Memory B cells contribute to rapid Bcl6 expression by memory follicular helper T cells

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In primary humoral responses, B-cell lymphoma 6 (Bcl6) is a master regulator of follicular helper T (TFH) cell differentiation; however, its activation mechanisms and role in memory responses remain unclear. Here we demonstrate that survival of CXCR5+ TFH memory cells, and thus subsequent recall antibody response, require Bcl6 expression. Furthermore, we show that, upon rechallenge with soluble antigen Bc6 in memory TFH cells is rapidly induced in a dendritic cell-independent manner and that peptide-class II complexes (pMHC) on cognate memory B cells significantly contribute to this induction. Given the previous evidence that antigen-specific B cells residing in the follicles acquire antigens within minutes of injection, our results suggest that memory B cells present antigens to the cognate TFH memory cells, thereby contributing to rapid Bc6 reexpression and differentiation of the TFH memory cells during humoral memory responses.

The development of high-affinity B-cell memory is essential in most effective vaccines that are in use today. Because most protein antigens require T-cell help to induce B-cell responses, understanding the mechanisms by which memory T and B cells are generated and maintained, as well as how their swift activation is executed, is of fundamental importance for vaccine development. In primary immune responses, it is widely accepted that among several differentiated helper T-cell subsets follicular helper CD4+ T cells (TFH cells) are the major subset to deliver help to B cells (1). TFH cells express CXC-chemokine receptor 5 (CXCR5), the chemokine receptor for the B-cell homing chemokine CXCL13. Surface expression of CXCR5 enables TFH cells to migrate into B-cell follicles, where they provide help to B cells to form germinal centers. In addition, TFH cells are needed for the crucial affinity-maturation process of B cells in germinal centers, whereby Ag-specific B cells undergo repeated rounds of somatic hypermutation and positive selection by TFH cells to rapidly evolve high-affinity somatically mutated B-cell receptors. B-cell lymphoma 6 (Bcl6) has recently been identified as a TFH lineage regulator (2–4); it is highly expressed by TFH cells and is required for their development. According to the current view, during a primary response Bcl6 expression by T cells is induced by priming with dendritic cells (5–7) and ICOS is a key coreceptor molecule for induction of Bcl6 (5, 8). The initial Bcl6 induction and subsequent CXCR5 expression allow CD4+ T cells to migrate toward the T-B border, where TFH cells interact with antigen-specific B cells. According to this model, cognate B cells are not required for the induction of Bcl6 but support the expansion of TFH cells (9).

Although the importance of Bcl6 and its expression kinetics in naïve T-cell differentiation have been well elucidated, its role and activation mechanisms in TFH memory cells still remain obscure. Hence, in this paper we first focus upon the roles of Bcl6, demonstrating its importance for maintenance of TFH memory cells. Then, we show that Bcl6 in memory TFH cells was rapidly induced upon rechallenge with soluble antigen and that this response was mainly mediated through antigen presentation by the cognate memory B cells. Given the good association between Bcl6 with IL-21 expression in differentiated memory TFH cells, our results suggest that memory B cells are the primary antigen-presenting cells (APC) to induce the rapid differentiation of memory TFH cells toward effector cells, further accelerating memory B-cell responses during recall.

Results

CXCR5+ Memory T Cells Provide Potent Help to Memory B Cells.

To identify TFH memory cells we set up adoptive transfer experiments in which naïve T cells purified from TEsA T-cell receptor transgenic (TCR Tg) mice were transferred into congenically marked mice and immunized with (4-hydroxy-3-nitrophenyl) acetyl (NP)-Ea-GFP in alum. Six weeks after immunization, expression of several surface molecules on the surviving memory T cells was examined. TEsA memory T cells were found to be heterogeneous in their expression of CXCR5 or CD62L (Fig. L4); 20–30% of memory TEsA T cells were CXCR5+ with low or high expression of CD62L.

To determine the capacity of the above distinct memory populations to help IgG1+ memory B cells, purified CXCR5+ or CXCR5− memory TEsA cells were transferred into congenic WT mice, together with B1-8hi IgG1+ memory B cells, followed by immunization with soluble NP-Ea-GFP. As shown in Fig. L8, naïve TEsA T cells could not induce differentiation of IgG1+ memory B cells into plasma cells. By contrast, CXCR5+ memory B

Significance

Follicular helper T (TFH) cells have emerged as the key cell type required for the formation of germinal centers and subsequent long-lasting antibody responses. It has been demonstrated that TFH cells enter the memory pool. However, it is unclear how the generation, survival, or activation of those TFH memory cells is regulated. Here we show that B-cell lymphoma 6 (Bcl6), a master regulator of TFH generation, is required for maintenance of TFH memory cells and subsequent humoral memory. In these recall responses, antigen-specific memory B cells majorly contribute to the quick induction of Bcl6 in TFH memory cells. Our results reinforce the importance of cognate interaction between memory TFH and memory B cells and give important implications for development of better vaccines.

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This differential B-cell helper activity was similarly observed when these two subsets exhibited differential memory B-cell helper activity, CXCR5⁺ or CXCR5⁻ 2WS-specific memory T cells were transferred together with B1-8hi IgG1⁺ memory B cells into congenic mice, followed by immunization with soluble NP-2WS-OVA. As shown in Fig. S2 E and F, CXCR5⁺ 2WS-specific memory T cells were superior to their CXCR5⁻ counterparts in inducing IgG1 response by memory B cells. Thus, the similarity between the endogenous and TCR-transgenic T cells validated the use of the adoptive transfer approach for subsequent studies of memory T cells.

**CXCR5⁺ Memory T Cells Are Derived from TFH Effectors.** Several recent studies have provided evidence that TFH effector T cells can further differentiate into resting memory CD4⁺ T cells (12–16). To confirm that CXCR5⁺ memory T cells in our system are derived from TFH effectors, we purified CXCR5⁺PD1⁺ (TFH) or CXCR5⁺PD1⁻ (non-TFH) TEa effector cells, transferred them into congenic naive mice, and then analyzed the phenotype of the surviving cells 4 wk later. As shown in Fig. S3A, the majority of the cells derived from TFH effectors maintained high levels of CXCR5 expression and were localized in the T-cell area, T-B border, or B-cell follicles, whereas non-TFH-derived cells gave rise to cells with lower CXCR5 expression and were found exclusively in the T-cell area. More importantly, TFH-effector-derived memory T cells had potent helper activity for antibody responses when B1-8hi memory B cells were transferred into the same recipients (Fig. S3 B and C). The TFH-effector-derived memory T cells elicited stronger plasma cell differentiation and IgG1 response than their non-TFH-derived counterparts. Thus, we concluded that the majority of CXCR5⁺ memory T cells originate from TFH effector cells.

**Bcl6 Is Expressed in CXCR5⁺ Memory T Cells.** Bcl6 is a master regulator of TFH generation, and TFH cells express high levels of Bcl6 (1). However, it is not clear whether Bcl6 is also required for maintenance or function of memory T cells derived from TFH effector cells. Before addressing this question, we determined the expression status of Bcl6 by examining Bcl6 protein levels in Bcl6-YFP reporter mice (17). As shown in Fig. 2A, a fraction of CXCR5⁺ effector T cells at 1 wk after immunization expressed high levels of Bcl6-YFP. The Bcl6-YFP⁺ cells were not present in the memory T-cell population. CXCR5⁺ memory T cells expressed quite low but slightly higher levels of Bcl6-YFP than naive or CXCR5⁺ memory T cells. Bcl6 mRNA levels correlated well with its protein levels (Fig. 2B). Twenty-four hours after rechallenge with soluble NP-Eα-Ova, cells with high levels of Bcl6-YFP were induced. CXCR5 was also up-regulated and high levels of Bcl6-YFP were observed in CXCR5⁻ cells (Fig. 2C). Bcl6-YFP expression was detected in the T-B border and B-cell follicles in the spleen (Fig. 2D), where CXCR5⁺ memory T cells were also enriched, suggesting the involvement of B cells in the activation of CXCR5⁺ memory T cells.

To investigate whether CXCR5⁺ memory T cells are the cells that express Bcl6 upon rechallenge, CXCR5⁺ or CXCR5⁻ memory T cells were purified, transferred, and restimulated with soluble antigen. As shown in Fig. 2E, Bcl6-YFP expression was induced by CXCR5⁺ donor cells, but barely by CXCR5⁻
which allowed conditional deletion of the bcl6 gene from TEa memory T cells by administration of tamoxifen. TEa CD4+ T cells were purified from Cre-ERT2 or Cre-ERT2 × Bcl6/YFP mice and were adoptively transferred into C57BL/6 mice. Six weeks after immunization with NP-Exo-GFP/alum, tamoxifen was administered on three consecutive days to delete the bcl6 gene from the transferred T cells (Fig. 3A). We confirmed that 90% of loxP-flanked DNA was deleted in memory TEa T cells in Cre-ERT2 × Bcl6/YFP mice (Fig. 3B). The phenotype of TEa memory T cells was examined 10 d after the last tamoxifen treatment. Deletion of the bcl6 gene by tamoxifen administration did not affect the number of CXCR5+ memory T cells (Fig. 3C). However, we observed a significant decrease in the number of CXCR5+ memory T cells (Fig. 3C), suggesting that Bcl6 is required for the survival of CXCR5+ memory T cells.

The requirement of Bcl6 for the survival of CXCR5+ memory T cells was further confirmed. CXCR5+ memory TEa T cells derived from Cre-ERT2 × Bcl6/YFP mice were purified and transferred to congenic mice, followed by tamoxifen treatment. As shown in Fig. S6, bcl6 deletion by tamoxifen treatment significantly decreased the number of donor-derived cells, suggesting that loss of CXCR5+ memory T cells was due to cell death, but not to phenotypic change.

We purified surviving memory T cells 10 d after the last tamoxifen treatment and transferred them into C57BL/6 mice that had received B1-8hi memory B cells. Upon rechallenge with NP-Exo-OVA, generation of CXCR5hiPD1hi T cells from transferred memory T cells was strongly inhibited by bcl6 deletion (Fig. 3D). Consequently, generation of CD138+ plasma cells from NP-specific memory B cells was also compromised (Fig. 3E). Collectively, these results show that Bcl6 is essential for maintenance of CXCR5+ memory T cells and subsequent memory antibody response.

CD11c+ Cells Are Dispensable for the Activation of CXCR5+ Memory T Cells and Secondary Ab Responses. The induction of Bcl6 in naïve T cells has been reported to require two steps (5–7, 9, 19); Bcl6 is first induced by interactions between conventional dendritic cells (DCs) and T cells. Then, although cognate B cells are not required for the induction of Bcl6, they support the expansion of
Bcl6-expressing Tfh cells. Rapid induction of Bcl6 in memory T cells, mainly in the T−B border region as described above, suggested that Bcl6 in CXCR5+ memory T cells might be induced in a manner different from that in naïve T cells. Therefore, we first determined whether interactions between CXCR5+ memory T cells and conventional DCs are necessary for up-regulation of Bcl6 and subsequent secondary antibody responses. We transferred naïve TEa × Bcl6-YFP T cells into CD11c-DTR Tg mice, followed by immunization with NP-Eα-GFP/alum. Six weeks later, the mice were administered diphtheria toxin (DT) to ablate CD11c+ cells and then were rechallenged on the next day with soluble NP-Eα-OVA to induce memory T-cell activation (Fig. 4). We confirmed that DT treatment efficiently depleted CD11c+ cells (Fig. S7A). Rechallenge with NP-Eα-OVA induced up-regulation of Bcl6 in CD11c-DTR mice (Fig. 4A). Notably, depletion of CD11c+ cells in CD11c-DTR mice did not compromise Bcl6 up-regulation in CXCR5+ memory T cells. Along with these data, the secondary anti-NP IgG1 response was normally induced even after CD11c+ cells were depleted (Fig. 4B). When NP-Eα-Ova with alum were used for the rechallenge, Bcl6 up-regulation was again unaffected by depletion of CD11c+ cells (Fig. S7B). As has been previously demonstrated (7), ablation of CD11c+ cells resulted in impaired Bcl6 induction by naïve T cells (Fig. S7C). Thus, we concluded that upon rechallenge with soluble antigen CD11c+ cells are not required for generation of Bcl6+ T cells and subsequent memory antibody responses.

Antigen-Specific Memory B Cells Efficiently Present Antigen and Activate CXCR5+ Memory T Cells. We next attempted to determine which cells could present antigen to activate CXCR5+ memory T cells during secondary immune responses. Soluble NP-Eα-GFP antigen was administered to WT mice that were unprimed or previously primed with NP-CGG/alum. In this setting, presentation of the Ea peptide could be monitored with the Y-Ae mAb, which is specific for Eα-I-Aβ complexes. We examined antigen presentation by DCs (CD11c+ MHC class II(+) total B cells (B220+) or NP-specific naïve B cells (B220+ NIP+CD138−)), and NP-specific memory B cells (B220+ NIP+ CD138+CD273+). As demonstrated in Fig. 5A, when we compared total B220+ B cells and total CD11c+ DCs, 0.8% and 2.4%, respectively, presented Ea peptide 24 h after antigen injection. However, if we focus on NP-specific B cells, 8.9% of NP-specific naïve B cells present the antigen. Notably, NP-specific memory B cells presented the antigen more efficiently; 17% of them were Y-Ae positive.

To examine whether antigen-specific memory B cells could indeed contribute to the activation of CXCR5+ memory T cells, we transferred TEa × Bcl6-YFP T cells into congenic mice, followed by immunization with Eα-GFP/alum. Then, we transferred NP-specific or NP-nonspecific memory B cells into the primed mice, just before the rechallenge with NP-Eα-OVA. As shown in Fig. 5B, transferred NP-specific memory B cells were able to enhance Bcl6-YFP expression by TEa memory T cells, whereas NP-nonspecific memory B cells failed to induce Bcl6-YFP. These results suggest that antigen-specific memory B cells...
activate cognate memory T cells to express Bcl6. This induction of Bcl6 was blocked by pretreatment of NP-specific memory B cells with anti-class II Ab (Fig. S8). Collectively, these results demonstrate that antigen-specific memory B cells indeed contribute to rapid Bcl6 up-regulation in CXCR5+ memory T cells by virtue of APC function.

Discussion

We previously demonstrated that IgG1 memory B cells require CD4 T cells to initiate secondary humoral immune responses (20). In this study, we have addressed two issues: which T-cell subset is mainly responsible for helping IgG1 memory B cells and how these T cells are activated upon secondary challenge, thereby inducing rapid and robust humoral responses. Here, we show that CXCR5+ TFH memory cells localize close to B-cell follicles and are superior in helping memory B-cell activation. Furthermore, we show that the CXCR5+ TFH memory cells promptly reexpress Bcl6 upon secondary challenge, and that this is primarily induced by the APC function of memory B cells. Thus, our results reinforce the importance of cognate interactions between TFH memory cells and memory B cells in humoral memory responses.

Regarding the commitment or plasticity of CXCR5+ TFH memory cells, several recent studies have come to different conclusions (13, 14, 21). Although our study has not directly addressed this issue, two lines of our evidence from our studies are more consistent with the idea that CXCR5+ TFH memory cells are committed populations that are poised for the lineage-specific reexpression of effector molecules upon rechallenge. First, testing TFH and non-TFH memory cells arose from the corresponding effector cells, respectively. Second, CXCR5+ TFH but not CXCR5− non-TFH memory cells up-regulated Bcl6 upon antigen rechallenge. In regard to the mechanistic aspect of the commitment of TFH memory cells, the low level of Bcl6 expression at the memory phase, as discussed below, might not allow the TFH memory cells to assume other lineages.

Based on studies using germ-line knockout or transgenic mice, Bcl6 has previously been suggested to be involved in CD8 and CD4 T-cell memory development (22, 23). However, it has not been clear whether Bcl6 is required for maintenance of memory T cells. By taking advantage of a conditional deletion system for the bcl6 gene we could demonstrate that TFH memory cells rely on Bcl6 for their survival. Inducible deletion of bcl6 from the antigen-specific memory T-cell compartment selectively decreased the number of CXCR5+ memory T cells. Consistent with a previous report (24), CXCR5+ TFH memory cells have quite low levels of Bcl6, only slightly higher than those in their CXCR5− counterparts or in naïve T cells. Conceivably, such low levels of Bcl6 are sufficient and required for survival of these cells. The molecular mechanisms by which Bcl6 controls survival of TFH memory cells are currently speculative. Given that Blimp-1 and Bcl6 are antagonistic transcription factors, repression of Blimp-1 by Bcl6 might be one of the potential survival mechanisms. Indeed, in the case of Blimp-1−/−/deficient CD8 T cells, memory precursor cells survived better (25).

We and others previously proposed that memory B cells are the primary APCs in the memory response and that locally confined TFH memory cells are the cognate regulators of the memory B-cell response (26, 27). These proposals are well substantiated by the following two lines of evidence presented in this study. First, memory B cells present antigens with high efficiency upon soluble antigen rechallenge compared with naïve B cells. Furthermore, memory B cells are significant contributors to the rapid up-regulation of Bcl6 on CXCR5+ TFH memory cells upon rechallenge. Second, the rapid and robust Bcl6 expression in CXCR5+ TFH memory cells was observed in locally confined regions (at the T−B border or in B-cell follicles), strongly suggesting the occurrence of cognate interactions between memory B cells and locally confined TFH memory cells. Although our data define memory B cells as the major APCs, it still remains possible that other APCs, such as DCs, can participate at least to some extent. Indeed, a recent report shows that even in a B-cell−deficient condition recall TFH-like response can occur. In these studies in a lymphocytic choriomeningitis virus infection system in B-cell−deficient μMT mice, TFH-like memory cells were able to recall a TFH-like response, although the efficiency was lower compared with WT mice (21). These observations, at first glance, seem to contradict our conclusion. However, in the life-long B-cell−deficient condition there may be some compensation and other APCs probably play a more crucial role in activating TFH memory cells.

Because the kinetics of IL-21 and Bcl6 up-regulation in CXCR5+ TFH memory cells upon rechallenge are correlated, it is likely that rapid Bcl6 up-regulation is a primary inducer of rapid differentiation of TFH memory cells toward effector cells. In regard to the rapid Bcl6 up-regulation, three mechanisms can be
envisioned. First, memory B cells with relatively high-affinity B-cell antigen receptors are able to rapidly capture low levels of secondary antigen and present this antigen to the cognate TFH memory cells. In this context, increased levels of CD80 and MHC class II on memory B cells could contribute to efficient activation of TFH memory cells (28). Second, cognate memory TFH cells reside in close proximity to memory B cells, which should facilitate their interactions. Finally, TFH memory T cells might undergo positive epigenetic modification of genes that allow them to swiftly up-regulate Bcl6. For instance, in the case of Tfh1 memory cells, Hale et al. recently demonstrated the epigenetic modification of the granzyme B locus (21).

Given the functional heterogeneity of memory B-cell subsets (e.g., IgM+ vs. IgG1+ memory B cells) (29, 30), it is possible that each subset might differentially contribute to activation of TFH memory cells. Thus, better understanding of the regulatory mechanisms in the interactions of these memory B-cell subsets and TFH memory cells should provide important insights for development of better vaccines.

Materials and Methods

Mice. Mice were maintained under specific pathogen-free conditions in accordance with the guidelines of the Animal Care and Use Committee of Osaka University. C57BL/6 mice were purchased from CLEA Japan. Tfh1 TCR transgenic mice (CD45.2/CD45.1 or CD45.1/CD45.2) (31), Bcl6-YFP reporter mice (17), CD11c-GFP-pTrcHis2) was kindly provided by Dr. Dan Brubaker (University of California, San Francisco) for critical reading of our manuscript, and H. Masuda for technical assistance. This work was supported by grants to W.I. and T.K. from the Ministry of Education, Culture, Sports, Science, and Technology and by a grant to T.K. from Japan Science and Technology Agency, Core Research for Evolutionary Science and Technology.


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