

Bcl6 regulates Th2 type cytokine productions by mast cells activated by FcεRI/IgE cross-linking

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Abstract

Bcl6-deficient ($Bcl6^{-/-}$) mice displayed Th2 type inflammation, which caused by abnormality of non-lymphoid cells. However, initiators for the Th2 type inflammation were not clear. In order to elucidate the initiators, we investigated property and function of mast cells derived from $Bcl6^{-/-}$ mice. Mast cells were developed from bone marrow cells cultured with IL-3 (BMMCs). Although the development of BMMCs from $Bcl6^{-/-}$ mice was similar to that from wild-type mice, proliferation of $Bcl6^{-/-}$ BMMCs stimulated with IL-3 was slightly lower than that of wild-type BMMCs. When these BMMCs were stimulated by FcεRI/IgE cross-linking, $Bcl6^{-/-}$ BMMCs produced Th2 cytokines more than wild-type BMMCs did. Thus, $Bcl6^{-/-}$ mast cells are one of the initiators for Th2 type inflammation in $Bcl6^{-/-}$ mice, and Bcl6 may be a molecular target for Th2 type allergic diseases.

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Keywords: Bcl6; Mast cells; Th2 type cytokines; FcεRI

1. Introduction

The human proto-oncogene *Bcl6* has been identified from chromosomal breakpoints involving 3q27 in diffuse large B-cell lymphomas (Kerchaert et al., 1993; Ye et al., 1993; Miki et al., 1994). The *Bcl6* gene encodes a 92- to 98 kDa nuclear phosphoprotein that contains the BTB/POZ domain in the NH₂-terminal region and Krüppel-type zinc finger motifs in the COOH-terminal region. Since the NH₂-terminal half of Bcl6 can bind to silencing mediator of retinoid and thyroid receptor protein (SMRT) and recruit the SMRT/histone

deacetylase complex to silencer regions of target genes to repress expression of these genes, *Bcl6* can function as a sequence specific transcriptional repressor. To observe physiological functions of *Bcl6*, this gene was disrupted in the mouse germ line. Bcl6-deficient ($Bcl6^{-/-}$) mice showed growth retardation and abnormal immune responses. They completely lack germinal center formation (Dent et al., 1997; Fukuda et al., 1997; Ye et al., 1997) and affinity maturation of antibodies specific for immunized antigens (Toyama et al., 2002).

$Bcl6^{-/-}$ mice also displayed inflammatory responses in multiple organs, especially in heart and lungs, characterized by infiltration of eosinophils and IgE bearing B lymphocytes (Th2 type inflammation), and frequently die at an early adult age (Dent et al., 1997; Ye et al., 1997; Yoshida et al., 1999). Immunization of $Bcl6^{-/-}$ mice with a protein antigen in adjuvant accelerated induction of the Th2 type inflammation (Ye et al., 1997). These data suggested that the Th2 type inflammation was originated by antigen-activated cells, especially

Abbreviations: BMMCs, bone marrow derived mast cells; $Bcl6^{-/-}$, Bcl6-deficient; SMRT, silencing mediator of retinoid and thyroid receptor protein; WT, wild-type; DNP, dinitrophenyl; HAS, human serum albumin; PI, propidium iodide

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T cells. Indeed, we have recently reported that the *IL-5* gene is one of the molecular targets of Bcl6 in T cells (Arima et al., 2002). Furthermore, recent analysis of the mechanism for the Th2 type inflammation using Bcl6^{-/-} embryonic stem cell-derived chimeric mice indicated that the functional dominance of Th2 cells in Bcl6^{-/-} mice is due to non-lymphoid cells including macrophages, dendritic cells, and mast cells (Toney et al., 2000). However, initiators for the Th2 type inflammation were not clear.

Mast cells are distributed throughout the vascularized tissues and play a key role in allergic reactions by releasing proinflammatory mediators such as histamine, leukotrienes and prostaglandin by antigen stimulation through a FcεRI/IgE complex. Mast cells also play a central role in innate immunity (Supajatura et al., 2002) or induce autoantibody-mediated vasculitis (Malaviya et al., 1996) by TNFα production. Furthermore, mast cells effect on immune competent cells, such as survival of eosinophils, maturation of dendritic cells, and activation of B and T cells by production of cytokines (Levi-Schaffer et al., 1998; Skokos et al., 2001; Skokos et al., 2003). Mast cells stimulated by FcεRI/IgE cross-linking produce the large amount of IL-4, which is essential for the induction of Th2 development (Schmitz et al., 1994; Fallon et al., 2002), and chemokines such as MCP-1, MIP1α, RANTES and Eotaxin, which work on Th2 development and eosinophil recruitment (Nakajima et al., 2002). These results suggest that mast cells seem to be an initiator of the Th2 type inflammation in Bcl6^{-/-} mice. Therefore, we investigated property and function of Bcl6^{-/-} mast cells derived from bone marrow cells cultured with IL-3 (BMMCs). When BMMCs were stimulated by FcεRI/IgE cross-linking, Th2 type cytokine productions by the Bcl6^{-/-} BMMCs were larger than those by the wild-type (WT) BMMCs. We discuss a role for Bcl6 in function of mast cells.

2. Materials and methods

2.1. Mice

Bcl6-deficient (Bcl6^{-/-}) (Yoshida et al., 1999), (C57BL/6 × DBA2) F1(Japan SLC), C57BL/6 mice (Japan SLC) were housed in microisolate cages under pathogen free conditions. All experiments were performed according to the guidelines of Graduate School of Medicine, Chiba University (Chiba, Japan).

2.2. Culture of BMMCs

Femoral bone marrow cells of 8–12-week-old Bcl6^{-/-} and WT mice were isolated and cultured in tissue culture plates as described previously (Suzuki et al., 2000). These bone marrow cells were cultured in RPMI 1640 medium containing 10% FCS and 5–10% of murine IL-3 conditioned medium (x63-IL3; kindly provided from Dr. H. Karasuyama, Tokyo Medical and Dental University) at 37 °C. Non-

adherent cells in the culture were harvested every week and continuously cultured in the new plates with fresh medium.

2.3. Flow cytometry

For detection of c-Kit and FcεRI on BMMCs, BMMCs were first incubated with un-conjugated anti-CD32/16 monoclonal antibody (2.4G2; BD PharMingen, San Diego, CA) at 4 °C for 10 min, then with IgE anti-dinitrophenyl (DNP) monoclonal antibody (SPE-7; Sigma, St Louis, MO) for 50 min, followed by the incubation with biotin conjugated anti-mouse IgE antibody (BD PharMingen) for 10 min. These cells were further incubated with streptavidin-conjugated allophycocyanin (BD PharMingen) and phycoerythrin conjugated anti-c-Kit monoclonal antibody (BD PharMingen) for 10 min. Flow cytometric analysis was performed using a FACSCalibur (Becton Dickinson, San Jose, CA).

2.4. Cell survival assay

BMMCs were washed 3 times with PBS and cultured in a 96-well-plate (1.5 × 10⁶ cells/ml) with RPMI 1640 medium without IL-3 at 37 °C for 8 days. Viability of these cultured cells was assessed by trypan blue exclusion method, and the number of viable cells was counted on day 0, 1, 3, 5 and 8 after stimulation. For analysis of apoptosis, BMMCs were incubated with FITC-labeled annexin V and propidium iodide (PI) (Bender Medsystems, Vienna, Austria) for 15 min according to the manufacturer's instructions. Analysis of apoptosis was performed using a FACSCalibur.

2.5. Proliferation assay

BMMCs were washed 3 times with PBS and cultured in a 96-well-plate (1.0 × 10⁶ cells/ml) with RPMI 1640 medium containing 0–10% of IL-3 at 37 °C for 36 h. These cells were incubated with 1 μCi [³H]thymidine (Amersham International, Aylesbury, UK) during the last 12 h. These cells were harvested onto glass fiber filters, and [³H]thymidine was counted with a liquid scintillation counter.

2.6. Stimulation of BMMCs by FcεRI/IgE cross-linking

BMMCs (1 × 10⁶ cells/ml) were incubated with IgE anti-DNP antibody (1 μg/ml) overnight at 37 °C and washed twice with RPMI medium (Malaviya and Uckun, 2002). These cells were then challenged with various concentrations of DNP–human serum albumin (DNP–HSA; Sigma) for 30 min.

2.7. β-hexosaminidase release assay

BMMCs were stimulated by FcεRI/IgE cross-linking in Tyrode buffer (130 mM NaCl, 5 mM KCl, 1.4 mM CaCl₂, 1 mM MgCl₂, 5.6 mM glucose, 10 mM HEPES, and 0.1% BSA, pH 7.4). The amount of β-hexosaminidase in culture supernatants and cell lysates of the activated BMMCs

was measured by hydrolysis of *p*-nitrophenyl-*N*-acetyl- β -D-glucopyranoside (Sigma) in 0.1 M sodium citrate buffer (pH 4.5) for 2 h at 37 °C. The reaction was stopped by addition of 0.2 M glycine and the optical density at 405 nm of the reaction mixture was measured by a densitometry. The percentage of specific β -hexosaminidase release was calculated as follows: the percentage released = $100 \times (\text{activity in supernatants} / \text{activity in supernatants and lysates})$ as previously described (Watanabe et al., 1999).

2.8. RT-PCR for cytokine mRNAs

Total RNA was isolated from BMMCs using TRIzol Reagent (GIBCO-BRL, Burlington, Canada) according to the manufacturer's instructions. The cDNA was synthesized from total mRNA (1 μ g) with oligo(dT)_{12–18} as a primer and Superscript II RNase H reverse transcriptase (Life Technologies, Rockville, MD). Levels of cytokine mRNA were assessed by semi quantitative RT-PCR. The 20 cycles of PCR for β -actin and 30 cycles of PCR for cytokines were carried out using the following conditions: denaturation at 94 °C for 30 s, annealing at 55 °C for 40 s and polymerization at 72 °C for 60 s. PCR primers for the cDNA amplification were as follows: *IL-4* primers, 5'-ACTGACGGCACAGAGCTATTGATG-3' and 5'-GGA-CTCATTCATGGTGCAGCTTATC-3'; *IL-5* primers, 5'-AGGATGCTTCTGCACTTGAGTGTTC-3' and 5'-CCC-TTGCAATTGACAGTTTTG-3'; *IL-6* primers, 5'-GTT-CTCTGGGAAATCGTGGA-3' and 5'-TGTA CTCCAGG-TAGCTATGG-3'; *IL-13* primers, 5'-CAGTCCTGGCTCT-TGCTTGC-3' and 5'-AAGTGGGCTACTTCGATTTTGG-3'; *TNF α* primers, 5'-TCTCATCAGTTCTATGGCCC-3' and 5'-GGGAGTAGACAAGGTACAAC-3'; β -actin primers, 5'-GTTTGAGACCTTCAACACC-3' and 5'-GTGGCCAT-CTCCTGCTCGAAGTC-3'.

2.9. ELISA

Concentration of IL-6 (OptEIA, San Diego, CA) and IL-13 (R&D Systems, Minneapolis, MN) in culture supernatants was measured by ELISA according to the manufacturer's instructions.

2.10. Statistical analysis

Statistical analysis was made using unpaired *t*-test. *P*-values of <0.05 were considered to be significant.

3. Results

3.1. Development of BMMCs from *Bcl6*^{−/−} bone marrow cells stimulated with IL-3

Bcl6^{−/−} mouse peritoneal mast cells were stained with antibodies to Fc ϵ RI and to c-Kit, and analyzed on a FACS.

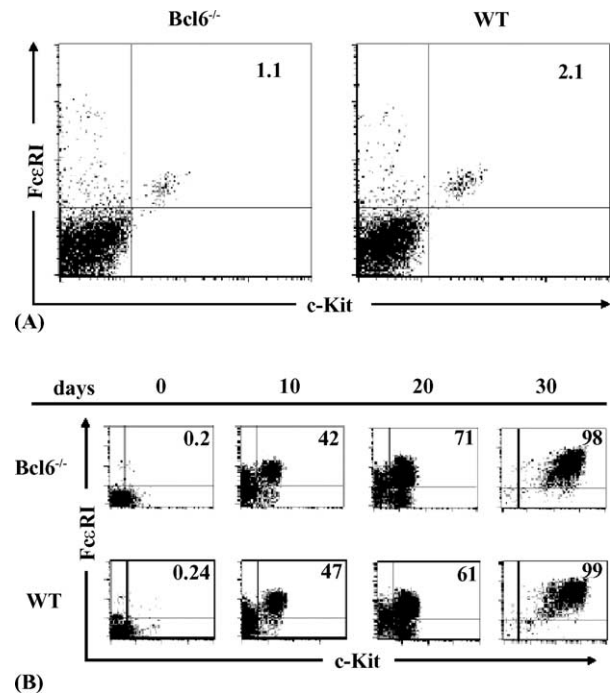


Fig. 1. Development of *Bcl6*^{−/−} BMMCs. (A) Mast cells in peritoneal cavity of *Bcl6*^{−/−} and WT mice were assessed by FACS analysis. The numbers in the corners indicate the percentages of mast cells in total peritoneal cells. (B) Bone marrow cells of *Bcl6*^{−/−} and WT mice were cultured with IL-3. Development of mast cells was assessed by FACS analysis. The numbers in the corners indicate the percentages of mast cells in cultured cells.

Although the percentage of *Bcl6*^{−/−} peritoneal mast cells was less than that of WT ones because of the larger cell infiltrations in the peritoneal cavity of *Bcl6*^{−/−} mice, mast cells with normal surface phenotypes were observed in *Bcl6*^{−/−} mice as well as WT littermates (Fig. 1A). In order to examine development of mast cells from bone marrow cells, *Bcl6*^{−/−} bone marrow cells were cultured with IL-3 for 30 days. Mast cells developed in the culture were stained with anti-c-Kit and anti-Fc ϵ RI antibodies and analyzed on a FACS every 10 days. Mast cells, with more than 98% of purity, were developed in the *Bcl6*^{−/−} bone marrow cell cultures as well as in the WT ones within 30 days (Fig. 1B). The level of Fc ϵ RI and c-Kit on *Bcl6*^{−/−} BMMCs was similar to that on WT BMMCs, and percentages of *Bcl6*^{−/−} BMMCs (c-Kit⁺, Fc ϵ RI⁺) in the cultures from day 10 to day 30 were similar to those of WT BMMCs. Thus, the deficiency of *Bcl6* in bone marrow cells may not affect the development of mast cells.

3.2. Functional property of *Bcl6*^{−/−} BMMCs

Since IL-3 is known to be an important mast cell growth factor, proliferation of *Bcl6*^{−/−} BMMCs to IL-3 stimulation was examined by thymidine uptake. Proliferation of *Bcl6*^{−/−} BMMCs stimulated with IL-3 was slightly lower than that of WT BMMCs (Fig. 2).

Apoptosis is induced in BMMCs by IL-3 starvation (Suzuki et al., 2000), and almost all of *Bcl6*^{−/−} and WT BMMCs died within 8 days after IL-3 starvation (Fig. 3A).

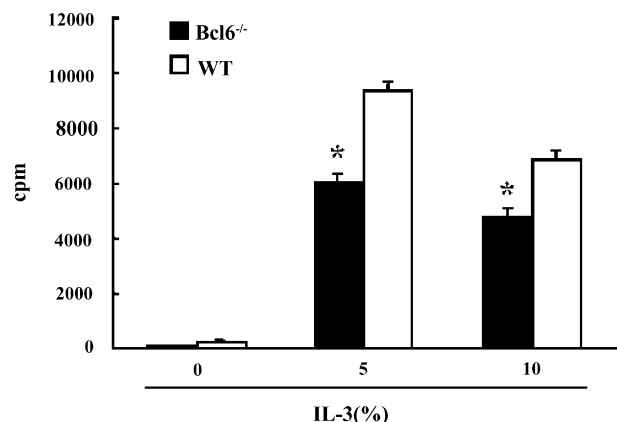


Fig. 2. Proliferation of Bcl6^{-/-} BMMCs. Bcl6^{-/-} (closed bar) and WT (open bar) BMMCs were cultured in the presence of various doses of IL-3 for 36 h. Data represents the mean $\pm$ S.D. of triplicate cultures. * $P < 0.0001$. Results are representative of three independent experiments.

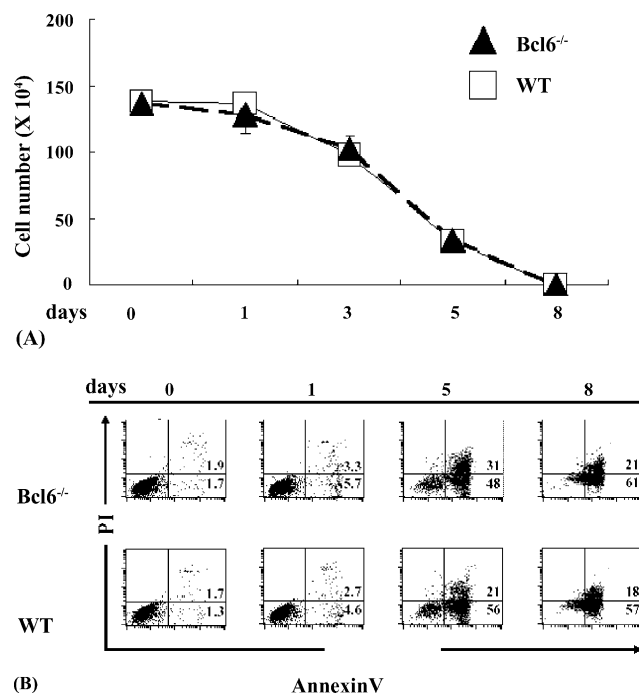


Fig. 3. Apoptosis of Bcl6^{-/-} BMMCs by IL-3 starvation. (A) Live cell numbers of Bcl6^{-/-} (closed triangle) and WT (open square) BMMCs were measured by the exclusion method after IL-3 starvation. (B) Apoptosis of Bcl6^{-/-} and WT BMMCs after IL-3 starvation was detected by staining with annexin V and PI. The numbers in the corners indicate the percentages of cells in each quadrant. Results are representative of three independent experiments.

Thus, apoptosis of Bcl6^{-/-} BMMCs after IL-3 starvation was examined by annexin V and PI staining. Percentages of apoptotic cells in Bcl6^{-/-} BMMCs were similar to those in WT BMMCs until day 8 after starvation (Fig. 3B).

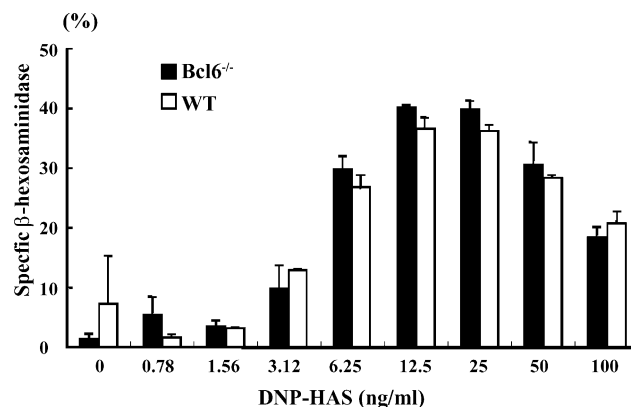


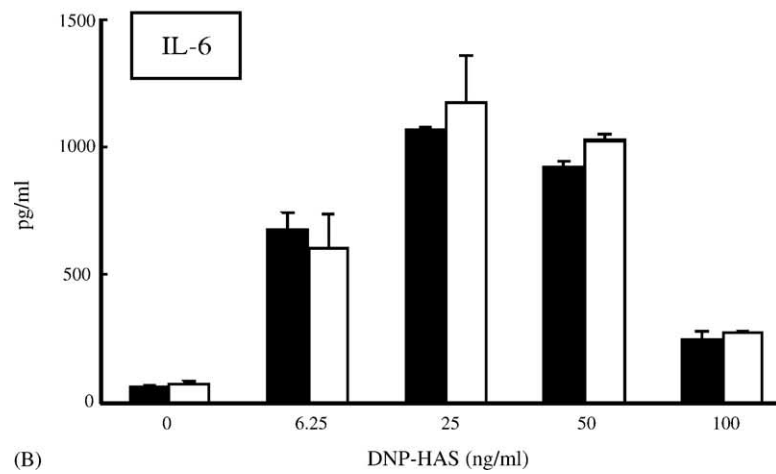
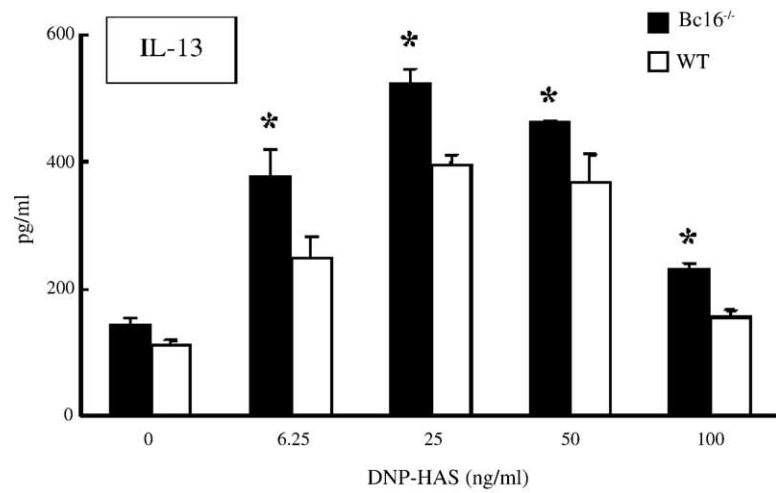
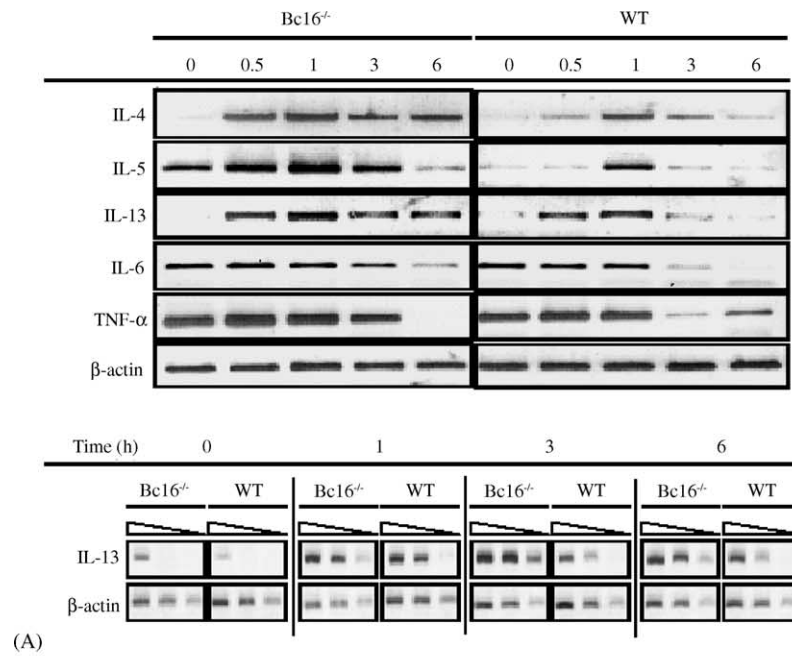
Fig. 4. Degranulation of Bcl6^{-/-} BMMCs. Degranulation of BMMCs stimulated by Fc ϵ RI/IgE cross-linking was assessed by the β -hexosaminidase release assay. After Bcl6^{-/-} (closed bar) and WT (open bar) BMMCs were stimulated by Fc ϵ RI/IgE cross-linking with various doses of DNP-HAS, the release of β -hexosaminidase in the culture supernatants was measured. Data represents the mean $\pm$ S.D. of triplicate cultures. Results are representative of three independent experiments.

Fc ϵ RI/IgE cross-linking is one of the major stimulations of mast cells, induces degranulation and chemical mediators release from mast cells. Since mouse mast cells release β -hexosaminidase as the result of Fc ϵ RI/IgE cross-linking (Watanabe et al., 1999; Suzuki et al., 2000), a release of β -hexosaminidase by Bcl6^{-/-} BMMCs after Fc ϵ RI/IgE cross-linking was examined by the β -hexosaminidase release assay. As shown in Fig. 4, Bcl6^{-/-} BMMCs released the similar amount of β -hexosaminidase as WT BMMCs did after Fc ϵ RI/IgE cross-linking with any amounts of DNP-HSA examined. Thus, degranulation of mast cells after Fc ϵ RI/IgE cross-linking is Bcl6-independent.

3.3. Th2 cytokine productions of Bcl6^{-/-} BMMCs stimulated by Fc ϵ RI/IgE cross-linking

Since Th2 cytokine mRNA expression is augmented in Bcl6^{-/-} T cells (Dent et al., 1997), we examined Th2 cytokine mRNA expression in Bcl6^{-/-} BMMCs stimulated by Fc ϵ RI/IgE cross-linking by RT-PCR. Expression of *IL-4* and *IL-5* mRNA was clearly augmented in Bcl6^{-/-} BMMCs after stimulation (Fig. 5A). Since expression of *IL-13* mRNA in Bcl6^{-/-} BMMCs seemed to be more than that in WT BMMCs 3 and 6 h after stimulation, the amount of *IL-13* mRNA in those activated BMMCs was carefully examined by semi-quantitative RT-PCR. The amount of *IL-13* mRNA in Bcl6^{-/-} BMMCs was 3- to 10-fold more than that in WT BMMCs 3 and 6 h after stimulation. However, there was no significant difference of *IL-6* and *TNF α* mRNA expression between Bcl6^{-/-} BMMCs and WT BMMCs after stimulation.

Fig. 5. Production of Th2 cytokines by Bcl6^{-/-} BMMCs. BMMCs were stimulated by Fc ϵ RI/IgE cross-linking with DNP-HAS (25 ng/ml). (A) Expression of Th2 cytokines in these BMMCs was assessed by RT-PCR (upper panel). The amount of *IL-13* mRNA was measured in cDNA with 3-fold dilutions (lower panel). (B) Production of IL-13 and IL-6 in the culture supernatants 24 h after stimulation was assessed by ELISA. Data represents the mean $\pm$ S.D. of triplicate cultures. Results are representative of three independent experiments. * $P < 0.01$.



Next, we examined cytokine productions in culture supernatants of Bcl6^{-/-} BMMCs stimulated by FcεRI/IgE cross-linking by ELISA. The amount of IL-13 in the culture supernatants of Bcl6^{-/-} BMMCs was more than that of WT BMMCs after FcεRI/IgE cross-linking with any amounts of DNP-HSA examined (Fig. 5B). The amount of IL-6 in the culture supernatants of Bcl6^{-/-} BMMCs was similar to that of WT BMMCs after stimulation. However, we could not detect IL-4 and IL-5 in the culture supernatants of Bcl6^{-/-} and WT BMMCs after stimulation (data not shown).

4. Discussion

Bcl6^{-/-} mice showed abnormal Th2 type inflammation (Dent et al., 1997) and the inflammation is initiated by non-lymphoid cells (Toney et al., 2000). In order to elucidate an initiator of the Th2 type inflammation, we examined functional property of mast cells from Bcl6^{-/-} mice as an initiator. There was no significant difference between Bcl6^{-/-} BMMCs and WT BMMCs in expression of surface FcεRI and c-Kit, in apoptosis induced by deprivation of IL-3, and in degranulation (release of β-hexosaminidase) induced by FcεRI/IgE cross-linking. However, Bcl6^{-/-} BMMCs stimulated by FcεRI/IgE cross-linking expressed more amounts of Th2 cytokine mRNAs such as *IL-4*, *IL-5* and *IL-13* than WT BMMCs did. The increased expression of *IL-13* mRNA in Bcl6^{-/-} BMMCs after stimulation was reflected to the amount of IL-13 protein in culture supernatants of the Bcl6^{-/-} BMMCs. However, we could not detect IL-4 and IL-5 protein in the culture supernatants of Bcl6^{-/-} and WT BMMCs after stimulation. These cytokines may be difficult to detect in supernatants of the BMMC culture by ELISA, because their immunoreactivity may be changed by the heparin produced by BMMCs or these cytokines may be degraded by protease chymase produced by BMMCs as previously reported (Okayama et al., 1995).

Mast cells have been focused on as a source of IL-4 in allergic diseases, and IL-4 is the most potent factor that induces Th2 development (Fallon et al., 2002). However, cell types that produce the initial IL-4 for the induction of Th2 development are controversial. It has been speculated that IL-4 from non-Th cells, such as mast cells and basophiles, may be required for Th2 development. For example, mast cells rapidly release large amounts of Th2 cytokines including IL-4 by FcεRI/IgE cross-linking (Kalesnikoff et al., 2001) or with lipopolysaccharide stimulation (Supajatura et al., 2002), and 90% of IL-4 producing cells in the mucosa of patients with allergic rhinitis are mast cells (Bradding et al., 1992). Since Th2 cytokines, not only IL-4 but also IL-13, are known to be important for Th2 development (McKenzie et al., 1998; Chiamonte et al., 1999), the mast cells are one of the initiators for Th2 dominance in Bcl6^{-/-} mice.

Mast cells do not mature in the bone marrow, circulate as committed progenitors and complete their maturation, and are activated in the peripheral tissues. Thus, mast cells are

considered to play an important role in peripheral tissues. Mast cells activated by various stimuli including FcεRI/IgE cross-linking and lipopolysaccharide (Kalesnikoff et al., 2001; Nakajima et al., 2002; Supajatura et al., 2002) rapidly release a number of cytokines and chemokines, and recruit and activate other immune competent cells (Levi-Schaffer et al., 1998; Skokos et al., 2001; Skokos et al., 2003). Chemical mediators produced by activated mast cells are also essential for allergic reactions. For example, histamine is an essential mediator for type I allergy and anaphylaxis (Dombrowicz et al., 1993; Naclerio, 1999; White, 1999), leukotrienes are essential for vascular permeability and bronchial reconstruction (Kanaoka et al., 2001; Maekawa et al., 2002), prostaglandin works on microvascular and also bronchial reconstruction (Diaz et al., 2002). Since the number of intraepithelial mast cells increased in Bcl6^{-/-} mice (Toney et al., 2000) and the production of Th2 cytokines was augmented in activated Bcl6^{-/-} mast cells, Bcl6^{-/-} mast cells may also be a major effector for Th2 type inflammation in Bcl6^{-/-} mice.

In summary, Bcl6 regulates Th2 type cytokine productions by mast cells stimulated by FcεRI/IgE cross-linking. Thus, Bcl6^{-/-} mast cells are one of the initiators for Th2 type inflammation in Bcl6^{-/-} mice, and Bcl6 may be a molecular target for Th2 type allergic diseases.

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