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B Cell Receptor Crosstalk: B Cells Express Osteopontin Through the Combined Action of the Alternate and Classical BCR signaling Pathways

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Abstract

Classical BCR signaling requires a number of signalosome mediators that are bypassed when BCR signaling follows an alternate pathway produced by prior exposure of B cells to IL-4. The two pathways, classical and alternate, co-exist in IL-4-treated B cells. Here we report that operation of the IL-4-induced alternate pathway in combination with the classical pathway changes the nature of the B cell response to BCR engagement so that the cytokine, osteopontin (Opn), is produced and secreted. Although Opn expression by B cells has not previously been noted, anti-Ig-induced secretion by IL-4-treated B cells amounts to levels comparable to those secreted by activated T cells. However, unlike T cell Opn expression, B cell expression of Opn is not mediated by TBet. Because elevated levels of IL-4 occur in association with severe illness, and because Opn is strongly associated with autoimmunity, these results suggest that the IL-4-induced alternate BCR signaling pathway may participate in the pathophysiology of autoimmune dyscrasias.

Keywords

B cells; signal transduction; cell activation

Introduction

Surface immunoglobulin constitutes the antigen binding component of the B cell receptor (BCR) complex, engagement of which initiates downstream signaling. In naïve B cells, the BCR-initiated signaling cascade involves, and requires, a number of mediators that have been collected together in a conceptual framework termed the signalosome (Fruman et al., 2000). Absence or inhibition of any of these signalosome elements terminates BCR-triggered signaling and aborts downstream outcomes such as ERK phosphorylation. This signalosome-dependent route we term the classical BCR signaling pathway. The situation is quite different for B cells that have been exposed to IL-4. IL-4 induces an alternate pathway for BCR signal propagation to pERK that bypasses the need for multiple signalosome elements (Guo and Rothstein, 2005). These two BCR signaling pathways, the classical pathway and the IL-4-induced alternate pathway, are distinct by a number of criteria. Whereas the classical pathway operates independently of Lyn, is PI3K-, phospholipase C- and PKC β -dependent, and is not diminished by rottlerin inhibition (Chan et al., 1997; Guo et al., 2007; Guo and Rothstein,

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2005; Leitges et al., 1996; Mecklenbrauker et al., 2002; Miyamoto et al., 2002; Su et al., 2002), the alternate pathway is Lyn-dependent, PI3K-, phospholipase C- and PKC β -independent, and rottlerin-sensitive (Guo et al., 2007; Guo and Rothstein, 2005). The classical and alternate pathways co-exist in IL-4-treated B cells. Thus, when BCR engagement follows IL-4, the classical and the alternate pathways are both activated and operate in parallel separately of each other, such that inhibition of both, at the same time, is required to completely block distal events (Guo et al., 2007).

The addition of the alternate BCR signaling pathway to the pre-existing classical BCR signaling pathway as a result of IL-4 exposure raises the question of what the former contributes that is not effected by the latter. Possibilities include strengthening of known BCR signaling events, or, alternatively, stimulation of novel outcomes. To address this issue, we focused on Osteopontin (Opn), a polyclonal B cell activator and autoimmunity-associated cytokine secreted by T cells in response to antigen receptor triggering but not known to be produced by B cells. We report here for the first time that Opn is expressed and secreted by BCR-stimulated, IL-4-treated B cells, through the combined action of the alternate and classical BCR signaling pathways.

Methods

Animals

Male BALB/cByJ mice, C57BL/6 mice, and T-bet-deficient mice, at 8-14 weeks of age were obtained from The Jackson Laboratory (Bar Harbor, ME, USA). Mice were cared for and handled in accordance with National Institutes of Health and institutional guidelines, and studies with these mice were approved by institutional review committees.

Lymphocyte isolation

B cells were prepared from spleen cell suspensions by negative selection and cultured at 2×10^6 per ml in RPMI medium as previously described (Rothstein et al., 1995). T cells were prepared from spleen cell suspensions by negative depletion using magnetic-activated cell sorting (Miltenyi Biotec, Auburn, CA, USA). B cell subpopulations (Fig 2) were sort-purified from immunofluorescently stained splenocytes (FO, MZ) and peritoneal washout cells (B1) as follows: FO, B220+CD21^{int}CD23^{hi}; MZ, B220+CD21^{hi}CD23^{lo}/int; B1, B220^{lo}CD5⁺.

Lymphocyte stimulation

B cells were stimulated by F(ab')₂ goat anti-mouse IgM (anti-Ig) after incubation in medium for 3 hours (naïve), or after treatment with IL-4 for 24 hours followed by incubation in medium for 3 hours (IL-4-treated), as previously described (Guo et al., 2007). Inhibitors were added one hour prior to stimulation with anti-Ig. T cells were stimulated by plate-bound anti-CD3 and anti-CD28 antibody as described (Shinohara et al., 2005).

Real-time PCR

RNA was prepared from B cells using Ultraspec reagent (BiotecX, Houston, Tx, USA) and was DNase treated. cDNA was prepared using AMV reverse transcriptase (Roche Applied Sciences, Indianapolis, IN, USA). Opn gene expression was assessed and normalized to β 2-microglobulin expression by real-time PCR using a MX3000P quantitative PCR machine (Stratagene, La Jolla, CA, USA) with the following primers (forward/reverse): Opn, GACAACAACGGAAAGGGCAG/GATCGGCACTCTCCTGGCT; β -microglobulin, CCCGCCTCACATTGAAATCC/GCGTATGTATCAGTCTCAGTGG.

Western immunoblot analysis

Proteins were extracted from B cell pellets with RIPA lysis buffer. In each experiment, equal amounts of protein for each condition (15–30 µg) were subjected to SDS-PAGE followed by immunoblotting as previously described (Schneider et al., 1997).

ELISA

Supernatant fluid was obtained from cultures of B cells or T cells at the indicated times and tested for Opn by ELISA according to the manufacturer's instructions (R&D Systems, Minneapolis, MN, USA).

Reagents

Affinity-purified F(ab')₂ polyclonal goat anti-mouse IgM (anti-Ig) was obtained from Jackson ImmunoResearch Laboratories (West Grove, PA, USA). Anti-Opn antibody was obtained from R&D Research Systems (Minneapolis, MN, USA). Anti-CD3, anti-B220, anti-CD5, anti-CD21, anti-CD23, and anti-CD28 antibodies were obtained from BD Pharmingen (San Diego, CA, USA). Anti-actin antibody and cycloheximide were obtained from Sigma Aldrich (St. Louis, MO, USA). LY294002 and Rottlerin were obtained from Calbiochem (Gibbstown, NJ, USA). Recombinant murine IL-4 was obtained from BD Pharmingen (San Diego, CA, USA).

Results

Osteopontin/ETA-1/SPP1 Expression is a Specific Outcome of Alternate Pathway Signaling

We considered that the alternate pathway for BCR signaling might encode a different kind of B cell response as compared to responses triggered by signaling via the classical pathway. To address this issue, we focused on Opn expression by B cells under 4 conditions: naïve; anti-Ig-stimulated for 4 hours; IL-4-treated for 24 hours; and, IL-4-treated for 24 hours and then anti-Ig-stimulated for 4 hours. We found that Opn RNA expression was markedly upregulated by anti-Ig in IL-4-treated B cells (increase over unstimulated naïve B cells: 40 ± 2.5, mean ± SEM, n=3), whereas Opn RNA was only minimally increased by anti-Ig in naïve B cells (3.2 ± 0.70) (Fig 1a). Along the same lines, Western blotting showed that Opn protein expression was upregulated by anti-Ig in IL-4-treated B cells as compared to the minimal expression observed with anti-Ig-stimulated naïve B cells (Fig 1b). We then evaluated B cell secretion of Opn protein by ELISA. In line with the results above, we found that Opn secretion was markedly induced when anti-Ig was added to IL-4-treated B cells whereas little or no Opn secretion was observed when anti-Ig was added to naïve B cells (Fig 1c). Together these results indicate that IL-4 treatment changes the outcome of BCR signaling by promoting the expression and secretion of Opn, which is not upregulated by BCR triggering via the classical pathway. Additional evaluation of B cell subpopulations showed that Opn was upregulated by anti-Ig in IL-4-treated follicular and marginal zone B cells, but not in B1 cells (Fig 2).

Secretion of Osteopontin by B Cells Compares Favorably with the Level Produced by Stimulated T Cells

The results described above represent the first documentation that B cells express and secrete Opn. It was of interest to determine the general magnitude of B cell-derived Opn expression. To address this issue we compared the amount of Opn secreted into culture medium by sort-purified B cells (IL-4-treated/anti-Ig stimulated) and by T cells (plate bound anti-CD3/anti-CD28-stimulated) over a 4 day period, detected by ELISA. We found that the levels produced by appropriately stimulated B and T cells were similar, although gene expression (not shown) and protein secretion occurred more rapidly in the former than in the latter (Fig 3).

Osteopontin Expression Requires Signaling Via Both the Classical and Alternate Pathways

Because the alternate pathway operates in parallel with the classical pathway, according to the dual pathway model for IL-4-treated B cells (Guo et al., 2007), Opn expression specific to IL-4-treated/anti-Ig-stimulated B cells could derive from the alternate pathway alone, or from contributions by both pathways. To address this issue, we examined Opn RNA expression in B cells wherein anti-Ig stimulation was accompanied by either LY294002 (to block the classical pathway, (Guo and Rothstein, 2005)), rottlerin (to block the alternate pathway, (Guo et al., 2007)), or both. We found that LY294002 alone, and rottlerin alone, blocked Opn expression (detected by real-time PCR), as did the two together (Fig.4). These results strongly suggest that elements of both the alternate and the classical pathways for BCR signaling are required for upregulated Opn expression. This conclusion is further supported by results indicating that Opn expression induced by anti-Ig in IL-4-treated B cells was blocked by Go6976 (which blocks classical, but not alternate, pathway signaling, reference (Guo et al., 2007)) added concurrently with anti-Ig (data not shown).

Induction of B Cell Osteopontin Expression Is Independent of T-bet

The need for two signaling pathways to bring about Opn expression in response to anti-Ig stimulation of IL-4-treated B cells raises the question of what distal factor or factors is directly responsible. Inasmuch as B cell Opn expression has not been reported before, nothing is known regarding the elements that might regulate transcription specifically in stimulated B cells, although a number of transcription factors have been reported to influence Opn expression in non-lymphoid cell types. In T cells the transcription factor T-bet is required for Opn expression (Shinohara et al., 2005). With this as a guide, we evaluated the need for T-bet in B cell Opn expression by examining B cells obtained from T-bet-deficient mice. We found that Opn expression produced in IL-4-treated/anti-Ig-stimulated B cells was unaffected by loss of T-bet at either the level of gene expression (Fig.5A) or at the level of secreted protein (Fig.5B), indicating that T-bet is not responsible for Opn expression in B cells.

Induction of B Cell Osteopontin Depends on Protein Synthesis

Because peak osteopontin gene expression in IL-4 treated/anti-Ig stimulated B cells peaked between 4 and 8 hours. We questioned whether Opn was activated by a preexisting transcription factor or, alternately, required protein synthesis for induction. To evaluate this, we exposed IL-4-treated B cells to the protein synthesis inhibitor, cycloheximide (10 μ M), during anti-Ig stimulation. As noted above, after stimulation by anti-Ig, naïve B cells expressed very little Opn mRNA whereas IL-4 treated B cell expressed substantial amounts. However, this induction was completely reversed when cycloheximide was present along with anti-Ig (Fig. 6). Thus, Opn expression in IL-4-treated B cell depends on synthesis of one or more activating factors.

Discussion

Osteopontin (Opn) is an approximately 60 kDa RGD-containing phosphoglycoprotein, also known and studied under the names early T lymphocyte activation 1 (Eta-1) and secreted phosphoprotein 1 (SPP1), that plays a role in diverse physiological and pathological processes involving multiple tissues (O'Regan et al., 2000b). Opn is secreted by many cell types, including T cells, dendritic cells, and macrophages, although until now expression by B cells has not been reported. In contrast to T cells, production of osteopontin by B cells does not require T-Bet, but relies instead on one or more other factors that depend on protein synthesis. Among the different transcription factors that have been implicated in Opn expression by various cell types is AP-1, whose induction by anti-Ig in primary B cells requires protein synthesis (Chiles et al., 1991). In recent work we have shown that anti-Ig upregulates c-Jun in IL-4-treated B cells (data not shown), whereas anti-Ig fails to induce c-Jun in naïve B cells

(Huo and Rothstein, 1995). Thus, B cell osteopontin expression may be fostered by alternate pathway-specific induction of c-Jun containing AP-1 heterodimers.

Aside from its role as a matrix protein affecting wound healing and bone deposition, Opn functions as a cytokine, activating dendritic cells, enhancing Th1 and inhibiting Th2 cytokine expression, and costimulating T cell proliferation (Ashkar et al., 2000; Liaw et al., 1998; O'Regan et al., 2000a; Oldberg et al., 1986; Shinohara et al., 2005). Moreover, Opn has important effects on B cells. Opn is a polyclonal B cell activator; recombinant and biochemically purified Opn stimulates immunoglobulin production by B cells in vitro, and transgenic, overexpressing Opn mice contain elevated serum levels of several isotypes (Iizuka et al., 1998; Lampe et al., 1991). In keeping with the broad effects of Opn on the immune system, Opn-deficient mice are unusually susceptible to several infectious agents such as *L. monocytogenes*, *M. bovis*, and HSV-1 (Ashkar et al., 2000; Nau et al., 1999). In addition, the gene encoding Opn maps to the murine rickettsial resistance (*Ric^r*) locus, and *Ric^r* alleles that yield deficient production of Opn are associated with susceptibility to *O. tsutsugamushi*, the etiologic agent of scrub typhus (Patarca et al., 1989).

Further, Opn is strongly associated with autoantibody production and autoimmunity. This is evidenced by an impressive and diverse set of findings. 1) Opn is elevated in murine *lpr/lpr* lupus-like disease, experimental autoimmune encephalomyelitis (EAE), and anti-collagen antibody-induced arthritis (CAIA), and is elevated in human systemic lupus erythematosus (SLE), multiple sclerosis (MS), and rheumatoid arthritis (RA) (Chabas et al., 2001; Comabella et al., 2005; Hudkins et al., 2000; Ohshima et al., 2002; Patarca et al., 1990; Xu et al., 2005; Yumoto et al., 2002); 2) Polymorphism of Opn is associated with human SLE (Forton et al., 2002); 3) Deficiency of Opn results in delayed onset of polyclonal B cell activation in *lpr/lpr* disease and amelioration of EAE and CAIA (Chabas et al., 2001; Jansson et al., 2002; Weber and Cantor, 2001; Yumoto et al., 2002); and, most directly, 4) Overexpression of Opn, both lymphoid-specific and non-lymphoid-specific, results in the spontaneous production of anti-dsDNA antibodies (Iizuka et al., 1998). Thus, B cell production of Opn is likely to produce significant autocrine effects.

Operation of the IL-4-induced alternate pathway changes the nature of the B cell response to BCR engagement so that osteopontin is produced, Opn being a cytokine-like gene/protein whose expression by B cells has not previously been reported. Elevated levels of IL-4 are associated with severe infectious, allergic and other disorders, and thus at various times throughout life B cells are likely to be exposed to IL-4 in an antigen non-specific fashion. This would generate the machinery for alternate pathway signaling by BCR engagement (Guo et al., 2007; Guo and Rothstein, 2005), leading to Opn production, which, through polyclonal B cell activation, would be expected to produce enhancement of immune responsiveness but at the risk of serological autoreactivity. The results reported herein thus suggest a potential new route to autoimmunity.

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Abbreviations

BCR, B cell receptor
Opn, osteopontin
FO, follicular
MZ, marginal zone
ELISA, Enzyme-Linked ImmunoSorbent Assay

IL-4, interleukin-4
 ETA-1, Early T lymphocyte activator 1
 SPP1, secreted phosphoprotein 1
 EAE, experimental autoimmune encephalomyelitis
 CAIA, collagen antibody-induced arthritis
 SLE, systemic lupus erythematosus
 MS, multiple sclerosis
 RA, rheumatoid arthritis
 CHX, cycloheximide

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Figure 1.

The IL-4-induced alternate pathway for BCR signaling leads to osteopontin expression and secretion. **A.** B cells were cultured in medium alone for 3 hours (MED) or with IL-4 at 10 ng/ml for 24 hours and then washed and “rested” for 3 hours (IL-4), after which B cells were stimulated with F(ab')₂ fragments of goat anti-mouse IgM antibody at 15 μ g/ml (α Ig) for the indicated times. RNA was prepared from treated B cells and reverse transcribed. The level of osteopontin relative to $\beta 2$ microglobulin was determined by real-time PCR. **B.** B cells were treated as described above, after which whole cell extracts were prepared and Western blotted with anti-Opn antibody. Blots were stripped and reprobed with anti-actin antibody to verify equal loading. **C.** B cells were treated as described above, after which supernatant fluids were

collected and assayed for Opn by ELISA. For each of A, B, and C, one of 3 comparable experiments is shown.

Figure 2.

Stimulated follicular and marginal zone B cells express osteopontin. Sort purified follicular (FO), marginal zone (MZ) and B1 cells were cultured in medium alone for 3 hours (MED) or with IL-4 at 10 ng/ml for 24 hours and then washed and “rested” for 3 hours (IL-4), after which B cells were stimulated with F(ab')₂ fragments of goat anti-mouse IgM antibody at 15 μ g/ml (α Ig) for the indicated times. RNA was prepared from treated B cells and reverse transcribed. The level of osteopontin relative to β 2microglobulin was determined by real-time PCR. One of two comparable experiments is shown.

Figure 3.

Stimulated B and T cells secrete similar amounts of osteopontin. B cells were cultured with IL-4 at 10 ng/ml for 24 hours, washed, rested, and stimulated with F(ab')₂ fragments of goat anti-mouse IgM antibody at 15 μg/ml for 1-4 days (IL-4/αIg). T cells were cultured with plate adherent anti-CD3 and anti-CD28 for the same periods. Supernatant fluids were collected and assayed for Opn by ELISA. One of 3 comparable experiments is shown

Figure 4.

Osteopontin expression by B cells depends on both the classical and alternate BCR signaling pathways. B cells were cultured in medium alone for 3 hours (MED) or with IL-4 at 10 ng/ml for 24 hours and then washed and “rested” for 3 hours (IL-4), after which B cells were stimulated with F(ab')₂ fragments of goat anti-mouse IgM antibody at 15 μ g/ml (α Ig) for 4 hours, in the presence or absence of rotterlin at 10 μ M (Ro) and/or LY294002 at 20 μ M starting 60 minutes before addition of α Ig. RNA was prepared from treated B cells and reverse transcribed. The level of osteopontin relative to β 2microglobulin was determined by real-time PCR. One of 3 comparable experiments is shown.

Figure 5.

Osteopontin expression by B cells is independent of T-bet. B cells were obtained from T-bet knock-out mice (T-bet KO) or from wild-type littermate control mice (WT). B cells were cultured in medium alone for 3 hours (MED) or with IL-4 at 10 ng/ml for 24 hours and then washed and “rested” for 3 hours (IL-4), after which B cells were stimulated with F(ab')₂ fragments of goat anti-mouse IgM antibody at 15 μ g/ml (α Ig). **A.** RNA was prepared from B cells treated with α Ig for 4 h and reverse transcribed. The level of Opn relative to $\beta 2$ microglobulin was determined by real-time PCR. **B.** Supernatant fluids were collected after α Ig treatment for 2 d and assayed for Opn by ELISA. One of two comparable experiments is shown.

Figure 6.

Opn transcription requires new protein synthesis. B cells were cultured in medium alone for 3 hours (MED) or with IL-4 at 10 ng/ml for 24 hours and then washed and “rested” for 3 hours (IL-4), after which B cells were stimulated with F(ab')₂ fragments of goat anti-mouse IgM antibody at 15 μg/ml (αIg) with or without cycloheximide. RNA was prepared from B cells treated with αIg for 4 h and reverse transcribed. The level of Opn relative to β2microglobulin was determined by real-time PCR. One of three comparable experiments is shown