Expression of Interleukin-4 Receptors on Early Human B-Lineage Cells

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Interleukin-4 (IL-4) regulates multiple stages of the antigen-dependent phase of B-cell development. However, its precise role in regulating B lymphopoiesis in bone marrow is not as well defined. We examined whether surface IgM-normal and leukemic human B-cell precursors (BCP) expressed IL-4 receptors using biotinylated IL-4. Constitutive expression of IL-4 receptors was detected on both normal and leukemic BCP. A higher percentage of normal BCP (82% ± 15%) expressed IL-4 receptors compared with leukemic BCP (44% ± 8%). Using mean fluorescent intensity as an indicator of receptor level on the IL-4 receptor positive cells, normal (91 ± 41) and leukemic (44 ± 37) BCP expressed comparable numbers of receptors. IL-4 induced the expression of CD23 on 30% of the leukemic BCP cases examined. IL-4 induced CD23 on surface IgM+ fetal bone marrow lymphoid cells but not on the surface IgM-normal BCP, despite the presence of detectable receptors on the surface IgM+ cells. IL-4 did not stimulate proliferation of normal BCP, nor could it enhance the effect of recombinant IL-7 or low molecular weight B-cell growth factor. However, IL-4 increased the expression of surface IgM and surface IgG on in vitro differentiated pre-B cells. Our collective results identify no role for IL-4 in the proliferation of normal or leukemic BCP, but identify a role in the enhancement of surface IgG expression during pre-B to B-cell differentiation.

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MATERIALS AND METHODS

Cells. Newly diagnosed fresh BCP-acute lymphoblastic leukemia (ALL) bone marrow aspirates expressing at least two of the three B-cell-associated antigens CD10, CD19, and CD24 were obtained from the Cell Marker Laboratory, Department of Laboratory Medicine and Pathology, University of Minnesota, Minneapolis. These specimens consisted of ≥80% (and frequently >90%) leukemic blasts. Normal fetal bone marrow lymphoid cells were obtained by flushing fetal femoral bones with 10 mL of RPMI-1640, 5% fetal calf serum (FCS). Mononuclear cells were isolated by centrifugation over Ficoll-Hypaque (Pharmacia Fine Chemicals, Piscataway, NJ) and washed thrice with RPMI-1640, 5% FCS. The preparations were adherent cell-depleted by incubation on plastic at 37°C, 5% CO2, for at least 3 hours. The resulting cells were greater than 95% viable by trypan blue exclusion. The use of all human tissue had the approval of the University of Minnesota Committee on the Use of Human Subjects in Research. BCP-ALL cell lines NALM-6, HPB-NULL, and BLIN-1, the pre-B lymphoblastic lymphoma cell line JM-1, and the Burkitt’s lymphoma cell line Raji were routinely maintained in RPMI-1640, 10% FCS, supplemented with 100 U/mL penicillin and 100 μg/mL streptomycin.

Antibodies. 13B3 (IgG2a)/anti-CD2 was produced and characterized in this laboratory. EBVSC2 (IgG1)/anti-CD23 was a gift from Dr Bill Sugden (University of Wisconsin, Madison). Polyclonal rabbit antihuman IL-4 antibody was a gift from Dr John Williams (Genzyme, Boston, MA). Anti-My8 (IgG2a) and anti-M9 (IgG2b)/anti-CD33 were purchased from Coulter Immunology (Hialeah, FL). OKM-1 (IgG2b)/anti-CD11b, HB57 (IgG1)/anti-IGM, HB61 (IgG1)/anti-k, and 10F7/MN (IgG1)/anti-glycoporphin

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were obtained from the American Type Culture Collection (ATCC) (Rockville, MD). J5 (IgG2a/-anti-CD10-phycoerythrin (PE) and IgG2a/-PE control myeloma protein were purchased from Coulter Immunology. IgG1 (MOPC) control myeloma proteins were purchased from Cappell (Durham, NC). FITC-goat antimouse IgG1 (FITC-GAM IgG1) was purchased from Southern Biotechnology Associates, Inc. (Birmingham, AL). FITC conjugation and biotinyl-
lation of HB57, HB61, and IgG1 myeloma protein was accom-
plished using the methods described by Goding.6

**Flow cytometry.** Two-color analysis was used to examine the expression of CD23 and CD10 on BCP-ALL. Cells, 1 × 10⁶, were stained with saturating amounts of J5 (IgG2a)/anti-CD10-PE and EBVCS2 (IgG1)/anti-CD23 in 50 μL of fluorescence buffer (1x phosphate buffered saline [PBS], 2.5% newborn calf serum) for 30 minutes at 4°C. Cell-bound anti-CD23 was then detected by FITC-GAM IgG1 at a final dilution of 1:40. Negative controls consisted of cells incubated with identical concentrations of IgG2a-PE and IgG1 myeloma protein, followed by counterstaining with FITC-GAM IgG1. A similar method was used to examine the expression of CD23 and surface IgM on fetal bone marrow lymphoid cells. Cells, 1 × 10⁶, were stained with a saturating amount of EBVCS2 (IgG1)/anti-CD23, and cell-bound anti-CD23 was detected by FITC-GAM IgG1. Unbound sites on FITC-GAM IgG1 were blocked by incubating cells with IgG1 myeloma protein at 40 μg/mL for 30 minutes at 4°C. Surface IgM was then detected using a saturating amount of biotinylated HB57 (IgG1)/anti-IgM, followed by counterstaining with streptavidin-PE (Caltag, San Francisco, CA). For negative controls, EBVCS2 and biotinyl-
ated HB57 were substituted with IgG1 myeloma protein and biotinylated IgG1 myeloma protein, respectively. Single-color analysis using FITC-conjugated HB57 and HB61 was used to detect surface IgM and surface IgK, respectively. Stained cells were washed thrice with fluorescence buffer and fixed with 0.5 mL of 1% paraformaldehyde in PBS before analysis on a FACScan (Becton Dickinson, Mountain View, CA).

**IL-4 receptor binding assay.** Human recombinant IL-4 was biotinylated as previously described.5 Five hundred nanograms of biotinylated IL-4 in 25 μL of staining buffer consisting of PBS, 0.5% bovine serum albumin (BSA) was incubated with 5 × 10⁶ cells at 4°C for 30 minutes. Cells were washed once in staining buffer, counterstained with SA-PE (dilution of 1:40) at 4°C for 30 minutes, and then analyzed on a FACScan. Competition assays were conducted by preincubating cells with log concentrations of unlabeled IL-4 before staining with biotinylated IL-4.

**Induction of CD23.** Fresh BCP-ALL or fetal bone marrow low-density leukocytes were incubated in RPMI-1640, 10% FCS, 2 mmol/L L-glutamine, 50 μmol/L L-mercaptoethanol with or without 1 to 800 U/mL of IL-4 (Genzyme). Expression of surface proteins was analyzed by flow cytometry after 24 to 48 hours of incubation.

**Magnetic bead depletion.** Fetal bone marrow nonadherent low-density leukocytes were suspended to 15 × 10⁶ cells/mL in RPMI-1640, 2% FCS containing a cocktail of monoclonal antibod-
ies (MoAbs) at saturating concentrations against myeloid/ monocytic cells (My 8, CD11b, CD33), erythroid precursors (glycophorin A), T cells (CD2), and mature B cells (surface IgM). Cells were incubated at 4°C for 45 minutes, and washed twice in RPMI-1640, 2% FCS. They were then incubated with goat antimo-
use IgG-coated Dynabeads M-450 (Robbins Scientific, Mountain View, CA) at a concentration of 5 to 10 beads/containing cell with constant rotation at 4°C for 60 to 90 minutes. The suspension was diluted twofold and placed next to a flat plate magnet for 5 to 10 minutes. Cells not bound to the Dynabeads were subsequently aspirated and used for various experiments. These cells were ~95% CD10⁺ and less than 2% surface Ig⁺. We consider these cells to represent the total BCP pool consisting of both cytoplasmic mu⁺ pro-B cells, cytoplasmic mu⁺ pre-B cells, and potentially other minor populations, as previously described.11

**Proliferation assay.** CD10⁺/surface IgM⁺ fetal bone marrow lymphoid cells purified by magnetic bead depletion were seeded at 0.5 to 1 × 10⁶ cells/200 μL of medium (RPMI-1640, 10% FCS, 2 mmol/L L-glutamine, 50 μmol/L L-mercaptoethanol [2-ME], 100 U/mL penicillin, and 100 μg/mL streptomycin) in 96-well round-bottom plates, in the presence of different stimulating agents. IL-4 was used at 1 to 100 U/mL, low molecular weight B-cell growth factor (L-BCGF; Cellular Products Inc. Buffalo NY) was used at 5% vol/vol, and IL-7 (PreproTech Inc, Rocky Hill, NJ) was used at 1 to 10 ng/mL. All experiments were conducted in triplicate. Cultures were incubated for 96 hours at 37°C, 5% CO₂, and pulsed with 1 μCi/well of [³H]-TdR (Amersham, Arlington Heights, IL; specific activity of 20 Ci/mmol) for the last 16 to 18 hours of incubation. The cells were then harvested onto glass fiber filters, the filters were dried overnight, and [³H]-TdR incorporation was quantitated by liquid scintillation spectrometry. The Student's unpaired t-test was performed to determine the significance of growth stimulation.

In vitro differentiation of normal BCP. In vitro differentiation of normal BCP was set up according to our previously published procedure.12 Briefly, CD10⁺/surface IgM⁺ fetal bone marrow lymphoid cells were seeded at 5 × 10⁶ cells/200 μL of RPMI-1640, 10% FCS, 2 mmol/L L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin. IL-4 was used at 1, 10, or 100 U/mL. In blocking experiments IL-4 was incubated with 4 μg/mL of rabbit antihuman IL-4 at 37°C for 30 minutes before addition to cells. After 48 hours of incubation the cells were analyzed for expression of surface Ig by flow cytometry.

**RESULTS**

Expression of IL-4 receptors on normal and leukemic human BCP. Successful application of biotinylated IL-4 in the evaluation of receptor expression using flow cytometry has been reported using both mouse and human B cells.13,14 To facilitate the comparison of relative levels of receptor expression based on the percentage of positive cells and mean fluorescent intensity (MFI), a single batch of biotinyl-
ated, recombinant human IL-4 was used throughout this study. Figure 1 illustrates the binding of biotinylated IL-4 to the pre-B lymphoblastic lymphoma cell line JM-1.13 Binding was effectively blocked by the presence of a 200-fold excess of unlabeled IL-4, confirming the specificity of biotinylated IL-4. A survey of IL-4 receptor expression on BCP-ALL cell lines, a Burkitt's lymphoma cell line, and fresh BCP-ALL is presented in Fig 2. Clearly positive signals were obtained using BCP-ALL cell lines (NALM 6, HPB-NULL, and BLN-1) and the Burkitt's lymphoma cell line RAJI (Fig 2A). IL-4 receptor positive cells were arbitrarily defined by subtracting the fluorescence signal obtained in the absence of biotinylated IL-4 from the fluorescence signal obtained in the presence of biotinylated IL-4. Using this definition, greater than 90% of the cells were IL-4 receptor positive. As determined by MFI, the level of recep-
tors was in the order of BLN-1 > RAJI > NALM-6 > HPB-NULL. Using the aforementioned definition for receptor positivity, only 44% ± 8% of the cells from any given fresh BCP-ALL expressed IL-4 receptors (Fig 2B), compared with 95% ± 1% of the cells from any given leukemic cell line (Fig 2A).
IL-4 receptor expression was then examined on normal BCP. CD10+/surface IgM⁻ fetal bone marrow cells were isolated by magnetic bead depletion. Results obtained from two fetal bone marrow donors (FBM-1, FBM-2) are presented in Fig 3. Approximately 85% and 95% of the CD10+/surface IgM⁻ fetal bone marrow lymphoid cells stained with biotinylated IL-4. Specificity of IL-4 binding was evaluated by cold competition using cells obtained from an additional donor (FBM-3, Fig 3). CD10+/surface IgM⁻ cells were incubated with biotinylated IL-4 plus unlabeled IL-4 at ratios of 1:200 (FBM-3, left histogram) and 1:1,000 (FBM-3, right histogram). At a ratio of 1:200, unlabeled IL-4 reduced the percentage of positive cells from 64 to 41, and the MFI from 41 to 31. At a ratio of 1:1,000, unlabeled IL-4 reduced the percentage of positive cells from 58 to 6, and the MFI from 21 to background.

IL-4-induced CD23 expression occurs on some BCP-ALL but not on normal BCP. The major outcome of IL-4/IL-4 receptor interaction on mature B cells is the induction of cell surface CD23. Hence, the ability of IL-4 to upregulate CD23 on BCP-ALL was assessed to determine the functional status of the IL-4 receptors on these cells. Ten of the 34 cases (30%) of BCP-ALL tested responded to IL-4 stimulation by expressing CD23. Figure 4 shows the response of one representative case. Greater than 90% of the BCP-ALL cells were CD10⁺. Very few CD23⁺ cells were detectable in the fresh cells or cells incubated in medium alone for 24 or 48 hours. Addition of IL-4 induced expression of CD23 by 24 hours with a more striking induction by 48 hours, and virtually all the CD23⁺ cells were CD10⁻. This
result provided direct evidence that CD10⁺ leukemic BCP expressed CD23 in response to IL-4. The specificity of IL-4 was confirmed using a rabbit anti-IL-4 neutralizing antibody. When IL-4 was incubated with anti-IL-4 for 30 minutes before the addition of BCP-ALL cells, all the CD23-inducing activity was negated (Fig 4). Moreover, substituting IL-4 with IL-1β, -2, -3, -6, -7, and L-BCGF did not result in CD23 upregulation on leukemic BCP (data not shown). A major polypeptide of 46 Kd was immunoprecipitated from an IL-4-treated BCP-ALL using the EBVCS2/anti-CD23 MoAb, confirming that the IL-4-induced antigen detected by flow cytometry had the expected molecular weight of CD23 (data not shown). Of the five cases of BCP-ALL assessed for IL-4 receptor expression in Fig 2, only two (patients LC and MD) responded to IL-4 by expressing CD23.

Adherent cell-depleted fetal bone marrow low-density leukocytes were then examined for their response to IL-4 stimulation. After 48 hours in culture with 800 U/mL of IL-4, 20% of the surface IgM⁺ cells became CD23⁺ (Fig 5A). In contrast, surface IgM⁻ fetal bone marrow lymphoid cells did not show an increase in CD23 expression (Fig 5A). Figure 5B shows the IL-4 dose-dependent induction of CD23 on fetal bone marrow lymphoid cells. Consistent with the data in Fig 5A, CD23 was only induced on surface IgM⁺ cells. IL-4 also did not induce CD23 on purified CD10⁻/surface IgM⁻ fetal bone marrow BCP (data not shown). This result ruled out the possibility that cells of other hematopoietic lineages present in fetal bone marrow low-density leukocytes interfered with the response of BCP to IL-4 stimulation.

Function of the IL-4 receptor on normal BCP. The functional status of IL-4 receptors on normal BCP was further investigated using proliferation and differentiation assays. CD10⁺/surface IgM⁺ fetal bone marrow cells were incubated with 1 to 100 U/mL of IL-4. These concentrations were effective in inducing CD23 in BCP-ALL (Fig 4) and surface IgM⁺ B cells (Fig 5). The ability of IL-4 to stimulate [³H]-TdR incorporation alone, or in combination with other growth factors (L-BCGF or IL-7), is shown in Table 1. IL-4 alone did not deliver any significant, dose-dependent, reproducible stimulation of normal BCP in three experiments. CD40 is expressed on approximately 2/3 of CD10⁻/surface IgM⁻ normal BCP. Because IL-4 plus anti-CD40 is a potent growth stimulus for mature B cells, the efficacy of this combination in stimulating proliferation of normal BCP was also tested. Consistent with our other studies, anti-CD40 did not enhance [³H]-TdR incorporation, nor did anti-CD40 plus 1 to 100 U/mL of IL-4. In contrast, L-BCGF (5% vol/vol) and IL-7 (1 or 10 ng/mL) significantly stimulated [³H]-TdR incorporation in CD10⁻/surface IgM⁻ BCP, with stimulation indices ranging from 1.4 to 6.6. IL-4 did not significantly enhance the effects of L-BCGF or IL-7.

Because IL-4 has been reported to play a role in the differentiation of pre-B to B cells in both humans and mice, the effect of IL-4 on the expression of surface IgM during in vitro differentiation of normal pre-B cells was examined. Using our recently described short-term culture for studying differentiation of normal human pre-B cells, IL-4 did not exert any appreciable effect on the acquisition of surface IgM⁺ cells, compared with medium alone (Table 2). However, incubation with IL-4 resulted in B cells
expressing a higher level of surface IgM and surface IgG. This effect was IL-4 specific, because it was totally abolished by rabbit anti–IL-4 antibody (Table 2). IL-4 also increased the level of cell surface IgG (data not shown).

**Discussion**

Biotinylated IL-4 and flow cytometric analysis were used to assess the expression of IL-4 receptors on normal and leukemic BCP in the current study. Although data on binding affinity and quantitation of receptor number per cell cannot be derived using biotinylated ligands, they can provide rapid evaluation of receptor expression on a cell-per-cell basis. Accordingly, this approach is particularly well suited for studying receptor expression on small numbers of cells. The validity of the biotinylated IL-4 binding assay used in this study is predicated on the ability of unlabeled IL-4 to effectively block the binding of biotinylated IL-4 to the pre-B lymphoblastic lymphoma cell line JM-1 (Fig 1), and normal BCP (Fig 3). Analysis of BCP-ALL cell lines (NALM-6, HPB-NUL, and BLIN-1), the Burkitt's lymphoma cell line RAJI, and CD10+/surface IgM- normal BCP showed clear positive staining for IL-4 receptors on nearly all cells (Figs 2A and 3). On the other hand, only 44% of the leukemic cells from any given fresh BCP-ALL were IL-4 receptor positive according to the definition used in this study. Nevertheless, when MFI was used to compare the level of IL-4 receptors expressed on the receptor positive cells, the number of receptors expressed on different BCP populations was comparable, ie, 58 ± 28% for BCP-ALL cell lines, 91 ± 44 for CD10+/surface IgM- normal BCP, and 44 ± 37 for fresh BCP-ALL.

The human IL-4 receptor is a member of a newly described receptor superfamily characterized by four conserved cysteine residues and a double tryptophan-serine...
that the low fluorescence signal is a true reflection of IL-4
NALM-6 Analysis of NALM-6 cells showed an easily
NALM-6 and RAJI (Fig 2A), and underwent a functional
motif located proximal to the transmembrane region.26
Binding assays using 125I-labeled IL-4 have detected 2,280 ±
210 IL-4 receptors/RAJI cell and 115 ± 15 IL-4 receptors/
NALM-6 cell.26 Analysis of NALM-6 cells showed an easily
detectable signal using biotinylated IL-4, comparable with
that obtained from RAJI cells (Fig 2A). This may reflect a
higher level of IL-4 receptors expressed on the NALM-6
cells used in this study. Leukemic cells from patient LC (Fig
2B) gave a biotinylated IL-4 fluorescence signal less than
NALM-6 and RAJI (Fig 2A), and underwent a functional
response to IL-4 by expressing CD23. This result argues that
the low fluorescence signal is a true reflection of IL-4
receptor expression on cells from patient LC, and biotinyl-
ated IL-4 can detect IL-4 receptors ≤200/cell.

The results in this study constitute the first report of the
ability of IL-4 to induce expression of CD23 on some
BCP-ALL. This is an intriguing observation in light of the
recent reports describing an autoproteolytic action of
CD23,27,28 and the reported ability of soluble CD23 to
stimulate proliferation of cells from different lineages
including T cells,29 B cells,30,31 and myeloid precursors.32
Soluble CD23 can also promote the functional maturation
of prothymocytes.33 The potential of CD23 induced on
BCP-ALL to be shed from cell surface and the response of
BCP-ALL to soluble CD23 remains to be determined.
Several mechanisms can be proposed to explain the vari-
able response of BCP-ALL to IL-4, despite the constitutive
expression of IL-4 receptors on all cases examined. Human
IL-4 mediates transient polyphosphoinositide hydrolysis,
Ca2+ mobilization, and cAMP generation in tonsillar B
cells, all of which are required for CD23 induction.34,35 Hence,
it is conceivable that in those cases of BCP-ALL that did
not respond to IL-4 by expressing CD23, this full comple-
ment of signals was not generated. Alternatively, transcrip-
tion or translation of CD23 may be specifically altered in
some leukemic cells, whereas changes in the expression of
other (undefined) molecules may be intact. The difference
in response to IL-4 may also reflect a structural difference
in the IL-4 receptor itself.

A major distinction between normal and leukemic BCP is
the inability of normal BCP to express CD23 (Fig 5),
despite the expression of IL-4 receptors (Fig 3). The
capacity of normal fetal bone marrow lymphoid cells to
express CD23 is correlated with the expression of surface
IgM (Fig 5). The acquisition of surface IgM may be
necessary but not sufficient for B cells to upregulate CD23
in response to IL-4 stimulation because not all fetal bone
marrow surface IgM+ B cells could express CD23 (Fig 5).
When CD10+/surface IgM+ BCP were cultured with or
without IL-4 some of them differentiated into surface IgM+
cells within 24 hours (Table 2), but CD23 could not be
detected on these differentiated surface IgM+ cells (data
not shown). This finding suggests that a full complement of
CD23-inducing signals is only generated in some surface
IgM+ fetal bone marrow B cells, and appears to be
restricted to mature B cells expressing higher levels of
surface IgM. It is also conceivable that an extremely minor
subpopulation of normal BCP may be capable of expressing
CD23 in response to IL-4, but were not detected by flow
cytometry. The BCP-ALL capable of expressing CD23 may
then represent leukemic transformation and clonal expan-
sion of such rare BCP. On the other hand, asynchronous
programming of maturation in leukemic cells25 may under-
lie the difference between normal and leukemic BCP.

The absence of an agonistic effect of IL-4 on the
proliferation of leukemic BCP agrees with previously pub-
lished data. Touw et al26 observed no proliferative effect of
IL-4 on BCP-ALL. Cell cycle analysis of the IL-4-
responsive BCP-ALL in this study showed no increase in
cells in S/G2 + M phases of the cell cycle compared with
cells cultured in medium alone (data not shown). The
ability of IL-4 to induce proliferation in CD10+/surface
IgM+ normal BCP was evaluated in three fetal bone
marrow donors (Table 1). IL-4 delivered no significant
stimulation and did not enhance the agonistic action of IL-7
or L-BCGF. IL-4 plus anti-CD40 delivers a poten
tial stimulus to mature B cells,22,23 but this combination did not stimulate
normal BCP (Table 1). IL-4 acts at multiple stages of
murine B-cell ontogeny by stimulating proliferation in early
precursors and differentiation in late precursors. Thus, it
maintains long-term cultures of pro-B cell clones,3 and
supports self-renewal in the early precursor population
during which the cells retain their immature phenotype.3
Although purified according to our definition of CD10
expression and lack of surface IgM, the normal BCP used in
this study constitute a heterogeneous population encompass-
ing cytoplasmic mu+ pro-B cells, cytoplasmic mu+ pre-B
cells, and potentially other minor subpopulations.3 Therefore,
it is conceivable that IL-4 may have limited prolifera-
tion enhancing actions on a very minor population of
human BCP.

Data presented in Table 2 demonstrate that CD10+/surface
IgM+ fetal bone marrow normal BCP were capable of
differentiating into surface IgM+/surface κ+ cells in the
absence of exogenous growth factors. We have recently
published a detailed appraisal of this in vitro differentiation
system for normal BCP.11 IL-4 did not significantly increase
or decrease the percentage of surface IgM+ and surface
Igκ+ cells compared with medium alone in the three donors
examined. However, cells cultured in IL-4 had levels of surface IgM and surface IgK on the in vitro differentiated cells that were consistently higher than cells maintained in medium alone. IL-4 may act on surface IgM- cells so that on successful differentiation cells will express higher levels of surface IgM. In this circumstance IL-4 may transduce a message to surface IgM- BCP. Alternatively, the expression of surface IgM may render cells responsive to IL-4, leading to an increase in surface Ig and, potentially, an increased sensitivity to antigenic stimulation.

In conclusion, we have described the constitutive expression of IL-4 receptors on normal and leukemic BCP. Our collective data indicate that IL-4 does not directly promote proliferation of human BCP. Progress has recently been made in our laboratory in modifying the murine Whitlock-Witte culture to support self-renewal of human BCP under serum-free conditions (Wolf ML, Buckley JA, Goldfarb A, Law C-L, LeBien TW: submitted for publication). Using this culture system, experiments are currently in progress to more critically assess the role of IL-4 on in vitro B lymphopoiesis. Likewise, the potential application of this system to study the role of IL-4 and CD23 in the self-renewal of leukemic BCP is also under investigation.

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