Bcl6 is essential for the generation of long-term memory CD4$^+$ T cells

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Abstract

Bcl6 plays a role in the generation and maintenance of memory CD8$^+$ T cells. We analyzed here a role for Bcl6 in the generation of long-term memory CD4$^+$ T cells. Naive CD45RB$^+$ CD4$^+$ T cells from Bcl6-deficient DO11.10 (KJ1.26$^+$) transgenic mice were transferred into BALB/c mice and immunized with ovalbumin peptide and LPS. Long-term memory KJ1.26$^+$ CD4$^+$ T cells from wild-type mice were detected in the spleen, lungs and liver during 10 weeks after immunization; however, Bcl6-deficient KJ1.26$^+$ CD4$^+$ T cells were vanished completely in those organs 4 weeks after immunization. Since memory CD4$^+$ T cells can be generated from effector CD4$^+$ T cells, properties of Bcl6-deficient effector CD4$^+$ T cells were compared with those wild-type effector CD4$^+$ T cells 10 days after immunization. Numbers of IFN-γ-non-producing CD45RB$^-$, CD62L$^+$ or IL-7Rα$^+$ effector CD4$^+$ T cells in the spleen, lungs and liver were similar between Bcl6-deficient and wild-type CD4$^+$ T cells. However, the percentage of apoptotic cells in Bcl6-deficient effector CD4$^+$ T cells was higher than that in wild-type effector CD4$^+$ T cells. At the late effector phase, the number of IFN-γ-non-producing cells and the percentage of apoptotic cells in Bcl6-deficient CD4$^+$ T cells were smaller and higher than those in wild-type CD4$^+$ T cells, respectively. These data suggest that Bcl6 in CD4$^+$ T cells plays a role in protection of memory precursor CD4$^+$ T cells from apoptosis and may involve in survivability of long-term memory CD4$^+$ T cells.

Introduction

Molecular mechanisms involved in the generation of immune memory cells are largely unknown and their elucidation is the key to design strategies to control the generation of immunity. Many factors are involved in the generation of immune memory cells, including memory B cells, CD4$^+$ T cells and CD8$^+$ T cells (1–6), and each factor controls the generation of each lineage of memory cells. However, no general factors for the generation of immune memory cells have been demonstrated. We have recently demonstrated that the proto-oncogene Bcl6 is essential for the generation of high-affinity memory B cells in germinal centers (7, 8) and also controls the generation and maintenance of antigen-specific and lymphopenia-induced memory CD8$^+$ T cells (9, 10). Thus, Bcl6 may be one of the general factors involved in the generation of immune memory cells although the role for Bcl6 in the generation of memory CD4$^+$ T cells has never been reported.

The human proto-oncogene Bcl6 has been identified from chromosomal breakpoints involving 3q27 in diffuse large B cell lymphomas (11–13). The Bcl6 gene encodes a 92- to 98-kDa nuclear phosphoprotein (14, 15) that contains the BTB/POZ domain in the NH$_2$-terminal region and Krüppel-type zinc finger motifs in the COOH-terminal region (11–13, 16). Since the NH$_2$-terminal half of Bcl6 can bind to silencing mediator of retinoid and thyroid receptor proteins which can recruit histone deacetylase (17, 18), Bcl6 can function as...
a sequence-specific transcriptional repressor (19–24). Some of the target genes for Bcl6 in T cells (25) and germinal center B cells (26) have been identified. Bcl6 is ubiquitously expressed in various tissues, including mature CD4+ T cells (16), and all hematopoietic lineages, including mature lymphocytes, can develop in Bcl6-deficient mice (16). Thus, we studied the role for Bcl6 in the generation of memory CD4+ T cells, especially long-term memory CD4+ T cells, using Bcl6-deficient CD4+ T cells from DO11.10 transgenic mice.

After mice are immunized with antigen in vivo, naive antigen-specific CD4+ T cells resided in secondary lymphoid tissues proliferate and differentiate into effector CD4+ T cells which migrate to lungs and liver within 10 days after immunization. Long-term memory CD4+ T cells can be generated from effector CD4+ T cells 4 weeks after immunization (27, 28). IFN-γ-producing effector CD4+ T cells are short-lived and long-term memory CD4+ T cells can be generated from IFN-γ-non-producing CD45RB+CD62L+ or IL-7Rα+ effector CD4+ T cells (5, 6, 29). But the mechanisms of generation and maintenance of IFN-γ-non-producing effector CD4+ T cells are not known. We show here that Bcl6-deficient effector CD4+ T cells were normally generated until 3 weeks after immunization. However, IFN-γ-non-producing Bcl6-deficient effector CD4+ T cells were not maintained in the spleen, lungs and liver for >4 weeks after immunization. Furthermore, the number of apoptotic cells in Bcl6-deficient CD4+ T cells was larger than that in wild-type CD4+ T cells at the effector phase and the late effector phase. Thus, Bcl6 in CD4+ T cells plays a role in protection of memory precursor CD4+ T cells from apoptosis and may involve in survivability of long-term memory CD4+ T cells.

Methods

Animals

BALB/c mice were purchased from Japan SLC Co. Ltd (Hamamatsu, Japan). Bcl6-deficient mice (7) were back-crossed to BALB/c mice >10 generations and crossed to the DO11.10 transgenic mice, which express the TCR specific for ovalbumin peptide (OVA) OVA323–329 in context of the MHC class II molecule I-Aq (30). The TCR can be detected by anti-KJ1.26 antibody (30). These mice were maintained under specific pathogen-free conditions in the animal center of Chiba University School of Medicine.

Flow cytometric analysis

Single cells were stained with various mAbs as follows: 10^6 cells were first blocked with unconjugated anti-CD32/16 (2.4G2, BD PharMingen, San Diego, CA, USA), followed by incubation with biotinylated antibodies and then incubated with directly conjugated antibodies and streptavidine–PerCP (BD PharMingen). These cells were analyzed on a FACSCalibur. The following antibodies were used for staining: CD4 (RM4-1)–allophycocyanin, CD44 (IM7)–PE, CD62L (MEL-14)–FITC, CD62L–PE, CD62L–biotin, CD45RB (16A)–FITC, CD45RB–PE, CD127 (B12-1)–biotin, CD132 (JORO50)–PE (BD PharMingen), annexin V–biotin (Bender MedSystems, San Bruno, CA, USA), KJ1.26–FITC (a gift from Taki, Shinshu University, Japan) and KJ1.26–biotin (a gift from Nakayama, Chiba University, Japan).

In vitro T cell differentiation cultures

Naive CD45RB+B+ T cells were sorted from spleen cells of DO11.10 transgenic mice using a FACSVantage. These sorted CD4+ T cells (1.5 × 10^5) were labeled with carboxyfluorescein diacetate succinimidyl ester (CFSE; Molecular Probes, Eugene, OR, USA) and stimulated with OVA (OVA323–329, 10 μM) and irradiated (3000 rads) BALB/c spleen cells as antigen-presenting cells (APCs) (1 × 10^5) in the presence of IL-2 (25 U ml⁻¹; PeproTech EC, London, UK), IL-12 (100 U ml⁻¹) and anti-IL-4 mAb (11B11; 25% culture supernatant) for T1,1-skewed condition (31). For T2-skewed condition, CD4+ T cells were stimulated as above in the presence of IL-2 (25 U ml⁻¹), IL-4 (100 U ml⁻¹) and anti-IFN-γ mAb (R4.6A2; 25% culture supernatant) (31).

Adaptive transfer of CD4+ T cells

Sorted splenic CD45RB+B+ T cells (5 × 10^6) from DO11.10 transgenic mice were transferred intravenously into BALB/c mice. These mice were injected intravenously with OVA (OVA323–329, 100 μg) plus LPS (25 μg) on the next day of transfer (27).

Intracellular staining

Immunized BALB/c mice transferred with CD4+ T cells were re-stimulated with OVA (OVA323–329, 100 μg) intravenously 10 days after the primary immunization. Spleen, lungs and liver were isolated from the mice 2 h after re-stimulation and then minced to make a single-cell suspension. These cells were incubated in medium supplemented with collagenase (1 mg ml⁻¹) and monensin (2 μM) for 20 min at 37°C. After washing the cells with PBS, these cells were stained with various antibodies for surface staining and then subjected to intracellular staining for IFN-γ (anti-IFN-γ–PE, BD PharMingen), using the CytoFix/Cytoperm kit (BD PharMingen), according to the manufacturer’s instructions. In indicated experiments, spleen cells from immunized mice or cultured cells were re-stimulated with OVA (OVA323–329, 10 μM) for 6 h, and then these cells were subjected for intracellular staining. For bromodeoxyuridine (BrdU) analysis, immunized mice were injected with BrdU intra-peritoneally and spleen cells were harvested 3 h after injection. These cells were stained with various antibodies for surface staining and then subjected to intracellular staining for BrdU (anti-BrdU–FITC, BD PharMingen) according to the manufacturer’s instructions.

Proliferation assay

Spleen cells from immunized mice were stimulated with OVA (OVA323–329, 10 μM) and cultured for 7 days using complete medium supplemented with IL-7 (10 ng ml⁻¹; PeproTech EC). Live cell numbers were counted under a microscope after staining with trypan blue (Invitrogen Corporation, Auckland, New Zealand).

Statistical analysis

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

A role for Bcl6 in the generation of long-term memory CD4+ T cells in vivo

In order to examine the generation of long-term memory CD4+ T cells, we performed transfer experiments. Naive CD45RB+ KJ1.26+ CD4+ T cells from spleens of Bcl6-deficient DO11.10 transgenic mice or wild-type DO11.10 transgenic mice were transferred into BALB/c mice intravenously. Mice were immunized with OVA and LPS on the next day of transfer. The presence of KJ1.26+ CD4+ T cells was examined in the spleen, lungs and liver of these BALB/c mice 10 weeks after immunization. KJ1.26+ CD4+ T cells were clearly detected in the spleen, lungs and liver of BALB/c mice transferred with wild-type KJ1.26+ CD4+ T cells (Fig. 1A and B). However, no KJ1.26+ CD4+ T cells were found in those organs of BALB/c mice transferred with Bcl6-deficient KJ1.26+ CD4+ T cells. We then examined the presence of KJ1.26+ CD4+ T cells in the spleen, lungs and liver of BALB/c mice transferred with Bcl6-deficient KJ1.26+ CD4+ T cells at various time points after immunization. Bcl6-deficient KJ1.26+ CD4+ T cells were detected in these organs 10 days after immunization and their numbers in the spleen and lungs were similar to those of wild-type KJ1.26+ CD4+ T cells (Fig. 1C). The number of Bcl6-deficient KJ1.26+ CD4+ T cells in the liver was significantly larger than that of wild-type KJ1.26+ CD4+ T cells. Bcl6-deficient KJ1.26+ CD4+ T cells could be detected in those organs until 3 weeks after immunization although their number in the spleen was smaller than that of wild-type KJ1.26+ CD4+ T cells. However, Bcl6-deficient KJ1.26+ CD4+ T cells were not detected in those organs.

Fig. 1. Survival of Bcl6-deficient KJ1.26+ CD4+ T cells after activation in vivo. Naive CD45RB+ KJ1.26+ CD4+ T cells from Bcl6-deficient or wild-type mice were transferred intravenously into normal BALB/c mice. These BALB/c mice were injected intravenously with OVA plus LPS. Numbers of CD45RB+ KJ1.26+ T cells were assessed in the spleen, lungs and liver at various time points after immunization on a FACS. (A) Transferred naive CD45RB+ KJ1.26+ CD4+ T cells and remaining KJ1.26+ CD4+ T cells in the spleen, lungs and liver 10 weeks after immunization were shown. Numbers indicated the percentages of KJ1.26+ T cells in total CD4+ T cells. (B) Numbers of KJ1.26+ CD4+ T cells in each organ 10 weeks after immunization. Closed and open bars indicate CD45RB+ KJ1.26+ T cells of Bcl6-deficient and wild-type CD4+ T cells, respectively. Data are presented as the mean ± SD from five independent experiments (***P < 0.005). (C) Numbers of KJ1.26+ CD4+ T cells in each organ 10 days and 3 weeks after immunization. Closed and open bars indicate CD45RB+ KJ1.26+ T cells of Bcl6-deficient and wild type, respectively. Data are presented as the mean ± SD from five independent experiments (NS, not significant; ***P < 0.005). (D) Closed and open circles indicate CD45RB+ KJ1.26+ T cells in the spleen, respectively. Closed and open triangles indicate CD45RB+ KJ1.26+ T cells in the liver, respectively. Data are presented as the mean ± SD from five independent experiments.
4 weeks after immunization (Fig. 1D). These results indicate that Bcl6 in CD4+ T cells is essential for the generation of long-term memory CD4+ T cells.

A role for Bcl6 in the generation of effector CD4+ T cells in vitro and in vivo

Tn1/Tn2 skewing of naive CD4+ T cells affects the differentiation of long-term memory CD4+ T cells (29). Thus, a role for Bcl6 in the generation of Tn1 and Tn2 cells was examined in vitro. Naive Bcl6-deficient and wild-type CD4+ T cells from DO11.10 transgenic mice after labeled with CFSE were cultured under either Tn1- or Tn2-skewed condition for 4 days. IFN-γ or IL-4 production in those CD4+ T cells was analyzed by FACS. As shown in Fig. 2, proliferation of CD4+ T cells and differentiation to cytokines (+) effector CD4+ T cells under the Tn1- or Tn2-skewed condition were indistinguishable between Bcl6-deficient and wild-type CD4+ T cells.

Since memory T cells develop from effector T cells (27, 28), the generation of effector CD4+ T cells from naive Bcl6-deficient CD4+ T cells was examined in vivo. BALB/c mice transferred with naive CD45RB+ CD4+ T cells labeled with CFSE were immunized with OVA and LPS on the next day of transfer. The presence of KJ1.26+ CD4+ T cells in the spleen, lungs and liver of those mice was examined 10 days after immunization. Percentages of KJ1.26+ CD4+ T cells recovered from the spleen and lungs of BALB/c mice transferred with Bcl6-deficient T cells were similar to those of BALB/c mice transferred with wild-type T cells (Fig. 3A). However, the percentage of Bcl6-deficient KJ1.26+ CD4+ T cells recovered from the liver was twice as large as that of wild-type KJ1.26+ CD4+ T cells. All the CD4+ T cells examined lost CFSE fluorescence (data not shown) and showed down-expression of CD45RB, suggesting that these CD4+ T cells were stimulated with the antigen. We also analyzed amounts of CD62L, IL-7Rα and IL-2/7γ on KJ1.26+ CD4+ T cells in the spleen. These expression levels were similar between Bcl6-deficient and wild-type KJ1.26+ CD4+ T cells (Fig. 3B).

Proliferation capacity and cell death were examined using BrdU incorporation and annexin V staining of KJ1.26+ CD4+ T cells in the spleen, respectively. Fig. 3(C) shows that percentages of BrdU+ cells were compatible between Bcl6-deficient and wild-type KJ1.26+ CD4+ T cells, but the percentage of annexin V+ cells in Bcl6-deficient KJ1.26+ CD4+ T cells was higher than that in wild-type KJ1.26+ CD4+ T cells (P = 0.015). We also examined cytokine production of Bcl6-deficient effector CD4+ T cells in the spleen, lungs and liver by FACS. BALB/c mice transferred with KJ1.26+ CD4+ T cells were boosted with OVA 10 days after the primary immunization, and the presence of IFN-γ-producing cells in those mice was examined. Percentages and cell numbers of IFN-γ+ Bcl6-deficient KJ1.26+ CD4+ T cells in the spleen and lungs were similar to those of wild-type KJ1.26+ CD4+ T cells (Fig. 3D and E). Cell numbers of IFN-γ+ but not IFN-γ- Bcl6-deficient KJ1.26+ CD4+ T cells in the liver were more than those of wild-type KJ1.26+ CD4+ T cells. Numbers of the IFN-γ-non-producing KJ1.26+ CD4+ T cells at the effector phase, which contain memory precursors (29), in the spleen were similar between Bcl6-deficient and wild-type CD4+ T cells. These results indicate that differentiation of Bcl6-deficient naive CD4+ T cells to effector CD4+ T cells is largely normal.

Next, we examined IFN-γ production and apoptosis of Bcl6-deficient CD4+ T cells in the late effector phase. The spleen of immunized mice was harvested 15 days after immunization. These spleen cells were re-stimulated with OVA for 6 h and IFN-γ production of CD4+ T cells was analyzed by FACS. The number of Bcl6-deficient KJ1.26+ CD4+ T cells was less than that of wild-type KJ1.26+ CD4+ T cells, but the number of IFN-γ-producing Bcl6-deficient CD4+ T cells was more than that of wild-type CD4+ T cells (Fig. 4A and B). However, numbers of IFN-γ-non-producing KJ1.26+ CD4+ T cells were smaller than those of wild-type KJ1.26+ CD4+ T cells. The percentage of annexin V+ cells in Bcl6-deficient KJ1.26+ CD4+ T cells was also higher than that in wild-type KJ1.26+ CD4+ T cells.

We also analyzed proliferation capacity of KJ1.26+ CD4+ T cells in the spleen of immunized mice 15 days after immunization using in vitro culture. These spleen cells were re-stimulated with OVA and cultured with medium supplemented with IL-7 for 7 days. Wild-type KJ1.26+ CD4+ T cells 20-fold but Bcl6-deficient KJ1.26+ CD4+ T cells proliferated 10-fold within 7 days after re-stimulation (Fig. 5A and B). Although the number of IFN-γ-producing cells in Bcl6-deficient KJ1.26+ CD4+ T cells was compatible to that in wild-type
Bcl6 plays an important role in the generation and maintenance of memory cells, especially central memory CD8+ T cells (9, 10) and memory B cells with high affinity in germinal centers (8, 16). In this present study, we also showed the essential role for Bcl6 in the generation of long-term memory CD4+ T cells. The adoptive transfer experiments revealed that Bcl6-deficient KJ1.26+ CD4+ T cells could be identified in the spleen, lungs and liver of transferred BALB/c mice at least 3 weeks after immunization. However, no Bcl6-deficient KJ1.26+ CD4+ T cells could be observed in any organs examined of transferred BALB/c mice 4 weeks after immunization. Long-term memory T cells can be detected in the spleen of mice between 3 and 6 weeks after immunization by the kind of antigens and adjuvants (6, 27). These results suggest that Bcl6 expression in CD4+ T cells is essential for the generation of long-term memory CD4+ T cells.

IL-7 signals are essential to maintain effector CD4+ T cells and to promote generation of memory CD4+ T cells after immunization (5, 6). Then, we examined IL-7 receptor α and common γ expression on Bcl6-deficient KJ1.26+ CD4+ T cells at various time points after stimulation. These levels were comparable to those on wild-type KJ1.26+ CD4+ T cells at any time points examined. Although the number of CD62L+ CD4+ T cells in the spleen of wild-type mice is more than that of IL-7-deficient mice at the late effector phase after immunization (5, 6), the percentage of CD62L+ cells in Bcl6-deficient effector CD4+ T cells was similar to that in wild-type effector CD4+ T cells. These results suggest that Bcl6 does not affect IL-7 signals required for the generation of long-term memory CD4+ T cells.

Discussion

Bcl6 plays an important role in the generation and maintenance of memory cells, especially central memory CD8+ T cells (9, 10) and memory B cells with high affinity in germinal centers (8, 16). In this present study, we also showed the essential role for Bcl6 in the generation of long-term memory CD4+ T cells. The adoptive transfer experiments revealed that Bcl6-deficient KJ1.26+ CD4+ T cells could be identified in the spleen, lungs and liver of transferred BALB/c mice at least 3 weeks after immunization. However, no Bcl6-deficient KJ1.26+ CD4+ T cells could be observed in any organs examined of transferred BALB/c mice 4 weeks after immunization. Long-term memory T cells can be detected in the spleen of mice between 3 and 6 weeks after immunization by the kind of antigens and adjuvants (6, 27). These results suggest that Bcl6 expression in CD4+ T cells is essential for the generation of long-term memory CD4+ T cells.

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Long-term memory T cells develop from effector T cells (28), and IFN-γ+ effector cells are short-lived and do not efficiently develop into long-term memory T cells (29). Although there was a significant difference in the number of IFN-γ-producing
A role for Bcl6 in cytokine production and survival of CD4+ T cells in the late effector phase. Naive CD45R0+ KJ.126+ CD4+ T cells were labeled with CFSE and transferred intravenously into normal BALB/c mice. These BALB/c mice were injected intravenously with OVA plus LPS. Cell numbers and IFN-γ production of KJ.126+ T cells in the spleen were assessed 15 days after immunization. Harvested spleen cells were stimulated with OVA for 6 h and then IFN-γ+ CD4+ T cells were analyzed using FACS. Annexin V+ KJ.126+ CD4+ T cells of spleen cells were also analyzed. (A) Numbers indicate percentages of KJ.126+ T cells and IFN-γ+ T cells within total CD4+ T cells or annexin V+ T cells within total KJ.126+ CD4+ T cells. Data are presented as a representative of three independent experiments. (B) Cell numbers of KJ.126+ T cells, IFN-γ+ and IFN-γ+ KJ.126+ T cells within the spleens or percentages of IFN-γ+ T cells and annexin V+ T cells within total KJ.126+ CD4+ T cells were shown. Closed and open bars indicate results of Bcl6 deficient and wild type, respectively. Data are presented as the mean ± SD from three independent experiments (*P < 0.05, **P < 0.01 and ***P < 0.005).

At the late effector phase, percentages of IFN-γ+ non-producing and producing Bcl6-deficient CD4+ T cells decreased and increased more than those of wild-type CD4+ T cells, respectively. Moreover, proliferation capacity of Bcl6-deficient CD4+ T cells was half of that of wild-type CD4+ T cells at the late effector phase although Bcl6-deficient CD4+ T cells could proliferate similar to that of wild-type CD4+ T cells at the effector phase (data not shown). These results indicate that Bcl6-deficient CD4+ T cells change their properties at the late effector phase and that Bcl6 plays a role for protection of IFN-γ+ non-producing effector CD4+ T cells from apoptosis in vivo. Thus, Bcl6 expression in CD4+ T cells is essential for protection of effector CD4+ T cells including long-term memory precursors from apoptosis and may be required for survivability of long-term memory CD4+ T cells. Further study is required to elucidate the molecular mechanisms of Bcl6 on the generation of long-term memory CD4+ T cells.
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Abbreviations

APC antigen-presenting cell
BrdU bromodeoxyuridine
CFSE carboxyfluorescein diacetate succinimidyl ester
OVA ovalbumin peptide

References