BAZF is required for activation of naive CD4 T cells by TCR triggering

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Keywords: cellular activation, knockout, T cell receptor, T lymphocytes, transcription factors, transgenic

Abstract

BAZF, a member of the Bcl6 gene family, acts as a sequence-specific transcriptional repressor in association with Bcl6. However, the tissue expression pattern of BAZF differs from that of Bcl6, suggesting a Bcl6-independent function of BAZF. In order to examine the physiological function of BAZF, we generated BAZF-deficient mice and transgenic mice with BAZF-cDNA under the control of the lck proximal promoter (lck-BAZF). These mice were viable and no gross anatomical abnormalities were observed after birth. Since Bcl6 is a key molecule for the generation of memory T cells, we examined the function of T cells of these mice. We show here that cell proliferation of naive CD4 T cells, but not memory ones, of BAZF-deficient mice to anti-CD3 antibody stimulation was impaired. Conversely, cell proliferation of naive CD4 T cells, but not memory ones, of lck-BAZF mice was augmented. Since cell proliferation of naive CD4 T cells of lck-Bcl6 mice to anti-CD3 antibody stimulation was severely impaired, BAZF may attenuate the regulatory effect of Bcl6 on antigenic activation of naive CD4 T cells by Bcl6/BAZF heterodimer formation. These results suggest that BAZF is necessary for activation of naive T cells to antigenic stimulation.

Introduction

We have cloned a novel Bcl6 family gene, BAZF (1). The predicted amino acid sequence of BAZF indicated that the BTB/POZ domain and five repeats of the Krüppel-type zinc finger motif are located in the N-terminal and C-terminal regions, respectively. The BTB/POZ domain is important for protein–protein interactions (2,3) and the zinc finger motifs bind to the specific DNA sequence in vitro (4,5). At the amino acid level, zinc finger motifs of BAZF are 94% identical to those of Bcl6, and BAZF binds to the DNA sequences similar to those for Bcl6 (1,6). The BTB/POZ domain of BAZF is 65% identical to that of Bcl6 and binds to that of Bcl6. Since the BTB/POZ domain and the middle portion of Bcl6 can bind to the silencing mediator of retinoid and thyroid receptor protein (SMRT) (7–10) and mSin3A (8), respectively, and SMRT and mSin3A form a repressive complex with histone deacetylase 1 (HDAC1) (11,12), Bcl6 recruits the SMRT/mSin3A/HDAC1 complex to specific Bcl6-binding regions of target genes and represses expression of these genes. Thus, BAZF might also function as a transcriptional repressor.

We have identified an identical 17-amino acid sequence in the middle portion of Bcl6 and BAZF, and the reporter gene assay indicates that a 27-amino acid sequence of BAZF, including the 17-amino acid region, expresses transrepressor activity in NIH3T3 cells (13). We established a yeast two-hybrid system to isolate binding molecules of the BTB/POZ domain or the middle portion of BAZF, including the 17-amino acid region (14). These portions of BAZF bound to the BTB/POZ domain or the middle portion of Bcl6, respectively. However, these bound to neither SMRT nor mSin3A. Thus, we examined repressor activity of BAZF in Bcl6-deficient cells. BAZF cannot display its repressor activity in Bcl6-deficient cells, and that repressor activity was recovered in Bcl6-deficient cells replenished with the BTB/POZ domain or the middle portion of Bcl6. Thus, BAZF may display its transrepressor activity by recruiting the SMRT/mSin3A/HDAC1 complex through association with Bcl6. Therefore, the biochemical property of BAZF is similar to that of Bcl6 and its function seems to be dependent on Bcl6. However, the tissue expression pattern of BAZF differs from that of Bcl6, although Bcl6 and BAZF mRNAs are induced in activated lymphocytes as an immediate early gene (1,15). These results also suggest a Bcl6-independent function of BAZF.

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Transmitting editor: S. Koyasu
Received 18 February 2004, accepted 15 July 2004
BAZF function in naive CD4 T cells

We have recently reported that Bcl6 plays a role in the generation and maintenance of activated CD8 T cells at the memory cell stage during both antigen- and lymphopenia-induced activation (16). We showed that a transient increase in the number of memory phenotype CD8 T cells occurs under physiological conditions in the lymphopenic environment of the spleen of very young mice, and that the magnitude of this increase is correlated with the amount of Bcl6 in T cells. Moreover, Bcl6 controls generation and maintenance of antigen-specific memory CD8 T cells in the spleen of immunized mice. Thus, Bcl6 is a key molecule for establishment of memory T cells. However, a role for BAZF in generation of memory T cells is largely unknown. In order to examine physiological functions of BAZF, especially for generation of memory T cells, we generated BAZF-deficient (BAZF-KO) and BAZF-transgenic (Ick-BAZF) mice. Both BAZF-KO and Ick-BAZF mice were viable, and no gross anatomical abnormalities were observed. The percentage of memory phenotype T cells in the spleen of these mice was normal. However, activation of naive CD4 T cells of BAZF-KO and Ick-BAZF mice to anti-CD3 antibody stimulation was inversely correlated with the amount of BAZF in T cells. Furthermore, the activation of naive CD4 T cells of Bcl6 transgenic (Ick-Bcl6) mice was also severely impaired, suggesting that BAZF attenuates the effect of Bcl6 by Bcl6/BAZF heterodimer formation. We discuss a role for BAZF in TCR signaling in naive CD4 T cells.

Methods

Animals
C57BL/6 Cr.Slc, (C57BL/6 × DBA/2) F1 and ICR mice were purchased from Japan SLC Co. (Hamamatsu, Japan) and Japan CLEA Co. (Tokyo, Japan), respectively. Transgenic mice carrying the mouse Bcl6 gene under the control of the Ick proximal promoter (Ick-Bcl6) have been described previously (16). All mice were maintained under specific pathogen-free conditions in the animal center of the Graduate School of Medicine, Chiba University. All experiments were approved by the Institutional Animal Care and Use Committee at the Graduate School of Medicine, Chiba University.

Gene targeting in embryonic stem cells
A targeting vector was generated from a 11 kb genomic clone containing the BAZF gene isolated from a genomic DNA library of mouse strain 129/SV. A 2.3 kb fragment from –20 bp in the 5′ flanking region of the BAZF gene to +120 bp of exon 5 was replaced by a neomycin resistant gene cassette (pMC1-neo). For negative selection, a herpes simplex thymidine kinase gene cassette was fused at the 5′ end of the short arm. R1 embryonic stem cells were transfected with the linearized targeting vector by electroporation and subjected to positive and negative selection using G418 and gancyclovir for 14 days. Approximately 160 clones were examined by Southern blots and homologous recombination was detected in 10 clones. Chimeric mice were generated using the aggregation method with some modification (17), and two independent targeted clones were transmitted to the germline. Tail DNAs from agouti pups obtained from mating with C57BL/6 mice were analyzed by Southern blots. Heterozygous mutant mice were backcrossed with C57BL/6 mice for more than five generations. Homozygous mutant pups were generated by intercrossing heterozygous mutant mice. Mice derived from two independent targeted ES clones were used in the subsequent studies.

Construction of the Ick-BAZF gene
BAZF cDNA in pGEM-4Z (1) was digested by EcoRI and SalI and the fragment of BAZF cDNA was inserted into the SalI and XhoI site in pACT2. BAZF cDNA in pACT2 was digested by BglII and the BglII fragment of BAZF cDNA was inserted into the BamHI site of the Ick-hGH plasmid (18).

DNA microinjection and screening of transgenic mice
Transgenic mice were produced by the method described by Hogan et al. (19). Briefly, (C57BL/6 × DBA/2) F1 mice were used to obtain fertilized eggs, and the Ick-BAZF transgene was microinjected into a male pronucleus of the fertilized egg. The injected eggs were returned to the oviducts of pseudo-pregnant mothers of ICR strain. When the mice were 4 weeks of age, total DNA was extracted from the tail of each mouse. The transgenes in genomic DNA isolated from transgenic mice were detected by slot blot analysis using the human growth hormone probe as described previously (20). The transgenic mice were backcrossed with C57BL/6 mice for more than five generations. Mice derived from two independent lines were used in the subsequent studies.

Southern blot analysis
Southern blot analysis was performed as described previously (20). Briefly, genomic DNA isolated from the mutant offspring was digested with BamHI, separated on a 1% agarose gel and the fragment of BAZF was detected by slot blot analysis using the human growth hormone probe as described previously (20). The probe was labeled with DIG (Roche Diagnostics) by PCR. The antibody reaction was performed using an enhanced chemiluminescent detection system (Roche Diagnostics). The antibody reaction was performed using an enhanced chemiluminescent detection system (Roche Diagnostics). As a probe, a 0.3 kb DNA fragment, which is external to the targeting vector, was labeled with DIG (Roche Diagnostics) by PCR (Fig. 1A). The probe detected the wild-type allele as a 1.5 kb fragment and the mutant allele as a 3.8 kb fragment.

Northern blot analysis
Total RNAs were extracted from mouse tissues with the Trizol reagent (Life Technologies, Gaithersburg, MD). Northern blot analysis was done as described previously (21). Briefly, total RNAs (20 μg) were electrophoresed through a 1.0% agarose gel containing formaldehyde and transferred to a nylon membrane. The filter was hybridized with a DIG-labeled probe and followed by the method described for Southern blot analysis. A 481 bp fragment (+626 to +1107) of the BAZF

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cDNA was used as a probe. This DIG-labeled BAZF middle probe (481 bp) was synthesized by PCR on BAZF cDNA using primers (5'-aggaagcctagttggggagagttc-3' and 5'-aggttagcaggtcggttaaaacg-3').

**RT–PCR and Southern blot analysis**

RT–PCR was performed as described elsewhere (22). Briefly, cDNAs were made by reverse-transcribing total RNAs with oligo(dT) primers and amplified 22–28 times with 2.5 units of Taq DNA polymerase, 1.5 mM MgCl2, 5% formamide and the primers (described above). The PCR products were separated on a 1% agarose gel and transferred onto a nylon membrane (Amersham Pharmacia). The filter was pre-hybridized for 1 h and hybridized with DIG-labeled probes overnight at 42°C. The DIG-labeled probes were detected using the same method described above. PCR primers for the cDNA amplification were the following: Bcl6, 5'-agcaagaacgcctgcatccttc-3' and 5'-catctctgtatgctgtggggactg-3'; BAZF, 5'-aggaagcctagttggggagagttc-3' and 5'-aggttagcaggtcggttaaaacg-3'; β-actin, 5'-gtggccatctcctgctcgaagtc-3' and 5'-gtggccatctcctgctcgaagtc-3'.

**Antibodies and flow cytometry analysis**

Thymocytes and spleen cells were stained with various mAbs as follows (16). Cell suspensions were first blocked with unconjugated anti-CD32/16 (2.4G2, PharMingen, San Diego, CA) followed by incubation with biotinylated antibodies and then incubation with directly conjugated antibodies and PerCP-streptavidine (PharMingen). The following antibodies were used for staining: PE–anti-CD8, PE–anti-TCRγδ, FITC–anti-Ly6c, APC–anti-B220, FITC–anti-IgM, FITC–anti-CD21, FITC–anti-IgD, FITC–anti-CD122, PE–anti-CD122, PE–anti-NK1.1,

Cell suspensions were treated with lysing buffer (ACK; 0.155 M ammonium chloride, 0.1 M disodium EDTA, 0.01 M potassium bicarbonate) to lyse erythrocytes before staining. Single-cell suspensions were prepared in staining medium (PBS with 3% FCS and 0.1% sodium azide) and were stained with the mAbs described above. After 20 min incubation on ice, cells were washed twice with staining medium and resuspended in staining medium supplemented with propidium iodide (PI, 1 μg/ml) to exclude dead cells, and analyzed by a FACSCalibur (Becton Dickinson).

Preparation of subsets of T cells from thymocytes and spleen cells

CD4 or CD8 double negative (DN), double positive (DP) and single positive (SP) T cells were sorted from thymocytes stained with a mixture of FITC anti-CD4 and PE anti-CD8 antibodies by a FACS Vantage (Becton Dickinson). For isolation of naive or memory CD4 T cells, spleen cells were incubated with a mixture of antibodies to CD8, B220, Mac-1, Gr-1 and CD44 for naive CD4 T cells or antibodies to CD8, B220, Mac-1, CD62L, Gr-1 and NK1.1 for memory CD4 T cells. These spleen cells were followed by treatment of antibody-coated microbeads (Miltenyi Biotec, Auburn, CA) with two beads/cell, then purified by negative selection on a SuperMacs cell sorter (Miltenyi Biotec). The purity of magnetically purified CD4 T cells was >90%.

Cell culture

Thymocytes (2 × 10^5 cells per well) or purified T cells (1 × 10^5 cells per well) were cultured with RPMI1640 medium supplemented with 10% FCS (Intergen, New York, NY), 50 μM 2-ME, 10 mM HEPES, streptomycin and penicillin in a flat-bottomed 96-well plate (Costar Co., Cambridge, MA) pre-coated with anti-CD3 mAb (10 μg/ml) (Cedarlane, Ontario, Canada) in the presence or absence of hamster anti-mouse CD28 mAbs (1 μg/ml) (Southern Biotechnology Associates, Birmingham, AL). These T cells were also cultured with PMA (10 ng/ml) (Sigma, St Louis, MO) and ionomycin (1 μM) (Calbiochem, Darmstadt, Germany).

Proliferation assay

T cells were cultured at 37°C in 5% CO₂ for 3 days, and 1 μCi [³H]thymidine (Amersham International, Aylesbury, UK) was added to the wells for the last 8 h of culture. T cells in the wells were harvested onto glass fiber filters and [³H]thymidine in these T cells was counted with a liquid scintillation counter.

ELISA for cytokines

T cells were cultured at 37°C in 5% CO₂ for 2 days. The amount of IL-2 in culture supernatants was measured by ELISA (BD PharMingen) as described previously (23).

Cell cycle analysis (PI staining)

T cells were cultured at 37°C in 5% CO₂ for 3 days. These cultured cells were incubated in 200 μl of Krishan's reagent (0.05 mg/ml PI, 0.1% sodium citrate, 0.02 mg/ml RNase A, 0.3% Nonidet P-40, pH 8.3), then fluorescence from propidium iodide-nuclear DNA complexes was analyzed with a FACSCalibur (Becton Dickinson).

In vitro apoptosis assay

T cells were cultured at 37°C in 5% CO₂ for 18 h. These cells were resuspended in 1X annexin V binding buffer (Bender Medsystems, Vienna, Austria) and stained with annexin V FITC (Bender Medsystems) and PI. Apoptotic and dead cells were detected on a FACSCalibur.

Statistical analysis

Statistical analysis was performed by unpaired t-test. P-values of <0.05 were considered significant.

Results

Generation of BAZF-KO and Ick-BAZF mice

Exons from 1 to 5, including the ATG translation start codon of the BAZF gene, were replaced by the neomycin resistant gene in embryonic stem cells (Fig. 1A). Heterozygous mutant (BAZF+/−) mice were interbred to obtain homozygous mutant (BAZF-KO) mice and their progeny were genotyped by Southern blot analysis (Fig. 1B). BAZF mRNA was not detected in total RNA from any tissues of BAZF-KO mice examined by northern blot analysis (data not shown). Analysis of >200 offspring revealed that growth curves and body weight of BAZF-KO mice were indistinguishable from those of BAZF−/− and BAZF+/− littermates (data not shown), and BAZF-KO mice were fertile. Although BAZF is expressed in heart and lungs and induced in activated lymphocytes (1), histological analysis of these tissues from BAZF-KO mice revealed no abnormalities (data not shown). The Ick-BAZF gene was injected into fertilized eggs to generate transgenic mice (Ick-BAZF). We established two lines of Ick-BAZF mice. Thymus and spleen of Ick-BAZF mice expressed the exogenous gene (Fig. 1C).

Thymocytes and spleen cells of BAZF-KO and Ick-BAZF mice were stained with various antibodies and analyzed on a FACSCalibur. Profiles of CD4/8 staining of thymocytes and spleen cells of BAZF-KO and Ick-BAZF mice were similar to those of control littermates (Fig. 2A). The percentage of CD8 SP thymocytes of Ick-Bcl6 mice was higher than that of control mice. The percentages of CD4 T and CD8 T cells in the spleen of Ick-Bcl6 mice were lower than those of control mice. The percentages of memory phenotype (CD44+CD62L−) CD4 T cells and (CD44+Ly6C+) CD8 T cells (data not shown) in the spleen of BAZF-KO and Ick-Bcl6 mice were at normal level, but lower than that of Ick-Bcl6 mice. However, levels of TCRββ and CD3 on thymocytes (Fig. 2B) and splenic T cells (data not shown) of these mice were similar to those of control littermates.

Since the percentage of CD8 SP thymocytes from BAZF-KO and Ick-BAZF mice was different from that of Ick-Bcl6 mice,
Fig. 2. Thymocytes, splenic T cells and memory phenotype (CD44^+CD62L^-) CD4 T cells in the spleen of BAZF-KO and Ick-BAZF mice. (A) Thymocytes and spleen cells of BAZF-KO, Ick-BAZF and Ick-Bcl6 mice were stained with anti-CD4, anti-CD8, anti-CD44 and anti-CD62L mAbs, and analyzed on a FACS. The numbers in the corners indicate the percentages of T cells in each quadrant. These results are representative of three independent experiments. (B) Levels of TCRαβ and CD3 on thymocytes of BAZF-KO, Ick-BAZF and Ick-Bcl6 mice. These results are representative of two independent experiments. (C) Expression of BAZF and Bcl6 mRNA in subsets of thymocytes from normal mice was analyzed by RT-PCR. These results are representative of two independent experiments.
expression of BAZF and Bcl6 mRNA in subsets of thymocytes from normal mice was analyzed by RT-PCR. BAZF expression was detected slightly in SP thymocytes but not in DN and DP thymocytes, whereas Bcl6 expression was detected in the four subsets of thymocytes and strongly in DN and CD8-SP thymocytes (Fig. 2C). These results suggest a role for Bcl6 in CD8-SP thymocytes.

**BAZF is required for cell proliferation of thymocytes activated with anti-CD3 antibody**

Thymocytes of BAZF-KO and Ick-BAZF mice were stimulated with anti-CD3 antibody for 3 days and their cell proliferation was analyzed on day 2 and 3 after stimulation. Cell proliferation of BAZF-KO thymocytes was less than 1/3 of control thymocytes (Fig. 3A). Cell proliferation of Ick-BAZF thymocytes was slightly higher than that of control littermates. However, the impairment of BAZF-KO and Ick-BAZF mice was not repeated by stimulation of PMA and ionomycin. Cell proliferation of both CD4-SP and CD8-SP thymocytes of BAZF-KO mice was lower than that of control littermates (Fig. 3B). The cell cycle of these stimulated thymocytes was analyzed by PI staining on a FACSCalibur. The percentages of both CD4-SP and CD8-SP thymocytes of BAZF-KO mice in the S/G2/M phase were <20% and lower than that (~28%) of control thymocytes (data not shown). When apoptosis of the activated CD4-SP and CD8-SP thymocytes of BAZF-KO and Ick-BAZF mice 18 h after stimulation was detected by annexinV staining, there was no significant difference between these thymocytes and control littermate thymocytes (Fig. 3C).

**BAZF is required for cell proliferation of naive phenotype splenic CD4 T cells but not memory ones activated with anti-CD3 antibody**

Naive phenotype (CD44-) CD4 T cells were isolated from spleen cells by negative selection on a SuperMACS cell sorter and stimulated with anti-CD3 antibody for 3 days. As shown in Fig. 4(A), cell proliferation of naive CD4 T cells of BAZF-KO mice was clearly lower than that of control littermates. When these CD4 T cells were stimulated with anti-CD3 and anti-CD28 antibodies, their cell proliferation was also lower than that of control thymocytes. However, stimulation of these cells with PMA and ionomycin did not show the difference. On the contrary, cell proliferation of naive phenotype CD4 T cells of Ick-BAZF mice stimulated with anti-CD3 antibody was slightly higher than that of control thymocytes. When these CD4 T cells were stimulated with anti-CD3 and anti-CD28 antibodies but not with PMA and ionomycin, cell proliferation of Ick-BAZF mice was higher than that of control thymocytes. Cell cycle of these stimulated CD4 T cells of BAZF-KO mice was analyzed by PI staining on a FACSCalibur, and the percentages of CD4 T cells in the S/G2/M phase were correlated with the cell proliferation results (Fig. 4B). However, cell cycle analysis of these stimulated CD4 T cells of Ick-BAZF mice did not detect a significant difference from that of control thymocytes (data not shown).

When memory phenotype (CD44+) splenic CD4 T cells of BAZF-KO and Ick-BAZF mice were isolated by negative selection on a SuperMACS cell sorter and stimulated with anti-CD3 antibody or with anti-CD3 and anti-CD28 antibodies for 3 days, their cell proliferation was similar to or slightly higher than that of control littermates (Fig. 4C). The stimulation of these T cells with PMA and ionomycin did not show any difference. Cell cycle analysis of these stimulated CD4 T cells of BAZF-KO and Ick-BAZF mice was also correlated with their cell proliferation results (data not shown).

**Overexpression of Bcl6 suppresses cell proliferation of naive phenotype splenic CD4 T cells activated with anti-CD3 antibody**

We have demonstrated that BAZF requires Bcl6 to display its repressor activity (14). In order to examine the relation between the function of BAZF in proliferation of naive CD4 T cells and heterodimer formation of Bcl6/BAZF, thymocytes and naive phenotype splenic CD4 T cells of Ick-Bcl6 mice were stimulated with anti-CD3 antibody for 3 days. Their cell proliferation was lower than that of control littermates (Fig. 5). However, this impairment of T cell proliferation of Ick-Bcl6 mice was not repeated by stimulation of PMA and ionomycin. Furthermore, cell proliferation of memory phenotype splenic CD4 T cells of Ick-Bcl6 mice to anti-CD3 antibody stimulation was similar to that of control thymocytes (data not shown). These results suggest that the function of BAZF in naive CD4 T cells stimulated with anti-CD3 antibody is related to Bcl6.

IL-2 can be produced by splenic CD4 T cells stimulated with anti-CD3 antibody (24,25). Thus, IL-2 production in the culture supernatants of splenic T cells of BAZF-KO mice stimulated with anti-CD3 antibody was measured by ELISA. As shown in Fig. 6, the amounts of IL-2 in culture supernatants of naive phenotype splenic CD4 T cells of BAZF-KO mice stimulated with anti-CD3 antibody or anti-CD3 and anti-CD28 antibodies, but not with PMA and ionomycin, were lower than those of control thymocytes. However, the amounts in the culture supernatants of memory phenotype splenic CD4 T cells stimulated with anti-CD3 antibody were similar between BAZF-KO mice and control littermates, although the amounts in memory CD4 T cells were 10-fold higher than those in naive CD4 T cells.

**BAZF expression is induced in naive phenotype splenic CD4 T cells but not in memory ones after anti-CD3 antibody stimulation**

BAZF expression in naive and memory phenotype splenic CD4 T cells of normal mice after anti-CD3 stimulation was analyzed by RT-PCR. BAZF mRNA in naive phenotype (CD44-) CD4 T cells was not detected before stimulation, induced within 1 h after stimulation and maintained until 6 h after stimulation (Fig. 7). On the other hand, BAZF expression in memory phenotype (CD44+) CD4 T cells was detected before stimulation and maintained until 6 h after stimulation. Bcl6 expression in both naive and memory CD4 T cells was detected before stimulation, induced 1 h after stimulation, and slightly down-regulated 6 h after stimulation.

**Discussion**

BAZF and Bcl6 are strongly expressed in lungs and heart, and induced in lymphocytes activated with PMA and ionomycin as
immediate early genes (1,15). The interaction between the BTB/POZ domain of Bcl6 and that of BAZF can make a heterodimer. Physiologically relevant DNA-binding sites have been reported for Bcl6 (4,26) and BAZF (6). Thus, heterodimer formation of Bcl6/BAZF may be important for binding to the target DNA site, and is essential for BAZF to display its transrepressor activity (14). Although Bcl6-KO mice display growth retardation and severe inflammation in lungs and heart with eosinophilic infiltration (21), BAZF-KO mice were viable and displayed no growth retardation and inflammation after birth. Furthermore, Bcl6 is a key molecule for generation of memory CD8 T cells (16), and the percentages of memory phenotype CD4 T cells (Fig. 2A) and CD8 T cells (data not shown) in the spleen of BAZF-KO and lck-BAZF mice were normal. These results suggest that a large part of the abnormalities detected in Bcl6-KO and lck-Bcl6 mice are independent of BAZF.

We show here that activation of naive CD4 T cells of BAZF-KO and lck-BAZF mice to anti-CD3 antibody stimulation was normal, though activation of memory CD4 T cells of these mice to anti-CD3 antibody stimulation was normal. This abnormality in naive CD4 T cells included cell proliferation, IL-2 production and cell cycle progression, although CD25 expression was normal in these activated naive CD4 T cells (data not shown). A part of this abnormality was repeated in CD8-SP thymocytes (Fig. 3B) and naive CD8 T cells (data not shown) of BAZF-KO mice. Stimulation of naive CD4 T cells of BAZF-KO and lck-BAZF mice with anti-CD3 and anti-CD28 antibodies still showed the abnormality. Since naive CD4 T cells of BAZF-KO and lck-BAZF mice can respond well to PMA and ionomycin stimulation, this abnormality may be unique to TCR signaling. Thus, BAZF may be required for full activation of naive CD4 T cells and CD8 T cells to TCR stimulation. Furthermore, this abnormality was detected in naive CD4 T cells of BAZF-KO and lck-Bcl6 mice, suggesting that BAZF function in activation of naive T cells by TCR stimulation is opposite to Bcl6 function. The function of BAZF on activation of naive T cells to TCR stimulation may attenuate the regulatory function of Bcl6 by heterodimer formation.

BAZF itself can bind to the specific DNA sequence (6), but cannot display its repressor activity (14). Thus, BAZF function in activation of naive T cells to TCR stimulation may not be due to its repressor activity. The BTB/POZ domain is important for protein–protein interaction and that of BAZF cannot recruit the SMRT/mSin3A/HDAC1 complex (14), suggesting that the domain of BAZF binds to other important molecules to activate TCR signaling in naive T cells. Further study is required for elucidation of the molecular mechanisms of BAZF function in naive T cells.

Activation of naive T cells to TCR stimulation is affected by the amount of BAZF and Bcl6 in the T cells. Although positive

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**Fig. 3.** Activation of thymocytes of BAZF-KO and lck-BAZF mice stimulated with anti-CD3 antibody. Thymocytes of BAZF-KO and lck-BAZF mice or CD4-SP and CD8-SP thymocytes of BAZF-KO mice were stimulated with anti-CD3 antibody, or PMA and ionomycin. (A) Cell proliferation of thymocytes of BAZF-KO (closed squares), lck-BAZF (closed triangles) and normal control (open circles) mice stimulated with anti-CD3 antibody, or PMA and ionomycin. Data represent the mean ± SD of triplicate cultures. **P < 0.01. Results are representative of three independent experiments. (B) Cell proliferation of CD4-SP and CD8-SP thymocytes of BAZF-KO (closed squares) and normal control (open circles) mice stimulated with anti-CD3 antibody. Data represent the mean ± SD of triplicate cultures. *P < 0.05, **P < 0.01. Results are representative of three independent experiments. (C) Apoptosis of CD4-SP and CD8-SP thymocytes of BAZF-KO mice stimulated with anti-CD3 antibody. CD4-SP and CD8-SP thymocytes were stained with annexin V and PI, and apoptosis was detected by FACS. The numbers in the corners indicate the percentages of T cells in each quadrant. Results are representative of three independent experiments.
selection of thymocytes in the thymus is also affected by TCR signaling (27), the percentage of DP and SP thymocytes was normal in the thymus of BAZF-KO and Ick-BAZF mice. The positive selection occurs in DP thymocytes and BAZF mRNA is undetectable in DP thymocytes from normal mice, suggesting that BAZF is not related to thymic positive selection.

The function of BAZF in TCR signaling can be ignored in memory CD4 T cells because this abnormality was not detected in memory CD4 T cells of BAZF-KO and Ick-BAZF mice. After antigenic activation, memory T cells take less time to enter the cell cycle, differentiate into effector T cells more rapidly and express a wider repertoire of effector molecules than naive T cells (28,29). A recent report demonstrated that the amount of p27Kip1 and activation of CDK6 are different between naive and memory CD8 T cells (30). However, the difference in molecular mechanisms of TCR signaling between

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**Fig. 4.** Activation of naive and memory phenotype CD4 T cells in the spleen of BAZF-KO and Ick-BAZF mice stimulated with anti-CD3 antibody. Naive and memory phenotype splenic CD4 T cells of BAZF-KO and Ick-BAZF mice were stimulated with anti-CD3 antibody, anti-CD3 and anti-CD28 antibodies, or PMA and ionomycin. (A) Cell proliferation of naive phenotype (CD44-CD255) CD4 T cells of BAZF-KO (closed squares), Ick-BAZF (closed triangles) and normal control (open circles) mice. Data represent the mean ± SD of triplicate cultures. *P < 0.05, **P < 0.01. Results are representative of three independent experiments. (B) Cell cycle analysis of naive phenotype (CD44-) CD4 T cells of BAZF-KO (closed squares) and normal control (WT) mice. The percentages of cells in the S/G2/M phase are shown. Results are representative of three experiments. (C) Cell proliferation of memory phenotype (CD44+) CD4 T cells of BAZF-KO (closed squares) and normal control (open circles) mice. Data represent the mean ± SD of triplicate cultures. Results are representative of three independent experiments.
naive and memory T cells is largely unknown. Since BAZF expression was detected in resting memory CD4 T cells but not in resting naive CD4 T cells, the exogenous BAZF in naive CD4 T cells of lck-BAZF mice may play a role in TCR signaling as BAZF in memory CD4 T cells of wild-type mice, suggesting a role for BAZF in full activation of CD4 T cells to TCR stimulation. Unraveling functions of BAZF and Bcl6 in naive T cells at molecular levels would shed new light on the activation.

Fig. 5. Activation of thymocytes and naive phenotype CD4 T cells in the spleen of lck-Bcl6 mice stimulated with anti-CD3 antibody. Thymocytes and naive phenotype (CD44+/C255-) splenic CD4 T cells of lck-Bcl6 (open squares) and normal control (open circles) mice were stimulated with anti-CD3 antibody, anti-CD3 and anti-CD28 antibodies, or PMA and ionomycin. Data represent the mean ± SD of triplicate cultures. *P < 0.05, **P < 0.01. Results are representative of three independent experiments.

Fig. 6. Cytokine production by naive and memory phenotype splenic CD4 T cells of BAZF-KO mice stimulated with anti-CD3 antibody. Naive (CD44+) and memory (CD44+) phenotype CD4 T cells of BAZF-KO (black bars) and normal control (white bars) mice were stimulated with anti-CD3 antibody, anti-CD3 and anti-CD28 antibodies, or PMA and ionomycin for 2 days. IL-2 production in culture supernatants were determined by ELISA. Data represent the mean ± SD of triplicate cultures. **P < 0.01. Results are representative of three independent experiments.
Expression of CD4 T cells of normal mice were stimulated with anti-CD3 antibody. Naive (CD44−) and memory (CD44+) phenotype splenic CD4 T cells of normal mice were stimulated with anti-CD3 antibody. Expression of BAZF and Bcl6 mRNA in these T cells various hours after stimulation was detected by RT-PCR and Southern blot analysis. β-actin mRNA was used as an amount control of mRNA. Results are representative of three independent experiments.

Acknowledgements

We are grateful to Drs S. Okada, S. Okabe and N. Takeda for discussion. We also thank H. Satake for skillful technical assistance and N. Kakinuma for secretarial services. This work was supported in part by Grants-in-Aid from the Ministry of Education, Science, Technology, Sports and Culture of Japan and the Uehara Memorial Foundation.

Abbreviations

APC aliphophycocyanin
BAZF-KO BAZF-deficient
DIG digoxigenin
DN double negative
DP double positive
HDAC1 histone deacetylase 1
PerCP perididine chlorophyll protein iodide
PI propidium iodide
SMRT silencing mediator of retinoid and thyroid receptor protein
SP single positive

References

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