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Bcl6 Is Required for the Development of Mouse CD4+ and CD8α+ Dendritic Cells

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Th2-type inflammation spontaneously shown in Bcl6-knockout (KO) mice is mainly caused by bone marrow (BM)-derived non-lymphoid cells. However, the function of dendritic cells (DCs) in Bcl6-KO mice has not been reported. We show in this article that the numbers of CD4+ conventional DCs (cDCs) and CD8α+ cDCs, but not of plasmacytoid DCs, were markedly reduced in the spleen of Bcl6-KO mice. Generation of cDCs from DC progenitors in BM cells was perturbed in the spleen of irradiated wild-type (WT) mice transferred with Bcl6-KO BM cells, indicating an intrinsic effect of Bcl6 in cDC precursors. Although cDC precursors were developed in a Bcl6-KO BM culture with Fms-like tyrosine kinase 3 ligand, the cDC precursors were more apoptotic than WT ones. Also p53, one of the molecular targets of Bcl6, was overexpressed in the precursors. The addition of a p53 inhibitor to Bcl6-KO BM culture protected apoptosis, suggesting that Bcl6 is required by cDC precursors for survival by controlling p53 expression. Furthermore, large numbers of T1/ST2+ Th2 cells were naturally developed in the spleen of Bcl6-KO mice. Th2 skewing was accelerated in the culture of WT CD4 T cells stimulated with Ags and LPS-activated Bcl6-KO BM-derived DCs, which produced more IL-6 and less IL-12 than did WT DCs; the addition of anti–IL-6 Abs to the culture partially abrogated the Th2 skewing. These results suggest that Bcl6 is required in cDC precursors for survival and in activated DCs for modulating the cytokine profile. The Journal of Immunology, 2011, 186: 000-000.

The human proto-oncogene Bcl6 has been identified from chromosomal breakpoints involving 3q27 in diffuse large B cell lymphomas (1–3). The Bcl6 gene encodes a 92–98-kDa nuclear phosphoprotein (4, 5) that contains the BTB/POZ domain in the NH2-terminal region and Krüppel-type zinc finger motifs in the C-terminal region (1–3, 6). Because the NH2-terminal half of Bcl6 can bind to silencing mediator of retinoid and thyroid receptor protein and recruit the histone deacetylase complex to silencer regions of target genes to repress expression of these genes (7, 8), Bcl6 acts as a sequence-specific transcriptional repressor (9–14). To observe physiological functions of Bcl6, this gene was disrupted in the mouse germ line. Bcl6-knockout (KO) mice showed growth retardation (15–17) and displayed Th2-type inflammatory responses in multiple organs, especially in the heart and lungs, characterized by infiltration of eosinophils at a young adult age (15, 16, 18). This Th2 skewing of splenic CD4 T cells in Bcl6-KO mice is due to CD4 T cells and bone marrow (BM)-derived nonlymphoid cells, and one of the nonlymphoid cells is macrophages that present Ags to T cells (19). Dendritic cells (DCs) are also the most effective APCs to stimulate naïve T cells in vivo and influence Th1/Th2 differentiation. However, the functions of Bcl6-KO DCs and their role in this Th2 skewing have not been reported.

Mouse splenic DCs consist of two populations; B220+ plasmacytoid dendritic cells (pDCs) and CD11c+B220− conventional DCs (cDCs). cDCs are subdivided into CD4+ cDCs, CD8α+cDCs, and CD8α−CD4− cDCs (20) in a steady state. These DCs have different immune functions. CD4+ cDCs have not been found to produce cytokines, but they effectively present Ags to CD4 T cells (21). CD8α+cDCs perform cross-presentation of foreign Ags to CD8 T cells and are a major producer of IL-12 (21–24). CD8α−CD4− cDCs produce IFN-γ (21). cDCs and pDCs can be generated from their progenitor cells in BM cells in vitro by Fms-like tyrosine kinase 3 ligand (FLT3L) stimulation (25). Transcription factors required for generation of these subsets of DCs have been extensively studied. IRF-2, IRF-4, and RelB are essential for CD4+ cDC generation (26–28), and IRF-8 and Id2 are critical for CD8α+ cDC generation (29, 30). Because the Bcl6 gene is one of the molecular targets of IRF-8 (31) and IRF-4 (32), generation of DC subsets may be perturbed in Bcl6-KO mice. This perturbation may cause the Th2 skewing in Bcl6-KO mice.

Furthermore, as a transcriptional repressor, Bcl6 regulates expression of genes, which are related to functions of Th2 cells and their skewing, such as the IL-5 gene in CD4 T cells (33), the IL-18 gene (34), and the IL-6 gene (19) in macrophages. These results also suggested that abnormal functions of Bcl6-KO DCs cause the Th2 skewing in Bcl6-KO mice. In this study, we examined differentiation and functions of DCs in the spleen of Bcl6-KO mice and in Bcl6-KO BM culture with FLT3L. We show that numbers of CD4+ and CD8α+cDCs were dramatically reduced.
in the spleen of Bcl6-KO mice. BM cell-transfer experiments clearly showed that this reduction in cDCs was due to the intrinsic effect of Bcl6 in their precursor cells. Moreover, the profile of cytokines produced by Bcl6-KO DCs activated with LPS was different from that produced by activated wild-type (WT) DCs. We discuss the roles for Bcl6 in cDC generation and DC functions that are related to Th2 skewing.

Materials and Methods

Mice

C57BL/6 and BALB/c mice were purchased from Japan SLC (Hamamatsu, Japan). Bcl6-KO (17), Ick-Bcl6–transgenic (35), DO11.10-transgenic (36), and p53-KO (37) mice were described previously. Bcl6-KO mice were backcrossed to C57BL/6 or BALB/c mice for >10 generations. C57BL/6 (B6-Ly5.1) mice were purchased from Charles River Japan (Yokohama, Japan). All mice were maintained under specific pathogen-free conditions in the animal center of the Graduate School of Medicine, Chiba University. The care of all animals was in accordance with Chiba University Animal Care Guidelines.

BM-derived DCs

Lineage-negative (B220–, Gr-1–, CD11b–, CD8–, CD4–, CD5–, and TER119–) BM cells were magnetically separated and then used for DC induction. Lineage-negative BM cells were cultured with RPMI 1640 medium (Sigma-Aldrich, St Louis, MO) supplemented with 100 ng/ml FLT3L (R&D Systems, Minneapolis, MN), 10% FCS (Intergen, New York, NY), 50 μM 2-ME, 10 mM HEPEs, 100 μg/ml streptomycin (Wako Chemical, Osaka, Japan), and 100 U/ml penicillin G (Banyu Pharmaceutical, Tokyo, Japan) at 37°C with 5% CO2. In the indicated experiment, lineage-negative BM cells were labeled with CFSE (Molecular Probes, Eugene, OR), according to the manufacturer’s instructions, or with 10 μM pMithiophrin-α (PFTα) (Calbiochem, San Diego, CA).

Immunization of mice with Ag-pulsed BM-derived DCs

BALB/c mice were injected i.v. with DO11.10 CD4 T cells (5 × 10^6) 24 h before immunization. BM-derived DCs from Bcl6-KO and WT mice were stimulated with LPS (10 μg/ml; Sigma-Aldrich) for 12 h and incubated with OVA peptide (OVA323–329). These OVA-pulsed DCs (5 × 10^4) were injected into the footpads of reconstituted mice. Popliteal lymph node cells were harvested at day 4 after immunization and cultured with WT spleen APCs and OVA peptide for 72 h. The amount of cytokines in the culture supernatants was measured using a Cytometric Bead Array (CBA) kit (BD Pharmingen, San Diego, CA), according to the manufacturer’s instructions.

Stimulation of CD4 T cells with BM-derived DCs

DO11.10 CD4 T cells (1 × 10^6) were cultured with LPS-activated BM-derived DCs (5 × 10^4) and OVA peptide for 7 days. These cultured CD4 T cells were stimulated with PMA (1 μg/ml) and ionomycin (1 μg/ml) for 6 h; monensin (2 μM) was added for the last 4 h at 37°C. Restimulated CD4 T cells and BM-derived DCs were stained with various Abs for surface staining and then were subjected to intracellular staining for various Abs using the CytoFix/Cytoperm kit (BD Pharmingen), according to the manufacturer’s instructions. Abs used for intracellular staining included Bcl2–FITC (BD Pharmingen), Bcl-x–biotin (Chemicon International, Temecula, CA), p53–PE (BD Pharmingen), IFN-γ–allophycocyanin (BD Pharmingen), and IL-4–PE (BD Pharmingen).

Assay for cytokines and chemokine

The amounts of cytokines and chemokines in culture supernatants were measured with the CBA.

Statistical analysis

Statistical analysis was performed using the unpaired t test; p values <0.05 were considered significant.

Results

Numbers of CD4+ and CD8α+ cDCs are reduced in the spleen of Bcl6-KO mice

We examined subsets of DCs in the spleen of Bcl6-KO mice with a C57BL/6 background. As shown in Fig. 1A, the percentage and number of cDCs (CD11c+) in Bcl6-KO mice were less than those in WT mice. In contrast, the percentage and number of pDCs (B220+ mPDCA-1+) were compatible between them. The percentages and numbers of CD4+ and CD8α+ cDCs in Bcl6-KO mice were less than those in WT mice (Fig. 1B). However, the number of CD4+ CD8α–cDCs in Bcl6-KO mice was similar to that in WT mice. Although there were a few CD4+ and CD8α+ cDCs in Bcl6-KO mice, the amount of CD11b on Bcl6-KO CD4+ cDCs was more than that on WT CD4+ cDCs (Fig. 1C). There were many CD8α+ cDCs in Bcl6-KO mice, and those corresponded to the CD24+CD11b+ cDC population. A few CD8α+ cDCs were also detected in WT mice. CD135 (FLT3) expression was rather low on CD4+ cDCs, CD4+ CD8α+ cDCs, and pDCs, but it was high on CD8α– cDCs in WT mice (Fig. 1D). CD135+CD4+ cDCs were missing in Bcl6-KO mice. The amount of CD135 on Bcl6-KO CD4+ CD8α– cDCs was more than that on WT CD4+ CD8α– cDCs. A similar impairment of cDC generation was observed in the spleen of Bcl6-KO mice with a different genetic (BALB/c) background (data not shown).
We analyzed the amount of Bcl6 mRNA in FACS-isolated DC subsets from the spleen of WT mice by real-time RT-PCR. The relative amounts in CD4+ and CD8α+ cDCs were 5.1- and 9.8-fold greater, respectively, than in CD4−CD8α− cDCs (Fig. 1E). The amount of Bcl6 in pDCs was comparable to that in CD4−CD8α− cDCs. These results suggested that Bcl6 is required for the generation of CD4+ and CD8α+ cDCs, which express a larger amount of Bcl6 in the spleen.

**Bcl6 in cDC precursor cells is required for generation of cDCs**

We examined the presence of DC progenitor cells, which express CD135 or Ly49Q, in BM cells of Bcl6-KO mice. The percentages of CD135+ or Ly49Q+ cells in Bcl6-KO BM cells were comparable to those in WT BM cells (Fig. 2A). To examine whether the impaired cDC generation in the spleen of Bcl6-KO mice was due to a defect of Bcl6 in cDC precursor cells, we transferred lineage-negative Bcl6-KO (Ly5.2) and WT (Ly5.1) BM cells at a ratio of 1:1 into irradiated (Ly5.1×Ly5.2) F1 WT mice. As shown in Fig. 2B, the percentage of CD11c+ cDCs derived from Bcl6-KO BM cells was less than that from WT BM cells in the recipient spleen. However, the percentage of B220−mPDCA-1+ pDCs derived from Bcl6-KO BM cells was comparable to that from WT BM cells. The percentages and numbers (data not shown) of CD4+ and CD8α+ cDCs derived from Bcl6-KO BM cells were also less than those from WT BM cells.

BM culture with FLT3L is a well-established system to examine the in vitro generation of DCs, especially CD8α− cDCs and B220+ pDCs (25). To confirm the role of Bcl6 in cDC generation, lineage-negative Bcl6-KO and WT BM cells were cultured with FLT3L for 7 d. WT BM cells differentiated into B220+ pDCs, as well as CD24+ CD11bnull and CD24+CD11b+ DCs in the B220− (cDC) population (Fig. 3A). In the Bcl6-KO BM culture, the percentage of CD24+ CD11bnull cDCs, but not CD24+CD11b+ cDCs and B220+ pDCs, was very low. The number of B220− cDCs, but not B220+ pDCs, in the Bcl6-KO BM culture was significantly less than in the WT BM culture. There were fewer Bcl6-KO CD24+CD11bnull cDCs than...
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FIGURE 3. Differentiation of DCs from DC progenitor cells in Bcl6-KO BM cells in vitro. Lineage-negative BM cells from Bcl6-KO and WT mice were cultured with FLT3L for 7 d. DCs in the culture were analyzed by FACS. A, A representative FACS profile. The numbers indicate the percentage of each gate. Open bars: WT BM cells. Filled bars: Bcl6-KO BM cells. Data are a representative of five independent experiments. Results are mean ± SD of triplicate cultures. **p < 0.005. B, BM cells from Bcl6-KO (Ly5.2) and WT (Ly5.1) mice were mixed and cultured with FLT3L. DCs (Ly5.1+ or Ly5.2+) in the culture were analyzed by FACS. The numbers indicate the percentage of each gate. Data are presented as a representative of three independent experiments.

WT ones. A similar impairment of cDC generation was observed in the BM culture of Bcl6-KO mice with a BALB/c background (data not shown).

To examine the intrinsic effect of Bcl6 in cDC precursor cells on cDC generation, lineage–negative Bcl6-KO (Ly5.2) and WT (Ly5.1) BM cells were mixed at a 1:1 ratio and stimulated with FLT3L for 7 d. The Ly5.1+ WT subset accounted for 82% of the DCs (Fig. 3B). Bcl6-KO CD24CD11b cDCs were fewer in percentage and number compared with the WT cDCs (0.29 ± 0.02 × 10⁵/well versus 9.4 ± 0.4 × 10⁵/well, respectively; p = 0.00048). Although the percentage of Bcl6-KO CD24CD11b cDCs was greater than WT cDCs, the number (4.1 ± 0.3 × 10⁵/well) of Bcl6-KO CD24CD11b cDCs was significantly less than that of WT ones (7.1 ± 0.1 × 10⁵/well; p = 0.0014), supporting the intrinsic effect of Bcl6 in cDC precursor cells on the generation of cDCs.

Bcl6 is required for protecting apoptosis of cDC precursor cells

Kinetics of Bcl6 mRNA expression in DCs generated in WT BM culture with FLT3L was analyzed by real-time RT-PCR. Bcl6 mRNA expression was induced in BM-derived DCs from day 2 of culture, and the amount at day 2 of culture was the greatest until day 5 of culture (Fig. 4A), suggesting the perturbation of DC generation in the Bcl6-KO BM culture after day 2 of culture. The kinetics of DC generation in the Bcl6-KO BM culture with FLT3L was compared with that in the WT BM culture. Lineage-negative B220CD24CD11b cDCs at day 0 of culture differentiated into CD24CD11b+ cDCs in Bcl6-KO and WT BM culture at day 2 of culture (Fig. 4B). CD24CD11b and CD24CD11b cDC precursors were generated in the WT BM culture after day 4. CD24CD11b cDC precursors, but not CD24CD11b cDC precursors, were generated in the Bcl6-KO BM culture after day 4. B220 pDC precursors were generated from DC progenitor cells in Bcl6-KO and WT BM cultures after day 4 (data not shown).

The relationship between cell proliferation and generation of CD24CD11b cDC precursors was examined using CFSE-labeled BM cells. Similar to WT BM cells, Bcl6-KO BM cells proliferated quickly after day 2 of culture (Fig. 4C). CD11b cDCs developed in the WT BM culture following cell-cycle progression. Bcl6-KO CD11b cDCs proliferated well, but differentiation of CD11b cDCs in the proliferating cells was impaired. Furthermore, the percentage of CD11b cDCs in the nondividing cells (dotted square) increased in the Bcl6-KO BM culture at day 5.

To detect apoptotic cells in the Bcl6-KO BM culture, CFSE-labeled or unlabeled BM-derived DCs were stained with Annexin V at day 5 of culture. The percentage of Annexin V+ cells in the proliferating Bcl6-KO DC precursors was greater than in the WT DC precursors (Fig. 4D). The percentage of Annexin V+ cells in Bcl6-KO CD24CD11b cDC precursors and CD24CD11b cDC precursors was greater than in WT ones. However, there was no difference in the percentages in B220 pDCs between those cultures. These results indicate that Bcl6 protects cell death of cDC precursor cells developed in BM culture with FLT3L.

We analyzed the amounts of apoptosis-related proteins (Bcl-2, Bcl-xL, and p53) in CD24CD11b cDC precursors, CD24CD11b cDC precursors, and B220 pDCs developed in the BM culture at day 5. The mean fluorescent intensity (MFI) of Bcl-2 in Bcl6-KO CD24CD11b cDC precursors was slightly greater than that in WT ones (Fig. 4E). The amounts of Bcl-xL and p53 in three subsets of Bcl6-KO DCs were greater than in WT DCs. The difference in the MFI of Bcl-2, Bcl-xL, and p53 between Bcl6-KO and WT CD24CD11b cDC precursors was the largest among three BM-derived DC subsets. Because the p53 gene is one of the molecular targets of Bcl6 (39), the amount of p53 mRNA in Bcl6-KO BM-derived DCs was analyzed by real-time RT-PCR; it was 5-fold greater than in WT BM-derived DCs (data not shown).

PFTα (p53 inhibitor) was added to the Bcl6-KO BM culture at the beginning of culture to examine the generation of CD24CD11b cDC precursors at day 7. Although its addition did not affect DC generation in the WT BM culture, it increased the percentage and number of CD24CD11b cDC precursors in the Bcl6-KO BM culture (Fig. 4F). Furthermore, the number of splenic DCs in (p53-KO × Bcl6-KO) double-KO mice was compared with that in p53-KO and Bcl6-KO mice. The numbers of CD11c+ cells and CD4+ and CD8+ DCs in double-KO mice were similar to those in p53-KO mice and were greater than those in Bcl6-KO mice (Fig. 4G). These results indicated that downregulation of p53 expression by Bcl6 is important for normal CD11c+ DC generation.

Th2 cells are dominantly induced from naive WT CD4 T cells stimulated with activated Bcl6-KO DCs and Ags

Bcl6-KO mice spontaneously display Th2-type inflammation (18). Indeed, T1/ST2, which is a marker for Th2 cells (40), was detected in significant percentages of CD4 T cells in the spleen of naive Bcl6-KO mice with a C57BL/6 or BALB/c background (Fig. 5A). However, T1/ST2 CD4 T cells were not detected in the spleen of naïve C57BL/6 or BALB/c mice. T1/ST2 CD4 T cells in the spleen of Bcl6-KO mice were CD44+ (Fig. 5B), CD62L+, and CD45RB− (data not shown) memory phenotype CD4 T cells. When Bcl6-KO mice were mated with lck-Bcl6–transgenic mice to generate Bcl6-KO-Rescue mice, in which the exogenous Bcl6 is expressed only in
T cells (35), the percentage of T1/ST2⁺ CD4 T cells in the spleen of Bcl6-KO Rescure mice was similar to that of Bcl6-KO mice, suggesting that non-T cells are the main cells responsible for inducing Th2 skewing in Bcl6-KO mice.

Lineage-negative Bcl6-KO (Ly5.2) and WT (Ly5.1) BM cells were transferred into irradiated (Ly5.1⁺Ly5.2) F1 WT mice. We examined the percentage of T1/ST2⁺ CD4 T cells in the spleen of BM chimeric mice. When mice were simultaneously transferred with Bcl6-KO and WT BM cells, the percentages of T1/ST2⁺ CD4 T cells derived from Bcl6-KO and WT BM cells were greater than in mice that received WT BM cells (Fig. 5C). Next, OVA-pulsed BM-derived DCs were injected into the footpads of mice reconstituted with DO11.10 CD4 T cells. Popliteal lymph node cells from the immunized mice were cultured with WT splenic APCs and OVA peptide for 72 h. The amount of IL-4, but not IFN-γ, in culture supernatants of lymph node cells from mice injected with Bcl6-KO DCs was greater than in mice injected with WT DCs (Fig. 5D). These results indicate that BM-derived DCs are responsible for inducing Th2 skewing in Bcl6-KO mice.

To examine the function of BM-derived DCs, lineage-negative BM cells from Bcl6-KO mice with a BALB/c background were cultured with FLT3L for 7 d. A, Bcl6 mRNA expression in differentiating WT DCs was analyzed by real-time RT-PCR. B, Differentiating cDCs [B220⁺ gated] were analyzed by FACS. The numbers indicate the percentage of each gate. Data are representative of five independent experiments. C, CFSE-labeled lineage-negative BM cells were cultured. CFSE and CD11b expression on DCs in the culture was analyzed by FACS. The numbers indicate the percentage of each square. Data are presented as a representative of three independent experiments. D, Annexin V expression on CFSE-labeled DCs; each subset of DCs in the culture was analyzed on day 5. Shaded graphs: WT mice. Open graphs: Bcl6-KO mice. The numbers indicate the percentage of each quadrant and Annexin V⁺ DCs. Data are a representative of three independent experiments. E, Apoptosis-related proteins in each subset of DCs were examined by FACS. Shaded graph: WT mice. Open graph: Bcl6-KO mice. The numbers indicate the MFI for each subset of DCs. Data are a representative of three independent experiments. F, PFTα was added to the BM culture at the beginning of culture. Representative FACS profile and the cell number of each subset of DCs. The MFI on activated Bcl6-KO DCs was similar to those on activated WT DCs 24 h after stimulation (Fig. 6A). Cytokine and chemokine production by activated Bcl6-KO DCs was also greater than that secreted by activated WT DCs, and TNF-α production by Bcl6-KO DCs was also greater than that of WT DCs. **p < 0.005. G, DCs in the spleen of p53-KO, Bcl6-KO, and (p53-KO × Bcl6-KO) double-KO mice were analyzed by FACS. The numbers indicate the percentage of each square or quadrant.
of WT DCs (Fig. 6B). However, IL-12 secretion by Bcl6-KO DCs was less than that of WT DCs. These results were repeated using BM-derived DCs from Bcl6-KO mice with a C57BL/6 background (data not shown). Furthermore, these results, with the exception of TNF-α production, were confirmed using FACS-sorted splenic CD11c+ DCs stimulated with LPS for 24 h (Fig. 6C).

Naive CD4 T cells from D011.10-transgenic mice were stimulated with LPS-activated BM-derived DCs and OVA peptides for 14 d. The percentage of T1/ST2+ CD4 T cells in the CD4 T cells stimulated with Bcl6-KO DCs was greater than that with WT DCs (Fig. 7A). Because IL-6 (41) and IL-12 (42) affect Th2 and Th1 skewing, respectively, Abs against IL-6 or IL-12 were added to the CD4 T cell-activation culture at the beginning of culture. The addition of anti–IL-6 Abs to the culture partially abrogated the Th2 skewing with Bcl6-KO DCs but not that with WT DCs (Fig. 7B). The neutralization of IL-12 in the culture by anti–IL-12 Abs increased the percentage of T1/ST2+ CD4 T cells stimulated with WT DCs but not that stimulated with Bcl6-KO DCs. To confirm Th2 skewing of CD4 T cells stimulated with BM-derived DCs, those cultured CD4 T cells were restimulated with PMA and ionomycin at day 7. IFN-γ and IL-4 production in the activated CD4 T cells and in the culture supernatants was examined by FACS and CBA, respectively. CD4 T cells stimulated with Bcl6-KO DCs contained less IFN-γ-producing cells and more IL-4-producing cells than did those stimulated with WT DCs (Fig. 7C). The amounts of IL-4 and IFN-γ in the culture supernatants correlated with the percentages of IL-4+ and IFN-γ-producing cells. These results indicated that Bcl6-KO BM-derived DCs act as effective Th2 inducers by producing greater amounts of IL-6 and lesser amounts of IL-12.

Discussion

The number of cDCs, especially CD4+ and CD8αα cDCs, was significantly reduced in the spleen of Bcl6-KO mice. The numbers of hematopoietic stem cells, common lymphoid precursors, common myeloid precursors (data not shown), and lineage-negative FLT3+ cells in BM cells were comparable between Bcl6-KO and WT mice. Recently, a single progenitor was reported for pDCs and cDCs (43, 44), and the number of pDCs in the spleen of Bcl6-KO mice was similar to that in WT mice. These results suggested that DC progenitors develop normally in the BM of Bcl6-KO mice. The abnormal development of cDCs in Bcl6-KO mice was confirmed by BM culture with FLT3L. GM-CSF is also an important cytokine for DC development (45), and the number of cDCs that developed in the Bcl6-KO BM culture with GM-CSF instead of FLT3L was similar to that in the WT BM culture (data not shown). Because the BM culture with FLT3L is more physiological for the development of splenic DC precursor than is the BM culture with GM-CSF (46), Bcl6 may be required for generation of cDCs in vivo from DC progenitors with FLT3L stimulation.

There were few CD8αα cDCs but detectable CD8ααCD11bnull cDCs in the spleen of Bcl6-KO mice, and CD8ααCD11bnull cDCs belonged to the CD24+CD11bnull cDC population. A few CD8ααCD11bnull cDCs were also detected in the spleen of WT mice. CD8αα on cDC precursors is upregulated by the maturation process in the spleen of WT mice (47), suggesting that CD8ααCD11bnull cDCs in the spleen are precursors of CD8αα cDCs and that Bcl6 is required for the differentiation of CD8αα cDCs from CD8ααCD11bnull cDCs in the spleen. Kinetics of DC generation in the BM culture with FLT3L supports the differentiation of CD24+CD11bnull→CD8αα cDCs from CD24+CD11bnull→CD8αα→CD11bnull cDC precursors. CD24+CD11b+ BM cells differentiated into CD24+CD11b+ cDC precursors at day 2 of culture and into CD24+CD11bnull→CD11bnull cDC precursors at day 4 of culture. Furthermore, the cell-proliferation analysis using CFSE-labeled BM cells demonstrated that CD11bnull cDCs developed from CD11b+ cDC precursors following cell-cycle progression. These results suggested that CD24+CD11bnull→CD8αα→CD11bnull→CD11bnull→CD11bnull→CD11bnull→CD8αα→cDCs. Because the differentiation of CD24+CD11bnull cDC precursors from CD24+CD11bnull cDC precursors was perturbed in the Bcl6-KO BM culture, Bcl6 is also required for the differentiation of CD24+CD11bnull cDC precursors in vitro.

The largest percentage of Annexin V+ cells was observed within CD24+CD11bnull cDC precursors in the Bcl6-KO BM culture with
FLT3L. Although a large percentage of CD24+CD11bdullCD8^^a+CD4 cDCs was detected in the spleen of Bcl6-KO mice, their number (0.7 ± 0.02 × 10^7/spleen) in the spleen of Bcl6-KO mice was less than that (1.9 ± 0.16 × 10^7/spleen) of CD24+CD11b^dull CD8^a+^dull cDCs in WT mice (p = 0.0047). These results suggested that differentiating CD24^+^CD11b^dull^CD8a^+^dull cDC precursors in the spleen of Bcl6-KO mice die by apoptosis. Bcl6 mRNA was strongly detected in CD8^a+ cDCs in the spleen and induced in CD24^+^CD11b^+ cDC precursors in the BM culture at day 2 of culture, suggesting that Bcl6 is required for differentiating CD8a^+^dull cDC precursors to protect apoptosis. Among the three DC subsets, the difference in the MFI of Bcl-2, Bcl-xL, and p53 expression between the Bcl6-KO and WT cDC population was greatest in the CD24^+^CD11b^+ cDC precursors. The percentage of CD11b^+ cDC precursors within the nondividing cells increased in the CFSE-labeled Bcl6-KO BM culture at day 5. Furthermore, the addition of the p53 inhibitor (PFTα) to the Bcl6-KO BM culture increased the percentage and number of CD24^+^CD11b^+ cDC precursors.

**FIGURE 6.** Function of activated Bcl6-KO DCs. Lineage-negative BM cells from Bcl6-KO and WT mice with a BALB/c background were cultured with FLT3L for 7 d. These cells were stimulated with LPS for 24 h. Open bars: WT BM-derived DCs. Filled bars: Bcl6-KO BM-derived DCs. A, IA^d^ and CD86 expression (MFI) on those activated DCs were examined by FACS. Data are a representative of three independent experiments. B, Cytokine secretion from those activated DCs was examined in the culture supernatant. Results are mean ± SD of triplicate cultures. C, CD11c^+^ DCs from spleens of Bcl6-KO and WT mice were stimulated with LPS for 24 h. IA^d^ and CD86 expression (MFI) on those activated DCs were examined by FACS. The amount of cytokines in the culture supernatant was examined. Results are mean ± SD of triplicate cultures. *p < 0.05; **p < 0.005. ND, not detected.

**FIGURE 7.** Effect of Bcl6-KO DCs on Th2 skewing of CD4 T cells. Naive WT CD4 T cells of DO11.10 mice were cultured with LPS-activated DCs derived from Bcl6-KO or WT BM culture with FLT3L and OVA peptides for 14 d. T1/ST2 expression on activated CD4 T cells was examined by FACS. A, Numbers indicate the percentage of each gate. Data are a representative of three independent experiments. B, Anti–IL-6 or anti–IL-12 Abs were added to the culture at the beginning of culture. Open bars: WT BM cells. Filled bars: Bcl6-KO BM cells. Results are mean ± SD of triplicate cultures. C, At day 7 of culture, CD4 T cells were restimulated with PMA and ionomycin for 6 or 24 h. IFN-γ and IL-4 production in activated CD4 T cells and in the culture supernatant were examined by FACS and CBA, respectively. Representative FACS profiles (left panels). Open graphs: restimulated CD4 T cells. Shaded graphs: nonrestimulated CD4 T cells. The numbers indicate the percentage of each gate. Percentage of cytokine-producing Th cells in the culture (middle panel). Open bars: WT DCs. Filled bars: Bcl6-KO DCs. Results are mean ± SD of three independent experiments. Amount of cytokines in the culture supernatant (right panels). Open bars: WT DCs. Filled bars: Bcl6-KO DCs. Results are mean ± SD of triplicate cultures. *p < 0.05; **p < 0.01; ***p < 0.005.
The impairment of DC development in Bcl6-KO mice was rescued in the (p53-KO × Bcl6-KO) double-KO mice. Because p53 is one of the molecular targets of Bcl6 (39), p53 expression controlled by Bcl6 in CD24 lowCD11b dimDC precursors may be critical for DC development, and Bcl-2 and Bcl-xL may be produced in CD24 lowCD11b dimDC precursors to protect their apoptosis.

Differentiation of CD4 + DCs can be traced by the expression of CD24 and CD11b on their cell surface. The majority of CD4 + DCs in the spleen belong to the CD24 lowCD11b + cDC population. BM culture with FLT3L demonstrated that CD24 +CD11d BM cells differentiated into CD24 +CD11b + DC precursors at day 2 and into CD24 lowCD11b + DC precursors at day 4, suggesting that CD24 +CD11b + DC precursors differentiate into CD24 lowCD11b +CD4 + DCs. CD24 lowCD11b +CD4 + DCs were barely detected in the spleen of Bcl6-KO mice. However, the number of CD24 lowCD11b + cDC precursors in the Bcl6-KO BM culture was slightly less than in the WT BM culture. Thus, Bcl6 is strongly required for generation of CD4 + DCs in vivo but not for the generation in vitro with FLT3L stimulation. These results suggested that CD24 lowCD11b + DCs that developed in the BM culture are still precursors of CD4 + DCs in the spleen and that Bcl6 is required for the differentiation of CD24 lowCD11b + DCs into CD24 lowCD11b +CD4 + DCs in vivo.

Bcl6 mRNA was induced in DC precursors developed in the BM culture with FLT3L, and the generation of DCs was perturbed in the Bcl6-KO BM culture with FLT3L but not in that culture with GM-CSF (data not shown), suggesting a role for Bcl6 in the signal transduction initiated by FLT3. FLT3L-KO mice showed reduced numbers of DCs and NK cells (48), and Bcl6-KO mice have a normal number of NK cells (data not shown), suggesting that Bcl6 is important for FLT3-mediated DC cell development but not for FLT3-mediated NK cell development. Several transcription factors were demonstrated to be critical for DC development. IRF-2–KO (26), IRF-4–KO (27), and RelB-KO (28) mice showed impaired generation of CD4 + DCs. IRF-8–KO (29) and Id2-KO (30, 49) mice lacked CD8a + DCs. However, the type of impairment of DC development in the spleen of those KO mice was not similar to that in Bcl6-KO mice, and the amounts of those transcription factors (IRF-2, IRF-4, RelB, IRF-8, and Id2) in Bcl6-KO BM-derived DCs were similar to those in WT DCs (data not shown). Further study is required to elucidate the molecular targets of Bcl6 (39), p53 expression controlled by Bcl6 in the (p53-KO × Bcl6-KO) double-KO mice. Because p53 is one of the molecular targets of Bcl6, p53 expression controlled by Bcl6 in the (p53-KO × Bcl6-KO) double-KO mice may be critical for the Th1/Th2 balance via the development of normal DC subsets and the regulation of DC functions, including IL-6 and IL-12 production.

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Disclosures
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