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# OX40 Costimulatory Signals Potentiate the Memory Commitment of Effector CD8+ T Cells

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## Abstract

A T cell costimulatory molecule, OX40, contributes to T cell expansion, survival, and cytokine production. Although several roles for OX40 in $CD8^+$ T cell responses to tumors and viral infection have been shown, the precise function of these signals in the generation of memory $CD8^+$ T cells remains to be elucidated. To address this, we examined the generation and maintenance of memory $CD8^+$ T cells during infection with *Listeria monocytogenes* in the presence and absence of OX40 signaling. We used the expression of killer cell lectin-like receptor G1 (KLRG1), a recently reported marker, to distinguish between short-lived effector and memory precursor effector T cells (MPECs). Although OX40 was dispensable for the generation of effector T cells in general, the lack of OX40 signals significantly reduced the number and proportion of $KLRG1^{low}$ MPECs, and, subsequently, markedly impaired the generation of memory $CD8^+$ T cells. Moreover, memory T cells that were generated in the absence of OX40 signals in a host animal did not show self-renewal in a second host, suggesting that OX40 is important for the maintenance of memory T cells. Additional experiments making use of an inhibitory mAb against the OX40 ligand demonstrated that OX40 signals are essential during priming, not only for the survival of $KLRG1^{low}$ MPECs, but also for their self-renewing ability, both of which contribute to the homeostasis of memory $CD8^+$ T cells.

The adaptive immune responses to infection are mounted by the optimal differentiation of naive T and B cells into effector cells, and subsequently into long-lived memory cells. Memory $CD8^+$ T cells represent the major effector arm of the adaptive immune system to maintain long-lived protective immunity against intracellular bacteria, protozoa, and viruses (1,2). Upon a subsequent infection by the same pathogen, pathogen-specific memory $CD8^+$ T cells that have been maintained in the absence of Ag are poised to respond quickly, specifically, and with sufficient amplitude to protect the host (3–5). Therefore, understanding the mechanisms underlying the generation and maintenance of memory $CD8^+$ T cells is critical, not only for contributing to basic immunology, but also for improved clinical applications, such as the design of new vaccines.



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Effector $CD8^+$ T cells are phenotypically diverse, with different fates and memory potentials (6–10). To identify memory precursor cells from among the effector T cells, Kaech and colleagues (8) recently demonstrated, using acute viral infection models, that killer cell lectin-like receptor G1 (KLRG1)[4] is a good marker for distinguishing between short-lived effector $CD8^+$ T cells ($KLRG1^{high}$) and the memory precursor effector $CD8^+$ T cells (MPECs; $KLRG1^{low}$). The same paper also demonstrated that long-lived $CD8^+$ T cells that have the ability to homeostatically turn over have the $KLRG1^{low}$ phenotype. In this study, we used this marker to examine the process of memory T cell generation, during which MPECs survive, expand, and finally differentiate into memory T cells.

Accumulating evidence shows that TCR signals and homeostatic cytokines, such as IL-7 and IL-15, are critical regulators of the generation and maintenance of $CD8^+$ memory T cells (11,12). TCR signals are essential for the survival of naive T cells and the generation of functional memory T cells. Signals through the receptors for IL-7 and IL-15 of memory T cells have been well studied and shown to mediate not only the acute homeostatic proliferation of T cells, but also the basal homeostatic proliferation of memory T cells. The basal homeostatic proliferation mediates Ag-independent self-renewal of memory T cells in a full T cell environment (13,14). However, the roles played by costimulatory signals in the generation and maintenance of $CD8^+$ memory T cells are still unclear. Among the T cell costimulatory molecules are several TNF receptor superfamily members, including OX40 (CD134), CD27, and 4-1BB, which contribute to the survival and expansion of effector T cells (15–22). Some of these T cells subsequently differentiate into long-lived memory T cells. The role of OX40 in the generation of memory $CD4^+$ T cells has been intensively studied and convincingly demonstrated to be important (23,24). In addition, recent studies suggest that OX40 signals are also important for the generation of memory $CD8^+$ T cells (19,25–27), although how and when OX40 signals are required remain to be elucidated.

In the present study, we took advantage of a useful model for acute bacterial infection. This model combines infection by recombinant *Listeria monocytogenes* expressing OVA (r*LM*-OVA) (28), which mimics the bacterial Ag during infection, with the MHC class I-restricted OVA-specific TCR-transgenic OT-I system (29). We also relied on the $KLRG1^{low}$ phenotype as a specific marker for memory precursor T cells, which helped us to investigate the role of OX40 signals during infection, to determine when the effector $CD8^+$ T cells undergo memory commitment. Finally, we used extensive adoptive transfer experiments to demonstrate the critical roles played by OX40 signals in both the generation and the maintenance of memory $CD8^+$ T cells.

## Materials and Methods

### Mice

Six- to 8-wk-old female wild-type C57BL/6 mice were purchased from Japan SLC. OX40 ligand (OX40L)-deficient mice and $Ly5.1^+$-C57BL/6 mice were previously described (21, 30,31). OT-I TCR-transgenic mice were a gift from W. Heath (Walter and Eliza Hall Institute, Melbourne, Australia) and were used as a source of $CD8^+$ T cells specifically responsive to the $OVA_{257–264}$ peptide (29). $Ly5.1^+$ wild-type OT-I and $Ly5.1^+$ $OX40^{-/-}$ OT-I mice were generated in-house by intercrossing OT-I mice with $Ly5.1^+$ wild-type and $Ly5.1^+$ $OX40^{-/-}$ mice, respectively. All of the mice were on a C57BL/6 background, and they were bred and maintained under specific pathogen-free conditions at Institute for Animal Experimentation, Tohoku University Graduate School of Medicine. All procedures were performed according

[4]Abbreviations used in this paper: KLRG1, killer cell lectin-like receptor G1; DC, dendritic cell; MPEC, memory precursor effector T cell; OX40L, OX40 ligand; r*LM*-OVA, recombinant *Listeria monocytogenes* expressing OVA.

to protocols approved by the Institutional Committee for the Use and Care of Laboratory Animals of Tohoku University.

### Microorganism, immunization, and assessment of bacterial burden

r*LM*-OVA was previously described (28,32). Mice were infected via the tail vein with a sublethal dose of r*LM*-OVA ($1 \times 10^4$ CFU; 0.1 $LD_{50}$)in 0.2 ml of PBS. To examine the protective function of memory T cells, the recipient mice that harbored long-lived wild-type or $OX40^{-/-}$ OT-I cells were rechallenged with a higher dose of r*LM*-OVA ($1 \times 10^5$ CFU). To evaluate the bacterial burden, the spleen and liver were removed and separately homogenized in 3 ml of PBS. Serial dilutions of each tissue extract were spread on Brain-Heart Infusion agar plates containing erythromycin, and the number of colonies was counted after incubation for 24–48 h at 37°C.

### Antibodies

The following Abs and reagents were purchased from BD Biosciences: anti-CD8 allophycocyanin, anti-TCR Vα2-PE, anti-TCR Vβ5-FITC, anti-Ly5.1 biotin, anti-Ly5.1 allophycocyanin, anti-CD11b FITC, anti-CD11c PE, anti-CD44 PE, anti-CD62L FITC, anti-CD62L biotin, anti-OX40 biotin, anti-granzyme B-PE, anti-IL-2 PE, anti-IFN-γ PE, streptavidin-allophycocyanin, and annexin V-FITC. Anti-mouse KLRG allophycocyanin was purchased from eBioscience. The inhibitory anti-OX40L mAb (MGP34; rat IgG2c) was previously described (21,33). The anti-IL-7R α-chain mAb (A7R34; rat IgG2a) was a gift from S. Nishikawa (Center for Developmental Biology, RIKEN, Kobe, Japan). Control rat Ig was purchased from Cappel. For staining OX40L and IL-7Rα, MGP34 and A7R34 were biotinylated and visualized using streptavidin-allophycocyanin.

### Lymphocyte isolation, cell sorting, and adoptive transfer

Naive $CD8^+$ T cells ($1 \times 10^4$) were purified from the spleen of Ag-naive wild-type OT-I or $OX40^{-/-}$ OT-I mice with the $Ly5.1^+$ congenic marker. They were then injected into the lateral tail vein of naive $Ly5.2^+$ congenic mice that were infected with r*LM*-OVA 24 h after the cell transfer (first host). In some experiments, to block the OX40-OX40L interaction, the recipient mice were given control rat IgG (300 μg) or blocking anti-OX40L mAb (MGP34) (300 μg) by i.p. injection 1 day before, and on days 1, 3, 5, and 7 after the infection. For the secondary adoptive transfer of effector T cells, the $Ly5.1^+$ effector donor cells were isolated from the spleen of the first host ($Ly5.2^+$) 6 days after the r*LM*-OVA infection by adding biotinylated anti-Ly5.1 mAb, followed by anti-biotin MicroBeads (Miltenyi Biotec) and sorting on an AutoMACS cell sorter (Miltenyi Biotec). The enriched $Ly5.1^+$ donor cells were further separated by a FACSAria cell sorter (Nippon BD) to purify $KLRG1^{low}$TCR-$V\alpha2^+CD8^+$ and $KLRG1^{high}$TCR-$V\alpha2^+CD8^+$ effector T cells. These cells ($2 \times 10^6$ each), from the first host, which harbored either the wild-type OT-I or $OX40^{-/-}$ OT-I donor cells, were adoptively transferred into an $Ly5.2^+$ second host (wild-type mouse) that had been infected with wild-type *L. monocytogenes* 6 days previously to prepare an appropriate physiological environment for the effector donor cells. For another secondary transfer of memory T cells, $Ly5.1^+$ memory donor cells were isolated 40 days after infection, as described above, and labeled with CFSE (Molecular Probes), as described previously (34,35). The CFSE-labeled memory T cells ($1 \times 10^6$) that had been generated in the first host were transferred into a nonirradiated (wild type or OX40L deficient).

### Flow cytometry analysis and intracellular staining

Before being stained, the cells were washed and resuspended in a staining buffer consisting of 1× PBS, 2% BSA, and 0.01% $NaN_3$. To block non-specific staining, the 2.4G2 anti-CD16/32 mAb was added. Abs for cell surface markers were added, and cells were incubated for 30 min

on ice. For intracellular cytokine staining, whole splenocytes ($5 \times 10^5$) were stimulated with the $OVA_{257–264}$ peptide for 4 h in the presence of brefeldin A (10 μg/ml; Invitrogen). Surface staining was performed with the indicated fluorochrome-conjugated mAbs. The cells were then resuspended in fixation/permeabilization solution (BD Cytofix/Cytoperm kit; BD Biosciences), and intracellular cytokine staining was performed according to the manufacturer's protocol. For intracellular staining of granzyme B, whole splenocytes were stained with Ly5.1 FITC and washed with chilled PBS. The cells were resuspended in the fixation/permeabilization solution (BD Cytofix/Cytoperm kit; BD Biosciences), and intracellular staining of granzyme B was performed, as described above. The samples were analyzed with a FACSCalibur flow cytometer (BD Immunocytometry Systems). The analyses were conducted using the CellQuest program (BD Immunocytometry Systems).

### Determination of apoptosis

Whole splenocytes ($5 \times 10^5$) that included effector donor T cells were cultured for 8 h in a complete medium (RPMI 1640 containing 10% FCS). The cells were then harvested and stained with anti-Ly5.1 mAb, anti-KLRG1 mAb, annexin V, and propidium iodide, according to the manufacturer's instructions. The intensity of the annexin V staining on the Ly5.1-gated cells was measured with a FACSCalibur flow cytometer.

### Measurement of cytolytic activity

Splenocytes from recipient mice ($n = 3$ each) that possessed wild-type or $OX40^{-/-}$ effector OT-I cells were collected 10 days after the infection. The cells collected (effector cells) were cocultured with $^{51}Cr$-labeled EL-4 cells ($4 \times 10^4$) (target cells) at the indicated ratios in the presence (10 μg/ml) or absence of $OVA_{257–264}$ peptide for 6 h. After the incubation, the radioactivities of the supernatants were determined with a γ counter. The results are expressed as the percentage of specific lysis = ((release in test – spontaneous release)/(release by detergent – spontaneous release)) × 100.

### Statistical analysis

Statistical analyses were performed with Student's *t* test. Values of $p < 0.01$ were considered significant. * and ** in any graph represent $p < 0.01$ and $p < 0.001$, respectively.

## Results

### *Expression of OX40 and OX40L during* Listeria *infection*

OX40 is transiently expressed on activated T cells upon antigenic stimulation, and its ligand, OX40L, is induced on mature APCs and activated T cells (19,21,35,36). To address when OX40 and OX40L interact during *Listeria* infection, we examined their expression profiles on $CD8^+$ T cells from OVA-specific TCR-transgenic (OT-I) mice. Naive $Ly5.1^+$ OT-I T cells were adoptively transferred into congenic $Ly5.2^+$ wild-type mice, followed by immunization with a sublethal dose of *rLM*-OVA. At various time points after the infection, the expression of OX40 and OX40L on donor T cells and recipient APCs, respectively, was examined. Although naive donor $CD8^+$ T cells did not express OX40, its expression was induced on activated donor T cells on day 2, peaked on day 3, and had become undetectable again by day 10 after the infection (*left*,Fig. 1). OX40L expression on several types of APCs, including $CD11b^+CD11c^-$ macrophages, $CD11b^+CD11c^+$ myeloid dendritic cells (DCs), and $CD11b^-CD11c^+$ lymphoid DCs, was also investigated. OX40L was transiently expressed on myeloid DCs, but not on macrophages or lymphoid DCs during *Listeria* infection (Fig. 1). Although in vitro activated $CD4^+$ T cells express OX40L (35), OX40L expression on donor $CD8^+$ T cells could not be detected at any time during the *Listeria* infection (data not shown).

These data suggest that OX40 signals were provided through T cell-myeloid DC interactions during the acute phase of the *Listeria* infection.

## OX40 signals are dispensable for the expansion and function of activated CD8$^+$ T cells, but determine the number of the KLRG1$^{low}$ MPEC population

OX40 signals are not essential for the initial proliferation of CD4$^+$ T cells during the priming phase of Ag stimulation, but significantly promote the survival and clonal expansion of effector CD4$^+$ T cells in the later effector phase (16,22,30). To examine whether OX40 might have a similar function in CD8$^+$ T cell responses, naive wild-type and OX40$^{-/-}$ OT-I CD8$^+$ T cells were adoptively transferred into congenic wild-type mice that were subsequently immunized with a sub-lethal dose of *rLM*-OVA. The recipient mice harboring wild-type and OX40$^{-/-}$ donor cells showed a similar kinetics of bacterial burdens after *rLM*-OVA infection, and cleared the bacteria equally well from the spleen and liver by day 6 postinfection (Fig. 2*A*). This suggests that both wild-type and OX40$^{-/-}$ donor cells were activated and differentiated under a similar inflammatory environment mediated by *Listeria* infection. At various time points postinfection, the donor cells were recovered from the spleens of the recipient mice. Both wild-type and OX40$^{-/-}$ OT-I donor cells could be detected in the spleen on day 2 after the infection, reached their peak number on day 6, and declined thereafter (Fig. 2*B*), much as described for CD4$^+$ T cells. The cytotoxic activity of spleen cells derived from recipient mice harboring OX40-KO OT-I donor cells was comparable to that of spleen cells derived from recipient mice harboring wild-type donor cells (Fig. 2*C*). Although previous reports demonstrated that OX40 signals enhanced granzyme B expression in effector CD8$^+$ T cells (25), Fig. 2*D* shows that wild-type and OX40$^{-/-}$ donor cells produced a comparable amount of granzyme B. Furthermore, these results indicate that the OX40 signals are dispensable for the expansion and function of activated CD8$^+$ T cells during the acute phase of infection.

To investigate the phenotypic heterogeneity of the effector CD8$^+$ T cell population, we used KLRG1 expression to distinguish between short-lived effector cells (KLRG1$^{high}$) and MPECs (KLRG1$^{low}$) (6,8), as well as the expression of the IL-7R α-chain (IL-7R) (high on MPECs) and CD62L. Flow cytometric analysis gated on KLRG1 expression at various time points after infection revealed phenotypic heterogeneity in the wild-type and OX40$^{-/-}$ effector T cells in the spleen. Six days after *Listeria* infection, effector CD8$^+$ T cells differentiated into the two distinct cell lineages, KLRG1$^{high}$ and KLRG1$^{low}$, in which the KLRG1$^{low}$ (IL-7R$^{high}$) population was expected to contain memory precursors (8). Although the total donor cell numbers in recipient mice harboring wild-type or OX40$^{-/-}$ OT-I cells were comparable even 10 days after infection, the subpopulation profiles were quite different in terms of KLRG1 expression. In particular, the percentage and the absolute number of KLRG1$^{low}$ MPECs in the OX40$^{-/-}$ OT-I cells were markedly lower than in the wild-type OT-I cells (Fig. 2, *E* and *F*). Compatible with a previous paper (8), almost all KLRG1$^{low}$ cells had the IL-7R$^{high}$ phenotype; we found that this was true regardless of OX40 signaling (Fig. 2*E*).

Because a recent report demonstrated that KLRG1$^{high}$ effector T cells more efficiently produce IFN-γ than KLRG1$^{low}$ effector T cells during viral infection (8), we further examined cytokine production of these effector T cells during *Listeria* infection. Unexpectedly, ex vivo Ag stimulation of KLRG1$^{high}$ and KLRG1$^{low}$ effector T cells from a 10-day infected recipient showed similar levels of their IFN-γ production even in the absence of OX40 signals. In contrast, a smaller population (25%) in OX40$^{-/-}$ KLRG1$^{low}$ T cells produced IL-2 compared with that (55%) in wild-type KLRG1$^{low}$ effector T cells (Fig. 2*G*), suggesting that OX40 signals promote IL-2 synthesis by MPECs. Similar results were found 8 days after infection (data not shown). Furthermore, flow cytometric analyses did not show significant differences in the expression profiles for other effector/differentiation markers, such as CD25, CD27, CD43, and CD44, between wild-type and OX40$^{-/-}$ OT-I donor cells (data not shown).

Collectively, these observations suggest that OX40 signals are necessary for the optimal generation of KLRG1$^{low}$CD8$^{+}$ MPECs, despite their dispensability for the effector function of activated CD8$^{+}$ T cells.

## OX40 signals are essential for the generation of functional memory CD8$^{+}$ T cells

We next assessed the accumulation of long-lived OT-I donor cells in the spleens of infected mice at the memory phase. Approximately 2-fold more wild-type OT-I donor cells had accumulated than OX40$^{-/-}$ OT-I cells on day 30 (Fig. 3*A*). Intriguingly, on day 60 and until day 150 after infection, the number of persisting OX40$^{-/-}$ donor T cells in the spleen was markedly reduced compared with wild-type donor T cells (Fig. 3*A*). There were also many fewer OX40$^{-/-}$ donor cells in the liver and bone marrow of recipient mice on day 150 than wild-type donor cells in these tissues (Fig. 3*B*). These results suggest that OX40 critically contributes to the generation and probably the maintenance of memory CD8$^{+}$ T cells.

Phenotypic analysis of the persisting donor cells on day 90 demonstrated that wild-type donor cells were predominantly KLRG1$^{low}$ IL-7R$^{high}$CD62L$^{high}$ (Fig. 3*C*). In contrast, in the absence of OX40, a substantial percentage of the IL-7R$^{high}$CD62L$^{high}$ donor CD8$^{+}$ T cells strongly expressed KLRG1, indicating that the majority of long-lived OX40$^{-/-}$ donor T cells was unable to acquire the memory phenotype (Fig. 3*C*). In addition, consistent with a previous report (37,38), these data imply that a high expression of IL-7R on OX40$^{-/-}$ OT-I cells is not sufficient to support their survival.

One of the cardinal properties of memory CD8$^{+}$ T cells is their ability to protect the host against secondary infection. To address whether the persisting CD8$^{+}$ T cells that were generated in the absence of OX40 signals are protective, 90 days after the first infection, the recipient mice that had harbored long-lived wild-type or OX40-KO OT-I donor cells were infected with a higher dose of rLM-OVA. As expected, recipient mice possessing the long-lived wild-type OT-I donor cells completely cleared the bacteria from their spleens by day 3 after reinfection (Fig. 3*D*). In comparison, mice harboring persisting OX40$^{-/-}$ OT-I cells were not able to clear bacteria from the spleen, and had 100,000-fold more bacteria in the liver (Fig. 3*D*). Because the impaired protection seen in the OX40$^{-/-}$ donor cell recipients correlated with the reduced number of functional memory T cells (Fig. 3*E*), we compared the in vivo protective function between wild-type and OX40$^{-/-}$ memory T cells. The equal number of long-lived wild-type and OX40$^{-/-}$ donor T cells was collected from the first hosts, and transferred into the naive second hosts. Even the second host receiving the OX40$^{-/-}$ memory donor cells showed no weight loss after infection with a lethal dose of rLM-OVA (data not shown). Bacterial clearance from the spleen of the two different second hosts was similar (Fig. 3*F*), suggesting the comparable protective function between wild-type and OX40$^{-/-}$ memory T cells generated in the first host.

Next, the functionality of long-lived wild-type and OX40$^{-/-}$ OT-I cells during in vitro recall responses was examined. Ninety days postinfection, whole splenocytes from each recipient mouse were collected and stimulated with OVA$_{257–264}$ peptide, and the synthesis of IL-2 and IFN-γ was analyzed by intracellular staining. Long-lived OX40$^{-/-}$ OT-I donor cells, especially the KLRG1$^{low}$ population, failed to produce IL-2, whereas wild-type memory CD8$^{+}$ T cells secreted substantial amounts of IL-2; both kinds of donor cells produced IFN-γ at the same level, regardless of KLRG1 expression (Fig. 3*G*). In addition, 60% of wild-type KLRG1$^{low}$ cells produced IL-2, whereas only 34% of OX40$^{-/-}$ KLRG1$^{low}$ population was positive for IL-2, suggesting the importance of OX40 in IL-2 secretion by each KLRG1$^{low}$ T cell. The absolute number of IL-2-producing KLRG1$^{low}$ cells derived from OX40$^{-/-}$ OT-I cells in vitro was also markedly reduced (Fig. 3*G*).

### OX40 signals contribute to the survival of KLRG1$^{low}$ memory precursor cells

Several previous papers have suggested that OX40 signals promote the survival of effector CD4$^+$ and CD8$^+$ T cells, probably leading to the effective generation of memory T cells. To elucidate the mechanisms for the OX40-mediated generation of memory CD8$^+$ T cells, we examined the susceptibility to apoptosis of effector CD8$^+$ T cells collected from the recipient mice 6 days after infection. Interestingly, half of the KLRG1$^{low}$ population in the OX40$^{-/-}$ effector CD8$^+$ T cells became apoptotic after only 8 h in vitro in complete medium, whereas almost all of the wild-type KLRG1$^{low}$ effector CD8$^+$ T cells remained alive (Fig. 4*A*), indicating an increased susceptibility of OX40$^{-/-}$ MPECs to apoptosis. In contrast to the MPEC population, the KLRG1$^{high}$ effector population did not show substantial apoptosis, even in the absence of OX40 signals (Fig. 4*A*).

To clarify the significance of the OX40-mediated survival of KLRG1$^{low}$ MPECs, we compared the process of memory T cell generation from purified KLRG1$^{high}$ and KLRG1$^{low}$ effector CD8$^+$ T cells in the presence or absence of OX40 signals. Equal numbers of isolated KLRG1$^{high}$ and KLRG1$^{low}$ effector CD8$^+$ T cells that were generated in the first host (harboring either wild-type or OX40-KO donor OT-I cells), during the 6 days following infection, were independently transferred into second recipients that had been infected with *Listeria* 6 days previously. The accumulation of donor cells in the spleen of the second recipients was assessed 7, 20, and 60 days after the transfer. As expected, the absolute number of persisting cells was greater on day 7 after the transfer of wild-type KLRG1$^{low}$ effector CD8$^+$ T cells than after the transfer of KLRG1$^{high}$ effector CD8$^+$ cells, and this difference was still observed on day 60 posttransfer (Fig. 4*B*). In addition, the transferred KLRG1$^{high}$ population from both wild-type and OX40$^{-/-}$ OT-I cells remained mainly KLRG1$^{high}$, whereas both KLRG1$^{high}$ and KLRG1$^{low}$ long-lived cells were observed when KLRG1$^{low}$ effector CD8$^+$ T cells were transferred (Fig. 4*C*), indicating that the KLRG1$^{low}$ effector CD8$^+$ T cells may give rise to both sets of cells. Notably, by day 60, no donor cells derived from the OX40$^{-/-}$ MPECs could be found (Fig. 4, *B* and *C*), indicating that OX40 signals play a critical role in the survival of KLRG1$^{low}$ MPECs.

### OX40 is essential for the self-renewal potential of memory CD8$^+$ T cells

Although the more rapid reduction of MPECs in OX40$^{-/-}$ T cells during the contraction phase is one of the possible mechanisms for the failure of OX40$^{-/-}$ donor cells to generate memory T cells, it cannot fully explain their decrease after the contraction phase (between days 60 and 150 in Fig. 3*A*). Therefore, we postulated that OX40 might also be implicated in the maintenance of memory T cells. Because the basal homeostatic proliferation contributes to the maintenance of memory CD8$^+$ T cells by mediating their Ag-independent self-renewal (8,13, 14), we have addressed whether OX40 might be involved in the basal homeostatic proliferation. Wild-type memory OT-I or OX40$^{-/-}$ memory OT-I cell populations from the first host were isolated, labeled with CFSE, and then adoptively transferred into Ag-naive wild-type second hosts. Although the KLRG1 expression profiles between wild-type and OX40$^{-/-}$ memory donor cells were different, their IL-7R expression levels were comparable (Fig. 5*A*). The basal homeostatic proliferation of these memory CD8$^+$ T cells was assessed by the dilution of CFSE intensity 30 days after transfer. When wild-type OT-I memory CD8$^+$ T cells were transferred, the KLRG1$^{low}$ population divided between one and four times, but most of the KLRG1$^{high}$ cells appeared to stop after one division (Fig. 5*B*). The significantly greater self-renewal potential of the KLRG1$^{low}$ long-lived CD8$^+$ T cells is concordant with their being memory CD8$^+$ T cells, which is consistent with the findings for viral infection. In striking contrast, long-lived KLRG1$^{low}$ OX40$^{-/-}$ donor cells failed to undergo homeostatic proliferation, and their KLRG1$^{high}$ population almost disappeared (Fig. 5*B*). Furthermore, the absolute number of long-lived OX40$^{-/-}$ donor cells in the second host was much lower than that of wild-type donor cells (Fig. 5*B*). These data indicate that OX40 signals critically mediate the basal

homeostatic proliferation of memory $CD8^+$ T cells, especially $KLRG1^{low}$memory $CD8^+$ T cells.

We next addressed whether OX40 signals during the memory phase are responsible for the self-renewal of memory T cells. Persisting wild-type OT-I donor cells were isolated from the first host 40 days after infection, and adoptively transferred into an Ag-naive wild-type or OX40L-deficient second host. In contrast to the $OX40^{-/-}$ long-lived $CD8^+$ T cells, which could not divide, wild-type memory OT-I cells in the OX40L-deficient host exhibited a robust homeostatic proliferation and similar cell accumulation as in the wild-type host (Fig. 5*C*). These transfer experiments imply that OX40 signals are not essential during the memory phase for the self-renewal of memory $CD8^+$ T cells, which may be programmed by previously provided OX40 signals.

### OX40 signals during priming are responsible for the generation and maintenance of memory $CD8^+$ T cells

As shown in Fig. 1, it is likely that OX40 and OX40L interactions occur through T cell-APC interactions at the T cell priming phase during *Listeria* infection. Given the dispensability of OX40 signals during the memory phase for memory T cell self-renewal (Fig. 5*B*), OX40 signals during priming thus might be essential and sufficient for the generation and maintenance of memory $CD8^+$ T cells. To address this possibility, we administered a blocking anti-OX40L mAb to recipient mice, during only the initial 7 days after the r*LM*-OVA infection, and the accumulation of donor cells in the spleen of the recipient mice was assessed. Similar to the initial expansion of the $OX40^{-/-}$ T cells (Fig. 2*B*), treatment with the anti-OX40L mAb did not have any effect on the expansion of OT-I donor cells during the priming and effector phases (Fig. 6*A*), confirming that OX40 signals are not crucial for the generation of Ag-specific effector $CD8^+$ T cells. However, as expected, the accumulation of long-lived OT-I donor cells in anti-OX40L mAb-treated mice was dramatically reduced (~4% of the level in control IgG-treated hosts on day 90) (Fig. 6*A*), and the kinetics for the decrease in the persisting donor cell number was similar to that of the $OX40^{-/-}$ donor cells (Fig. 2*B*). Anti-OX40L mAb treatment also suppressed the generation of $KLRG1^{low}IL\text{-}7R^{high}CD62L^{high}$memory $CD8^+$ T cells (Fig. 6*C*). Furthermore, the $KLRG1^{low}$memory subset of donor cells that had been generated in the host treated with the anti-OX40L mAb failed to produce IL-2 upon ex vivo Ag restimulation (Fig. 6*D*). These results were very similar to those obtained using $OX40^{-/-}$ donor cells.

Finally, to investigate whether blocking OX40 signals only during priming could suppress the self-renewal of memory $CD8^+$ T cells, long-lived wild-type OT-I donor cells were isolated from a first host that had been treated with inhibitory anti-OX40L mAb or control IgG during the priming phase of the *Listeria* infection, labeled with CFSE, and then adoptively transferred into Ag-naive wild-type second hosts. Similar to the defective self-renewal of the $OX40^{-/-}$ memory T cells (Fig. 5*A*), the long-lived OT-I donor cells derived from effector $CD8^+$ T cells generated in the absence of OX40 signals failed to show homeostatic proliferation in the second host (Fig. 6*E*). These results not only confirm that OX40 signals are required for the generation of $KLRG1^{low}$ memory $CD8^+$ T cells, but also suggest that OX40 signals are essential during priming for the self-renewal of memory $CD8^+$ T cells.

## Discussion

Recent findings have clearly demonstrated that effector $CD8^+$ T cells are phenotypically diverse in terms of KLRG1 expression (6,7), and indicate that $KLRG1^{low}$ effector $CD8^+$ T cells become committed to the memory fate during acute viral infection (8–10). The present study has confirmed these observations in an acute bacterial infection model and further obtained several new findings on the OX40 roles in the homeostasis of $KLRG1^{low}$ effector and memory T cells. With regard to the OX40 signals' roles in the generation and homeostasis of

memory $CD8^+$ T cells, several previous papers have suggested OX40 to be important for the survival of heterogeneous effector $CD8^+$ T cells (19,25–27). The present results demonstrate that OX40 signals promote the survival of $KLRG1^{low}$ $CD8^+$ MPECs, a subset of effector T cells. Because OX40 stimulation directly induces NF-κB-associated survival signals in activated T cells (23,24,39), the NF-κB activation may be involved in the OX40-mediated survival of $KLRG1^{low}$ $CD8^+$ MPECs.

We also show in this study that OX40 signals critically potentiated the maintenance of the memory $CD8^+$ T cells. Furthermore, the OX40 signals activated during priming appeared to be sufficient to imprint the self-renewal potential of $KLRG1^{low}$ memory $CD8^+$ T cells onto the MPECs (Figs. 5*B* and 6*E*). In addition, we previously demonstrated using an acute viral infection model that OX40 signals during Ag priming are required for the expansion of memory $CD8^+$ T cells during secondary infection (19). Therefore, OX40 signals during priming seem to imprint the memory competency onto $CD8^+$ MPECs. The OX40-mediated memory imprinting may indicate a novel role for T cell costimulatory signals apart from the conventional survival signals. However, the question of how OX40 signals imprint the commitment for self-renewal onto $CD8^+$ T cells that are Ag specifically activated remains unsolved. Our next study will therefore focus on the identification of the molecule(s) responsible for OX40-induced memory imprinting.

A higher proportion of $KLRG1^{high}$ cells in the first host was observed in $OX40^{-/-}$ donor cells than in wild-type donor cells in any time points during both the effector and the memory phases. However, $KLRG1^{high}$ long-lived cells from $OX40^{-/-}$ memory T cells were diminished when memory $OX40^{-/-}$ T cells, which initially consisted of both $KLRG1^{high}$ and $KLRG1^{low}$ populations, were transferred into Ag-naive second hosts (Fig. 5*B*). Therefore, a certain environment in the first host after *Listeria* infection might be important to maintain the $OX40^{-/-}$ $KLRG1^{high}$ long-lived T cells, although the maintenance of the wild-type $KLRG1^{high}$ long-lived T cells may not require the putative environment (Fig. 5*B*). A recent report suggests that $KLRG1^{high}$ effector T cells may be specifically induced by inflammatory signals (i.e., IL-12) (8). Therefore, inflammation-mediated environments induced by *Listeria* infection in the first host may be essential for survival and generation of $KLRG1^{high}$ $CD8^+$ T cells from $OX40^{-/-}$ donor T cells.

The deficiency of the $OX40^{-/-}$ donor T cells in survival and self-renewal may be owing to some abnormality in unidentified endogenous factors, because the circumstances for memory generation and maintenance in the recipient (such as the lymph node structure, $CD4^+$ T cells, APCs, stromal cells, and probably stromal cytokines, IL-7 and IL-15) were the same for wild-type and $OX40^{-/-}$ donor cells in our experimental setting. We examined the donor cell expression of receptors for the homeostatic cytokines IL-7 and IL-15, which are expressed by recipient cells, because these cytokines mediate the homeostatic proliferation of memory T cells. However, IL-7R and CD122 (the shared β subunit for IL-2Rs and IL-15Rs) levels on the $KLRG1^{low}$ population of $OX40^{-/-}$ $CD8^+$ T cells were similar to those on the $KLRG1^{low}$ population of wild-type $CD8^+$ T cells (Figs. 2*E*,3*C*, and 5*A*, and data not shown). In addition, the in vitro culture of activated $OX40^{-/-}$ OT-I T cells showed a robust proliferative response to exogenous IL-2 (data not shown), suggesting that a lack of signaling molecules involved in the γc/JAK3/STAT pathway may not cause their impaired survival and self-renewal, and indicating that defective IL-7 and IL-15 signals are probably not associated with the impaired generation and maintenance of $CD8^+$ T cells. We nevertheless found that $KLRG1^{low}$ long-lived T cells are the main producer of IL-2, and that the IL-2 production by $KLRG1^{low}$ MPECs and memory T cells that were generated in the absence of OX40 signals was severely impaired (Figs. 2*G* and 3*G*).

IL-2 is a well-known T cell-derived cytokine, which also controls several T cell responses, including their activation, expansion, and activation-induced cell death, and the generation of functional memory $CD8^+$ T cells, in an autocrine or paracrine manner. IL-2 thus might be a key factor for the OX40-mediated survival of MPECs and maintenance of memory $CD8^+$ T cells. This scenario is partially supported by a recent report showing that IL-2 signals during priming imprint functional memory properties onto $CD8^+$ T cells, although the same paper also demonstrated that IL-2 is dispensable for the generation of long-lived $CD8^+$ T cells (40). Based on this scenario, the ability of memory precursor and memory $CD8^+$ T cells to produce IL-2 might be conferred on activated $CD8^+$ T cells by OX40 signals during Ag priming. A precise understanding of OX40-mediated memory imprinting may provide us not only with important insights into the mechanisms of the development and homeostasis of memory T cells, but also with beneficial information for designing new vaccination strategies.

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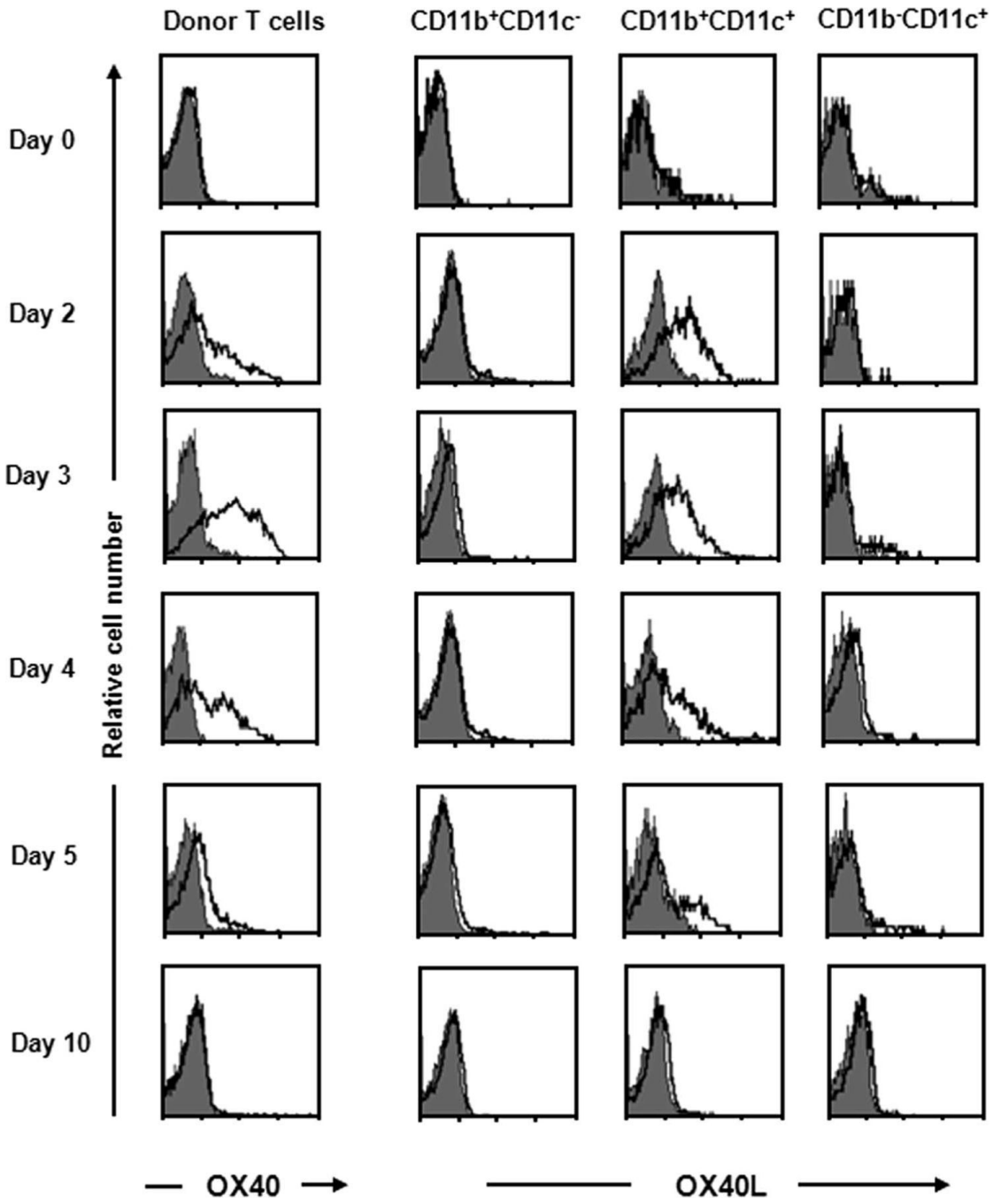


**FIGURE 1.**

Transient expression of OX40 and OX40L on T cells and myeloid DCs, respectively, during r*LM*-OVA infection. Purified naive Ly5.1$^+$ OT-I cells ($1 \times 10^4$) were adoptively transferred i.v. into Ly5.2$^+$ congenic recipient mice that were infected with r*LM*-OVA ($1 \times 10^4$ CFU) at 24 h after the cell transfer. The expression of OX40 on Ly5.1$^+$ TCR-Vα2$^+$ donor cells and the expression of OX40L on recipient APCs (CD11b$^+$CD11c$^-$, CD11b$^+$CD11c$^+$, and CD11b$^-$CD11c$^+$) in the spleen were analyzed on the indicated days after infection. Results shown are representative mouse from each time point from one representative experiment of three.

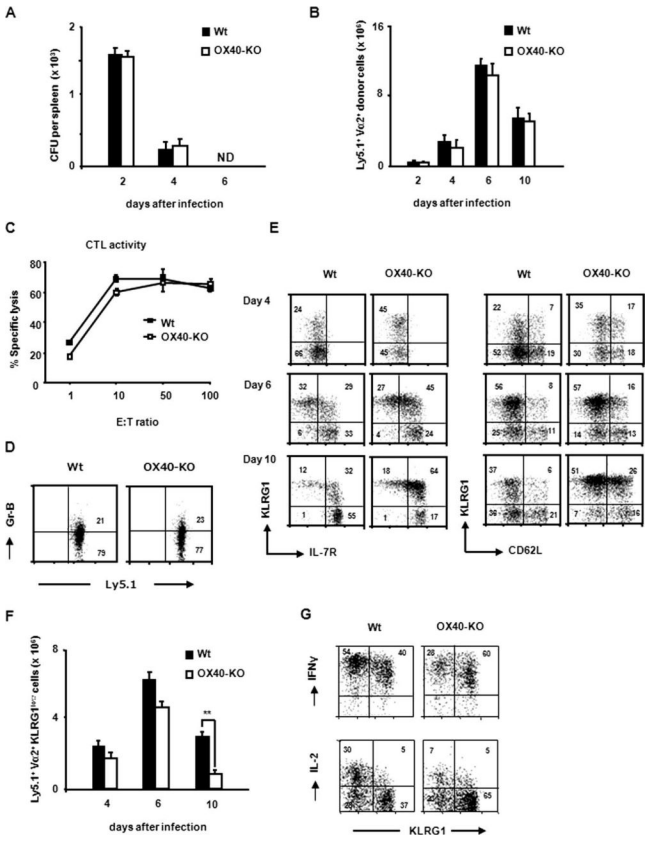


**FIGURE 2.**

OX40 signals are dispensable for the expansion and function of activated $CD8^+$ T cells, but determine the number of $KLRG1^{low}$ MPECs. Purified naive $Ly5.1^+$ OT-I or $Ly5.1^+$ $OX40^{-/-}$ OT-I cells ($1 \times 10^4$ each) were adoptively transferred i.v. into $Ly5.2^+$ congenic wild-type mice that were infected with r*LM*-OVA ($1 \times 10^4$ CFU) 24 h after transfer. *A*, On the indicated days postinfection, the spleen and liver were removed and separately homogenized in PBS. Each extract was cultured in triplicate on an erythromycin-containing plate, and the erythromycin-resistant bacteria colony number was measured. The average CFU per spleen and liver from each recipient was calculated. Results represent the mean ± SD of the average CFU from three mice per group. *B*, The absolute number of $Ly5.1^+$ TCR-$V\alpha2^+$ donor cells in the spleens of recipient mice ($n = 4$ in each group) was counted on the indicated days after infection. Results represent the mean ± SD of the donor cell numbers from four mice per group. Similar results were obtained in two independent experiments. *C*, Spleen cells from the recipient mice ($n = 3$ each) that harbored wild-type or $OX40^{-/-}$ OT-I cells were collected 10 days after the infection. The collected cells (effector cells) were cocultured with $^{51}Cr$-labeled EL-4 cells ($4 \times 10^4$) (target cells) at the indicated ratios in the presence (10 μg/ml) or absence of $OVA_{257–264}$ peptide for 6 h. After the incubation, the radioactivities of the supernatants were determined with a gamma counter. Results are the mean ± SD of percentage of lysis obtained from three mice per group. Similar results were obtained in two independent experiments. *D*, On day 10 postinfection, intracellular granzyme B expression in wild-type OT-I and $OX40^{-/-}$ OT-I cells from the recipient spleen was examined by flow cytometry. The number in each quadrant indicates the frequency of each subset. Results shown are representative mouse from one representative experiment of two. *E*, The kinetic expression of KLRG1 and IL-7R (*left*) and KLRG1 and CD62L (*right*) on $Ly5.1^+$ wild-type OT-I or $Ly5.1^+$ $OX40^{-/-}$ OT-I donor cells was examined on the indicated days postinfection. The number in each quadrant indicates the percentage of each subset. Results shown are the

expression profiles of $Ly5.1^+$ $V\alpha2^+$ cells in pooled splenocytes from three mice, and are representative from each time point from one representative experiment of two. Similar results were obtained in two independent experiments. *F*, The absolute numbers of $KLRG1^{low}$ cells in $Ly5.1^+$ wild-type (■) and $OX40^{-/-}$ (□) OT-I donor cells were counted on the indicated days infection. Results represent the mean ± SD of the donor cell number from four mice per group. Similar results were obtained in two independent experiments. *G*, Whole splenocytes from the recipient mice that harbored either wild-type or $OX40^{-/-}$ OT-I cells were collected on day 10 after r*LM*-OVA infection, and in vitro restimulated with $OVA_{257–264}$ peptide for 4 h. IFN-γ and IL-2 production by $KLRG1^{high}$ and $KLRG1^{low}$ subsets in $Ly5.1^+$ donor cells was analyzed by using intracellular staining. The number in each quadrant indicates the percentage of frequency of each subset in the $Ly5.1^+$ donor cells. Similar results were obtained in two independent experiments.

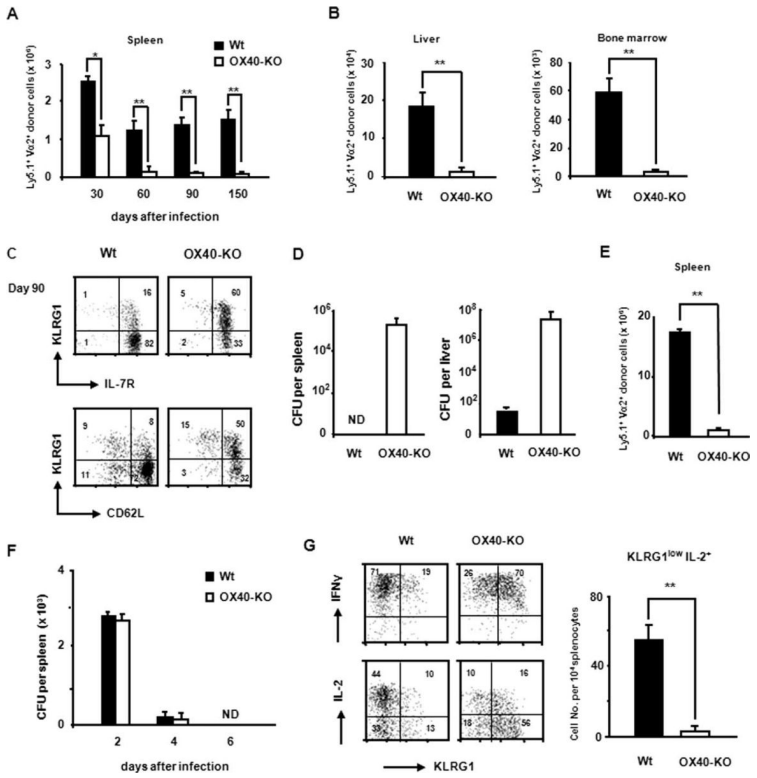


**FIGURE 3.**

OX40 signals are essential for the generation of functional memory CD8$^+$ T cells. Persisting wild-type or OX40$^{-/-}$ donor T cells, which were generated in the first host during *Listeria* infection, were tracked during memory phase. *A*, Purified naive Ly5.1$^+$ OT-I or Ly5.1$^+$ OX40$^{-/-}$ OT-I cells ($1 \times 10^4$ each) were adoptively transferred i.v. into Ly5.2$^+$ congenic wild-type mice that were infected with r*LM*-OVA ($1 \times 10^4$ CFU) 24 h after transfer. The absolute number of Ly5.1$^+$TCR-Vα2$^+$ donor cells in the spleens of recipient mice was counted on the indicated days after the infection. Results represent the mean ± SD of the donor cell numbers from four mice per group. Similar results were obtained in two independent experiments. *B*, The absolute number of donor cells in the liver (*left*) and bone marrow (*right*) of the recipient mice was measured on day 150 after infection. Results represent the mean ± SD from four mice per group. *C*, The expression of KLRG1 and IL-7R (*upper*), and KLRG1 and CD62L (*lower*) on Ly5.1$^+$ long-lived OT-I or OX40$^{-/-}$ OT-I cells in the spleen of the recipient mice on day 90 after infection was examined. Results are representative of four mice in each group. Similar results were obtained in the four mice in each group. *D*, On day 90 postinfection, the recipient mice ($n = 3$ in each group), which harbored either wild-type or OX40$^{-/-}$ donor cells, were rechallenged with a higher dose of r*LM*-OVA ($1 \times 10^5$ CFU). The spleen and liver were removed 3 days after the second infection, and separately homogenized in PBS. Each extract was cultured in triplicate on an erythromycin-containing plate, and the erythromycin-resistant bacteria colony number was measured. The average CFU per spleen and liver from each recipient were calculated. Results represent the mean ± SD of the average CFU from three mice per group. *E*, On day 90 postinfection, the recipient mice ($n = 4$ in each group) were rechallenged with r*LM*-OVA ($1 \times 10^5$ CFU), and the number of Ly5.1$^+$TCR-Vα2$^+$ donor OT-I cells in the spleens was examined 5 days after rechallenge. Results represent the mean ± SD of the donor cell numbers from four mice in each group. Similar results were obtained in two independent experiments. *F*, The long-lived Ly5.1$^+$ wild-type or OX40$^{-/-}$ donor cells ($5 \times 10^4$) generated in the first hosts were collected 45 days after infection, and transferred into the naive second recipients. The second hosts were infected with a higher dose of r*LM*-OVA ($1 \times$

$10^5$ CFU), and the bacterial burden in the spleen of each group was counted at days 2, 4, and 6 of infection. Results represent the mean ± SD of the average CFU from three mice per group. *G*, Production of IL-2 and IFN-γ by donor cells upon in vitro recall responses was examined, as described in Fig. 2*G*, on day 90 after infection. The absolute number of IL-2-producing KLRG1$^{low}$ donor cells in the culture was counted. The number in each quadrant of the dot plots indicates percentage of frequency of each subset in the Ly5.1$^+$ donor cells. Results in the dot plots are representative of three mice in each group. The graph represents the mean ± SD of the number of IL-2-producing KLRG1$^{low}$ donor cells per $10^4$ whole splenocytes from three mice in each group. Similar results were obtained in two independent experiments.

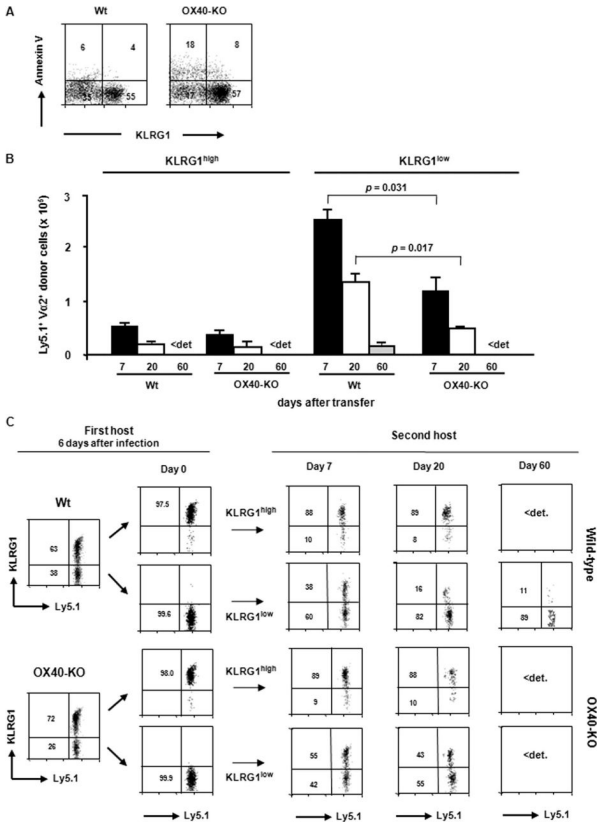


**FIGURE 4.**

OX40 signals promote the survival of KLRG1$^{low}$ memory precursor cells. *A*, Spleens were removed from the recipient mice that harbored wild-type or OX40$^{-/-}$ donor cells 6 days after the r*LM*-OVA infection. Whole splenocytes ($5 \times 10^5$) that included effector donor T cells were cultured for 8 h in complete medium. The cells were then harvested and stained with anti-Ly5.1 mAb, anti-KLRG1 mAb, and annexin V. The intensity of the annexin V staining on the Ly5.1-gated cells was measured with a FACSCalibur flow cytometer. The number in each quadrant indicates the percentage of frequency of each subset in the Ly5.1$^+$ donor cells. Results shown are representative mouse from each time point from one representative experiment of two. *B*, Ly5.1$^+$ effector donor cells were isolated from the spleen of the first host (Ly5.2$^+$) 6 days after the r*LM*-OVA infection with an AutoMACS cell sorter. The enriched Ly5.1$^+$ donor cells were further separated by a FACSAria cell sorter to purify KLRG1$^{low}$TCR-Vα2$^+$CD8$^+$ and KLRG1$^{high}$TCR-Vα2$^+$CD8$^+$ effector T cells. These cells ($2 \times 10^6$ each), from the first host, which harbored either the wild-type OT-I or OX40$^{-/-}$ OT-I donor cells, were adoptively transferred into an Ly5.2$^+$ second host (wild-type mouse) that had been infected with wild-type *L. monocytogenes* 6 days previously. The absolute number of the live Ly5.1$^+$TCR-Vα2$^+$ cells derived from each population in the spleen of each recipient was counted on the indicated days after transfer. The graph represents the mean ± SD of the number of the donor cells from three mice on each day in each group. Similar results were obtained in two independent experiments. <det., Below detection. *C*, Expression of KLRG1 on Ly5.1$^+$TCR-Vα2$^+$ donor cells was examined on the indicated days after transfer. Results shown are representative mouse from each time point from one representative experiment of two. Similar results were obtained in the two experiments. <det., Below detection.

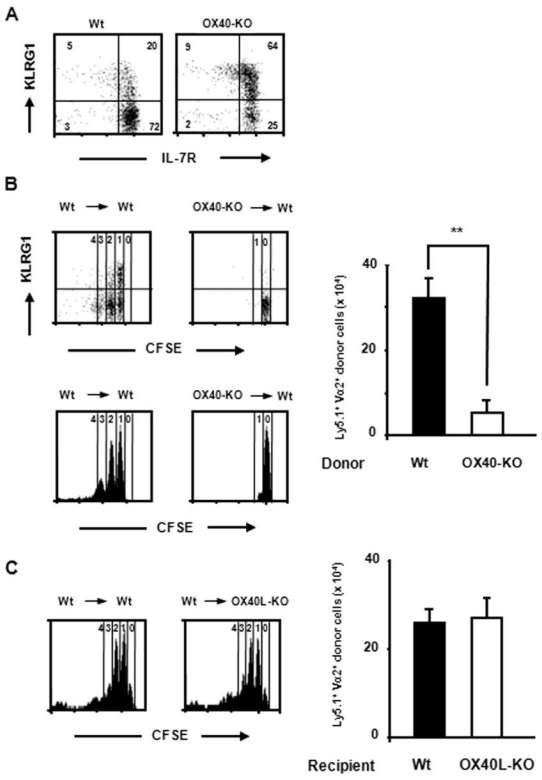


**FIGURE 5.**

OX40 is essential for the self-renewal potential of memory CD8$^+$ T cells. Long-lived Ly5.1$^+$ donor cells from the first host were transferred into Ag-naive second host, and their homeostatic proliferation was assessed. *A*, Ly5.1$^+$ donor cells in the first host, which possessed either wild-type or OX40$^{-/-}$ donor cells, were collected 40 days after infection by using an AutoMACS cell sorter. Before being transferred into second host, the donor cells were stained with anti-KLRG1 and IL-7R mAbs, and their expression profiles of KLRG1 and IL-7R were examined. The data shown are representative of independent four or five donor cell samples in each group. *B*, The purified long-lived Ly5.1$^+$ donor cells from the first host were labeled with CFSE and transferred ($1 \times 10^6$ in each group) into wild-type congenic mice ($n = 3$). The dilution of CFSE intensity of Ly5.1$^+$TCR-Vα2$^+$ donor cells in the spleen of recipient mice was assessed by flow cytometry. The absolute number of Ly5.1$^+$TCR-Vα2$^+$ donor cells in the spleen of the second host was counted. The data shown in the flow cytometric analysis are representative of three mice per each group. The graph represents the mean ± SD of the absolute numbers of the donor T cells in the spleen of recipient mice ($n = 3$ in each group) on day 30 posttransfer. Similar results were obtained in two independent experiments. *C*, CFSE-labeled wild-type long-lived donor T cells ($1 \times 10^6$) were transferred into naive Ly5.2$^+$ congenic wild-type or OX40L$^{-/-}$ mice ($n = 3$ each), as described above. Cell division of the donor cells in the spleen of the second host was assessed by a CFSE dilution assay. The data shown in the flow cytometric analysis are representative of three mice per each group in one representative experiment of two. The graph represents the mean ± SD of the donor cell numbers in the spleen from recipient mice ($n = 3$ in each group) at day 30 posttransfer.

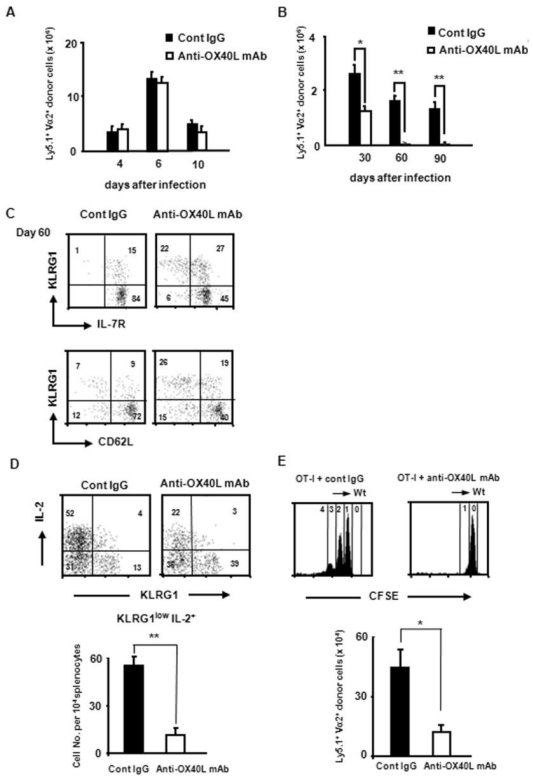


**FIGURE 6.**

Blocking of OX40 signals during priming suppresses both the generation and the maintenance of memory $CD8^+$ T cells. Purified naive $Ly5.1^+$ OT-I cells ($1 \times 10^4$) were adoptively transferred i.v. into $Ly5.2^+$ congenic recipient mice that were infected with r*LM*-OVA ($1 \times 10^4$ CFU) at 24 h after the cell transfer. To block the OX40-OX40L interaction, the recipient mice were given control rat IgG (300 μg) or blocking anti-OX40L mAb (MGP34) (300 μg) by i.p. injection 1 day before, and on days 1, 3, 5, and 7 after the infection. Accumulation of effector (*A*) and memory (*B*) donor cells was determined by tracking $Ly5.1^+$ TCR-$V\alpha2^+$ cells in the spleen of each recipient mice ($n = 3$ or 4 in each group). Results represent the mean ± SD of the absolute numbers of the donor cells in the spleens from three or four mice in each group on the indicated days, and are representative of two independent experiments. *C*, On day 60 postinfection, IL-7R and CD62L expression by $KLRG1^{high}$ and $KLRG1^{low}$ donor cells in the spleen of the recipient mice that were treated with control rat IgG or blocking anti-OX40L mAb during priming were examined by flow cytometric analysis. Results shown are representative mouse from three mice from one representative experiment of two. *D*, Production of IL-2 and IFN-γ by donor cells upon in vitro recall stimulation was examined on day 60 after infection, as described in Fig. 2*G*. The number in each quadrant of the dot plots indicates percentage of frequency of each subset in the $Ly5.1^+$ donor cells. Results in the dot plots are representative of four mice in each group. The graph represents the mean ± SD of the number of IL-2-producing $KLRG1^{low}$ donor cells per $10^4$ whole splenocytes from four mice in each group. Similar results were obtained in two independent experiments. *E*, Forty days after infection, $Ly5.1^+$ donor cells were purified from the first host that had been treated with anti-OX40L blocking mAb or control rat IgG (300 μg/mouse), as described above. The purified $Ly5.1^+$ donor cells ($1 \times 10^6$) were transferred into Ag-naive second host, and their homeostatic proliferation was assessed 30 days after the transfer by a CFSE dilution assay, as described in Fig. 5*B*. The data shown in the flow cytometric analysis are representative of three mice per each group in one representative experiment of two. The graph represents the mean ± SD of the absolute numbers

of Ly5.1$^{+}$TCR-Vα2$^{+}$ donor T cells in the spleen from three mice in each group at day 30 posttransfer. Similar results were obtained in two independent experiments.