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Bcl6 Acts as an Amplifier for the Generation and Proliferative Capacity of Central Memory CD8+ T Cells

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Central memory CD8+ T cells (T CM) are considered to be more efficient than effector ones (T EM) for mediating protective immunity. The molecular mechanism involved in the generation of these cells remains elusive. Because Bcl6 plays a role in the generation and maintenance of memory CD8+ T cells, we further examined this role in the process in relation to T CM and T EM subsets. In this study, we show that T CM and T EM were functionally identified in CD62L+ and CD62L− memory (CD44+Ly6C+)+ CD8+ T cell subsets, respectively. Although T CM produced similar amounts of IFN-γ and IL-2 to T EM after anti-CD3 stimulation, the cell proliferation capacity after stimulation and tissue distribution profiles of T CM differed from those of T EM. Numbers of T CM were greatly reduced and elevated in spleens of Bcl6-deficient and lck-Bcl6 transgenic mice, respectively, and those of T EM were constant in nonlymphoid organs of these same mice. The majority of Ag-specific memory CD8+ T cells in spleens of these mice 10 wk after immunization were T CM, and the number correlated with Bcl6 expression in T cells. The proliferation of Ag-specific memory CD8+ T cells upon secondary stimulation was dramatically up-regulated in lck-Bcl6 transgenic mice, and the adoptive transfer experiments with Ag-specific naive CD8+ T cells demonstrated that some of the up-regulation was due to the intrinsic effect of Bcl6 in the T cells. Thus, Bcl6 is apparently a crucial factor for the generation and secondary expansion of T CM.


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Abbreviations used in this paper: T CM, central memory CD8+ T cell; T EM, effector memory CD8+ T cell; Tg, transgenic; KO, knockout; WT, wild type; VV-ova, vaccinia virus encoding OVA; PI, propidium iodide.
We further characterized functional properties of memory CD8+ T cells generated in Bcl6-deficient and lck-Bcl6 Tg mice in relation to TCM and TEM subsets. Murine central and effector memory CD4+ T cells can be distinguished by CD62L expression (21, 30), albeit with low levels on TEM (31), and memory CD8+ T cells belong to the CD44+Ly6C+ subset (22). Thus, we divided the memory (CD44+Ly6C+) CD8+ T cells according to their CD62L expression, and then, we found that functional properties and tissue distribution of CD62L+ and CD62L− subpopulations of memory CD8+ T cells resembled those of TCM and TEM, respectively. Using a phenotypic analysis method and functional studies, we found the correlation between the number of TCM in the spleen and the amount of Bcl6 expression in T cells, even though few TCM were generated and maintained in the spleen of Bcl6-deficient mice. On the contrary, generation and maintenance of TEM in nonlymphoid organs were independent of the amount of Bcl6 in T cells. Furthermore, the proliferative capacity of phenotypic TCM activated with anti-CD3 and Ag-specific memory CD8+ T cells upon secondary stimulation correlated with the amount of Bcl6 expression in T cells. The role for Bcl6 in the generation and secondary expansion of TCM is discussed.

Materials and Methods

Animals

C57BL/6 mice were purchased from Japan SLC (Hamamatsu, Japan). Bcl6-deficient mice (28) were backcrossed to C57BL/6 mice >10 times. C57BL/6 mice carrying the murine Bcl6 DNA (25) under the control of the murine lck-proximal promoter (32) were generated (lck-Bcl6 Tg mice) (22). C57BL/6 (B6-Ly5.1) mice were purchased from Charles River (Kanagawa, Japan). All of the mice including OT-I Tg mice (33) were maintained under specific pathogen-free conditions in the animal center of Graduate School of Medicine, Chiba University.

Flow cytometric analysis of CD8+ T cells

Spleen cells were stained with various mAbs as follows: 106 spleen cells were first blocked with unconjugated anti-CD122/6 (2.4G2; BD Pharmingen, San Diego, CA), followed by incubation with biotinylated Abs, and then incubated with directly conjugated Abs and streptavidin-PerCP (BD Pharmingen). The following Abs (BD Pharmingen) were used for staining: CD8 (53-6.7)-allophycocyanin, CD8-FITC, CD44 (IM7)-PE, CD44-allophycocyanin, Ly6C (AL-21)-FITC, Ly6C-biotin, CD26L (MEL-14)-FITC, CD26L-biotin, Ly5.2 (104)-FITC, Ly5.2-biotin, H-2Kd tetramers bearing CD8 (53-6.7)-allophycocyanin, CD8-FITC, CD44 (IM7)-PE, CD44-allophycocyanin, Ly6C (AL-21)-FITC, Ly6C-biotin, CD26L (MEL-14)-FITC, CD26L-biotin, Ly5.2 (104)-FITC, Ly5.2-biotin, H-2Kd tetramers bearing the OVA peptide (SIINFEKL, OVA57–69-ova8) were kindly provided by Dr. P. Marrack (National Jewish Medical and Research Center, Denver, CO). Spleen cells (2 × 107) were incubated with 5–10 µg/ml tetramers at 37°C for 2 h. The remaining Abs were then added, and the spleen cells were stained with those Abs for 20–40 min before analysis on a FACS Calibur (BD Biosciences, San Jose, CA).

Induction and activation of Ag-specific memory CD8+ T cells

Vaccinia virus encoding OVA (OV-a) was propagated as described (34), and VV-OVA (1 × 104–2 × 105 PFU) were given i.v. to challenge the mice. At 10 wk after the primary challenge, 50 µg/ml OVA peptide (ova8) in PBS (200 µl) were given to these mice i.v.

IFN-γ and IL-2 production by CD8+ T cells

For IFN-γ and IL-2 production by TCM and TEM, phenotypic TCM and TEM were sorted from spleen cells using a FACS Vantage (BD Biosciences). Purified TCM and TEM were then incubated in wells coated with immobilized anti-CD3 (145-2C11) (10 µg/ml) in the presence of anti-CD28 (37.51) (1 µg/ml) for 24 h. The amounts of IFN-γ and IL-2 in culture supernatants were measured using ELISA (BD Pharmingen). For intracellular staining of IFN-γ, spleen cells from boosted mice were cultured for 5 h with Monensin (2 µM). These cells were then stained with Abs and subjected to intracellular staining for anti-IFN-γ Ab (XMG1.2-FITC, -PE; BD Pharmingen), using the CytoFix/Cytoperm kit, according to manufacturer’s instructions (BD Pharmingen).

Cell cycle analysis

Cell cycle analysis was done as described (35). Briefly, phenotypic TCM and TEM were sorted from spleen cells using a FACS Vantage, and purified TCM and TEM were incubated for 24 h in wells coated with immobilized anti-CD3 (10 µg/ml) in the presence of anti-CD28 (1 µg/ml). These TCM and TEM were incubated in hypotonic lysis buffer that contained propidium iodide (PI) (0.1% sodium citrate, 0.01% Triton X-100, 0.1 mg/ml RNase A, and 0.1 mg/ml PI). The DNA content in each nucleus was analyzed using FACSCalibur and CellQuest software (BD Biosciences).

Adaptive transfer of T cells

Splenoctyes from Bcl6-deficient × OT-I Tg, lck-Bcl6 × OT-I doubly Tg, and OT-I Tg mice were incubated for 2 h with 2 µg/ml OVA peptide (ova8). These cells were then incubated with a mixture of Abs to CD4, Mac-1, NK1.1, CD44, Ly6C, and B220, followed with Ab-coated microbeads (Miltenyi Biotec, Auburn, CA), using two beads per cell, and then purified on a MACS cell sorter (Miltenyi Biotec). Magnetically purified naive CD8+ T cells (>90% pure) were labeled with CFSE. Those CD8+ T cells (1 × 104) were given i.v. into C57BL/6 (B6-Ly5.1) mice. In some experiments, FACS-purified naive CD8+ T cells (1 × 105) from spleen cells of lck-Bcl6 × OT-I doubly Tg and OT-I Tg mice were injected into C57BL/6 (B6-Ly5.1) mice. These mice were immunized with VV-ova 1 day after transfer.

RT-PCR analysis

RNA was prepared from naive and memory CD8+ T cells of OT-I Tg mice using TRizol reagent (Invitrogen Life Technologies, Carlsbad, CA). Total RNA was reverse-transcribed using SuperScript RT (Invitrogen Life Technologies) and oligo(dT) (Pharmacia, Piscataway, NJ) in a final volume of 20 µl, and the cDNAs (1 µl) were used for PCR. After an initial 5-min incubation at 94°C, 30–35 cycles of PCR were run under the following conditions: denaturation at 94°C for 60 s, annealing at 55°C for 30 s, and polymerization at 72°C for 60 s. The primers used were as follows: Bcl6, 5′-AGAAAGAAGGCCGTGCATCCTC3′ and 5′-CATCTCTGATGCTGTTGGGACGT-3′; and β-actin, 5′-GTTGAGACCTCaACAC3′ and 5′-GTTGCCCATCCTCGTCTCAAGT-3′. The PCR products were separated on a 1.2% agarose gel and stained with ethidium bromide.

Results

Functional properties of CD62L+ and CD62L− memory CD8+ T cells

We examined functional properties of CD62L+ and CD62L− T cell subsets within memory (CD44+Ly6C+) CD8+ T cells obtained from spleens of Bcl6-deficient, lck-Bcl6 Tg, and WT mice. CD62L+ and CD62L− memory CD8+ T cells from the spleen cells were sorted using FACS. They were then stimulated in vitro in anti-CD3-coated wells in the presence of anti-CD28, and cell proliferation (cell cycle) and function (cytokine production) were assessed 24 h after stimulation. Less than 0.5% of the sorted CD8+ T cells were in the cell cycle before stimulation (data not shown). Assessment of cell proliferation by PI staining after stimulation showed that 7.9, 38, and 23% of CD62L+ memory CD8+ T cells of Bcl6-deficient, lck-Bcl6 Tg, and WT mice, respectively, were in cell cycle (Fig. 1A). Conversely, <2% of CD62L− memory CD8+ T cells of all the strains used were proliferating. Assessment of cytokine production in culture supernatants of stimulated cells revealed that IFN-γ productions by CD62L+ and CD62L− memory CD8+ T cells were similar (Fig. 1B). Also, IL-2 productions by the CD62L+ memory CD8+ T cells of these strains were similar, and higher than those produced by the CD62L− memory CD8+ T cells. Although the capacity of IFN-γ and IL-2 productions was similar, these results suggest that CD62L+ and CD62L− memory CD8+ T cells represent TCM and TEM, respectively.

Because TCM preferentially reside in lymphoid organs, whereas TEM home to nonlymphoid tissues such lungs and liver (8), we assessed percentages of CD62L+ (TCM) and CD62L− (TEM) memory CD8+ T cells in spleens, lungs, and livers of Bcl6-deficient, lck-Bcl6 Tg, and WT mice by FACS analysis. The cell number of each subpopulation was calculated based on the percentage and total CD8+ T cell number in each organ. The proportion of TCM in the spleen correlated well with Bcl6 expression in T cells
The S/G 2 M phase of cell cycle.

Furthermore, apoptotic cells within CD8⁺ T cells with annexin V staining showed no significant difference among these strains (data not shown).

We also examined the tissue distribution of Ly5.2⁺ CD8⁺ T cells in the spleen, lungs, and liver 7 days after transfer. Their numbers were calculated from the percentage and total Ly5.2⁺ CD8⁺ T cell number in each organ. Ly5.2⁺ CD8⁺ T cell numbers in the spleen directly correlated with Bcl6 expression in T cells, whereas the numbers in the lungs and liver inversely correlated with the expression (Fig. 3B). Analysis of ratios of Ly5.2⁺ CD8⁺ T cell numbers in the lungs and liver to those in the spleen showed that the ratios inversely correlated with Bcl6 expression in T cells (Fig. 3C). Similar results were obtained in these transferred mice 4 days after transfer (data not shown). These results suggest that Bcl6 in CD8⁺ T cells plays a role in the generation of Ag-specific T_CM at the very early phase of differentiation.

The effect of Bcl6 on the generation and maintenance of Ag-specific T_CM was further examined using Bcl6-deficient, lck-Bcl6 Tg, and WT mice. Mice were immunized with VV-ova, and Ag-specific memory (CD44⁺) CD8⁺ T cells in the spleen, lungs, and liver were detected by staining with OVA-MHC class I tetramers 10 wk after immunization. The proportions of OVA-specific CD44⁺ CD8⁺ T cells in the spleen, lungs, and liver were the highest in lck-Bcl6 Tg mice and the lowest in Bcl6-deficient mice (Fig. 4A). The number of OVA-specific CD44⁺ CD8⁺ T cells was calculated based on the percentage and total CD8⁺ T cell number in each organ. The results from these cell numbers were similar to those of the percentages (Fig. 4B). Ratios of the cell numbers in the lungs and liver to those in the spleen inversely correlated with Bcl6 expression in T cells (Fig. 4C). The large percentages (knockout (KO), 97 ± 1.4; WT, 93 ± 1.9; Tg, 93 ± 1.8; n = 5 mice in each line) of OVA-specific CD44⁺ CD8⁺ T cells in the spleen were Ly6C⁺. Furthermore, the numbers (KO, 0.59 × 10⁵ ± 0.22 × 10⁵; WT, 4.3 × 10⁶ ± 0.38 × 10⁶; Tg, 7.2 × 10⁴ ± 1.4 × 10⁴; n = 3 mice in each line) of CD62L⁺ (T_CM) cells within OVA-specific CD44⁺ CD8⁺ T cells in the spleen were much higher than those (KO, 0.26 × 10⁴ ± 0.11 × 10⁴; WT, 1.3 × 10⁴ ± 0.51 × 10⁴; Tg, 1.6 × 10⁴ ± 0.68 × 10⁴; n = 3 mice in each line) of T_EM, and also correlated with Bcl6 expression in T cells.

A role for Bcl6 in the function of Ag-specific T_CM

To examine functional properties of OVA-specific memory (CD44⁺) CD8⁺ T cells in Bcl6-deficient, lck-Bcl6 Tg, and WT mice 10 wk after immunization, these mice were boosted with OVA peptide. IFN-γ produced by OVA-specific CD44⁺ CD8⁺ T cells in the spleen 5 h after boosting was examined using intracellular staining. IFN-γ-producing CD44⁺ CD8⁺ T cells were detected in all spleens including those of Bcl6-deficient mice, although the percentage in Bcl6-deficient mice was the lowest among the mice (Fig. 5A). Ratios of the percentage of IFN-γ-producing CD44⁺ CD8⁺ T cells to that of OVA-specific CD44⁺ CD8⁺ T cells in the spleen of these mice were similar among the groups (Fig. 5B).

Expansion of OVA-specific CD44⁺ CD8⁺ T cells in the spleen, lungs, and liver 5 days after boosting was examined by FACS analysis. The percentage of OVA-specific CD44⁺ CD8⁺ T cells clearly increased in spleens, lungs, and livers of WT and lck-Bcl6
These mice were then immunized with VV-ova 1 day after this transfer. Ly5.2<sup>+</sup> T<sub>CM</sub> and Ly5.2<sup>+</sup> T<sub>EM</sub> were sorted from spleen cells of mice transferred with naive CD8<sup>+</sup> T cells of OT-I Tg mice 6 wk after the transfer. Bcl6 expression in these CD8<sup>+</sup> T cells was analyzed using RT-PCR. Although Bcl6 expression was not clearly detected in naive CD8<sup>+</sup> T cells from OT-I Tg mice, the expression was strongly detected in T<sub>CM</sub> and T<sub>EM</sub> and the amount in T<sub>CM</sub> was larger than that in T<sub>EM</sub> (Fig. 7A). The percentage and cell number (2.6 × 10<sup>4</sup> ± 0.47 × 10<sup>4</sup>) of Ly5.2<sup>+</sup> CD44<sup>+</sup> CD8<sup>+</sup> T cells in the spleen of mice transferred with naive CD8<sup>+</sup> T cells of lck-Bcl6<sup>+</sup> OT-I doubly Tg mice was similar to those (2.3 × 10<sup>4</sup> ± 0.14 × 10<sup>4</sup>) with naive CD8<sup>+</sup> T cells of OT-I Tg mice 10 wk after stimulation (Fig. 7B). Those mice were boosted with OVA peptide (ova8), and expansion of Ly5.2<sup>+</sup> CD44<sup>+</sup> CD8<sup>+</sup> T cells in the spleen 5 days after boosting had been analyzed using FACS. The percentage and cell number (36 × 10<sup>4</sup> ± 3.4 × 10<sup>4</sup>) of Ly5.2<sup>+</sup> CD44<sup>+</sup> CD8<sup>+</sup> T cells in the spleen of mice transferred with naive CD8<sup>+</sup> T cells of lck-Bcl6<sup>+</sup> OT-I doubly Tg mice were larger than those (16 × 10<sup>4</sup> ± 5.5 × 10<sup>4</sup>) with naive CD8<sup>+</sup> T cells of OT-I Tg mice (n = 3). The increase rate of this transfer experiment with lck-Bcl6<sup>+</sup> OT-I doubly Tg mice was lower than that of lck-Bcl6 Tg mice shown in Fig. 6B. These results suggest that part of the secondary expansion of T<sub>CM</sub> is due to the intrinsic effect of Bcl6 in the T cells.

The intrinsic effect of Bcl6 on secondary expansion of T<sub>CM</sub>

To confirm the effect of Bcl6 on the generation and secondary expansion of T<sub>CM</sub>, phenotypic naive (CD44<sup>−</sup>Ly6C<sup>−</sup>) CD8<sup>+</sup> T cells were sorted from spleen cells of lck-Bcl6 × OT-I doubly Tg and OT-I Tg mice, and transferred into Ly5.1 congenic mice. These mice were then immunized with VV-ova 1 day after this transfer. Ly5.2<sup>+</sup> T<sub>CM</sub> and Ly5.2<sup>+</sup> T<sub>EM</sub> were sorted from spleen cells of mice transferred with naive CD8<sup>+</sup> T cells of OT-I Tg mice 6 wk after the transfer. Bcl6 expression in these CD8<sup>+</sup> T cells was analyzed using RT-PCR. Although Bcl6 expression was not clearly detected in naive CD8<sup>+</sup> T cells from OT-I Tg mice, the expression was strongly detected in T<sub>CM</sub> and T<sub>EM</sub> and the amount in T<sub>CM</sub> was larger than that in T<sub>EM</sub> (Fig. 7A). The percentage and cell number (2.6 × 10<sup>4</sup> ± 0.47 × 10<sup>4</sup>) of Ly5.2<sup>+</sup> CD44<sup>+</sup> CD8<sup>+</sup> T cells in the spleen of mice transferred with naive CD8<sup>+</sup> T cells of lck-Bcl6<sup>+</sup> OT-I doubly Tg mice was similar to those (2.3 × 10<sup>4</sup> ± 0.14 × 10<sup>4</sup>) with naive CD8<sup>+</sup> T cells of OT-I Tg mice 10 wk after stimulation (Fig. 7B). Those mice were boosted with OVA peptide (ova8), and expansion of Ly5.2<sup>+</sup> CD44<sup>+</sup> CD8<sup>+</sup> T cells in the spleen 5 days after boosting had been analyzed using FACS. The percentage and cell number (36 × 10<sup>4</sup> ± 3.4 × 10<sup>4</sup>) of Ly5.2<sup>+</sup> CD44<sup>+</sup> CD8<sup>+</sup> T cells in the spleen of mice transferred with naive CD8<sup>+</sup> T cells of lck-Bcl6<sup>+</sup> OT-I doubly Tg mice were larger than those (16 × 10<sup>4</sup> ± 5.5 × 10<sup>4</sup>) with naive CD8<sup>+</sup> T cells of OT-I Tg mice (n = 3). The increase rate of this transfer experiment with lck-Bcl6<sup>+</sup> OT-I doubly Tg mice was lower than that of lck-Bcl6 Tg mice shown in Fig. 6B. These results suggest that part of the secondary expansion of T<sub>CM</sub> is due to the intrinsic effect of Bcl6 in the T cells.

FIGURE 2. Tissue distribution of CD62L<sup>+</sup> and CD62L<sup>−</sup> memory CD8<sup>+</sup> T cells. A–C, The percentage of CD62L<sup>+</sup> and CD62L<sup>−</sup> T cells within memory (CD44<sup>−</sup>Ly6C<sup>−</sup>) CD8<sup>+</sup> T cells was analyzed in spleens (A), lungs (B), and livers (C) of Bcl6-deficient (KO), lck-Bcl6 Tg, and WT mice, 6 mo of age, by FACS. Numbers in the corners indicate the percentage of CD8<sup>+</sup> T cells in each quadrant and the indicated markers. These results are representative of 10 independent experiments. D, Numbers of CD62L<sup>+</sup> (T<sub>CM</sub>) and CD62L<sup>−</sup> (T<sub>EM</sub>) memory CD8<sup>+</sup> T cells in each organ. The results represent the means and variations (SD) of 10 mice.
Discussion
The present study involved characterization of functional properties of memory CD8 T cells maintained in Bcl6-deficient and lck-Bcl6 Tg mice, and then compared them to those of T_{CM} and T_{EM} of WT mice. First, we defined the phenotype for murine T_{CM} and T_{EM} according to the surface marker CD44, Ly6C, and CD62L expression. Additionally, cell proliferation studies indicated that CD62L^{+} and CD62L^{-} memory (CD44^{+}Ly6C^{-}) CD8^{+} T cells are indeed T_{CM} and T_{EM}, respectively, and we also con-

**FIGURE 3.** Tissue distribution of Ag-stimulated CD8^{+} T cells. Spleen cells of Bcl6-deficient × OT-I Tg (■), lck-Bcl6 × OT-I doubly Tg (□), and OT-I Tg (□) mice were stimulated with OVA peptide (ova8) in vitro for 2 h. Phenotypic naive (CD44^{-}Ly6C^{-}) CD8^{+} T cells were sorted from these spleen cells and labeled with CFSE. These CD8^{+} T cells were then transferred into naive Ly5.1 congenic mice. A, Proliferation of Ly5.2^{-}CD8^{+} T cells in the spleen was analyzed based on CFSE expression 4 and 7 days after transfer. B, Numbers of Ly5.2^{-}CD8^{+} T cells in the spleen, lungs, and liver were calculated using FACS 7 days after transfer. C, Ratios of transferred cell numbers in the lungs and liver to those in the spleen. The results represent the means and variations (SD) of three mice.

**FIGURE 4.** Tissue distribution of Ag-specific memory CD8^{+} T cells. Bcl6-deficient (■), lck-Bcl6 Tg (□), and WT (□) mice were immunized with VV-ova. Ag-specific CD44^{+}CD8^{+} T cells in the spleen, lungs, and liver were detected by staining with OVA-MHC class I tetramers 10 wk after immunization. A, OVA-specific CD44^{+}CD8^{+} T cells in the spleen, lungs, and liver were detected by FACS. Numbers in the corners indicate the percentage of OVA-specific CD44^{+}CD8^{+} T cells in each oval. These results are representative of five independent experiments. B, Numbers of OVA-specific CD44^{+}CD8^{+} T cells in the spleen, lungs, and liver were assessed using FACS. C, Ratios of these cell numbers in the lungs and liver to those in the spleen. The results represent the means and variations (SD) of five to six mice.
CCR7 is an additional surface marker for human TCM (7); the phenotypic patterns on memory CD8\(^+\) T cells were similar to those of CD62L expression (data not shown). Although the cytokine production profiles could be used to distinguish TCM (CCR7\(^+\)) from TEM (CCR7\(^-\)) in human memory T cells (7), this is not always the case in murine memory CD8\(^+\) T cells (13). We also observed that both CD62L\(^-\) and CD62L\(^+\) memory CD8\(^+\) T cells generated in the spleen under homeostatic conditions or by Ag stimulation (data not shown) produced similar amounts of IFN-\(\gamma\) after stimulation. Thus, murine TCM and TEM, which are functionally discriminated based on their capacity for cell proliferation after secondary stimulation, can be identified by their surface phenotypes including CD44 and Ly6C together with CD62L and CCR7.

Bcl6-deficient and lck-Bcl6 Tg mice enabled elucidation of the molecular mechanism of the functional difference of TCM and TEM. As we have previously shown (22), the number of memory (CD44\(^+\)Ly6C\(^-\)) CD8\(^+\) T cells was reduced and elevated in spleens of Bcl6-deficient and lck-Bcl6 Tg mice, respectively.

**FIGURE 5.** IFN-\(\gamma\) production by Ag-specific memory CD8\(^+\) T cells after boosting. Bcl6-deficient (■), lck-Bcl6 Tg (□), and WT (□) mice were immunized with VV-ova. These mice were boosted with OVA peptide (ova8) 10 wk after immunization. A, IFN-\(\gamma\) production by CD44\(^+\)CD8\(^+\) T cells in the spleen was examined 5 h after boosting by intracellular staining. The numbers in the corners indicate the percentage of IFN-\(\gamma\)-producing CD44\(^+\)CD8\(^+\) T cells in each oval. B, Ratios of the percentages of IFN-\(\gamma\)-producing CD44\(^+\)CD8\(^+\) T cells to those of OVA-specific CD44\(^+\)CD8\(^+\) T cells in the spleen. The results represent the means and variations (SD) of five to six mice.

**FIGURE 6.** Proliferation of Ag-specific memory CD8\(^+\) T cells after boosting. Bcl6-deficient (■), lck-Bcl6 Tg (□), and WT (□) mice were immunized with VV-ova. These mice were boosted with OVA peptide (ova8) 10 wk after immunization. A, OVA-specific CD44\(^+\)CD8\(^+\) T cells in the spleen, lungs, and liver were detected 5 days after boosting by FACS. Numbers in the corners indicate the percentage of CD8\(^+\) T cells in each organ. B, Numbers of OVA-specific CD44\(^+\)CD8\(^+\) T cells in the spleen, lungs, and liver were calculated by the percentage and CD8\(^+\) T cell number in each organ. C, The rates of increase in cell numbers of OVA-specific CD44\(^+\)CD8\(^+\) T cells in the spleen, lungs, and liver after boosting. Data are represented as means and variations (SD) of three to four mice.
normal conditions, the number of T EM was not affected by Bcl6 expression in T cells. Notably, T CM even outnumbered T EM in the lungs and liver of immunized mice due to the dominance of T CM in contrast to homeostatically generated T CM mentioned above. When we analyzed Ag-specific memory CD8+ T cells from unprimed OT-I Tg mice by FACS. Bcl6 expression in T cells. Although we could not distinguish T CM from T EM in the lungs and liver of immunized mice due to the paucity of Ag-specific memory CD8+ T cells recovered, the similarity of tissue distribution profiles of Ag-specific memory CD8+ T cells to those of homeostatic ones led us to speculate that the number of Ag-induced T EM in nonlymphoid organs was more or less similar regardless of the amount of Bcl6 expression in T cells. Taken together, these results indicate that Bcl6 supports the generation of Ag-induced as well as lymphopenia-induced T CM but not that of T EM (Fig. 8).

The mechanism of Bcl6 that augments the generation of T CM could be explained by the protection of apoptosis and/or by the augmentation of cell proliferation. However, the number of apoptotic cells within differentiating CD8+ T cells in the spleen until 7 days after Ag stimulation was not affected by Bcl6 expression in T cells (data not shown). When lck-Bcl6 Tg and WT mice were fed BrdU for 3 days, the percentage of BrdU-labeling (proliferating) CD8+ T cells in the spleen of lck-Bcl6 Tg mice was ~3-fold larger than that of WT mice (data not shown). Furthermore, Bcl6 plays a role in proliferative capacity of phenotypic and Ag-specific memory CD8+ T cells upon secondary stimulation. Therefore, Bcl6 may augment cell proliferation of naive CD8+ T cells after activation to result in the increase of T CM. However, cell cycle progression of Ag-specific naive CD8+ T cells in the spleen until 7 days after Ag stimulation was not affected by Bcl6 expression in T cells. Thus, Bcl6 may play a role in continuation of cell proliferation of differentiating CD8+ T cells.

We observed dramatic difference in the tissue distribution of Ag-experienced CD8+ T cells according to Bcl6 expression in T cells. When we transferred Ag-specific naive phenotype CD8+ T cells of Bcl6-deficient, WT, and lck-Bcl6 Tg mice after Ag activation into Ly5.1 congenic mice, ratios of the transferred cell number in nonlymphoid organs to that in the spleen 7 days after transfer inversely correlated with Bcl6 expression in T cells. These ratios were similar to those of the Ag-specific memory CD8+ T cell numbers in Bcl6-deficient, WT, and lck-Bcl6 Tg mice 10 wk after immunization. The ratios within 4 days after transfer were already similar to those in the 7 days after transfer (data not shown). Because these transferred CD8+ T cells were in the effector/precursor cell stage within 8 days after activation (37) and the precursors to memory CD8+ T cells exist early during an infection (14–16, 37), the effector/precursor cells may already express the homing ability of T CM and T EM. Thus, Bcl6 in CD8+ T cells may play a role in the generation of T CM at the effector/precursor cell stage.

It is also interesting to speculate on the developmental relationship between T CM and T EM. When human memory CD4+ T cells

When we calculated the absolute number of T CM and T EM in the spleen, the dominance of T CM over T EM was more prominent in lck-Bcl6 Tg mice than WT mice. However, this dominance was no longer observed in the spleen of Bcl6-deficient mice. In contrast, within lungs and liver where T EM are abundantly observed under normal conditions, the number of T EM was not affected by Bcl6 expression in T cells. Notably, T CM even outnumbered T EM in those nonlymphoid organs of lck-Bcl6 Tg mice. These results suggest that Bcl6 plays a role in the generation of T CM but not T EM. Because phenotypic T CM were detected, albeit at a much-reduced level, in the spleen of Bcl6-deficient mice, the requirement for Bcl6 in the generation of T CM is not absolute. It seems likely that Bcl6 plays an auxiliary function to amplify the process of T CM generation.

We further explored the role for Bcl6 in the generation of Ag-specific T CM in contrast to homeostatically generated T CM mentioned above. When we analyzed Ag-specific memory CD8+ T cells 10 wk after immunization, the vast majority of Ag-specific memory CD8+ T cells in the spleen were expected to be T CM and the number of T CM found in the spleen was again parallel to the Bcl6 expression in T cells. Although we could not distinguish T CM from T EM in the lungs and liver of immunized mice due to the paucity of Ag-specific memory CD8+ T cells recovered, the similarity of tissue distribution profiles of Ag-specific memory CD8+ T

FIGURE 7. The intrinsic effect of Bcl6 on proliferation of Ag-specific memory CD8+ T cells after boosting. Phenotypic naive (CD44+Ly5.2+) CD8+ T cells were sorted from spleen cells of lck-Bcl6×OT-I doubly Tg and OT-I Tg mice. These CD8+ T cells were then transferred into naive Ly5.1 congenic mice. These mice were immunized with VV-ova 1 day after transfer, and boosted with OVA peptide (ova8) 10 wk after transfer. A, Ly5.2+ T CM and T EM in the spleen of mice 6 wk after immunization were isolated using FACS. Phenotypic naive CD8+ T cells were isolated from unprimed OT-I Tg mice by FACS. Bcl6 mRNA in these T cells was analyzed by RT-PCR. β-actin mRNA was used as a control. B, Transferred CD44+CD8+ T cells in the spleen of mice 10 wk after immunization and in the spleen of mice 5 days after boosting were detected by staining with anti-Ly5.2. Numbers in the corners indicate the percentage of Ly5.2+CD44+CD8+ T cells in each oval. These results are representative of three independent experiments.

FIGURE 8. The generation of T CM and T EM in lymphoid and nonlymphoid organs of lck-Bcl6 Tg mice.
are restimulated in vitro, central memory CD4+ T cells may give rise to effector memory CD4+ T cells (7). These support a linear differentiation pathway from TCM to TEM. However, data from a recent report (38) demonstrated a direct developmental pathway from TEM to TCM. These models do not seem to fit well with our current findings. Although the phenotypic TCM number in Bcl6-deficient mice was much lower than that in lck-Bcl6 Tg mice, similar numbers of phenotypic TCM were generated and maintained in nonlymphoid organs of Bcl6-deficient and lck-Bcl6 Tg mice. Furthermore, homing ability of TCM and TEM was induced in the effector/precursor CD8+ T cells. These results suggest the nonlinear differentiation model of TCM and TEM (39). A recent report (40) also supports the distinct differentiation pathway for TCM and TEM. Thus, our results support a model of memory T cell development (41); a short duration of antigenic stimulation favors the differentiation of TCM, whereas a longer duration of stimulation favors the differentiation of TEM. Although it is impossible to know which of the mechanisms is valid, we do believe that understanding of Bcl6 functions at the molecular level would facilitate deeper insights into the lineage relationship between TCM and TEM.

Ahmed and colleagues (38) reported that TCM are more effective mediators of protective immunity than TEM. Thus, Bcl6, an important amplifier of TCM generation as indicated in this study, is a key molecule in protective immunity by CD8+ T cells. Bcl6 plays a role in proliferative capacity of Ag-specific memory CD8+ T cells upon secondary stimulation. Because the large percentages (>80%) of Ag-specific memory CD8+ T cells in the spleen of lck-Bcl6 Tg mice before boosting were TCM, their higher growth rates at secondary expansion in lck-Bcl6 Tg mice strongly suggest a role for Bcl6 in the proliferation of TCM after boosting. This function of Bcl6 was also confirmed by the cell cycle analysis of phenotypic TCM activated with anti-CD3. Interestingly, the proliferation of Ag-specific memory CD8+ T cells was detected in Bcl6-deficient mice after boosting, suggesting that Bcl6 is not absolutely required for secondary expansion of TCM but instead plays an auxiliary function to amplify the process. Therefore, Bcl6 can augment the protective immunity by increasing TCM numbers at their generation and secondary expansion.

The adoptive transfer experiments suggested that secondary expansion of Ag-specific memory CD8+ T cells is due to the intrinsic effect of Bcl6 in CD8+ T cells. However, the expansion rates of Ag-specific memory CD8+ T cells in the transfer experiments were smaller than those in the spleen of lck-Bcl6 Tg mice, suggesting that some of the secondary expansion is due to the intrinsic effect of Bcl6 in the CD8+ T cells. Because memory CD8+ T cells generated in the absence of CD4+ T cells display poorer recall responses than do memory CD8+ T cells generated with CD4+ T cell help (42–45), CD4+ T cells of lck-Bcl6 Tg mice may enhance the generation of Ag-specific memory CD8+ T cells with a high proliferative potential. Further study is required to elucidate mechanisms of the development of Ag-specific memory CD8+ T cells with a high proliferative potential in lck-Bcl6 Tg mice.

The developmental pathway of memory B cells through the germinal center stage is affected by Bcl6 expression in B cells (28). Indeed, memory B cells with high-affinity Ab are not developed in Bcl6-deficient mice. However, memory B cells with nonmutated V_{H} gene can be generated without germinal center formation in Bcl6-deficient mice (46). Because one type (effector) of memory CD8+ T cells but a few of the other type (central) of memory CD8+ T cells can be generated and maintained in Bcl6-deficient mice, functions of Bcl6 in TCM are similar to those in the memory B cells with high-affinity Ab. Recently, Fearon et al. (47) outlined the comparable behavior of TCM and memory B cells as memory stem cells. Memory B cells derived from germinal centers seem to be memory stem cells like TCM, and Bcl6 may play a role in the generation and expansion of these memory stem cells. Therefore, our current findings shed new light on the generation and expansion of effective mediators of protective immunity by CD8+ T cells and B cells. As Bcl6 is a transcriptional regulator, it would likely function by regulating expression of a target gene(s). Identification of the gene(s) will help us to understand molecular mechanisms of memory responses and provide us with information useful to design effective vaccines.

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References

11. Reinhardt, R. L., A. Khrotuts, R. Merica, T. Zell, and M. K. Jenkins. 2001. Visualization of the generation and expansion of these memory stem cells. Therefore, our current findings shed new light on the generation and expansion of effective mediators of protective immunity by CD8+ T cells and B cells. As Bcl6 is a transcriptional regulator, it would likely function by regulating expression of a target gene(s). Identification of the gene(s) will help us to understand molecular mechanisms of memory responses and provide us with information useful to design effective vaccines.


