk-2 after stimulation with CD40L/IL-21.

**BMPs activate the Smad1/5/8-pathway in CD19+ B cells**

To investigate how the various BMPs mediate their functional effects in naive and memory B cells, we next investigated BMP-induced signaling. We stimulated peripheral blood CD19+ B cells with BMP-6 for various periods of time and examined activation of Smad1/5/8. BMP-6 induced phosphorylation of Smad1/5/8 after 30 min and reached maximum at 3 h of stimulation (Fig. 6A). The phosphorylation was still enhanced after 24 h. Furthermore, we tested the effects of BMP-2, -4, -6 and -7, and all BMPs induced activation of Smad1/5 (Fig. 6B). The BMPs also induced phosphorylation of pSmad1/5 in the presence of CD40L/IL-21 (Fig. 6B), although weaker as CD40L/IL-21 reduced the phosphorylation level of Smad1/5/8 (Supporting Information Fig. 6).

**BMP-6 induces expression of ID1 and ID3, and inhibits CD40L/IL-21-induced upregulation of XBP1**

As BMP-6 potently suppressed plasma cell differentiation and Ig production, we used this BMP to investigate the expression of key regulators of plasma cell differentiation, in addition to the BMP target genes ID1, ID2 and ID3. Real-time RT-PCR was performed on IgG-depleted memory B cells cultured for 2 or 4 days in the presence of CD40L/IL-21, with or without...
BMP-6. The expression of ID1 was increased 7.2- and 4.5-fold by BMP-6 after 2 and 4 days respectively (Fig. 7A). ID3 expression was increased 3.4-fold at day 4 in the presence of BMP-6, whereas ID2 was increased less than 2-fold. Furthermore, CD40L/IL-21 significantly increased the expression of IRF4, PRDM1 (gene encoding Blimp-1) and XBP1 at day 4 compared with day 2 (Fig. 7B). The strongest induction was seen for XBP1 with a 13-fold increase at day 4, relative to CD40L/IL-21-stimulated cells day 2, and this induction was markedly reduced by BMP-6. As the probe to detect XBP1 in these experiments detected the splice variants XBP1S as well as XBP1U, we also repeated this with a probe specific for the active form XBP1S. We found CD40L/IL-21-induced induction of XBP1S to be inhibited by BMP-6 to the same extent as XBP1 (Supporting Information Fig. 7). In contrast, IRF4 and PRDM1 expression levels were not affected by BMP-6. The expression of AICDA, the gene encoding AID, was not significantly changed by CD40L/IL-21-stimulated cells day 2, and this induction was markedly reduced by BMP-6. As the probe to detect XBP1 in these experiments detected the splice variants XBP1S as well as XBP1U, we also repeated this with a probe specific for the active form XBP1S. We found CD40L/IL-21-induced induction of XBP1S to be inhibited by BMP-6 to the same extent as XBP1 (Supporting Information Fig. 7). In contrast, IRF4 and PRDM1 expression levels were not affected by BMP-6. The expression of AICDA, the gene encoding AID, was not significantly changed by CD40L/IL-21 or BMP-6 (Fig. 7B). Taken together, these data indicate that BMP-6 inhibited plasma cell differentiation by suppressing CD40L/IL-21-induced upregulation of XBP1, possibly via upregulation of ID1 and ID3.

Discussion

The essential role of BMPs during embryogenesis and regulation of bone formation in adults is well established, but knowledge of their effects in the immune system is incomplete. We investigated how these growth factors affected human B-cell differentiation to plasmablasts. We found that BMP-2, -4, -6 and -7 all efficiently reduced CD40L/IL-21-induced Ig production in naive and memory B cells. However, how the different BMPs repressed Ig production varied. BMP-6 strongly inhibited plasma cell differentiation, in contrast to BMP-7 which mainly reduced Ig production via induction of apoptosis. We found GC B cells to express high levels of BMP7, but low levels of BMP6 (Supporting Information Fig. 8). BMP7 mRNA was also detected in B and T cells from peripheral blood [40], and normal and malignant plasma cells can express BMPs [27, 41]. This indicates that BMPs exist in lymphoid tissue and that the observed effects of BMPs on lymphocytes are of physiological relevance.

CD40L/IL-21 stimulated Ig production and induced differentiation to CD27⁺CD38⁺ plasmablasts in naive and memory B cells, as shown previously [7, 8]. The Ig production in memory B cells exceeded the production in naive B cells, which is expected since the differentiation of memory B cells was far more efficient than differentiation of naive B cells. The inhibitory effects of BMPs on Ig production have not previously been shown, but the role of TGF-β in Ig production is well studied. TGF-β inhibits production of IgM and IgG [34]. Furthermore, TGF-β directs IgA CSR in B cells [33], but since TGF-β is a strong inhibitor of cell growth [42], B cells depend on co-stimulation to induce efficient IgA secretion. For instance, TGF-β in combination with IL-10 induces secretion of IgA [3].

In CD40L/IL-21-activated B cells, BMP-6 strongly inhibited differentiation but had less potent effect on DNA synthesis, in contrast to BMP-7 which strongly inhibited DNA synthesis and induced apoptosis, but only slightly affected differentiation. This difference in functional effect is surprising considering that BMP-6 and BMP-7 belong to the same subgroup of BMPs, exhibiting 71% amino acid identity [43]. However, in adult kidney cells, BMP-7 but not BMP-6 was able to counteract TGF-β-induced epithelial to mesenchymal transition [44], indicating that BMP-6 and BMP-7 can differ in their effects despite high structural similarity. The preferred type I receptor for BMP-6 and BMP-7 is Alk-2, but they have also been shown to bind Alk-3 and Alk-6, depending on the cell type [45–47]. We found that Alk-2 was the only type I receptor with detectable expression, but we cannot rule out that other BMP receptors are expressed at levels sufficient for functional effects but below the detection limit. The findings of Seckinger et al. [27] support this hypothesis as they showed mRNA expression of ACVR1 (Alk-2), BMPR1A (Alk-3), as well as all type II receptors in peripheral blood memory B cells. Thus, we cannot rule out that BMP-6 and BMP-7 differ in their affinities for different heteromeric type I and type II receptor complexes, and that this partly can account for the different functional effects.

Upregulation of ID proteins have been shown to be important mediators of BMP effects in many cell systems [21]. We found
that BMP-6 induced upregulation of ID1 and ID3, suggesting a role for these genes as mediators of the BMP-6-induced inhibition of Ig production and plasma cell differentiation. We have previously shown that ID-1 is the mediator of BMP-6 inhibitory effects in T cells [38]. Several studies have shown a role for ID proteins in humoral immune responses through inhibition of E2A which is highly expressed in activated B cells and regulates CSR through direct induction of AID [48]. For instance, ID-1 has been shown to inhibit CSR [49], and inhibition of E2A by ID-2 or ID-3 leads to impaired Ig production [50]. Furthermore, a defect in BCR-induced proliferation has been seen in ID3 knock-out mice, leading to impaired humoral immune responses [51].

The transcription factors IRF-4, Blimp-1 and XBP-1 are all necessary for plasma cell differentiation, and as expected, CD40L/IL-21 increased the expression of these genes. BMP-6 inhibited the upregulation of XBP1, but did not affect the expression of IRF4 and PRDM1/Blimp-1 which are both upstream of XBP1. This suggests that BMP-6 affects late events in the plasma cell differentiation program. No previous studies have reported on the relationship between BMPs or ID proteins and these transcription factors. Even though the upregulation of ID1 and ID3 suggests that ID proteins mediate the inhibitory effect of BMP-6 on XBP1 expression, the exact mechanism involved needs to be further investigated. In addition to IDs, other candidate genes for mediating the suppressive effects of BMP-6 on XBP1 expression, could be the BMP target genes RUNX as these also have been shown to affect CSR and Ig production [52, 53].

To conclude, we have found that several BMPs have inhibitory effects on humoral immune responses in vitro. BMPs reduced Ig production by inhibiting plasma cell differentiation, reducing proliferation and inducing apoptosis. Differentiation to plasmablasts was inhibited by BMP-6 at a late stage, as expression of XBP1 but no upstream transcription factors were reduced.
These results open for further studies to elucidate the immuno-regulatory role of BMPs in B cells.

Materials and methods

Reagents and Abs

CD40L and Enhancer for Ligand were from Alexis Biochemicals, Enzo Life Sciences (NY, USA). Recombinant human (rh) IL-21 was from Invitrogen (CA, USA), whereas the following recombinant proteins and Abs were purchased from R&D Systems (MN, USA): rhBMP-2, rhBMP-4, rhBMP-6, rhBMP-7, mouse rNoggin and anti-human BMP-6 mAb (clone 74219). The following biotinylated Abs were from R&D Systems: anti-activin RIA, anti-BMPR-IA, anti-BMPR-IB, anti-BMPR-II, anti-activin RIIB, Streptavidin PE, anti-CD5 PECy7, anti-CD19 FITC, anti-CD19 PE, anti-CD20 aliphophocyanin-H7, anti-CD20 PerCPCy5.5, anti-CD27 aliphophocyanin, anti-CD27 PE, anti-CD38 FITC and anti-IgD PerCPCy5.5 were from BD (CA, USA), anti-CD19 PECy5 Ab were from Beckman Coulter (CA, USA), whereas anti-lambda PE anti-kappa aliphophocyanin were from Dako (Denmark). Goat serum was purchased from Sigma-Aldrich (MO, USA). Anti-phospho-Smad1/5/8 and anti-phospho-Smad1/5 Ab, was from Cell Signaling Technology (MA, USA), anti-IRF-4 (Mum1) and anti-Actin Ab were from Santa Cruz Biotechnology (CA, USA). Anti-XBP-1 Ab was from Abcam (UK) and Hoechst 33258 (2 μg/mL in PBS) was from Calbiochem (Germany).

Cell samples and immunomagnetic selection of B-cell subsets

Peripheral blood was collected from anonymous, healthy donors at The Blood Bank in Oslo, after informed consent and with approval from regional authorities (REK S-03280). B cells were isolated using positive selection with CD19⁺ Dynabeads (Invitrogen) as described previously [54]. IgD-depleted memory B cells were obtained by negative selection by incubating CD19⁺ B cells with Pan Mouse IgG Dynabeads (Invitrogen) coated with anti-mouse IgD Abs (BD) for 30 min at 4°C, followed by removal of beads.

Culture conditions

Purified B cells were cultured in X-VIVO15 (BioWhittaker, Switzerland). Proliferation and differentiation were induced by CD40L (used at 1 μg/mL and pre-incubated with Enhancer for Ligand (1 μg/mL) for 30 min at room temperature before adding to cells) and IL-21 (50 ng/mL) in the presence or absence of rhBMP-2 (300 ng/mL), rhBMP-4 (50 ng/mL), rhBMP-6 (500 ng/mL), rhBMP-7 (400 ng/mL), mouse rNoggin (5 μg/mL) or anti-human BMP-6 mAb (500 ng/mL). In some experiments, the number of cell divisions was tracked by labeling the cells with CFSE (Molecular Probes, OR, USA). CFSE (5 μM) in PBS was added to the cells (20 × 10⁶ cells/mL) and incubated for 10 min at 37°C. The reaction was stopped by adding ice-cold PBS with 20% FCS, followed by washing and culturing of the cells as described.

Cell proliferation

To measure DNA synthesis, B cells were cultured in triplicates (75,000 cells/200 μL in 96-well plates) for 3 days, and ³H-thymidine (American Radiolabeled Chemicals, MO, USA) was added for the last 16h of incubation. The cells were harvested on a Filtermate 196 harvester (Packard Instrument, CT, USA) and thymidine incorporation was measured on a Top Count microplate scintillation counter (Packard Instrument).
Cell death and apoptosis

Purified B cells were cultured for 3 days and stained with 5 μg/mL propidium iodide (PI; Invitrogen) or TUNEL (Roche, Switzerland) according to the manufacturer’s recommendations. The cells were analyzed on a FACS Calibur (BD).

Determination of Ig levels

Supernatants were collected from naive and memory B cells grown for 7 days (0.2 × 10⁶ cells/500 μL in 48-well plates). Secreted Igs were measured by Human IgA, IgM and IgG ELISA Quantitation Sets from Bethyl Laboratories (TX, USA). Absorbance was measured by a Sunrise Plate Reader (Tecan, Switzerland) set at 450 nm.

FACS analysis and cell sorting

All labeling reactions were performed by incubating cells with Abs for 30 min at 4°C. When an unconjugated primary Ab was used, the cells were washed twice before incubation with the secondary Ab. The cells were analyzed on a FACS Calibur Flow Cytometer, LSR II or FACS Canto (all from BD). Data were collected using FACS Diva software whereas analysis was performed using FlowJo (Tree Star, OR, USA) or Cytobank software (www.cytobank.org).

Western blot analysis

Cells were stimulated for 1 h or as indicated, before they were lysed in Tris lysis buffer as described previously [55]. Cell lysates were electrophoresed using 10% SDS-polyacrylamide gels (Pierce, IL, USA) and transferred to PVDF membranes (Millipore, MA, USA). The membranes were blocked for 60 min with 5% BSA (Sigma-Aldrich) with TBST before they were incubated with primary Abs overnight. After washing in TBST, the membranes were incubated for 60 min with HRP-conjugated anti-rabbit or anti-goat IgG Ab (Dako). Protein bands were visualized by the ECL or ECL-plus detection system (GE Healthcare, NJ, USA). Protein expression was quantified by scanning hyperfilms and using Quantity One Software (Bio-Rad, CA, USA).

Immunocytochemistry

Cells were fixed in 4% PFA (Electron Microscopy Sciences, PA, USA) in PBS, washed in PBS and permeabilized in 90% methanol in PBS at −20°C. After washing in PBS, cells were incubated in blocking buffer (1 mg/mL human γ-globulin (Sigma) in 0.9% NaCl) at room temperature for 30 min, followed by incubation with primary Abs (diluted in blocking buffer) in a humid chamber at 4°C overnight. Cells were washed in PBS the following day and subsequently stained with DyLight-conjugated secondary Abs (Jackson Immunoresearch, PA, USA; diluted in blocking buffer) at room temperature for 30 min in dark. Cells were washed in PBS and cytospin samples were made (Shandon Cytospin 2). Cells were mounted in fluorescent mounting medium (Dako) containing Hoechst 33258 and visualized in a Zeiss LSM710 confocal unit (Carl Zeiss, Germany), equipped with a 25 × /0.8 oil objective. Images were exported as tiff images and assembled in Illustrator (Adobe, CA, USA). Quantification of positive cells was performed by counting 150 cells pr. sample.

Real-time RT-PCR

RNA was isolated and cDNA was made as described previously [55]. Gene expression was analyzed by real-time quantitative RT-PCR using TaqMan Universal PCR master mix (Applied Biosystems) and the following TaqMan Gene Expression assays (Applied Biosystems): BMP6 (Hs00233470), BMP7 (Hs00233476), ID1 (Hs00704053), ID2 (Hs00747379), ID3 (Hs00171409), AICDA (Hs00221068), PRDM1 (Hs00153357), XBP1 (Hs00964359; which binds to both splicing variants), XBP1S (Hs01056534) and PGK1 (Hs99999906). The samples (containing 10 ng mRNA) were run on an ABI Prism 7000 Sequence Detection System (Applied Biosystems) as described previously [55]. Each measurement was done in duplicates and the threshold cycle (Ct) was determined. The gene expression was quantified using the comparative CT method as described in the ABI7700 User Bulletin 2 (Applied Biosystems).

Statistical analysis

The two-tailed Wilcoxon test for paired samples was applied to determine the level of statistical significance, using SPSS 16.0 (SPSS, IL, USA). In TUNEL experiments, a two-tailed, paired t-test was used. Data were regarded statistical significant at p.<0.05.

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Abbreviation: AID: activation-induced cytidine deaminase · Allc: activin receptor-like kinase · Blimp-1: B-lymphocyte-induced maturation protein 1 · BMPs: bone morphogenetic proteins · CSR: class switch recombination · ID: DNA-binding protein inhibitor · IRF-4: interferon regulatory factor 4 · XBP-1: X-box binding protein 1

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