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Differential Requirements for OX40 Signals on Generation of Effector and Central Memory CD4⁺ T Cells

Pejman Soroosh, Shouji Ine, Kazuo Sugamura, and Naoto Ishii

Memory T cells can be divided into effector memory (TEM) and central memory (TCM) subsets based on their effector function and homing characteristics. Although previous studies have demonstrated that TCR and cytokine signals mediate the generation of the two memory subsets of CD8⁺ T cells, the mechanisms for generation of the CD4⁺ TEM and TCM cell subsets are unknown. We found that OX40-deficient mice showed a marked reduction in the number of CD4⁺ TEM cells, whereas the number of CD4⁺ TCM cells was normal. Adoptive transfer experiments using Ag-specific CD4⁺ T cells revealed that OX40 signals during the priming phase were indispensable for the optimal generation of the CD4⁺ TEM, but not the CD4⁺ TCM population. In a different transfer experiment with in vitro established CD4⁺ CD44<sup>hi</sup>CD62L<sup>lo</sup> (TEM precursor) and CD4⁺ CD44<sup>hi</sup>CD62L<sup>hi</sup> (TCM precursor) subpopulations, OX40-KO TEM precursor cells could not survive in the recipient mice, whereas wild-type TEM precursor cells differentiated into both TEM and TCM cells. In contrast, TCM precursor cells mainly produced TCM cells regardless of OX40 signals, implying the dispensability of OX40 for generation of TCM cells. Nevertheless, survival of OX40-KO TEM cells was partially rescued in lymphopenic mice. During in vitro recall responses, the OX40-KO TEM cells that were generated in lymphopenic recipient mice showed impaired cytokine production, suggesting an essential role for OX40 not only on generation but also on effector function of CD4⁺ TEM cells. Collectively, the present results indicate differential requirements for OX40 signals on generation of CD4⁺ TEM and TCM cells. The Journal of Immunology, 2007, 179: 5014–5023.

Follow recognition of Ag, naive T cells begin a differentiation process that culminates in the generation of effector T cells, and a small number of the effector T cells survive and develop into memory T cells, which confer long-term immunity. Recent studies have shown that, similar to CD8⁺ memory T cells, CD4⁺ memory T cells are heterogeneous and can be divided into effector memory (TEM) and central memory (TCM) subsets based on their phenotype, function, and anatomic distribution (1, 2). TEM (CD44<sup>hi</sup>CD62L<sup>lo</sup>) cells reside in both lymphoid and nonlymphoid tissues, where they elicit immediate protection by producing effector cytokines at the site of Ag encounter. In contrast, TCM cells (CD44<sup>hi</sup>CD62L<sup>hi</sup>) mainly localize to the secondary lymphoid tissues, where they mediate long-lasting protection through efficient clonal expansion (3–5).

Accumulating evidences have shown that TCR signals and homeostatic cytokines, such as IL-2, IL-7, and IL-15, critically regulate the generation of CD8⁺ memory T cells (6, 7). TCR and IL-7 signals are indispensable for the generation of both CD8⁺ TEM and TCM subsets. In contrast, IL-2 and IL-15 have distinct effects on generation of CD8⁺ memory T cells (8). Although IL-15 preferentially promotes generation of TCM cells, IL-2 mainly contributes to development of TEM cells (9). Furthermore, recent evidences have demonstrated that generation of CD4⁺ and CD8⁺ memory T cells is controlled by distinct mechanisms. For example, despite crucial roles for IL-15 on the generation of CD8⁺ memory T cells, its function in homeostasis of CD4⁺ memory T cells is controversial (6, 7, 10). On the other hand, CD8⁺ memory T cells can be easily detected by TCR-specific MHC class I/peptide-tetramer, but it is unclear how CD4⁺ memory T cells are generated probably due to lack of MHC class II/peptide-multimer. Recent papers, nevertheless, have shown that IL-7 is essential for generation and survival of the both CD4⁺ TEM and TCM cell subsets. Blockade of IL-7 signals by treatment with anti-IL-7 receptor mAb markedly diminished memory T cell population (12). Moreover, survival of both CD4⁺ memory T cell subsets was completely suppressed in IL-7-deficient mice (13). However, it is still unknown whether the generation and homeostasis of the two subsets of CD4⁺ memory T cells is mediated by distinct mechanisms.

Signals through T cell costimulatory molecules are critically involved in eliciting optimal T cell activation during Ag priming (14, 15). Among the T cell costimulatory molecules, several TNF receptor superfamily molecules, such as OX40 (CD134), CD27, and 4-1BB also contribute to the generation of memory T cells, probably by promoting the survival and expansion of effector T cells (16–23). Furthermore, Croft and colleagues (24) have recently demonstrated that OX40 signals play a critical role not only in accumulation of effector T cells but also in the effector function of long-lived CD4⁺ T cells, suggesting a possible role for OX40 on CD4⁺ TEM cells. However, little is known about whether OX40 signals may preferentially contribute to generation of either CD4⁺ TEM or TCM cell subset, or equally promote the generation of both two subsets. In the present study, we demonstrate that OX40 signals during the Ag-priming phase promote the survival of CD4⁺ TEM cells. In contrast, generation of the CD4⁺ TCM subset is independent of OX40 signals.
Materials and Methods

Mice

Six- to eight-week-old female C57BL/6 mice were purchased from Japan SLC. OT-II TCR-transgenic mice were a gift from Dr. W. Heath (WEHI, Melbourne, Australia) and were used as a source of V_{a}^{12}V_{B5.1-2^{n}} CD4^{+} T cells responsive to the OVA_{323-339} peptide (23). OT-II OX40-KO mice were generated in-house by intercrossing OT-II mice with OX40-KO mice. To analyze polyclonal TEM and TCM populations, 16-wk-old OX40-KO and sex-matched control wild-type mice, both of which were bred and kept under the same condition in our facility, were used. All the mice were on a C57BL/6 background, and they were bred and maintained under specific-pathogen-free conditions at the Institute for Animal Experimentation, Tohoku University Graduate School of Medicine.

Isolation of lymphocyte populations

Single-cell suspensions were prepared from the lymph nodes and spleens. Lymphocytes were isolated from nonlymphoid tissues as previously described (24). The number of CD4^{+} T_{EM} and T_{CM} cells was calculated based on the percentage of each subpopulation that was CD44^{high}CD62L^{low} and CD44^{high}CD62L^{high}, respectively, and the total cell number
in each organ. Naive CD44hi CD4+ T cells were purified from the spleen of OT-II and OT-II OX40-KO mice by using mouse anti-CD4 MicroBeads and an AutoMACS magnetic cell sortor (Miltenyi Biotec) and an AutoMACS magnetic cell sortor (Miltenyi Biotec). To isolate Ly5.2+ donor T cells, the spleen cells from Ly5.1+ recipient mice were incubated with a biotin-labeled anti-Ly5.1 Ab (BD Biosciences) and a biotin-labeled mixture of Abs for specific for CD4+ T cells (provided with the mouse CD4+ T cell isolation kit (Miltenyi Biotec). Anti-biotin Ab-conjugated magnetic beads (Miltenyi Biotec) were then added and the donor T cells (negative fraction) were purified using an AutoMACS cell sortor. The TEM (CD2L2hi) and TCM (CD2L2lo) cell fractions in the Ly5.2+ fraction were further divided using mouse anti-CD2L2 MicroBeads (Miltenyi Biotec). The purity of the sorted samples was ≥90% for each population.

**Antibodies and flow cytometry**

The following Abs were purchased from BD Biosciences: anti-CD4, anti-CD8, anti-CD44, anti-CD25, anti-CD44, anti-CD4, anti-CD62L, anti-CD62, anti-Vα2-PE, anti-Vβ5-FITC, anti-Ly5.1, anti-Ly5.1, anti-CD44, anti-CD62L, and anti-Ly5.2. Cell staining was performed as described previously (27). Streptavidin-allophycocyanin (BD Biosciences) was used to visualize biotin-labeled Abs. Control rat IgG (Cappel) and inhibitory anti-CD40L mAb (MGP34) were used in some cell cultures (22). All the samples were analyzed with a FACSCalibur flow cytometer (BD Biosciences). The analyses were conducted using the CellQuest program (BD Biosciences).

**In vitro cell stimulation and cytokine assays**

Naive T. TEM, and TCM cells from OT-II and OT-II OX40-KO mice were stimulated with OVA13-23,39 (0.1 μM) in the presence of wild-type irradiated (30 Gy) T cell-depleted splenocytes (APCs). Proliferation was measured in triplicate by the incorporation of [3H]thymidine (1 μCi/well; ICN Pharmaceuticals) during the last 8 h of each culture. In vitro T cell survival was determined by Trypan blue exclusion and the percent recovery was calculated based on the input number of cells. Cytokine levels in cell culture supernatants were assayed using ELISA kits for IL-2, IL-4, IFN-γ (BD Biosciences), and IL-13 (R&D Systems), according to the manufacturer’s recommendations.

**Adoptive transfer experiments**

Purified naive CD4+ T cells (2.5–5 × 10^6) from the spleen of OT-II or OT-II OX40-KO mice with the Ly5.2+ congenic marker were injected into the tail vein of untreated Ly5.1+ congenic wild-type mice. One day later, the mice were immunized i.v. with 2 mg OVA protein (Worthington) plus 50 μg LPS. In a second experiment, 1 × 10^6 OT-II and OT-II OX40-KO T cells were primed with 0.1 μM OVA13-23,39 in the presence of irradiated wild-type splenocytes (4 × 10^6) for 3 days and then were re-stimulated for one more day in fresh medium in the absence of OVA. To block the OX40-OX40L interaction, an anti-OX40L mAb (MGP34) was added to the cell culture during priming, as previously described (28). The in vitro-activated Ly5.2+ OT II or OT-II OX40-KO T cells (2.5–5 × 10^6) were adoptively transferred into untreated or sublethally irradiated (500 Gy) Ly5.1+ congenic wild-type mice. In some cases in vitro activated Ly5.2+ OT-II or OT-II OX40-KO T cells were further divided to CD62L2hi and CD62L2lo subsets using mouse anti-CD62L MicroBeads (Miltenyi) and then transferred (1–2 × 10^6) into naive Ly5.1+ congenic wild-type mice. After the indicated number of days, the donor T cells in the lymphoid and nonlymphoid tissues were tracked by determining the percentage of each subpopulation and the total cell number.

**Statistical analysis**

Statistical analyses were done with Student's t test. Values of p < 0.01 were considered significant.

**Results**

**Tissue distribution of CD4+ memory T cell subsets in lymphoid and nonlymphoid organs of wild-type and OX40-deficient mice**

Although previous reports showed that the OX40-OX40L interaction promotes the generation of CD4+ memory T cells (16, 17, 21, 22), whether the TEM and TCM cell subsets require OX40 signals equally is unknown. We addressed this question by estimating these populations in OX40-deficient mice. Interestingly, the percentage and cell number of the CD4+ TEM population in the spleen and peripheral lymph nodes of old OX40-deficient mice (16 wk old) were significantly reduced, whereas those of the TCM cells were unchanged in the absence of OX40 expression (Fig. 1A). These results are compatible with previous observations that OX40L-KO mice have less CD44hiCD2L2loCD4+ T cell population in the spleen as compared with wild-type mice, whereas OX40L-transgenic mice show a significant increase in the splenic CD4+ TEM population (29). Because the major home for TEM cells is extralymphoid tissues, we examined the TEM population in these tissues. A marked reduction in CD4+ TEM cells was observed in the liver, lung, peritoneal cavity, and lamina propria of the colon in the OX40-deficient mice compared with wild-type mice (Fig. 1B). Although it is largely unknown how the polyclonal memory T cells arise naturally in unmanipulated mice, it has been proposed that immune responses to environmental Ags may lead to their generation. In fact, the reduction of CD4+ TEM cells seen in the OX40-deficient mice was most prominent in the lung and lamina propria of the colon (Fig. 1B) probably due to the active immune responses to environmental Ags that take place in these mucosal tissues. These observations suggest that OX40 signals may be involved in the generation or survival of CD4+ TEM cells in lymphoid and nonlymphoid tissues.

**OX40 signals contribute to the generation of Ag-specific effector memory but not central memory CD4+ T cells**

The complex repertoire of T cells in normal mice and the constitutive stimulation of endogenous T cells by environmental Ags, such as bacterial flora in the intestine and autoantigens, make it difficult to analyze the Ag-specificity of CD4+ memory T cells. Therefore, to address functional roles for OX40 in the generation and homeostasis of CD4+ memory T cells in an Ag-specific manner, we used OVA-specific TCR-transgenic T (OT-II; MHC class II restricted) cells. Naive OT-II or OT-II OX40-KO T cells (Ly5.2) were adoptively transferred into congenic wild-type Ly5.1 mice. The recipient mice were then given an i.v. injection of OVA plus LPS to induce the transient activation of the donor T cells, some of

**FIGURE 2.** OX40 signals selectively control size of Ag specific TEM but not TCM cells. To generate activated (3 days p.i) and memory (45 days p.i) T cells and naive Ly5.2 CD4+ OT-II and OT-II OX40-KO T cells were adoptively transferred into Ly5.1 congenic wild-type mice, followed by immunization with OVA plus LPS. A, the expression of CD44 and CD62L is depicted on naive, newly activated, and memory OT-II and OT-II OX40-KO CD4+ T cells in the spleen. The percentage (left) and absolute numbers (right) of CD44hiCD62Llo (●) and CD44hiCD62Lhi (□) populations in activated (3 days p.i) OT-II T cells is shown. The donor T cells in each quadrant indicate the percentage of each subset in Ly5.2 donor T cells. B, surface expression of CD25 and OX40 on CD44hiCD62Llo and CD44hiCD62Lhi (□) populations in activated (3 days p.i) OT-II T cells is shown. The donor T cells in each quadrant indicate the percentage of each subset in Ly5.2 donor T cells. C, expression of CD44 and CD62L is shown on memory (45 days p.i) OT-II or OT-II OX40-KO CD4+ T cells in the peripheral lymph nodes and nonlymphoid tissues. The percentage (left) and absolute numbers (right) of CD44hiCD62Llo (●) and CD44hiCD62Lhi (□) donor memory OT-II T cells were determined in the peripheral lymph node, splenocytes, and lungs of the recipient mice. Results from spleens (A) and peripheral lymph nodes represent the mean ± SD from four mice per group, and are representative of two independent experiments. For nonlymphoid tissues, cells from the lungs and livers were pooled separately from the same mice. The significance of the data was evaluated by Student’s t test (* and **, p < 0.01 and p < 0.001, respectively).
which can differentiate into OVA-specific memory T cells (3). Three and 45 days post immunization, donor CD4⁺ T cells were tracked on the basis of expression of the Ly-5.2 congenic marker. Activated OT-II cells expressed CD25 and CD44 (Fig. 2, A and B), whereas the long-lived donor OT-II cells lost CD25 expression despite the persisting CD44^{high} phenotype regardless of OX40 expression (Fig. 2, A and B). In addition, in agreement with previous results, CD25⁺ activated OT-II cells were larger than the memory OT-II cells (data not shown) (2). These markers thus enabled us to distinguish between activated OT-II and memory OT-II T cells.
both of which have high CD44 expression. Further flow-cytometric analyses revealed that the both activated and memory OT-II cells in the recipient mice were heterogeneous for CD62L expression, with two distinct populations, CD62L\text{low} and CD62L\text{high} (Fig. 2A). Because the CD62L\text{low} and CD62L\text{high} populations in CD44\text{high} memory T cells are the TCM and TEM cells, respectively, the two populations of activated OT-II donor cells are probably the precursors for the two memory T cell populations. Interestingly, at the early stage of Ag priming, activated OT-II and OT-II OX40-KO donor cells showed comparable numbers of these TEM precursors (CD44\text{high}CD62L\text{low}) (middle panel of Fig. 2A), suggesting that OX40 signals are dispensable for the generation of the TEM precursor cells during early stage of stimulation. In contrast, the number of long-lived OT-II OX40-KO TEM cells was significantly diminished as compared with OT-II TEM cells, while the TCM pools were equivalent between the OT-II and OT-II OX40-KO cells at the memory phase (lower panel of Fig. 2A). These findings are consistent with previous observations that, despite of the dispensability of OX40 signals on the initial generation of activated T

both of which have high CD44 expression, OX40 signals can promote accumulation of effector CD4+ T cells at later times (16, 21, 23). Similarly, in the peripheral lymph nodes, where TCM cells are dominant, a significant reduction of OT-II OX40-KO TEM, but not TCM cells, was observed (Fig. 2C). Furthermore, in the liver and lung, both of which are major homing sites for TEM cells, the accumulation of OT-II OX40-KO TEM cells was much lower than that of OT-II wild-type TEM cells (Fig. 2C). However, we could detect very few donor cells, even derived from OT-II wild-type mice, in the peritoneal cavity, bone marrow, and the lamina propria of the intestine of recipient mice (data not shown). These results suggest that OX40 signals preferentially contribute to the generation of CD4+ TEM rather than CD4+ TCM cells. To understand the distinct requirements for OX40 on the generation of CD4+ TCM and TEM cells, we further examined kinetic expression of OX40 on CD4+ TEM precursor in comparison with that on CD4+ TCM precursor during 7 days after Ag stimulation. However, no significant difference in OX40 expression levels between the two populations was observed (Fig. 2B and data not shown). Thus, OX40 expression
FIGURE 5. OX40 signals preferentially regulate the functional reactivity of T<sub>EM</sub> and not T<sub>CM</sub> cells. A, Persistent CD44<sup>high</sup>CD62L<sup>low</sup> and CD44<sup>high</sup>CD62L<sup>high</sup> donor T cells were separately purified from wild-type sublethally irradiated congenic hosts that had received in vitro-activated OT-II or OT-II OX40-KO CD4<sup>+</sup> T cells four weeks before (see Materials and Methods). The histograms were gated on Ly5.2 CD44<sup>high</sup> cells, and the resultant T cells were 85–95% pure. B, Sorted (CD62L<sup>low</sup> and CD62L<sup>high</sup>) memory OT-II and OT-II OX40-KO T cells pooled from five adoptive hosts (2.5 x 10<sup>5</sup>)
levels on the two populations are unable to explain the distinct requisites for OX40 on their generation. To confirm the role for OX40 on the generation of CD4⁺ TEM cells, we next used different type of the common method for developing long-lived CD4 T cells, in which OT-II and OT-II OX40 KO CD4 T cells were activated in vitro (Fig. 3A) and then transferred into naive wild-type or sublethally-irradiated naive wild-type congenic mice (30–32). Before the transfer and 6 wks after that, both the in vitro-activated T cells (Fig. 3A) and persisting donor T cells (Fig. 3B) up-regulated CD44 expression. In addition, compatible with the former in vivo results (Fig. 2A), the CD44<sup>high</sup> population in both in vitro-activated OT-II and long-lived donor cells could be divided into the CD62L<sup>low</sup> and CD62L<sup>high</sup> populations (Fig. 3, A and B). Similar to in vivo results the long-lived OT-II OX40-KO TEM cells were severely reduced, whereas the T<sub>CM</sub> numbers were comparable regardless of OX40 expression (Fig. 3B). Although the reduction of the donor TEM pool was statistically significant in either recipient, the lymphopenic status of the irradiated mice appeared to partially rescue the OX40-dependent TEM-cell generation in terms of the cell number and ratio to the T<sub>CM</sub> population (Fig. 3B). The requirement for OX40 signals for the survival of TEM cells in the lymphopenic environment might be less stringent than under the full-lymphocyte condition. The limited competition between donor and intrinsic T cells for homeostatic cytokines and self peptide-MHC II ligand under the lymphopenic conditions might elicit the OX40-independent compensatory effects on homeostasis of donor TEM cells. OX40 signals, nevertheless, are still dispensable for the generation and homeostasis of T<sub>CM</sub> cells, even under lymphopenic conditions (Fig. 3B).

To further analyze of whether OX40 signals during early Ag-priming are sufficient for long-term survival of CD4⁺ TEM cells Ag-primed OT-II cells were generated in vitro in the presence or absence of an inhibitory anti-OX40L mAb, and subsequently, equal numbers of the primed OT-II T cells were transferred into untreated naive wild-type mice. As expected, blockade of the OX40-OX40L interaction during the priming phase was sufficient to suppress the TEM cell pool, whereas the T<sub>CM</sub> cell number was unchanged (Fig. 3C). Thus, one of the major roles of OX40 signals may be to guarantee a long-lasting survival advantage in the TEM precursor cells, which ultimately translates into the optimal generation of the TEM population.

**OX40 signals are essential for expansion and survival of CD4⁺ TEM precursor cells**

We next investigated whether OX40 signals directly promote accumulation of CD4⁺ TEM precursor cells, or OX40 signals may induce conversion of CD4⁺ TEMCM precursor to TEM cells. To address this, CD4⁺CD62L<sup>low</sup>CD44<sup>high</sup> and CD4⁺CD62L<sup>high</sup>CD44<sup>high</sup> subsets were independently isolated from in vitro activated OT II and OT II OX40-KO T cells and transferred into naive wild-type congenic mice. Four weeks after the transfer, the population and the cell number of TEM and T<sub>CM</sub> cells derived from either transferred CD62L<sup>low</sup> or CD62L<sup>high</sup> memory precursor cells were evaluated in the recipient mice. Consistent with previous reports on CD8⁺ memory T cells (33, 34), the phenotype of CD4⁺CD62L<sup>low</sup>CD44<sup>high</sup> cells (T<sub>EM</sub> precursor) was almost stable, and they could potently expand in the absence of OX40 signals (Fig. 4). In contrast, wild-type CD4⁺CD62L<sup>low</sup>CD44<sup>high</sup> TEM cells (T<sub>EM</sub> precursor) phenotypically differentiated into both TEM and T<sub>CM</sub> cells (left, Fig. 4). Nevertheless, neither TEM nor T<sub>CM</sub> cell was detected when CD4⁺KO TEM precursor cells were transferred (right, Fig. 4). Collectively, OX40 signals play a critical role in expansion and survival of TEM precursor cells.

**OX40 signals preferentially regulate the functional reactivity of TEM rather than T<sub>CM</sub> cells**

With the goal of defining the functional roles of OX40 signals in the different subsets of memory T cells, we analyzed the recall responses of each memory T cell population in the presence or absence of OX40 signals. Because the OX40-KO TEM population almost vanished in intact hosts, we decided to use sublethally irradiated mice as recipients, in which we could detect 20 times more OX40-KO T<sub>EM</sub> cells than in unirradiated recipients (Fig. 3B). Four weeks after the transfer, the persisting donor CD4⁺CD62L<sup>low</sup> T<sub>EM</sub> and CD4⁺CD62L<sup>high</sup>T<sub>CM</sub> cells were sorted from the spleens of the recipient mice (Fig. 5A) and subjected to recall responses in vitro. Compatible with the common characteristics of T<sub>CM</sub> cells, upon in vitro Ag-stimulation donor CD4⁺CD62L<sup>high</sup>T<sub>CM</sub> cells showed a stronger proliferative activity, more IL-2 secretion, and lower effector cytokine production as compared with donor CD4⁺CD62L<sup>low</sup>T<sub>EM</sub> cells derived from the same recipient mice (Fig. 5, B and C). Interestingly, CD4⁺CD62L<sup>high</sup>T<sub>CM</sub> cells that were generated in the absence of OX40 signals showed comparable proliferation and cytokine production with wild-type CD4⁺CD62L<sup>high</sup>T<sub>CM</sub> cells, suggesting that OX40 signals are dispensable for the recall responses of T<sub>CM</sub> cells. However, the levels of IFN-γ, IL-4, and IL-13 in the culture supernatants of OT-II OX40-KO T<sub>CM</sub> cells were significantly lower than those of the OT-II wild-type T<sub>CM</sub> cells. These results suggest that OX40 contributes to optimal recall production of Th1 and Th2 cytokines from CD4⁺ TEM cells. Because OX40 signals are also involved in CD4⁺ T cell survival after Ag-priming (17, 28), each memory T cell subset was reactivated in vitro with Ag, and the accumulation of viable OT-II cells was monitored. The in vitro survival of TEM cells derived from OX40-KO donor cells was dramatically suppressed, even though their initial proliferation was not affected (Fig. 5, B and D). Again, the survival of the T<sub>CM</sub> cells was independent of OX40 signals. Taken together, these results suggest that OX40 signals are essential not only for the generation but also for functional reactivity of CD4⁺ TEM cells.

**Discussion**

The present study demonstrates that OX40 signals selectively promote the generation of CD4⁺ TEM cells but not CD4⁺ T<sub>CM</sub> cells. In contrast to the specific reduction of CD4⁺ TEM cells seen in the absence of OX40, constitutive interactions between OX40 and OX40L in OX40L-transgenic mice increase the number of CD4⁺ TEM cells but not CD4⁺ T<sub>CM</sub> cells (Refs. 29, 35, and N. Ishii and P. Soroosh, unpublished observations). Moreover, another study showed that the absence of OX40 signals in a rodent asthma model (using OX40-deficient Th2 cells or an inhibitory anti-OX40L mAb) significantly reduced the accumulation of CD4⁺ TEM cells and other inflammatory cells in the lung (36). These observations are consistent with the idea that the OX40-OX40L interaction is required for the optimal generation of TEM cells, which ultimately translates into the optimal generation of TEM cells. However, the levels of IFN-γ, IL-4, and IL-13 in the culture supernatants of OT-II OX40-KO T<sub>CM</sub> cells were significantly lower than those of the OT-II wild-type T<sub>CM</sub> cells. These results suggest that OX40 contributes to optimal recall production of Th1 and Th2 cytokines from CD4⁺ TEM cells. Because OX40 signals are also involved in CD4⁺ T cell survival after Ag-priming (17, 28), each memory T cell subset was reactivated in vitro with Ag, and the accumulation of viable OT-II cells was monitored. The in vitro survival of TEM cells derived from OX40-KO donor cells was dramatically suppressed, even though their initial proliferation was not affected (Fig. 5, B and D). Again, the survival of the T<sub>CM</sub> cells was independent of OX40 signals. Taken together, these results suggest that OX40 signals are essential not only for the generation but also for functional reactivity of CD4⁺ TEM cells.
specifically required for the generation of CD4+ TEM cells. Although TCR and IL-7 signals are essential for generation of the both memory T cell subsets (6, 7, 12, 13), differential roles of costimulatory signals for development of central and effector memory T cells has not been reported. Several studies have demonstrated that signals down-stream of OX40 costimulation promote expansion and survival of effector CD4+ T cells, which may lead to the optimal generation of TEM population (17, 23, 24, 28). Our results, however, suggest that OX40 signals are dispensable for generation of CD4+ TCM cells, indicating that the two distinct CD4+ memory populations have different requirements for OX40 costimulatory signals on their generation. Normal development and survival of TCM cells in the absence of OX40 signals may raise a question of whether TCM cells might be derived from a population of naive T cells that have never received optimal activation signals. However, comparable expression of CD25 and OX40 on both activated CD62Llow and CD62Lhigh populations during activation indicates that both subsets are efficiently activated (Fig. 2B and data not shown). Despite the optimal activation of CD62Lhigh subset, TCM precursors might be insensitive to OX40 costimulation probably due to redundant roles of other homeostatic signals (37, 38). In this regard, further investigation is required to dissect the specific signals that differentially control homeostasis of TEM and TCM cells.

The specific reduction of the CD4+ TEM pool seen in the absence of OX40 raises the question of how signals through OX40 selectively control the generation of TEM cells. Previous findings clearly demonstrated that the precursors of TEM and TCM cells develop from activated T cells as separate lineages, and confirmed the important observation that ‘late-arriving’ naive CD4+ T cells require further study.

reduction of TEM cells. According to this scenario, effective OX40 signals for development of naive T cells into effectors, resulting in the lack of OX40 signals attenuates the activation signals required for the differentiation of naive T cells into effectors, resulting in the reduction of TEM cells. According to this scenario, effective OX40 signals may be provided before or during the TEM/TCM commitment. However, the efficient development of TEM precursors (CD44highCD62Llow) in the absence of OX40 signals (Fig. 2A) suggests that OX40 signals might be dispensable for the lineage commitment of the CD62Llow and CD62Lhigh populations. Rather, presence of OX40 signals during initial Ag encounter imprint a survival program in the TEM cells. In these contexts, despite the importance of OX40 signals for the generation of CD4+ TEM cells, the precise functional role of OX40 in the TEM/TCM commitment requires further study.

Although in vivo lineage conversion from CD8+ TEM to TCM cells has been demonstrated in several murine models (33, 34), similar lineage conversion in CD4+ memory T cells has not been reported. As shown in Fig. 4, transferred CD4+CD44high CD62Llow cells in Ag-free recipient mice could phenotypically differentiate into both the TEM and TCM populations, while CD4+CD44highCD62Lhigh cells mainly maintained their phenotype. This result suggests that, similar to CD8+ memory T cells, CD4+ TEM precursors can be converted into CD4+ TCM cells under a certain condition. However, OX40-deficient TEM precursor cells are unable to persist or convert to TCM cells in the absence of antigenic stimulation (Fig. 4). Therefore, OX40 signals may mainly promote generation of TEM cells but not conversion of CD4+ TCM precursors to TEM cells.

In view of the functional difference between TEM and TCM cells, our present findings indicate that targeting OX40 and OX40L in vivo may lead to effective therapeutic strategies for T cell-mediated immune disorders, such as autoimmune, allergic, and inflammatory diseases. Conventional immunotherapies not only affect the T cells responsible for attacking diseases, but all the other T cells within the host as well, which can lead to an immunosuppressive state with the eventual development of opportunistic infections and cancer. In contrast, blockade of the OX40-OX40L interaction may specifically suppress ongoing Ag-specific T cell responses by preventing TEM cell generation. This notion is supported by a previous report using a viral model of lung inflammation, in which a blockade of OX40 signals was shown to modulate the disease process. In that study, the administration of OX40-Ig efficiently tempered influenza-induced lung inflammation even after the onset of disease manifestations, interestingly, without any adverse effects on either the viral clearance or memory T cell immune responses to viral rechallenge (42). Based on our present understanding of the distinct roles of TEM and TCM cells, it seems that in the viral model, blockade of OX40 signals inhibited TEM cell-mediated lung inflammation, but it did not affect TCM cell function, which mediates the robust vaccine effects. Thus, blockade of the OX40-OX40L interaction is a promising therapeutic strategy for treating T cell-mediated organ-specific inflammation without unwanted immunosuppression.


