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分担研究報告書

腫瘍細胞特異的遺伝子発現の経時的変化と治療の有効性についての研究

研究分担者　福島　敬　筑波大学大学院人間総合科学研究科 小児内科講師

研究要旨：　小児急性リンパ性白血病の治療成績向上には、治療の層別化(リスク分類)の果たしてきた役割が大きい。従来使用されてきた“発症年齢”と“白血球数”とによる層別化に加え、種々のリスク因子が検討されている。中でも治療開始初期の反応性（白血病細胞の減少速度）は、既に臨床試験として治療介入に使用されている。染色体の転座に伴うキメラ遺伝子は白血病細胞に特異的に発現される遺伝子であり、治療反応性を高精度に評価する指標となるかどうかを検証することを目的として、本研究を行った。微量残存白血病(MRD)追跡対象 38 例の内、19 症例は day29 までに検出感度未満まで減少した。予後不良因子とされる BCR-ABL の MRD は、8 症例中 5 症例で day29 以降まで検出された。寛解導入療法中に 2 週間ごとにキメラ遺伝子 MRD を評価することによって、治療の層別化・個別化に応用できる可能性があると考えられた。

研究協力者
南木融　筑波大学附属病院 検査部技師長

A．研究目的

小児 ALL の治療成績向上には層別化治療が大きな役割を果してきた。将来のより高精度な層別化・個別化の根拠となる新規リスク因子の抽出が検討される中、初期治療反応性が有望な候補の１つであるとされている。
染色体異常に伴うキメラ遺伝子に由来する mRNA 発現定量による初期治療反応性について、リスク因子としての臨床的意義を検証する。

B．研究方法

東京小児がん研究グループ(TCCSG)登録急性リンパ性白血病(ALL)症例のうち、2010 年 4 月 1 日から 2011 年 3 月 4 日までの期間に、188 症例に対して筑波大学附属病院において発症時キメラ遺伝子スクリーニングの中央診断を行った。各医療機関(表 1 = TCCSG 施設 59)から匿名化された骨髄または血液の送付を受け、標準的な方法によって mRNA 抽出、cDNA 作成、定量 PCR を行った。スキリーニング対象のキメラ遺伝子は以下のとおりである。

- TEL-AML1 (ETV6-AML1)
- E2A-PBX1
- Minor BCR-ABL
- Major BCR-ABL
- MLL-AF4
- MLL-AF9
- MLL-ENL
- SIL-TAL1

なお、検出感度は 20 コピー／ 100ngRNA に設定した。38 症例でいずれかのキメラ遺伝子が検出された。内訳は表 2 に示す。
発症時のスクリーニング結果のみ、担当医に報告した。キメラ遺伝子が検出された 38 症例では、寛解導入療法および早期強化療法期間にかけて、該当するキメラ遺伝子の定量による微量残存白血病細胞(MRD)を追跡した。治療は、TCCSG の ALL 16 次案に準拠した。
（倫理面への配慮）
臨床検体を用いた本研究の実施にあたり、関連法規を遵守し筑波大学附属病院倫理審査委員会ならびに病院長の承認を得た上で、更に診療を担当する各 TCCSG 施設においても倫理委員会または研究審査委員会（IRB）の承認を得て実施した。検体提供者への人権擁護および個人情報保護に細心の注意を払った。

C．研究結果

１．TEL-AML1 (ETV6-AML1)
本キメラ遺伝子が検出された 17 症例の内 14 症例は day29 の時点で MRD 検出感度未満まで減少したが、1 症例は day15 で陰性化した一方で、2 症例は day29 以降まで検出された（図１）。

2． BCR-ABL

本キメラ遺伝子が検出された 8 症例（ 7 症例の minor BCR-ABL、1 症例の major BCR-ABL ）の MRD は、全例 day29 以降まで検出された（図2）。

3． E2A-PBX1

本キメラ遺伝子が検出された 8 症例の内、3 症例は day15 で陰性化したが、それ以外の症例は、day29 以降まで検出された（図3）。

4． SIL-TAL1

本キメラ遺伝子が検出された 2 症例のうち、計画通りに追跡された 1 症例は、day15 で陰性化した（図4）。

5． MLL 再構成

本キメラ遺伝子が検出された 3 症例（ MLL-AF4、MLL-AF9、MLL-ENL が各 1 例ずつ ）のうち、MRD 追跡は 2 症例に対して実施された。1 症例は day15 で陰性化したが、他の 1 症例では day43 （寛解導入療法後）まで検出され、早期強化療法後に陰性化した（図5）。

D．考察

予後不良因子とされる BCR-ABL の MRD 陰性化までの期間は、他のキメラ遺伝子に比べて明らかに長い傾向が確認された。一方で、予後不良因子とはされていない TEL-AML1 および E2A-PBX1 では、多くの症例では MRD 陰性化が day29 の時点であるが、一部に day15 の早期に陰性化する反応良好例がある一方で、陰性化が day43 以降の反応不良例が存在する。治療開始初期の MRD の減衰率または、検出感度未満に減少する次期によって将来の再発を予測することが可能かどうか、可能であった場合に、既知の予後因子と独立した役割を担うことができるのか、本研究対象症例の今後の臨床経過を追跡することによって明らかにされよう。

E．結論

1．キメラ遺伝子による MRD 追跡対象 38 例の内、19 症例は day29 までに検出感度未満まで減少した。

2．予後不良因子とされる BCR-ABL の MRD は、8 症例中 5 症例で day29 以降まで検出された。

3．寛解導入療法中に 2 週間ごとにキメラ遺伝子 MRD を評価することによって、治療の層別化・個別化に応用できる可能性がある。

F．研究発表

1． **論文発表**

なし。

2． **学会発表**

なし。

G．知的財産権の出願・登録状況

（予定を含む）

1. 特許取得

無し

2. 実用新案登録

無し

3. その他

無し

表1　TCCSG参加施設（2010年度59施設）

茨城県立こども病院小児科
神奈川県立こども医療センター血液・再生医療科
熊本大学医学部付属病院小児科
熊本赤十字病院小児科
群馬大学医学部附属病院　小児科
前橋赤十字病院小児科
慶應義塾大学病院小児科
東京歯科大学市川総合病院小児科
足利赤十字病院小児科
横須賀共済病院小児科
総合太田病院小児血液腫瘍科
国立がんセンター中央病院小児科
国立病院機構熊本医療センター小児科
国立成育医療研究センター血液腫瘍科/固形腫瘍科
埼玉医科大学国立医療センター小児腫瘍科
埼玉医科大学総合医療センター小児科
埼玉県立小児医療センター血液・腫瘍科
東京慈恵会医科大学附属病院小児科
自治医科大学附属病院小児科
順天堂大学医学部附属順天堂病院小児科
昭和大学藤が丘病院小児科
信州大学医学部附属病院小児科
長野県立こども病院総合診療部
聖マリアンナ医科大学病院小児科
聖マリアンナ医大大学横浜市西部病院小児科
聖路加国際病院小児科
千葉大学医学部附属病院小児科
成田赤十字病院小児血液腫瘍科
松戸市立病院小児科
帝京大学ちば総合医療センター小児科
千葉県こども病院血液・腫瘍科
帝京大学医学部附属病院小児科
東海大学医学部附属病院小児科・細胞移植科
東京医科歯科大学医学部附属病院小児科
武蔵野赤十字病院小児科
東京医科大学病院小児科
東京大学医学部附属病院小児科
東京女子医科大学東医療センター小児科
東邦大学医療センター大森病院小児科
東邦大学医療センター大橋病院小児科
獨協医科大学病院小児科
東京都立小児総合医療センター血液腫瘍科
がん・感染症センター都立駒込病院小児科
日本医科大学附属病院小児科
日本医科大学千葉北総病院小児科
山梨大学医学部附属病院小児科
横浜市立大学附属病院小児科
済生会横浜市南部病院小児科
東京大学医科学研究所附属病院小児細胞移植科
筑波大学附属病院小児科
群馬県立小児医療センター血液・腫瘍科
杏林大学病院 小児科
昭和大学病院 小児科
東京西徳洲会病院 小児難病センター
防衛医科大学校 小児科
亀田総合病院 小児科
静岡県立静岡がんセンター 小児科
済生会横浜市東部病院 こどもセンター小児科
相模原協同病院小児科

表2　キメラ遺伝子スクリーニング症例数と結果

総症例数	188
いずれも検出せず	144
解析不能検体数	6
キメラ遺伝子検出症例数	38
TEL-AML1 検出症例数	17
E2A-PBX1 検出症例数	8
minor *BCR-ABL* 検出症例数	7
major *BCR-ABL* 検出症例数	1
MLL-AF4 検出症例数	1
MLL-AF9 検出症例数	1
MLL-ENL 検出症例数	1
SIL-TAL1 検出症例数	2

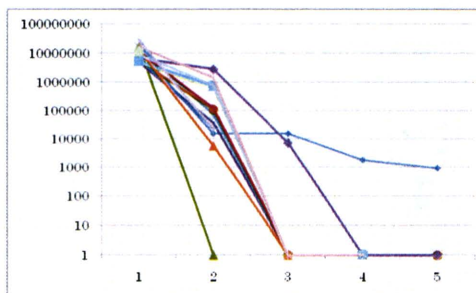

図1　*TEL-AML 1* (n=17) コピー数（ /100ng RNA ）の推移

1：発症時骨髄
2： day 15
3： day 29
4： day 43 （寛解導入療法終了時）
5：早期強化療法後（ week 12 相当）

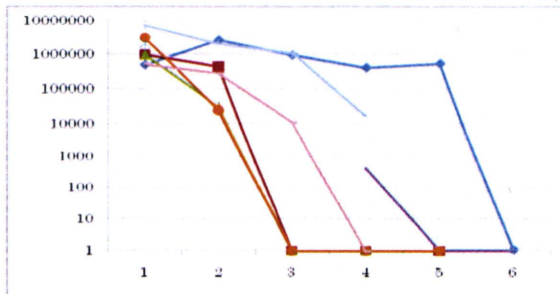

図2． *BCR* － *ABL* （ major n= 1, minor n= 7）コピー数（ /100ng RNA ）の推移

1 ：発症時骨髄
2 ： day 15
3 ： day 29
4 ： day 43 （寛解導入療法終了時）
5 ：早期強化療法後（ week 12 相当）
6 ：以後の強化療法後

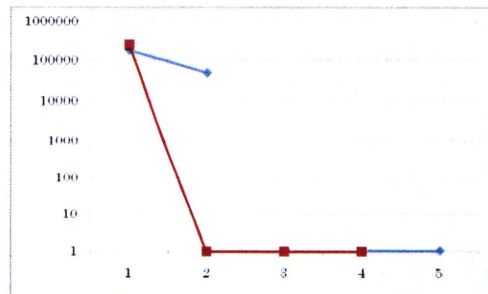

図4 *SIL* － *TAL1* (n=2) コピー数（ /100ng RNA ）の推移

1 ：発症時骨髄
2 ： day 15
3 ： day 29
4 ： day 43 （寛解導入療法終了時）
5 ：早期強化療法後（ week 12 相当）

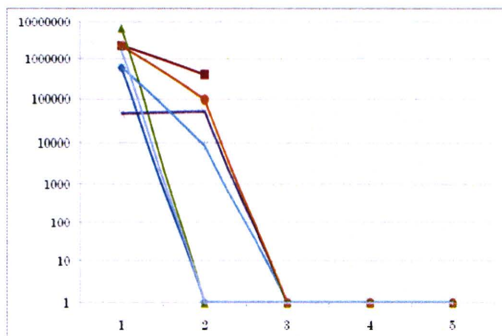

図3 *E2A* － *PBX1* (n=8) コピー数（ /100ng RNA ）の推移

1 ：発症時骨髄
2 ： day 15
3 ： day 29
4 ： day 43 （寛解導入療法終了時）
5 ：早期強化療法後（ week 12 相当）

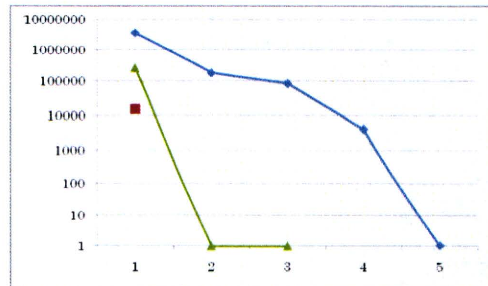

図5 *MLL* 再構成(*MLL-AF4* n=1、*MLL-AF9* n=1、*MLL-ENL* n=1)コピー数（ /100ng RNA ）の推移

1 ：発症時骨髄
2 ： day 15
3 ： day 29
4 ： day 43 （寛解導入療法終了時）
5 ：早期強化療法後（ week 12 相当）

研究成果の刊行に関する一覧表

書籍

著者氏名	論文タイトル名	書籍全体の編集者名	書　籍　名	出版社名	出版地	出版年	ページ
	該当なし						

雑誌

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ORIGINAL ARTICLE

Differential effects of BAFF on B cell precursor acute lymphoblastic leukemia and Burkitt lymphoma

Keiko Onda · Kazutoshi Iijima · Yohko U. Katagiri · Hajime Okita · Masahiro Saito · Toshiaki Shimizu · Nobutaka Kiyokawa

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Abstract B cell-activating factor belonging to the tumor necrosis factor superfamily (BAFF) is a crucial factor for B cell development and is involved in the survival of malignant B cells, but its effect on B cell precursors (BCPs) remains unclear. We investigated BCP acute lymphoblastic leukemia (-ALL) cells for BAFF receptor (-R) expression and compared the effect of BAFF on BCP-ALL cells and Burkitt lymphoma (BL) cells. Expression of BAFF-R was detected in some cell lines and some clinical specimens of both BL and BCP-ALL. BAFF acted on both BL and BCP-ALL cells and promoted proliferation by both. BAFF also inhibited apoptosis by BL cells induced by cross-linking of cell surface molecules and anticancer drugs, but failed to inhibit apoptosis by BCP-ALL cells. BAFF induced prompt and obvious activation of the NF-κB signaling pathway in BL cells, but only weak and delayed activation of the pathway in BCP-ALL cells. The results of this study indicate that some BCP-ALL cells and some BL cells express BAFF-R, but that the effects of BAFF on BCP-ALL cells are different from its effects on mature B cell malignancies.

Keywords BAFF · B cell precursor ALL · Burkitt lymphoma · Apoptosis

1 Introduction

B cell-activating factor belonging to the tumor necrosis factor (TNF) superfamily (BAFF), also called B-lymphocyte stimulator (BlyS), is a cytokine that is produced by monocytes, dendritic cells, and some T cells [1–6]. Transgenic mice overexpressing BAFF exhibit hyperplasia of the mature B cell compartment [7–9], whereas mice deficient in BAFF have a reduced number of peripheral B cells and exhibit an almost complete loss of follicular and marginal zone B cells in their secondary lymphoid organs [9–11]. This suggests that BAFF is a survival and maturation factor for B cells and crucial for B cell development [1, 12]. To date, three distinct BAFF receptors have been identified, namely, BAFF receptor (BAFF-R, also called BR3), B cell maturation antigen (BCMA), and transmembrane activator and CAML-interactor (TACI). While BAFF binds to all three with similarly high affinity, only BAFF-R is thought to be responsible for the survival and differentiation of B cells [6, 13–20].

It has been well documented that some of the malignant as well as normal B cells express BAFF-R, and thus BAFF-mediated signaling is involved in the survival and proliferation of malignant B cells [1]. Indeed, BAFF is thought to be produced in excess in patients with various B cell malignancies, including B cell chronic lymphocytic leukemia, multiple myeloma, Hodgkin lymphoma, and various types of non-Hodgkin lymphomas (NHLs), such as follicular lymphoma, diffuse large cell lymphoma, mantle cell lymphoma, and marginal zone lymphoma, either by the tumor cells themselves or by cells in their microenvironment, or by both,

K. Onda · K. Iijima · Y. U. Katagiri · H. Okita · M. Saito · N. Kiyokawa (✉)
Department of Pediatric Hematology and Oncology Research, National Research Institute for Child Health and Development, 2-10-1 Okura, Setagaya-ku, Tokyo 157-8535, Japan
e-mail: nkiyokawa@nch.go.jp

K. Onda · M. Saito · T. Shimizu
Department of Pediatrics, Juntendo University, School of Medicine, Bunkyo-ku, Tokyo, Japan

and to behave as an autocrine and paracrine survival and proliferation factor in these B cell malignancies [21–27].

The capacity of B cells to bind BAFF has been found to be correlated with their maturation stage, and the effect of BAFF on B cells is dependent on their degree of maturation. However, the precise role of BAFF in B cell development is still a matter of controversy [1, 28], and the role of BAFF in the development of B cell precursors (BCPs), in particular, remains largely unknown. In addition, the effect of BAFF on the neoplastic counterpart of BCPs, namely BCP acute lymphoblastic leukemia (-ALL)/lymphoma, is also still unclear. Since BCP-ALL is the most common malignancy in childhood, whether BAFF has any effect on BCP-ALL cells should be investigated.

In this study, we investigated the effect of BAFF on BCP-ALL cells in comparison with its effect on the cells of Burkitt leukemia/lymphoma (BL), which is a subtype of NHL and another common B-lineage malignancy in children. In this study, we report the findings that some BCP-ALL cells as well as some BL cells expressed BAFF-R, and that BAFF promoted proliferation by both. Interestingly, although BAFF inhibited apoptosis by BL cells, it failed to inhibit apoptosis by BCP-ALL cells, suggesting that BAFF acts on B-lineage malignant cells differentially in a maturation stage-related fashion. The molecular basis of the differential effects of BAFF on BCP-ALL and BL cells is also analyzed and discussed.

2 Methods

2.1 Reagents

Recombinant human BAFF was obtained from R&D Systems, Inc. (Minneapolis, MN). Dexamethasone (DEX) and etoposide (VP-16) were obtained from Sigma-Aldrich Co. (St. Louis, MO). The monoclonal Abs (mAbs) used were phycoerythrin (PE)-conjugated anti-BAFF-R from Santa Cruz Biotechnology (Santa Cruz, CA), fluorescein isothiocyanate (FITC) anti-CD10, FITC anti-CD21, FITC anti-HLA-DR, PE anti-CD40, PE-Texas Red (ECD) anti-CD45, PE-cyanin 5.1 (PC5) anti-CD33, and PE-cyanine 7 (PC7) anti-CD19 from Beckman/Coulter, Inc. (Westbrook, MA). The mouse mAbs used for the immunochemical analysis were anti-I-κB, anti-NF-κB p65, and anti-Bcl-2 from Santa Cruz and anti-β-actin (AC-15) from Sigma. The rabbit Abs used were anti-TRAF-3, anti-NF-κB1, anti-NF-κB2, anti-phospho-specific NF-κB p65 (Ser536), anti-phospho-specific NF-κB p105 (Ser933), and anti-phospho-specific I-κB-α (Ser32) from Cell Signaling Technology, Inc. (Danvers, MA). Purified anti-μ chain rabbit polyclonal Ab from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA) was used to cross-link B cell receptors (BCRs). Mouse anti-CD24 [29], anti-CD10, and anti-CD20 mAbs (Beckman) were used to cross-link each molecule. Secondary Abs, including enzyme-conjugated Abs, were purchased from Jackson and Dako (Denmark A/S).

2.2 Cells and cultures

The human BL-derived cell lines Daudi, Ramos, P32/ISH (Japanese Cancer Research Resource Bank, JCRB, Tokyo, Japan), EB-3, NAMALWA (Institution of Fermentation, Osaka, Japan), and BALM-18 (Dr. Y. Matsuo, Fujisaki Cell Center, Hayashibara Biochemical Labs, Inc.) and the human hairy cell leukemia cell line MLMA (JCRB) were used. The human BCP-ALL cell lines Reh (American Type Culture Collection), RS4;11, P30/OHK (JCRB), NALM-17, NALM-27 (Hayashibara), and NALM-6 (Tohoku University Cell Bank, Sendai, Japan) were also used. Cells were cultured at 37°C in RPMI 1640 medium (Sigma) supplemented with 10% FCS (Sigma) under a humidified 5% CO_2 atmosphere. Cells were cultured in the presence or absence of BAFF, and viable cell counts were made after staining with Trypan blue at the times indicated in the figures. In parallel, cells were plated on a 96-well culture plate (Corning, Inc., Corning, NY) and cell proliferation was also assessed by water-soluble tetrazolium salt (WST) assays (Cell Counting Kit-8, DojinDo, Kumamoto, Japan), a cell proliferation assay that colorimetrically determine dehydrogenase activity of the cells. Based on our preliminary experiments, we chose a concentration of 200 ng/ml as a sufficient dose of BAFF to achieve a maximum effect on cell proliferation.

Clinical specimens from pediatric patients, consisting of 20 patients with BCP-ALL, 10 with BL, and 2 with reactive lymphadenitis, were selected from the files of specimens collected in our laboratory between 1985 and 2001. The specimens are now kept under anonymous conditions, and all of the experiments included in this study adhered to the tenets of the Declaration of Helsinki and were performed with the approval of the local ethics committee.

The human bone marrow (BM) CD34+ cells used were purchased from Cambrex Bio Science Walkersville, Inc. (Walkersville, MD). A cloned murine BM stromal cell line, MS-5, was kindly provided by Dr. K. J. Mori (Niigata University, Niigata, Japan). BCP cells were induced from BM CD34+ cells by co-culture with MS-5 cells as described previously [30].

2.3 Immunofluorescence analysis and detection of apoptosis

To detect BAFF-R, cells were stained with fluorescein-labeled mAbs and analyzed by flow cytometry (FCM, FC500, Beckman) as described previously [31]. The frequency of apoptosis was quantified by MEBCYTO-

Apoptosis kit (Medical & Biological Laboratories, Co., Ltd., MBL, Nagoya, Japan) as described previously [29]. The caspase activation was quantified by APOPCYTOTM Intracellular Caspases Activity Detection Kit (MBL) and then analyzed according to the manufacturer's protocol.

2.4 Reverse-transcriptase (RT)-PCR

Total RNA was extracted from cultured cells with an RNeasy plus Mini Kit (QIAGEN, Valencia, CA), and cDNA was generated with a FirstStrand cDNA Synthesis Kit (Pharmacia Biotech, Uppsala, Sweden). A set of primers (5′-caaattcagctcttcactc-3′, and 5′-tttgaaggcacagga acag-3′) was used to amplify the 193-bp fragment of BAFF-R transcripts by PCR as described previously [32], and the products were separated on a 2.0% agarose gel. The transcripts of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were amplified as an internal control [32].

2.5 Statistical analysis

The statistical analysis was performed by means of a nonparametric Mann–Whitney test, and correlations were determined by using nonparametric statistics. A p value less than 0.05 was considered to be statistically significant.

2.6 Immunoblotting

Immunoblotting was performed as described previously [33]. Briefly, a 50 μg sample of each cell lysate was electrophoretically separated on an SDS-poly acrylamide gel and transferred to a nitrocellulose membrane. The membrane was incubated with the appropriate combination of primary and secondary Abs, washed, and examined with an enhanced chemiluminescence reagent system.

3 Results

3.1 Expression of BAFF-R in BL and BCP-ALL cells

First, we investigated whether the BL and BCP-ALL cell lines expressed BAFF-R. As shown in Fig. 1a, flow cytometric analysis revealed that most of the BL cell lines expressed BAFF-R. Of the 6 BL cell lines tested, 4, BALM-18, BALM-24, P32/ISH, and Ramos, exhibited a relatively high level of expression, but EB-3 cells did not express BAFF-R at all and the expression of BAFF-R by Daudi cells was very limited. On the other hand, testing the BCP-ALL cell lines for BAFF-R expression by FCM showed that most of the lines used in this study were positive, but expression by RS4;11 cells was very limited (Fig. 1b). As shown in Fig. 1c, hairy leukemia-derived MLMA cells exhibited high level expression of BAFF-R. We confirmed BAFF-R expression by these cell lines at the mRNA level by RT-PCR (Fig. 1c). Similar examination for expression of the other types of BAFF receptors by FCM showed no significant expression of BCMA or TACI protein by any of the BL or BCP-ALL cell lines (data not shown).

Next, we examined BL and BCP-ALL cells in the clinical material for BAFF-R expression. As shown in Fig. 2a, flow cytometric analysis revealed that the lymphoma cells in some of the BL cases expressed BAFF-R. Of the 10 cases tested, 3 were clearly positive for BAFF-R. We also confirmed that the leukemic cells in some of the BCP-ALL cases expressed BAFF-R, and 7 of the 20 cases were positive for BAFF-R (Fig. 2b). These findings indicate that some BL and BCP-ALL cells express BAFF-R proteins on their surface.

We also investigated whether non-leukemic B cells express BAFF-R. FCM of BCP cells induced from bone marrow CD34+ cells in vitro by co-culture with MS-5 cells revealed that some BCP cells expressed BAFF-R (Fig. 2c), indicating that the expression of BAFF-R is a characteristic of particular BCP cells. Similarly, some non-leukemic mature B cells in peripheral lymph nodes also expressed BAFF-R (Fig. 2d).

3.2 BAFF accelerates proliferation by both BL and BCP-ALL cells

Next, we investigated whether BAFF affects proliferation by BL and BCP-ALL cells. The Trypan blue dye exclusion assay showed that proliferation of BAFF-R-positive BL cell lines (BALM-18, P32/ISH, Ramos, and NAMALWA) increased slightly after BAFF was added to the culture, and the increase was statistically significant (Fig. 3a). A cell proliferation assay with WST revealed a similar tendency (Fig. 3a). In contrast, BAFF failed to induce a significant increase in cell proliferation by Daudi cells and EB-3 cells, which exhibited no significant BAFF-R expression (Fig. 3a), indicating that the proliferation-inducing effect of BAFF is specific to BAFF-R-positive cells. We investigated whether BAFF affects proliferation by BCP-ALL cell lines in a similar manner and observed a similar effect (Fig. 3b), suggesting that BAFF promotes the growth of both BAFF-R-positive BL cells and BCP-ALL cells.

3.3 BAFF inhibits apoptosis by BL cells, but not by BCP-ALL cells

It has been well documented that stimulation of cultured BL cells via particular surface molecules, including BCR and CD20, induces apoptosis [34–36]. It has also been reported that BAFF is responsible for B cell survival and prevents apoptosis by cultured B cells [37, 38]. We therefore investigated whether BAFF is able to inhibit BL cell apoptosis that

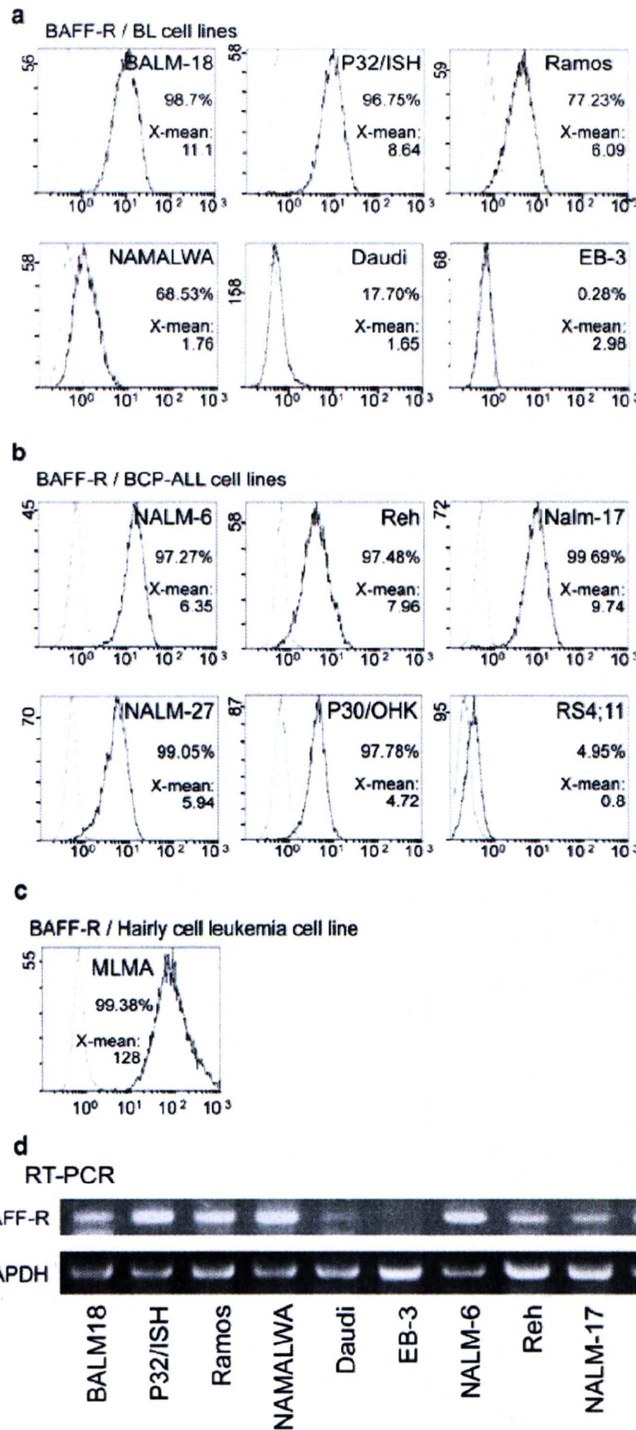

Fig. 1 Expression of BAFF receptor (BAFF-R) in Burkitt lymphoma (BL) cell lines and B cell precursor acute lymphoblastic leukemia (BCP-ALL) cell lines. **a** Six BL cell lines were stained with PE-labeled anti-BAFF-R antibody and analyzed by flow cytometry. The histograms obtained (*dark lines*) are shown superimposed on those of the negative control (cells stained with isotype-matched control mouse Ig, *light lines*). Positivity (%) and mean fluorescence (X-mean) values are shown. X-axis, fluorescence intensity; Y-axis, relative cell number. **b** Six BCP-ALL cell lines were examined for BAFF-R expression as described in **a**. **c** Hairy cell leukemia-derived cell line MLMA was tested as a positive control and presented as in **a**. **d** The expression of BAFF-R mRNA in both groups of cell lines and MLMLA cells was investigated by the reverse-transcriptase polymerase chain reaction (RT-PCR)

is mediated by cross-linking of CD20 and BCR. As shown in Fig. 4a, assessment by annexin-V binding showed that the incidence of P32/ISH cell and BALM-18 cell apoptosis induced by both BCR- and CD20-mediated stimuli was reduced to a statistically significant extent by the addition of BAFF to the culture. We also investigated the inhibitory effect of BAFF on apoptosis by BL cells mediated by DEX and observed a similar inhibitory effect, but this effect on the apoptosis mediated by VP-16 was very limited (Fig. 4a). Although a higher dose of BAFF tended to inhibit induction

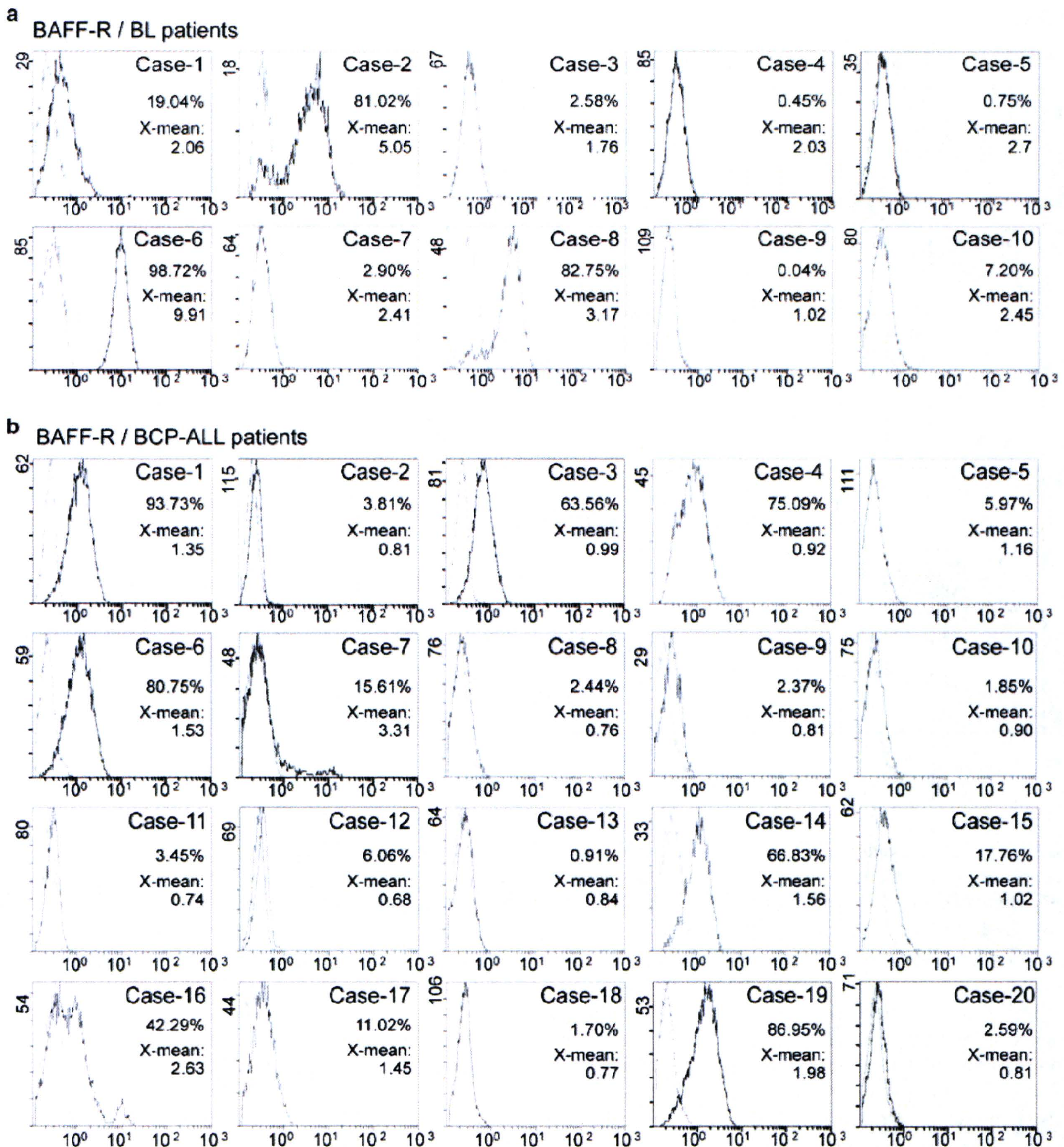

Fig. 2 Analysis of BAFF receptor (BAFF-R) expression in clinical material and non-leukemic samples. The histograms for BAFF-R in ten Burkitt lymphoma (BL) cases (**a**) and twenty B cell precursor acute lymphoblastic leukemia (BCP-ALL) cases (**b**) are shown as in Fig. 1a. **c** BCP cells induced from bone marrow CD34+ cells in vitro were examined for expression of BAFF-R and other leukocyte antigens by flow cytometry, and the cytograms are shown. Positivity (%) of each fraction and mean fluorescence (X-mean) values of BAFF-R-positive fractions are shown. **d** Mononuclear cells obtained from lymph nodes of two non-leukemic reactive lymphadenitis patients were examined for the expression of BAFF-R as in **c**

of apoptosis more effectively (Fig. 4b), the inhibitory effect of BAFF on induction of apoptosis was always partial (data not shown). As shown in Fig. 4c, when BAFF was added to the culture prior to the induction of apoptosis, its inhibitory effect on induction of apoptosis was greater than when added at the start of apoptosis induction. We also confirmed that

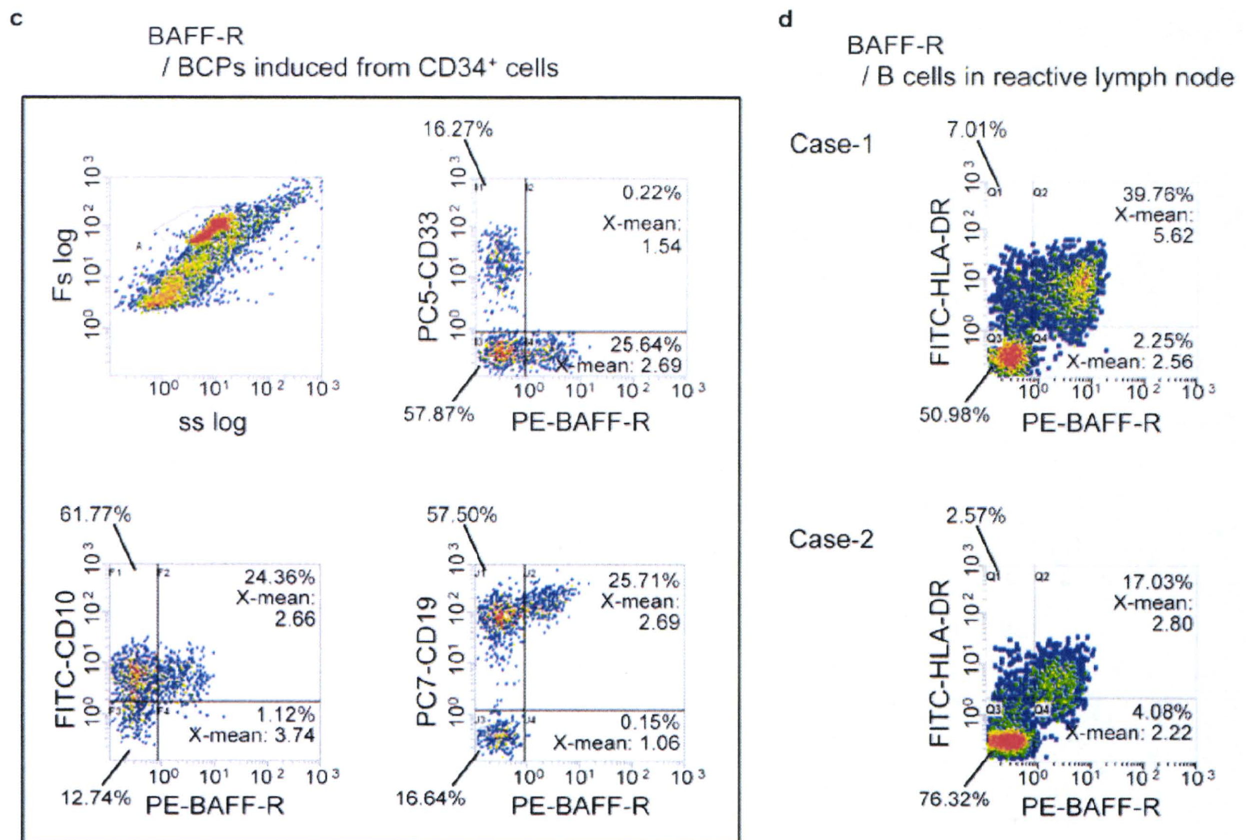

Fig. 2 continued

BAFF inhibits activation of caspase in BL cells during the course of its inhibition of apoptosis (Fig. 4d). As shown in Fig. 4e, BAFF failed to inhibit apoptosis in BAFF-R-negative EB-3 cells, indicating that the inhibitory effect of BAFF on induction of apoptosis is specific for BAFF-R-positive BL cells.

We then investigated whether BAFF would inhibit apoptosis by BCP-ALL cells. Since BCP-ALL cell lines do not express BCR and most of them are CD20-negative, BCP-ALL cell apoptosis is not induced by cross-linking these molecules. However, in a previous study we discovered that cross-linking of other molecules expressed on BCP-ALL cells, including CD24, can induce apoptosis by BCP-ALL cells [39]. When we investigated the effect of BAFF on CD24- and CD10-mediated apoptosis by NALM-6 cells, however, no inhibitory effect of BAFF was observed on apoptosis mediated by either of them (Fig. 5). We also discovered that BAFF was unable to inhibit NALM-6 cell apoptosis mediated by DEX or VP-16 (Fig. 5). We tested other BCP-ALL cell lines, including Reh and NALM-17, in a similar manner, but no inhibitory effect of BAFF on induction of apoptosis was observed in any of the BCP-ALL cells (Fig. 5 and data not shown). BAFF also failed to inhibit activation of caspase in BCP-ALL cells (data not shown). We therefore concluded that although BAFF is capable of inhibiting induction of apoptosis by BL cells, it does not have an inhibitory effect on induction of apoptosis by BCP-ALL cells.

3.4 Molecular basis of the differential effects of BAFF on BL and BCP-ALL cells

It has been reported that activation of the alternative NF-κB pathway (processing of NF-κB2 and nuclear translocation of p52/RelB heterodimers) is a major outcome of BAFF-R-stimulation, but that BAFF-R also weakly activates the classical NF-κB pathway (processing of NF-κB1 and nuclear translocation of p50/RelA) [1, 20]. When expression of signaling molecules related to the NF-κB pathways, including NIK, I-κB, NF-κB1, NF-κB2, and p65 NF-κB (RelA), was investigated by immunoblot analysis, no clear differences were found between the BL cell lines (Ramos, BALM-18, P32/ISH) and BCP-ALL cell lines (NALM-6, Reh, LC4-1) (data not shown). In contrast,

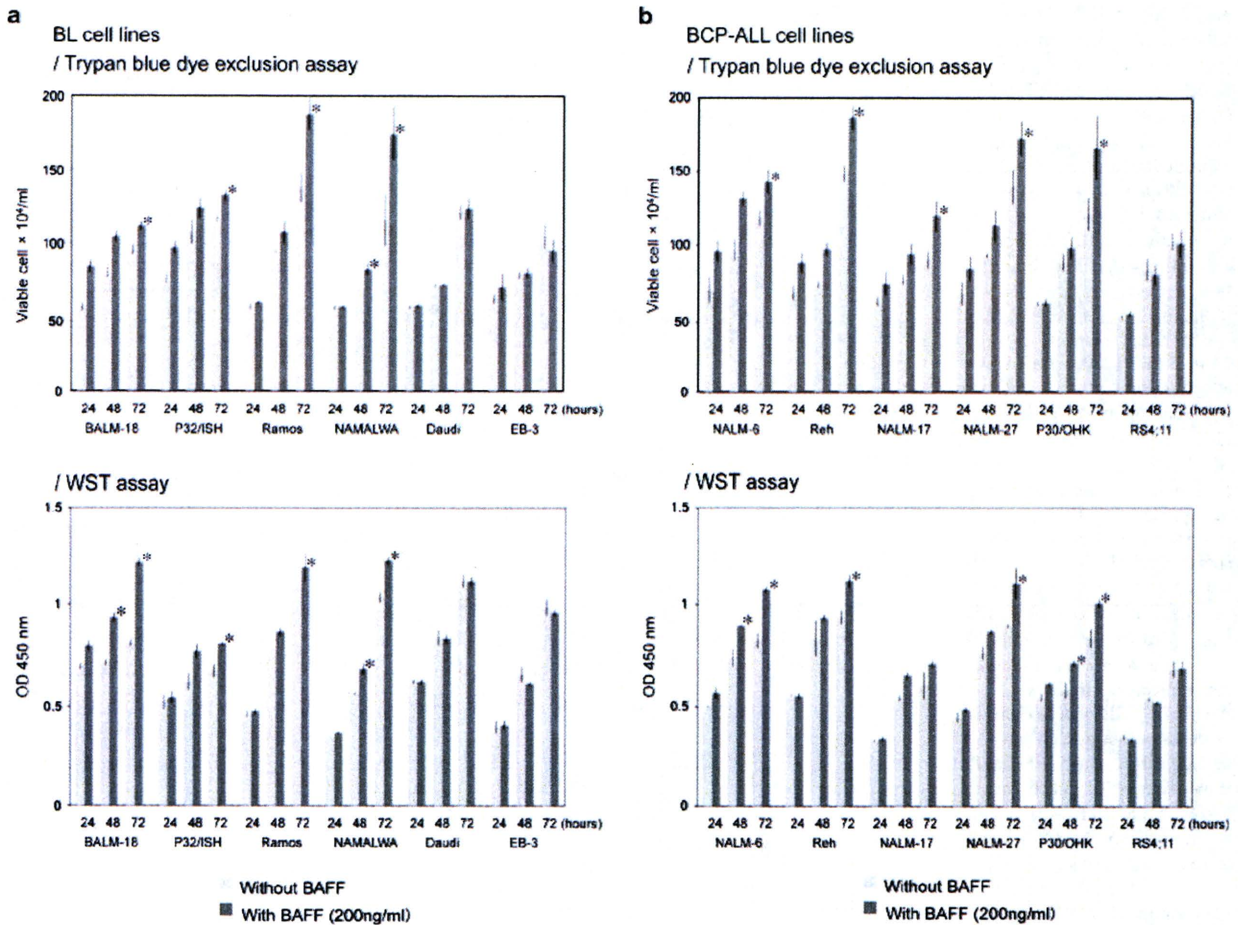

Fig. 3 Effect of BAFF on proliferation by Burkitt lymphoma (BL) cells and B cell precursor acute lymphoblastic leukemia (BCP-ALL) cells. The effect of BAFF (200 ng/ml) on proliferation by BL cell lines (**a**) and BCP-ALL cell lines (**b**), each at a starting cell concentration of 5×10^5/ml, was investigated by Trypan blue dye exclusion assay and WST assays. Each experiment was performed in triplicate, and the means ± SD of the data are shown. *Statistically significant ($p < 0.05$)

protein expression of TRAF-3 was detected in all three BL cell lines and in NALM-6 cells, but not in other BCP-ALL cell lines (data not shown).

Next, we investigated the activation of signaling molecules induced by BAFF stimulation. As shown in Fig. 6a, investigation of the phosphorylation state of I-κB-α, p65 NF-κB, and p105 NF-κB, the regulatory molecules of the NF-κB1 signaling cascade revealed a temporary increase in their phosphorylation state following BAFF stimulation in P32/ISH cells (peaking in 5–10 min), but not in NALM-6 cells. However, as shown in Fig. 6a, a slight increase in phosphorylation of p105 NF-κB was detected in NALM-6 cells at 60 min after BAFF stimulation. We also observed a clear increase in the active forms of both NF-κB1 (p50) and NF-κB2 (p52) in P32/ISH cells after stimulation with BAFF (Fig. 6b). In contrast, the increase in active forms of NF-κBs mediated by BAFF in NALM-6 cells was smaller than that in P32/ISH cells (Fig. 6b). Immunoblot analysis revealed a more marked increase in the active form of NF-κB1 than in the active form of NF-κB2 (Fig. 6b).

We also investigated the activation of signaling molecules induced by BAFF stimulation in hairy leukemia-derived MLMA cells in the same manner as a positive control for mature B cell malignancy. As shown in Fig. 6, BAFF induced prompt and sustained phosphorylation of I-κB-α, p65 NF-κB, and p105 NF-κB (Fig. 6a), whereas the active form of NF-κB1 (p50) was already present prior to stimulation with BAFF, and whether its level increased after the addition of BAFF was unclear (Fig. 6b). In contrast, BAFF significantly increased the level of active form of NF-κB2 (p52) in MLMA cells (Fig. 6b).

We previously reported finding that BAFF induces Bcl-2 expression in MLMA cells [40]. When we investigated the effect of BAFF on Bcl-2 expression in P32/ISH and NALM-6 cells by immunoblot analysis, we found that BAFF induced a small amount of Bcl-2 protein expression

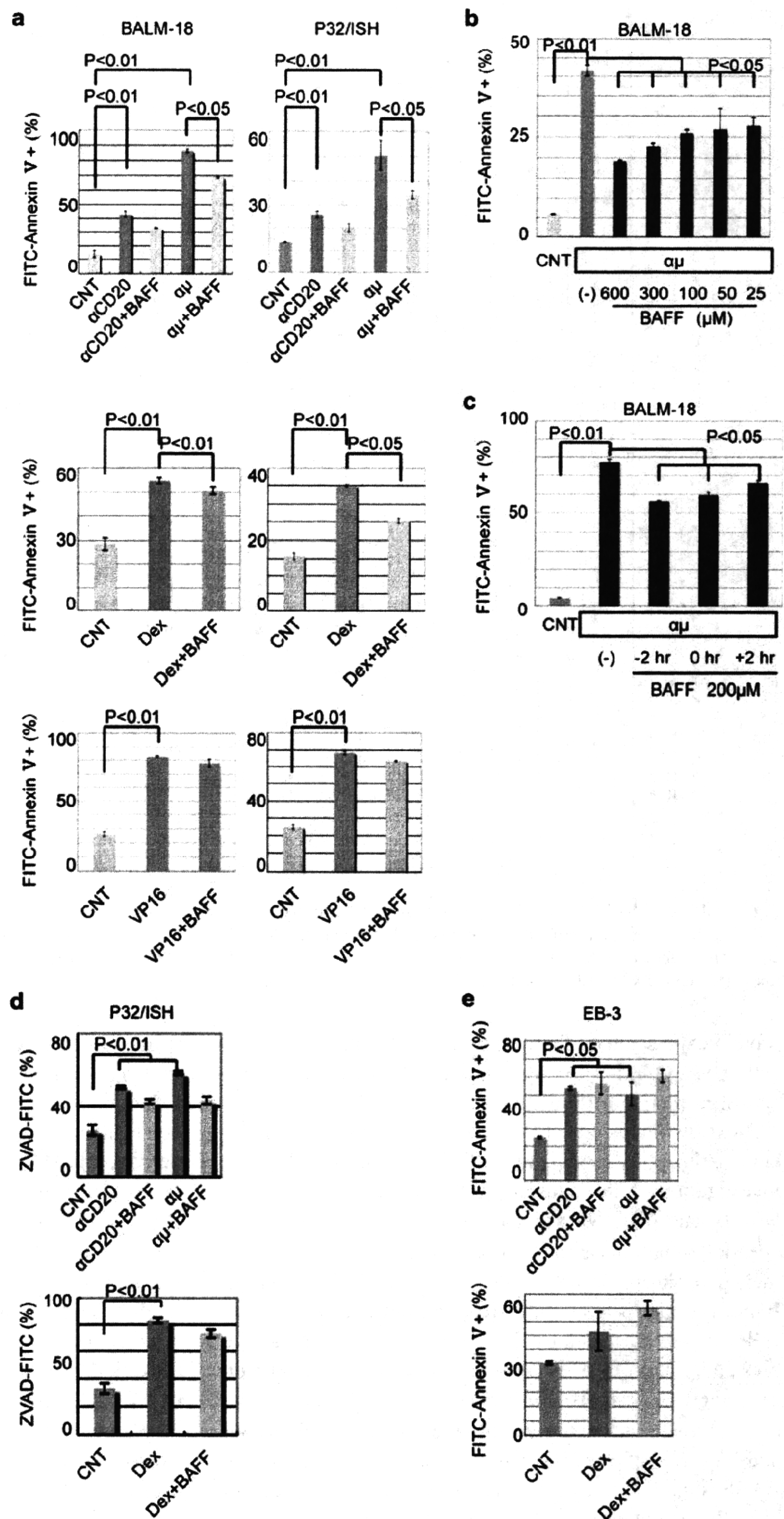

Fig. 4 Effect of BAFF on apoptosis by Burkitt lymphoma (BL) cells. **a** BL cell lines BALM-18 and P32/ISH were treated with either rabbit anti-μ heavy-chain ($\alpha\mu$) antibody (Ab), a combination of anti-CD20 Ab (αCD20) and secondary rabbit anti-mouse Ig Ab, dexamethasone (DEX, 2 and 100 μM for BALM-18 and P32/ISH, respectively), or VP-16 (0.5 and 12.5 μM for BALM-18 and P32/ISH, respectively) for 24 h. BAFF (200 ng/ml) was added to the culture at the same time, and apoptotic cells were identified by binding with FITC-conjugated annexin V. Each experiment was performed in triplicate, and the means ± SD of the data are shown. **b** BALM-18 cells were treated with rabbit anti-μ heavy-chain antibody ($\alpha\mu$) and BAFF was added to the culture at the same time as $\alpha\mu$ was added at different concentrations. Apoptotic cells were examined as described in **a**. **c** BAFF was added to the culture either at the same time as (0), 2 h before (−2), or 2 h after (+2) the addition of $\alpha\mu$. **d** P32/ISH cells were treated as in **a**, and caspase activation was assessed with FITC-conjugated z-VAD-fmk. The results are shown in the form of a graph (*left*). **e** BAFF-R-negative EB-3 cells were examined as a negative control as in **a**

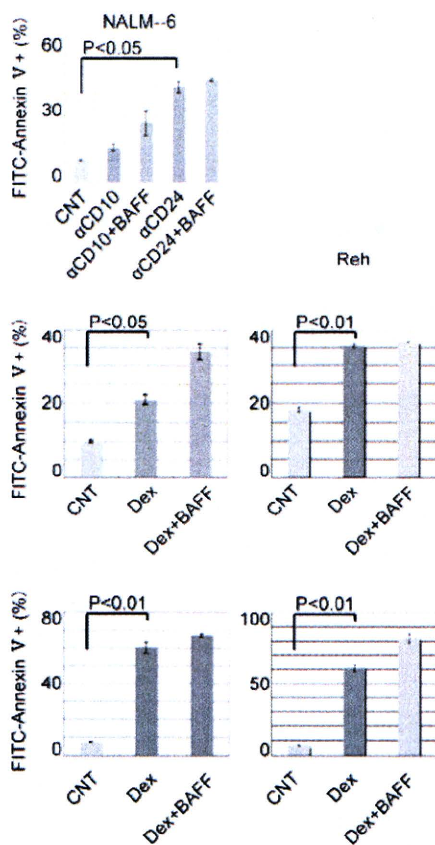

Fig. 5 BAFF does not inihibit apoptosis by B cell precursor acute lymphoblastic leukemia (BCP-ALL) cells. BCP-ALL cell lines NALM-6 cells and Reh cells were treated with DEX (20 and 200 μM for NALM-6 and Reh, respectively) or VP-16 (50 and 12.5 μM for NALM-6 and Reh, respectively) for 24 h, and the effect of BAFF on induction of apoptosis was assessed in the same manner as in Fig. 4. The effect of BAFF on induction of NALM-6 cell apoptosis induced by either anti-CD10 Ab (αCD10) or anti-CD24 Ab (αCD24) in the presence of secondary rabbit anti-mouse Ig Ab was also tested. Since CD24 and CD10 failed to induce apoptosis in Reh cells, the assay was not performed with Reh cells

in P32/ISH cells, whereas Bcl-2 was already expressed in NALM-6 cells, and that BAFF did not affect its expression (Fig. 6c).

3.5 Effects of BAFF on CD40 expression in BL and BCP-ALL cells

We also previously reported discovering that expression of CD40, a TNF-receptor family molecule critical for B cell survival, was up-regulated in hairy leukemia-derived MLMA cells after BAFF stimulation [40]. As shown in Fig. 7, flow cytometric analysis revealed that BAFF also induced up-regulation of CD40 in P32/ISH cells, but no clear change in CD40 expression was observed in NALM-6 cells.

4 Discussion

As stated above, expression of BAFF-R has been reported in some of B cell malignancies [21, 26, 27], whereas BAFF-R expression in BL and BCP-ALL/lymphoma has not been well characterized. The results of the present study clearly showed expression of BAFF-R by tumor cells of BL and BCP-ALL, both of which are major B-lineage hematologic malignancies in children, but that BAFF-R expression was limited in some cases for each of these diseases.

The results of this study have also shown that BAFF acts on both BL and BCP-ALL cells and promotes their proliferation. Furthermore, BAFF was found to inhibit the BL cell apoptosis mediated by the cross-linking of cell surface molecules, including BCR and CD20, and induced by anticancer drugs, including DEX. Interestingly, however, BAFF failed to inhibit apoptosis by BCP-ALL cells, indicating that BAFF has a differential effect on B-lineage malignancies that have originated at different stages of B cell development. As mentioned above, BAFF has been reported to act as a survival and proliferation factor in B cell malignancies and a significant correlation between clinical outcome and BAFF-R expression in tumor cells or serum BAFF levels has been demonstrated in a certain subtype of NHLs [21, 27, 41]. Studies to clarify the relations between BAFF-R expression and the clinical features of BL and BCP-ALL are now being prepared.

Expression of BAFF-R has been reported to increase with the stage of B cell maturation. Rodig et al. [26] reported that up-regulation of BAFF-R occurs during the transition from BCPs to mature B cells and that BAFF-R is present in most B cells circulating in peripheral blood. They also reported that BAFF-R expression appears to be highest on the naive B cells in the mantle zones, whereas BAFF-R is only detectable in a subset of B cells in the germinal centers (GCs) [26]. Since BL cells are thought to be a malignant counterpart of centroblasts present in germinal centers, our finding that some BL cells express BAFF-R seems to be consistent with the report by Rodig et al. In contrast, we found that BAFF-R is expressed in some BCP cells, indicating that BAFF may also affect the proliferation and development of some BCP cells. All of the above data indicate that distinct subsets, namely, a BAFF-R-positive subset and a BAFF-R-negative subset, are present in both BCP and GC B cells. Further analysis of the effect of BAFF on BCP and GC B cells should provide useful information with regard to the biological differences between BAFF-R-positive and BAFF-R-negative cases of both BL and BCP-ALL.

As reported in this study, although both BL and BCP-ALL cells express BAFF-R, BAFF was found to have an anti-apoptotic effect on BL cells, but not on BCP-ALL

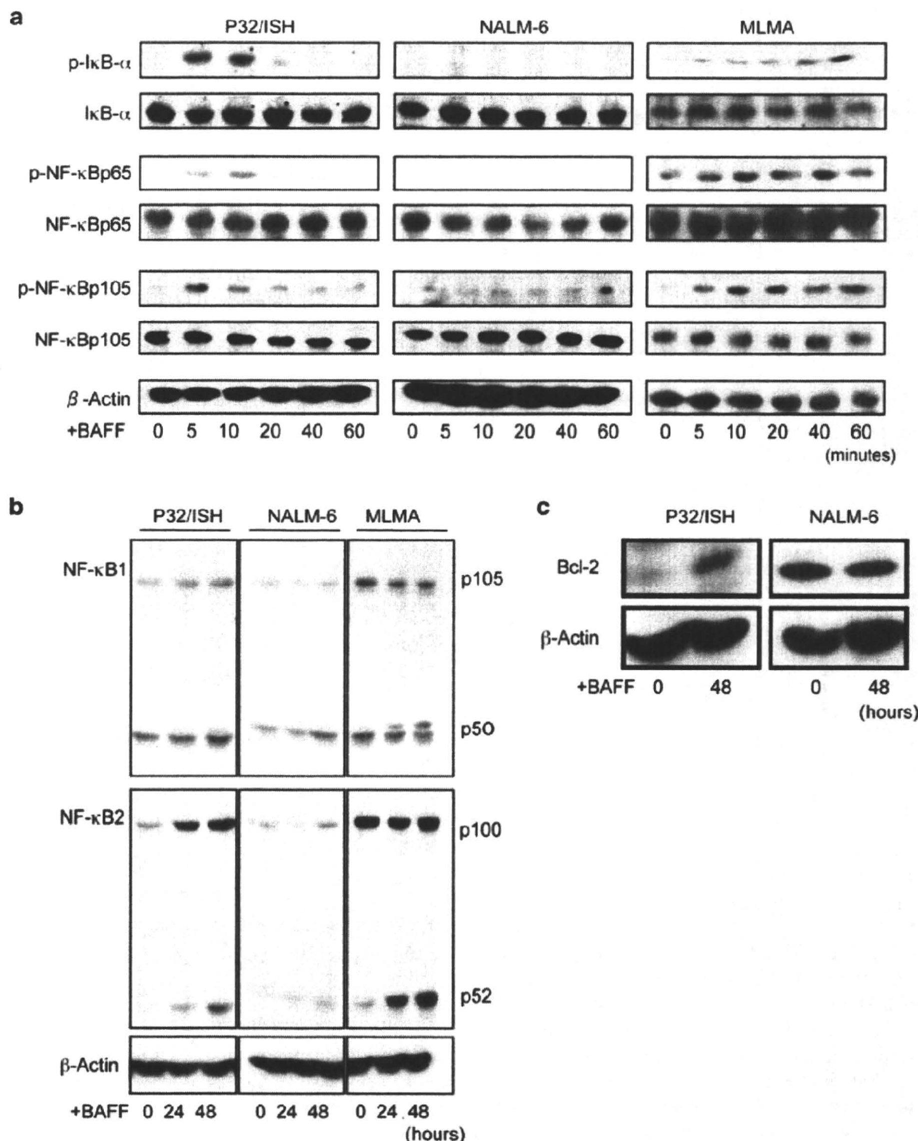

Fig. 6 Comparison between the BAFF-mediated signaling in Burkitt lymphoma (BL) cells and B cell precursor acute lymphoblastic leukemia (BCP-ALL) cells. **a** P32/ISH, NALM-6, and MLMA cells were treated with 200 ng/ml of BAFF, and cell lysates were prepared at the times indicated. Immunoblotting with phospho-specific Abs was performed to detect changes in the phosphorylation state of the proteins indicated. Whole molecules of each protein were included as internal controls. The β-actin protein was also included as internal control. **b** P32/ISH, NALM-6, and MLMA cells were treated with BAFF as in **a**, and cell lysates were prepared at the times indicated. Immunoblotting with Abs specific for either NF-κB1 (precursor form p105, activated form p50) or NF-κB2 (precursor form p100, activated form p52) was performed. The β-actin protein was also detected as internal control. **c** Cell lysates of P32/ISH and NALM-6 cells were examined for Bcl-2 expression as in **b**. The above experiments were repeated three times independently and similar results were obtained

cells. The precise mechanism responsible for the differential effect of BAFF on BL and BCP-ALL cells is unknown. As reported above, major signaling molecules located downstream of BAFF-R were detected in both BL and BCP-ALL cells. However, whereas clear activation of the NF-κB cascade was observed in P32/ISH BL cells, only limited activation was observed in NALM-6 BCP-ALL cells. Up-regulation of CD40 expression following BAFF stimulation was observed in P32/ISH cells, but not in NALM-6 cells. In our preliminary experiments, microarray analysis further suggested that BAFF mediates gene expression in BCP-ALL cells, which is distinct from the gene expression that it mediates in BL cells. Since BCP cells are reported to have a related but different signaling system from mature B cells, it can be speculated that the BCP cells transduce the BAFF-mediated stimuli in a manner that differs from mature B cells and that BAFF-mediated signaling does not mediate the expression of anti-apoptotic molecules in BCP-ALL cells. Further studies of the genes selected above will help to elucidate how BAFF affects BCP-ALL cells and how it is involved in the inhibition of apoptosis in BL cells.

In conclusion, we observed BAFF-R expression in both BL and BCP-ALL cells, which are derived from B cells at different stages of development, and found that BAFF affects tumor cells of these two B-lineage malignancies in a different manner. Since BAFF seems to be involved in survival and/or proliferation of tumor cells of these two B-lineage malignancies, it might be possible to develop a novel approach for the treatment of BL and BCP-ALL by

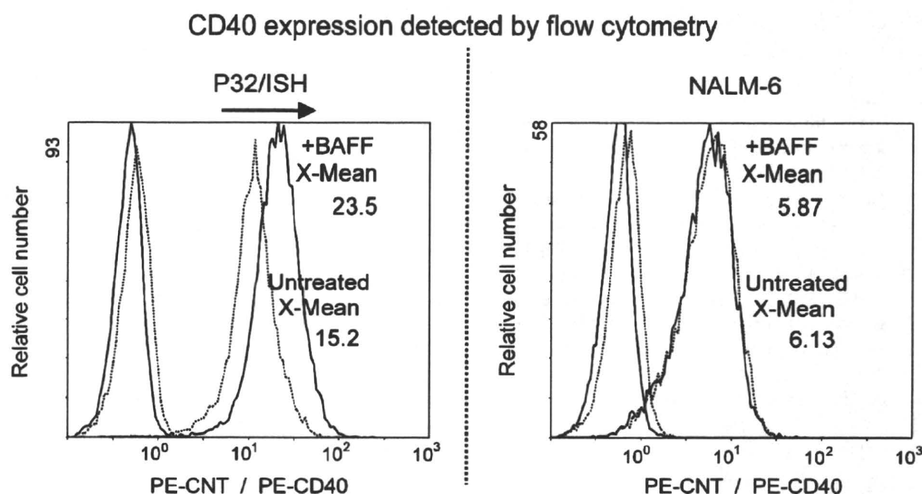

Fig. 7 Effect of BAFF on CD40 expression in P32/ISH and NALM-6 cells. Cells were cultured for 24 h with (+BAFF, *dark lines*) or without (untreated, *light lines*) BAFF 200 ng/ml and examined for the level of CD40 expression by flow cytometry. The histograms of the negative controls are superimposed and shown in each panel (on the *left side*)

targeting BAFF signaling. Although more detailed experiments are clearly needed, our findings in this study should provide a model for investigating the molecular basis of the developmental stage-dependent effect of BAFF on B cells in vitro and help elucidate how BAFF affects early B cell development.

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Conflict of interest statement We have no financial relationships or conflicts of interest related to this manuscript.

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