Human B Cell Precursors Proliferate and Express CD23 after CD40 Ligation

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Summary

The CD40 surface membrane molecule plays an important role in the activation of mature human B cells, but its role in earlier stages of B lineage development is unknown. Here, we have investigated the effects of triggering the CD40 antigen on B cell precursors (BCP) by crosslinking with anti-CD40 antibody presented by Fc'RII-receptor type II-transfected murine Ltk- cells (CD40 system). CD10+ surface immunoglobulin negative (sIg-) BCP, freshly isolated from fetal bone marrow or precultured on stromal cells, proliferated in the CD40 system. This effect required the presence of IL-3, which acted as a specific cosignal among a panel of cytokines examined. The association of IL-10 and IL-7 potentiated the observed IL-3 and CD40-dependent BCP proliferation, demonstrating that IL-10 can act on early B lineage cells. CD40-dependent activation of fetal BCP did not favor maturation to sIg+ B cells, but resulted in the induction of high levels of surface membrane CD23. The emerging CD23+ BCP lacked sIg and CD10, and represented an important proportion of the cycling cells in the CD40-dependent cultures. Taken together, our data demonstrate that stimulation of the CD40 antigen induces expression of the CD23 gene, and regulates cell proliferation during normal human B cell ontogeny.

The life history of B lymphocytes can be divided into two major steps. First, an antigen-independent process (B lymphopoiesis) permits constant generation of new immunocompetent surface (s)IgM+sIgD+ mature B cells in the bone marrow (for reviews see references 1 and 2). Then, mature B cells can be driven by antigens to proliferate and differentiate in secondary lymphoid organs (immunopoiesis) (for a review see reference 3). Both soluble cytokines and cell membrane–associated molecules regulate the various steps of B lymphocyte development. In this context, the CD40 B cell surface membrane molecule has recently been identified as playing a fundamental role in the process of immunopoiesis. Human CD40 is a 50-kD glycosylated phosphoprotein (4), that belongs to a new superfamily of receptors that includes the receptors for nerve growth factor and TNF (5, 6).

Anti-CD40 antibody presented by Fcγ receptor type II (FcγRII) (CDw32)–transfected murine fibroblastic Ltk- cells (CD40 system) induces strong and long-lasting B cell expansion in the presence of IL-4 (7, 8). The proliferation of anti-CD40-activated mature B cells is also enhanced by IL-10 (9). More strikingly, IL-10 allows CD40-activated B cells to produce large quantities of IgM, IgG, and IgA after their differentiation into plasma cells. Culturing CD40-activated naive sIgD+sIgM+ B cells in the presence of IL-4 or IL-10 and TGF-β induces the production of IgE or IgA, respectively, as a consequence of isotype switching (10–14).

Recent studies have indicated that CD40 crosslinking represents the first step of the interaction between T and B cells. A ligand to CD40 has recently been identified and expressed from activated T cells, and CD40-lg fusion proteins inhibit T cell–dependent B cell activation (15–20).

CD40 is known to be expressed on normal and leukemic B cell precursors (BCP), including the earliest B lineage cells bearing the progenitor cell antigen CD34 (21–23). However, although CD40 triggering has been shown to result in tyrosine phosphorylation of BCP intracellular proteins (24), no information is available on the physiological consequences of CD40 stimulation in B lymphopoiesis.

Here, we evaluated the effects of CD40 triggering on BCP cultured in the CD40 system. We demonstrate that BCP isolated from fetal bone marrow can be activated to undergo CD40-dependent proliferation and expression of CD23.

Materials and Methods

Isolation of BCP. Mononuclear cells were isolated from mid-term human fetal femoral bone marrow by Ficoll-Hypaque, and the CD10+ fraction was recovered by panning with anti-CD10

1 Abbreviations used in this paper: BCP, B cell precursor; BMSC, bone marrow stromal cell; FcγRII, Fcγ receptor type II; FSC, forward scatter; SCF, stem cell factor; sIg, surface Ig; SSC, side scatter.
mAb (ALB-1; Immunotech, Marseille, France) (25). slg- cells were depleted by anti-mouse Ig immunomagnetic beads (Dynabeads M450; Dynal, Oslo, Norway) after labeling with a cocktail of murine mAbs directed against μ, δ, α, γ, κ, and λ chains (all from Immunotech). The BCP population obtained was thus >98% CD19+ and lacked slg (see Fig. 4 A).

**BCP Cultures.** Cultures were performed in Opti-MEM liquid medium (Gibco Laboratories, Grand Island, NY), supplemented with 10% vol/vol heat-inactivated FCS, 10 mM Hepes, 2 mM glucose, 5 × 10-5 M 2-β-ME, penicillin (100 U/ml), and streptomycin (100 μg/ml) (Flow Laboratories, Irvine, UK). The effects of stimulation through the CD40 receptor were either evaluated on freshly isolated fetal CD10+ slg- BCP (see above), or after preculture of the CD10+ slg- cells on bone marrow stromal cells (BMSC) in the presence of 10 ng/ml purified recombinant human IL-7 (2.5 × 10^6 U/mg) (R & D Systems, Inc., Minneapolis, MN). The BMSC were isolated from adult human femur material by a procedure previously described in detail (26). Coculture of CD10+ slg- cells on BMSC results in BCP expansion (26), with obvious practical consequences for the present study.

The CD40 culture system has previously been described (7, 11). Freshly isolated or BMSC precultured fetal CD10+ slg- BCP were seeded onto irradiated (7,000 rad) adherent murine Ltk- cells transfected with CDw32/FcyRII (CDw32 L cell line), in the presence of 500 ng/ml of anti-CD40 mAb (mAb 89) (27) or an isotype-matched (IgG1) murine mAb. Cultures in the CD40 system were established at a ratio of one CDw32 L cell per two CD10+ slg- BCP.

**Cytokines.** Cell cultures were performed in the presence of purified recombinant human cytokines, at concentrations known to be saturating in various biological assays. IL-3 (sp act, 5 × 10^6 U/mg, batch 21294-72-40), IL-4 (sp act 10^6 U/mg, batch 81L-1002), and IL-10 (sp act 10^6 U/mg, batch 27785-82), were all provided by Schering-Plough Research Institute (Bloomfield, NJ). IL-1α (sp act 10^5 U/mg), IL-6 (sp act 10^5 U/mg), stem cell factor (SCF) (sp act 10^5 U/mg), and TNF-α (sp act 2 × 10^5 U/mg), were all purchased from Genzyme Corp. (Cambridge, MA). IL-2 (sp act 5 × 10^5 U/mg) was obtained from Amgen Biologicals (Thousand Oaks, CA). IFN-γ (5 × 10^5 U/mg) was purchased from Amersham (Les Ulis, France). Recombinant IL-7 (see above), and natural human TGF-β were from R & D Systems, Inc.

**Proliferation Assays.** For [3H]thymidine incorporation studies, BCP were seeded at 10^4 cells/well (100 μl) in 96-well flat-bottomed microtest tissue culture plates (Becton Dickinson & Co., Lincoln Park, NJ), with or without irradiated CDw32 L cells (5 × 10^3/well). At time points indicated, cultures were pulsed with 1 μCi methyl [3H]thymidine (Commissariat à l'Energie Atomique, Saclay, France; sp act 25 Ci/mmol) for 8 h, and subsequently harvested. Radioactivity was counted in a β-scintillation counter, and results were expressed as mean cpm ± SD observed in triplicate wells. In parallel, CD10+ slg- BCP were seeded at 10^6 cells/well (1 ml) in 24-well tissue culture plates (Nunc, Roskilde, Denmark) with or without 5 × 10^5 irradiated CDw32 L cells. Lymphoid cells were subsequently recovered by vigorous pipetting, a procedure that was sufficient for detachment from the adherent cells. Viable lymphoid cells were subsequently counted in triplicate culture wells using a hemocytometer.

**Cytofluorometric Analysis.** Phenotypic analysis was performed using standard surface membrane immunofluorescence technique, with FITC-labeled murine mAbs anti-CD10 and anti-CD19 (Becton Dickinson & Co., Mountain View, CA); anti-CD21 and anti-μ chain (Immunotech); anti-CD23 (28); and anti-CD40 (mAb 89) (27). Fluorescence was analyzed on a FACSScan® (Becton Dickinson & Co.), gating out both CDw32 L cells (large size), and nonviable lymphoid cells (incorporating propidium iodide). Analysis of DNA content was performed by staining cells with the DNA-specific dye Hoechst 33342 (Calbiochem-Novabiochem, La Jolla, CA) (10 μM, for 45 min at 37°C), followed by standard surface labeling using FITC-conjugated mAbs. Two-color fluorescence was subsequently analyzed on a FACStarPlus® flow cytometer (Becton Dickinson & Co.), after excluding CDw32 L cells.

**Results**

**Freshly Isolated BCP Proliferate in the CD40 System.** We have previously shown that mature human B cells enter a state of sustained proliferation when incubated with CDw32/FcyRII L cells and anti-CD40 antibody (CD40 system). Here, we investigated whether such a system would support the proliferation of purified human BCP. As illustrated in Fig. 1, no significant [3H]thymidine uptake was observed when highly purified CD10+ slg- fetal BCP were cultured in the presence of anti-CD40 and irradiated CDw32 L cells alone. However, the addition of IL-3 to such cultures induced [3H]thymidine uptake, as measured on day 12. This effect was specific for CD40 triggering, as it was not observed upon substitution of the anti-CD40 mAb with a control antibody (Fig. 1). Addition of soluble anti-CD40 antibody (tested at concentrations up to 10 μg/ml) did not stimulate cell proliferation, despite the presence of IL-3 (data not shown). Finally, as illustrated in Fig. 1, the proliferation of BCP cultured in the CD40 system in the presence of IL-3 was higher than in IL-3 alone in the absence of L cells.

BCP cultured in the CD40 system together with IL-3 were observed to grow in clumps, in contrast with BCP cultured on stromal cell layers. Interestingly, cells grown in the CD40 system without IL-3 did not generate such clumps.

**Figure 1.** Freshly isolated BCP proliferate in the CD40 system. 10^4 CD10+ slg- fetal BCP were cultured on 5 × 10^5 irradiated CDw32 L cells with anti-CD40 antibody (mAb 89) or a murine control mAb, with or without IL-3. In parallel, the BCP were seeded without CDw32 L cells, with or without IL-3. Data represent mean [3H]thymidine incorporation ± SD observed in triplicate wells on day 12. Anti-CD40 and control mAbs were used at 500 ng/ml, and IL-3 at 10 ng/ml.
Taken together, our results demonstrate that BCP display enhanced proliferation in response to IL-3 after CD40 cross-linking.

**BCP Precultured on Stromal Cells Proliferate in the CD40 System.** As human fetal BCP proliferate on BMSC (26, 29), we wondered whether such precultured cells would also proliferate in the CD40 system. Thus, fetal CD10⁺ slg⁻ BCP were expanded for 10 d on BMSC supplemented with IL-7, and 10⁴ precultured cells were seeded onto irradiated CDw32 L cells. As illustrated in Fig. 2 A, no significant [³H]thymidine uptake was observed in the absence of anti-CD40 mAb. However, addition of IL-3 to stroma-precultured BCP resulted in significant levels of [³H]thymidine uptake in the CD40 system (Fig. 2 A), whereas IL-1, IL-2, IL-4, IL-6, IL-10, SCF, IFN-γ, TNF-α, and TGF-β were inactive (Fig. 2 B). The marginal [³H]thymidine uptake observed in response to IL-7 (Fig. 2 B) was found to be CD40 independent.

Whereas IL-1, IL-2, IL-4, IL-6, SCF, IFN-γ, IFN-α, or TGF-β failed to enhance the anti-CD40/IL-3 response of stroma-precultured CD10⁺ slg⁻ BCP (data not shown), the association of IL-10 and IL-7 consistently potentiated the induced DNA synthesis (Fig. 2 A). In some experiments, addition of IL-10 or IL-7 alone enhanced the anti-CD40/IL-3 response, but this effect could not be reproducibly demonstrated.

The DNA synthesis observed in the CD40 system in response to IL-3 plus IL-10 plus IL-7 indeed reflected cell multiplication, as shown by an increase in viable cell numbers over a 2-wk culture period (Fig. 3). The CD40 system in combination with IL-3 was insufficient to expand the overall BCP seeded, although higher cell numbers were recovered than in the absence of IL-3 (Fig. 3), in line with the observed [³H]thymidine data (Figs. 1 and 2). Finally, BCP did not survive beyond 4–5 d on irradiated CDw32 L cells in the presence of a control mAb, despite the presence of cytokines (data not shown). Taken together, these data demonstrate that fetal BCP precultured on bone marrow stroma proliferate specifically in response to IL-3 after CD40 cross-linking, and that this effect is potentiated by IL-7 and IL-10.

**BCP Cultured in the CD40 System Express CD23 and CD21 but Lose CD10.** The phenotype of freshly isolated CD10⁺ slg⁻ fetal bone marrow BCP is illustrated in Fig. 4 A. The cells displayed CD19, CD10, and CD40 antigens, but did not express CD21, CD23, or slgM. In addition, freshly isolated CD10⁺ slg⁻ cells display a low 90⁺ light scatter distribution (side scatter, SSC) (Fig. 4 A).

![Figure 2](image-url)

**Figure 2.** Fetal BCP precultured on bone marrow stroma proliferate in the CD40 system. (A) CD10⁺ slg⁻ BCP were expanded 12 d on stromal cells + IL-7, and subsequently plated (10⁴ cells/well) on 5 × 10³ irradiated CDw32 L cells with anti-CD40 mAb or a control mAb. Cultures were performed in medium alone, in IL-3, in IL-3 plus IL-7, in IL-3 plus IL-10, or in IL-3 plus IL-7 plus IL-10. Data represent mean [³H]thymidine incorporation ± SD on day 9, as measured in triplicate wells. (B) CD10⁺ slg⁻ BCP were expanded 10 d on stromal cells plus IL-7, and subsequently plated (10⁴ cells/well) in the CD40 system, with various human cytokines: IL-1α, 20 U/ml; IL-2, 20 U/ml; IL-3, 10 ng/ml; IL-4, 50 U/ml; IL-6, 20 U/ml; IL-7, 10 ng/ml; IL-10, 100 ng/ml; SCF, 50 ng/ml; IFN-γ, 500 U/ml; TNF-α, 50 U/ml; and TGF-β, 2 ng/ml. Data represent mean [³H]thymidine incorporation ± SD on day 8 of culture, as measured in triplicate wells.
Upon culture in the CD40 system in the presence of IL-3, cells segregated into two distinct subsets: R1 with low forward scatter (FSC) and SSC, and R2 with high FSC and SSC (Fig. 4 B). This pattern was dependent upon CD40 triggering, as insignificant numbers of R2 cells were found when BCP were cultured in IL-3 in the presence of a control mAb (Fig. 4 C).

The R1 and R2 cell subsets identified in the CD40 system in the presence of IL-3 had substantially different phenotypes, although both expressed CD19 and CD21 antigens (Fig. 4 B). The R1 population was largely CD10+ and CD23-, whereas the R2 population expressed high levels of CD23 antigen, but no CD10. Furthermore, R2 cells were slgM- (Fig. 4 B), slgD- slgG- (data not shown), whereas the R1 population included a significant proportion of slgM+ cells (Fig. 4 B).

Induction of CD23 was specific for CD40 triggering, as cells in cultures containing a control mAb were CD23- (Fig. 4 C). BCP recovered after preculture of CD10+ slg- cells on BMSC were found to exhibit a similar phenotype upon secondary stimulation in the CD40 system (data not shown).

As illustrated in a representative experiment performed on stroma-precultured BCP, 9.3% of the cells were in cycle (S+G2M phases) after 12 d of secondary culture in the CD40 system plus IL-3 (Fig. 5 A). Notably, the cycling cells were mostly included within the CD23+ compartment (75.5% of total S+G2M cells; Fig. 5 B) and were slgM- (Fig. 5 C). The cells also lacked expression of other surface-membrane Ig isotypes, and did not display intracytoplasmic L chains (data not shown).

BCP grown in the CD40 system in a combination of IL-3 plus IL-10 plus IL-7, which resulted in maximal proliferation, also remained slgM- (Fig. 5 F), but a lower proportion of cells expressed CD23 when compared with cultures containing IL-3 alone (15.9% vs 33.2%) (Fig. 5 E). In addition, whereas a higher fraction of S+G2M cells was observed in the IL-3 plus IL-10 plus IL-7 cultures (15.8%, Fig. 5 D), as expected from the proliferation data, the cycling cells expressed less CD23 (48.5% of total S+G2M cells, Fig. 5 E) than those cultured with IL-3 alone.

These results indicate that activation of BCP through their CD40 antigen leads to proliferation of CD23+ slg- and CD23- slg- cells.

Collectively, our data demonstrate that BCP are induced to express CD23 and downregulate CD10 when cultured in the CD40 system in the presence of IL-3.

Discussion

In the present study, we have demonstrated that human BCP proliferate when cultured in the CD40 system in the presence of IL-3. However, major differences were observed between the responses of BCP and those of mature B cells in the CD40 system. First, unlike mature B cells, proliferation of BCP appears to be strictly dependent on the presence of exogenous cytokines. Interestingly, whereas mature B cell proliferation is boosted by IL-4 and to a lesser extent IL-10, that of BCP necessitates IL-3 and is further enhanced by addition of IL-7 and IL-10. Notably, IL-4 did not display any costimulation effect on BCP cultured in the CD40 system, a finding in line with our previous demonstration of the inhibitory effect of IL-4 on BCP proliferation (30).

Whereas BCP cultured in the CD40 system in the presence of IL-3, IL-7, and IL-10 expanded in number, the multiplication rate was much lower than that observed with mature B cells in the CD40 system together with IL-4. This may reflect the lack of a proliferation factor important for the multiplication of BCP or a lower proliferative capacity of BCP when compared with mature B cells. The latter is possible, as committed BCP appear to have a relatively limited proliferation potential in vivo (31), whereas mature B cells can expand several thousand-fold in response to antigen stimulation, most particularly in germinal centers. However, CDw32 L cells may produce factors inhibiting BCP proliferation, which could antagonize the stimulatory effect of CD40 crosslinking. In line with this notion would be the low responsiveness to the combination of IL-7 and IL-3 in the presence of CDw32 L cells and control antibody (Fig. 2 A), as compared with that of BCP cultured without L cells (25).

The stimulatory effect of IL-3 on BCP is not dependent on CD40 crosslinking, as it has already been reported on BCP cultured without antibody or accessory cells (25, 32). Whereas IL-10 has been shown earlier to enhance the proliferation of activated mature B lymphocytes (9), it failed to induce the proliferation of BCP cultured alone or in the CD40 system. Yet, IL-10 was found to enhance the proliferation of BCP cultured in the CD40 system together with IL-3 and IL-7. Thus, progenitor B cells represent another cellular target of human IL-10.

Activation of BCP through their CD40 antigen induces
the appearance of high SSC cells as analyzed by flow cytometry. This high SSC population was particularly conspicuous when CD40 activation was combined with IL-3, and accordingly, included the great majority of the proliferating cells in such cultures. The high SSC population expressed high levels of cell surface CD23, a broadly distributed 45-kD glycoprotein (33). CD23 has been described to represent a stage-specific marker on slgM + B cells before isotype switching in B cell ontogeny (34, 35). The present data, however, demonstrate that CD23 is inducible before acquisition of slgM.

In parallel to our results on BCP, it is of interest that mature B cells have recently been reported to acquire CD23 upon culture in the CD40 system (36). Unlike its effect on mature B cells (37), IL-4 does not appear able to promote CD23 expression on BCP (35) (our unpublished data). Thus, CD40-mediated induction of CD23 on BCP could be independent of IL-4. In this context, the present BCP cultures are unlikely to contain IL-4 which is essentially produced by human T cells (38) and basophil/mast cells (39). It will be interesting to determine whether the factor inducing CD23 on BCP represents a form of the CD40 ligand produced by activated T cells (19). Cycle analysis indicated that most of the S+G2M cells expressed CD23 in the CD40 system in the presence of IL-3, but that this proportion was lowered upon addition of IL-7 and IL-10 to the cultures. This may be due to an inhibitory effect of IL-10 on CD23 expression which we have observed on mature B cells (our unpublished data).

The potential role of CD23 in human B lymphopoiesis remains to be determined. In this context, the soluble 25-kD form of CD23, which is cleaved by autoprotoelysis from the
Figure 5. CD40-dependent activation induces the proliferation of CD23+ slgM- and CD23+ slgM- BCP. CD10+ slg- BCP were expanded 11 d on stromal cells plus IL-7, and then cultured 12 d in the CD40 system, with IL-3 alone (10 ng/ml), or with IL-3 (10 ng/ml) plus IL-7 (10 ng/ml) plus IL-10 (100 ng/ml). Data represent two-color flow cytometry FACStar analysis of DNA content (Hoechst 33342) vs expression of surface membrane markers. G0 + G1 cells (low Hoechst) and S + G2M (high Hoechst), as well as surface antigen positive cells, were determined from the fluorescence limits indicated on the dot plots. Negative surface membrane staining was provided by a control mAb of unrelated specificity.

cell surface (40), displays a broad spectrum of biological activities on hematopoietic cells. Notably, the combination of sCD23 and IL-1α induces the development of myeloid progenitors (41) and the maturation of prothymocytes (42). It will be thus be of interest to determine whether the combination of sCD23 and IL-1α displays any maturation effects on BCP.

The culture system described herein did not allow the transition of BCP into mature B cells even when proliferating cells were subsequently transferred into a CD40 system containing IL-4. In this context, the signal requirement for slgD expression is being actively searched for. It is possible that the majority of the BCP triggered in the CD40 system may be unable to mature because they are out of phase with the developmental stage where CD40 activation occurs in vivo.

We observed that BCP activated through CD40 downregulated their CD10 antigen. CD10 is a cell surface metallopeptidase that has recently been shown to negatively modulate BCP growth (43). Thus, downregulation of CD10 may confer a selective advantage in B cell ontogeny.

Although low levels of CD21 were induced in control cultures, CD21 expression was considerably enhanced by activation in the CD40 system. Of interest, CD21 has been demonstrated to represent a ligand for CD23 (44), raising the possibility of a CD21–CD23 interaction within our BCP cultures.

The significance of CD40-mediated activation of BCP deserves further investigation. In view of its well-documented role on mature B cells, it is tempting to speculate that CD40 is involved in a selection process at different stages in the life history of B lymphocytes. By extension, CD40 may participate in a more general network in hematopoiesis. In this context, we have observed CD40 expression on most CD34+ hematopoietic progenitors lacking B lineage markers (23). In addition, CD40 may participate in T cell selection as it is expressed on thymic epithelial cells (45).

Since activated T cells express a CD40 ligand, CD40 activation may represent a mechanism by which antigen-stimulated T lymphocytes could regulate hematopoietic development. Our present data demonstrating that IL-3 plays a particular role in CD40-dependent BCP proliferation should be considered in the context that activated T cells represent one of the few presently known physiological sources of IL-3. Several groups (46–49) have recently established that mutations in the gene encoding the T cell CD40 ligand are responsible for the X-linked HIGM1 hyper-IgM syndrome. As this condition results in a defect in the capacity for isotype switching, but not in primary B cell production, it would appear that a putative interaction between CD40 and its T cell ligand is not an obligate pathway in B cell ontogeny, consistent with a mere regulatory function. Alternatively, however, the possibility should be considered that CD40 plays a more central role in B lymphopoiesis by interacting with a second ligand, whose existence has been suggested (50), and which could be provided by the BMSC microenvironment. In this respect, it is interesting to note that other members of the CD40 antigen superfamily, such as the TNF receptors and the nerve growth factor receptor, bind to several ligands (6).
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