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IL-15-Dependent Induction of 4-1BB Promotes Antigen-Independent CD8 Memory T Cell Survival[1]

Gayle Pulle, Mariana Vidric, and Tania H. Watts[2]

Mice lacking CD137L (4-1BBL) show normal primary expansion and contraction of the CD8$^+$ T cell response to influenza virus, but exhibit a defect in Ag-specific CD8$^+$ T cell numbers at 3–6 wk postinfection. Previous results showed that the decrease in CD8$^+$ T cell numbers in this model is not due to a programming defect during primary expansion. Thus, it appears that 4-1BB/4-1BBL interactions control the number of surviving CD8$^+$ effector memory cells, late in the primary response. In this report, we asked how 4-1BB on T cells could play a role after Ag has apparently been cleared from the host. We show that IL-15, a cytokine involved in regulation of CD8$^+$ memory T cell survival, induces the expression of 4-1BB on CD8$^+$CD44^{high} memory phenotype T cells, but not on CD4$^+$ T cells. The Ag-independent induction of 4-1BB by IL-15 was dependent on MAPK p38 and ERK activation. Transfer of in vitro-generated OT-I CD8$^+$ memory T cells into unimmunized wild-type or 4-1BBL-deficient hosts revealed a 2- to 3-fold survival advantage when 4-1BBL was present, recapitulating the effect seen in the endogenous response to influenza in mice. Decreases in the overall number of memory CD8$^+$ T cells were also observed in the bone marrow of unmanipulated 4-1BBL-deficient mice. These data suggest a model whereby 4-1BB expression on memory CD8$^+$ T cells, perhaps due to encounter with IL-15 in the bone marrow, allows 4-1BB/4-1BBL interactions to maintain memory CD8 T cell survival in the absence of Ag. *The Journal of Immunology,* **2006, 176: 2739–2748.**

Memory responses are the hallmark of the acquired immune system. Long-term T cell immunity is based on increased numbers of Ag-specific T cell precursors after infection (1) and memory properties that include faster responsiveness to reinfection with more rapid acquisition of effector functions such as cytotoxicity and cytokine production (2, 3). In response to Ag, the majority of activated effector CD8$^+$ T cells undergo programmed expansion and contraction but a small subpopulation of these cells escape apoptosis and add to the stable memory T cell pool (4).

There is currently much interest in the factors that allow the establishment and maintenance of T cell memory. 4-1BB (CD137), a member of the TNFR family, and its ligand (4-1BBL, CD137L) are important in controlling the size of the CD8$^+$ T cell recall response to viruses (5–8). Mice lacking 4-1BBL show normal primary expansion and contraction of the CD8$^+$ T cell response to influenza virus, but show decreased T cell numbers at 3–5 wk postinfection (7). Previous studies have demonstrated that when T cells are primed in wild-type (WT)[3] or 4-1BBL$^{-/-}$ mice and equivalent numbers of Ag-specific T cells are transferred into WT mice, the primed CD8$^+$ T cells expand equivalently when re-exposed to virus, arguing against a role for 4-1BBL in programming T cell responses (8).

Substantial evidence supports a role for 4-1BB in the survival of CD8$^+$ T cells (7, 9–12) through the NF-κB-dependent induction of Bcl-x_L and Bfl-1 (13) and through the downstream activation of protein kinase B resulting in up-regulation of the antiapoptotic protein cellular FLIP short form (c-$FLIP_{short}$) (14). Taken together, the available evidence suggests that 4-1BB/4-1BBL interaction is involved in survival signaling in T cells and may play a role in maintenance of T cell responses after Ag has been cleared.

4-1BB is normally expressed only transiently after Ag-dependent T cell recognition in vivo (10, 15). Furthermore, treatment of 4-1BBL-deficient mice with agonistic anti-4-1BB Ab suggests that the effects of 4-1BB signaling are relatively transient (8). This raises the question of how 4-1BB could provide a signal for maintenance of T cell survival at several weeks after initial administration of Ag. Thus, we hypothesized that other signals may regulate 4-1BB expression once Ag is cleared.

Possible candidates for induction of 4-1BB on memory T cells include IL-7 and IL-15, two cytokines that regulate memory cell survival and homeostasis (16). Mice lacking IL-15 or its high-affinity receptor, IL-15Rα, showed reduced numbers of memory phenotype CD44^{high}CD8$^+$ T cells (17, 18). Furthermore, both IL-15$^{-/-}$ and IL-15R$\alpha^{-/-}$ mice mount normal initial responses to lymphocytic choriomeningitis virus and vesicular stomatitis virus, the virus is effectively cleared, and initially normal numbers of memory CD8$^+$ T cells were generated. However, the numbers of CD8$^+$ memory cells decline over time (19, 20). It is of interest that these IL-15-deficient mice show a similar phenotype to 4-1BBL-deficient mice with respect to declining numbers of virus-specific CD8$^+$ memory phenotype cells.

In this study, we treated murine T cells with IL-15 to examine 4-1BB expression. We generated memory cells in vitro and transferred them into WT or 4-1BBL$^{-/-}$ hosts to determine the effects of 4-1BB on memory cell survival in the absence of Ag in vivo. We report that IL-15, but not IL-7, induces 4-1BB on CD44^{high} CD8$^+$ memory T cells and not on CD4$^+$ T cells and that 4-1BBL in the unimmunized recipient plays a role in the survival of transferred memory T cells.

Department of Immunology, University of Toronto, Toronto, Ontario, Canada

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[2] Address correspondence and reprint requests to Dr. Tania H. Watts, Department of Immunology, University of Toronto, 1 King's College Circle, Toronto, Ontario M5S 1A8, Canada. E-mail address: tania.watts@utoronto.ca

[3] Abbreviations used in this paper: WT, wild type; neg, negative; int, intermediate.

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Materials and Methods

Mice

C57BL/6 WT mice were obtained from Charles River Laboratories. 4-1BBL$^{-/-}$ mice (5) backcrossed onto the C57BL/6 background ($n = 9$) were bred in our facility. OT-I mice (21) were provided by Dr. P. Ohashi (Ontario Cancer Institute, Toronto, Ontario, Canada). OT-I mice were crossed with CD45.1 congenic mice, obtained from Taconic Laboratories. CD40L$^{-/-}$ mice were provided by Dr. J. Gommerman (Medical Sciences Building, Toronto, Ontario, Canada). CD40L was detected by isolation of genomic tail DNA and PCR analysis. OT-I transgene expression in the mice was confirmed using fluorescently conjugated Abs to TCR Vα2 and Vβ5.1. CD45.1 was then used as a marker of donor T cells in adoptive transfer experiments. Mice were maintained under specific viral/pathogen-free conditions in microisolator cages. All studies involving mice were approved by the University of Toronto animal care committee in accordance with the regulations of the Canadian Council on Animal Care.

T cell isolation and cytokine stimulation

T cells were purified from mouse spleens by depletion of unwanted subsets using T Cell Immunocolumns (Cedarlane Laboratories) or using magnetic bead separation (Easy Sep; StemCell Technologies). Purity of T cells was checked using anti-CD3-PE (eBioscience) and was found to be >90% T cells. T cells (1×10^6cells/ml) were cultured in 24-well plates with or without various concentrations of recombinant human IL-15 (R&D Systems) or murine IL-7 from the supernatant of stably transfected J558 cells, a gift from Dr. C. Paige (Ontario Cancer Institute, Toronto, Ontario, Canada). Stock concentration of IL-7 from the supernatant was determined to be 2.5 μg/ml when standardized against known concentrations of rIL-7. T cells were cultured for 24 h with the following inhibitors: Ly294002, SB203580, U0126, and piceatannol (Sigma-Aldrich) dissolved in DMSO, at the concentrations indicated in the figures. Cells were preincubated with inhibitors for 1 h before IL-15 addition.

Ag-experienced OT-I cell generation and adoptive transfers

OT-I splenocytes (5×10^6cells/ml) were stimulated with 10 μg/ml OVA 257–264 peptide (SIINFEKL; purchased from Alberta Peptide Institute), for 16–18 h in RPMI 1640 supplemented with 10% FCS, excess peptide was then washed out, and cells were cultured for an additional 24 h. The nonadherent cells were then transferred to a tissue-culture flask and rested for 24 h at $1–2 \times 10^6$cells/ml in fresh medium. Viable lymphocytes were recovered using Lympholyte-M (Cedarlane Laboratories) density gradient centrifugation. The cells were then washed and resuspended at $1–2 \times 10^6$cells/ml in medium supplemented with 20 ng/ml rIL-15 (R&D Systems). At this stage, the cultures contained ~90% CD8$^+$ T cells. Cells were cultured in tissue flasks and medium with cytokine was replaced every 2 days. Total culture time was 8–9 days and cells were analyzed for various activation and memory phenotype surface markers at days 0, 2, and 8 of culture. At the end of culture, >95% of all viable cells were CD8$^+$, Vα2$^+$, Vβ5$^+$, and CD45.1$^+$. These cells were then washed and resuspended in Hank's medium at $1–3 \times 10^7$cells/ml and 100 μl were adoptively transferred into CD45.2 WT, 4-1BBL$^{-/-}$, or CD40L$^{-/-}$ hosts. Transferred cells were enumerated in the spleen at 1, 3, and 6 wk posttransfer. Donor cell recovery and memory phenotype CD8$^+$CD44^{high} cells were also analyzed in host mice femur bone marrow.

In vivo restimulation of OT-I memory cells

Mice were immunized s.c. with 2 mg of OVA (Sigma-Aldrich) and 50 μg of LPS (Sigma-Aldrich) 24 h or 3 wk posttransfer of memory OT-I cells. Donor cell expansion was analyzed in the spleen. Effector function was measured by IFN-γ staining.

Flow cytometry

For staining of T cells, spleen cell suspensions were prepared in PBS/2% FCS/0.01% sodium azide (FACS buffer) on ice. OT-I transgene expression was assayed using CD8$^+$, Vα2$^+$, and Vβ5.1$^+$ specific fluorescence-conjugated Abs (eBioscience). OT-I cells were also stained for the following activation and memory phenotype markers at various stages during in vitro memory cell generation: CD44-PE, biotinylated 4-1BB, and CD69-FITC (eBioscience) as well as CD8-FITC, CD4-FITC, CD62L- FITC, MR1 (CD40L), which were purified from hybridoma supernatants and labeled with FITC or biotin according to manufacturer's instructions. Appropriate isotype controls were used to define background staining. Cells were surface-stained with allophycocyanin-conjugated antimouse CD8α and FITC or PE-labeled CD45.1 (eBioscience) to identify transferred cells.

To measure intracellular IFN-γ, 5×10^6 splenocytes were restimulated in culture medium (RPMI 1640/10% FCS with antibiotics and 2-ME) for 5 h at 37°C with 1 μM of SIINFEKL peptide and Golgi-stop (BD Pharmingen), followed by surface-staining with FITC-anti-CD8 and PE-anti-CD45.1 as described above. Following surface staining, cells were fixed in Cytofix/Cytoperm solution (BD Pharmingen) and then stained with allophycocyanin-conjugated antimouse IFN-γ diluted in 1× Perm/Wash solution (BD Pharmingen).

For CFSE staining, purified T cells were washed twice with PBS and resuspended in prewarmed PBS at a concentration of 5×10^7cells/ml with 2.5 μM CFSE (Molecular Probes) and incubated at 37°C for 15 min. Cells were then washed once in medium with 10% FCS to bind excess CFSE. TO-PRO-3 iodide staining for analysis of cell death was performed as described previously (22). T cells were incubated poststimulation with TO-PRO-3 (Molecular Probes) at 0.1 μM in FACS buffer for 10 min at room temperature, immediately before analysis. Samples were analyzed using a FACSCalibur and CellQuest (BD Biosciences) or FlowJo software (Tree Star). All flow cytometry plots are gated on live cells as assessed by forward/side scatter and CD8$^+$ or CD4$^+$ T cells.

Statistical analysis

Where indicated, p values were obtained using the Student t test (unpaired, two-tailed, 95% confidence interval).

Results

IL-15 induces 4-1BB on memory phenotype CD8$^+$ T cells.

To test the role of cytokines in the induction of 4-1BB, we examined the effects of IL-7 and IL-15 on T cells, as these cytokines have been shown to play a role in T cell memory. Purified splenic T cells were cultured with low (20 ng/ml) and high (100 ng/ml) doses of IL-7 and IL-15. Treatment with IL-15 resulted in a dose- and time-dependent induction of 4-1BB expression selectively on CD8$^+$ T cells (Fig. 1*A*). 4-1BB was not detected on CD4$^+$ T cells, nor was it induced with IL-7 treatment at either dose (Fig. 1*A*). Expression of 4-1BB on IL-15-treated cells was limited to the CD44^{high} population and peaked at day 4 poststimulation (Fig. 1*B*). Approximately 50% of CD8$^+$CD44^{high} cells continued to express 4-1BB for as long as 10 days in continuous culture with 100 ng/ml IL-15 (data not shown). The effects of IL-15 were specific to 4-1BB, as the related TNFR family member OX40 was not induced on CD4$^+$ or CD8$^+$ T cells at 72 h of culture with 100 ng/ml IL-15 (Fig. 1*C*) nor was it induced at other time points (24, 48, or 96 h; data not shown). However, anti-CD3 (1 μg/ml) plus anti-CD28 (10 μg/ml) treatment showed positive staining for OX40 expression (data not shown). We also isolated bone marrow cells because they have been reported to contain a significant reservoir of CD8$^+$ memory cells (23, 24). Indeed, we observed that >50% of the CD8$^+$ T cells in the bone marrow were CD44^{high} and that IL-15 treatment induced expression of 4-1BB on 10–30% of these cells by 48 h (Fig. 1*D*).

Although IL-7 treatment did not result in 4-1BB expression, T cells still responded to this cytokine as observed by increased cell size and granularity in the forward scatter/side scatter profile (Fig. 2*A*). IL-7 increased cell survival (Fig. 2*B*) without affecting proliferation or activation of cells as measured by CFSE and CD69 staining, respectively (Fig. 2, *C* and *D*). IL-15, in contrast, enhanced cell viability (Fig. 2*B*), even at doses where minimal proliferation was seen (Fig. 2*C*, *top panel*). At higher doses (e.g., 100 ng/ml) IL-15 induced cell division (Fig. 2*C*) and expression of the activation Ag CD69, which is coexpressed with 4-1BB (Fig. 2*D*). These experiments demonstrate that IL-15 can act on memory phenotype CD8$^+$ T cells to induce surface expression of 4-1BB in the absence of Ag.

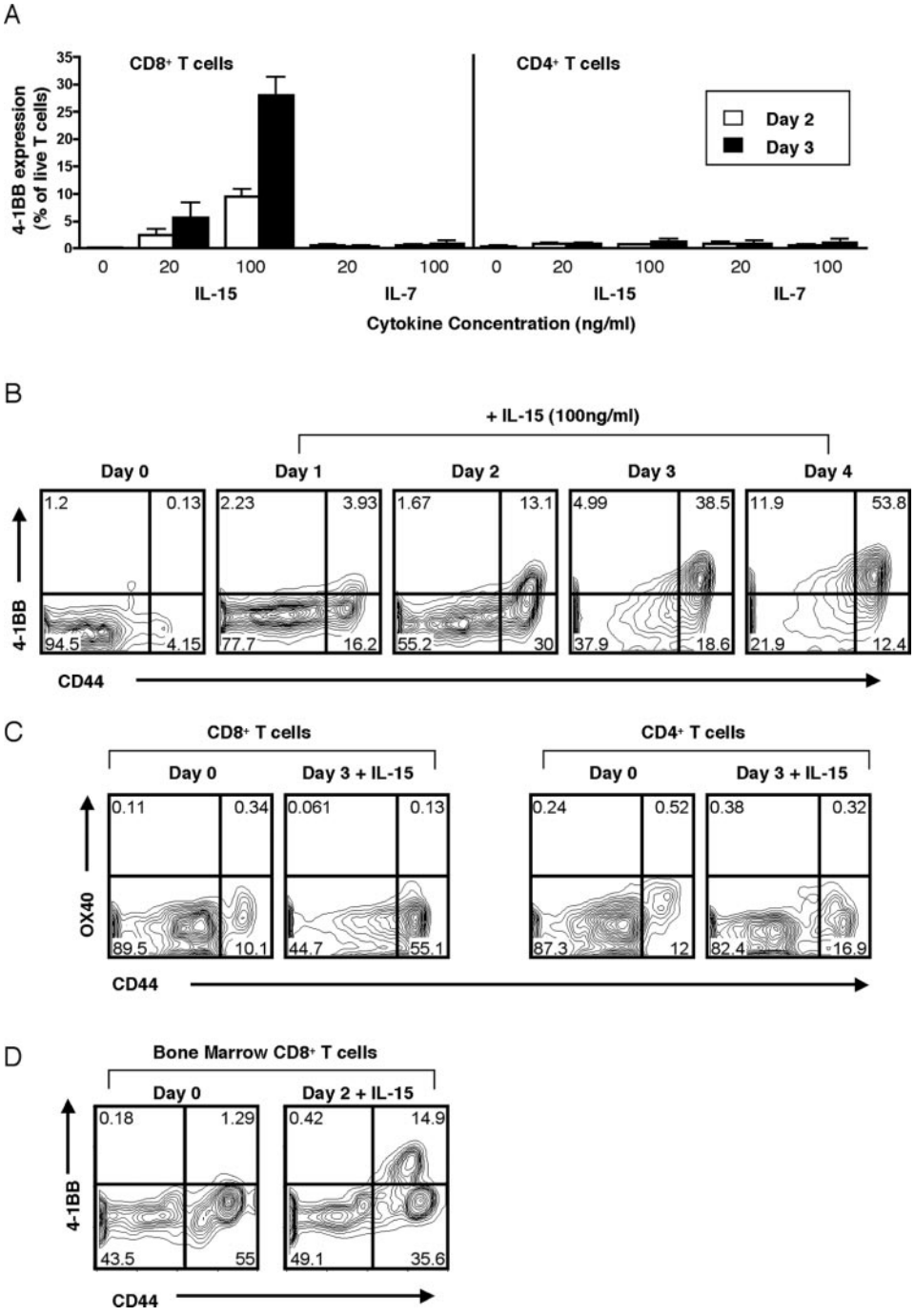

FIGURE 1. IL-15 induces 4-1BB surface expression on $CD8^+CD44^{high}$ T cells. *A*, Purified splenic murine total T cells were cultured at 10^6 cells/ml for 2–3 days with a low (20 ng/ml) and high (100 ng/ml) concentration of IL-15 and IL-7. Cells positive for 4-1BB surface expression were detected by flow cytometry. *Left panel*, 4-1BB expression on the live $CD8^+$ T cells; *right panel*, for live $CD4^+$ T cells. *B*, Murine splenic T cells were cultured at 10^6 cells/ml for 1–4 days with 100 ng/ml recombinant human IL-15 and analyzed for expression of CD8, CD44, and 4-1BB. *C*, Cells cultured as in *B* were stained for OX40 and CD44 on $CD4^+$ and $CD8^+$ T cells at 72 h. *D*, Total bone marrow cells were isolated from mice femurs and cultured with 100 ng/ml IL-15 for 48 h and analyzed for expression of CD8, CD44, and 4-1BB. Data are representative of at least three separate experiments.

IL-15-mediated 4-1BB expression is MAPK dependent

To determine the mechanisms involved in IL-15 induction of 4-1BB expression, we blocked several of the IL-15 cytokine receptor signaling pathways. IL-15 and IL-2 share the IL-2Rβ-chain, which elicits three main signaling pathways: JAK-STAT, MAPK, and PI3K (25). In addition, there is also a report of involvement of the tyrosine kinase syk in IL-15R signaling (26). The JAK-STAT pathway is critical to the common γ-chain family of cytokines and would not confer specificity. Thus, we inhibited two MAPK pathways, ERK and p38, with U0126 and SB203580, respectively, as well as the PI3K pathway with Ly294002 and the syk pathway with piceatannol, to determine their involvement in 4-1BB induction by IL-15 (Fig. 3*A*). All inhibitors were added to purified murine splenic T cells 1 h before IL-15 treatment. An early time point (24 h poststimulation) was examined to reduce possible toxic effects of inhibitors. IL-15 induction of 4-1BB was inhibited in a dose-dependent manner by the MAPK inhibitors SB203580 (p38) and U0126 (ERK) (Fig. 3*A*). In contrast, increasing doses of the syk inhibitor piceatannol caused no inhibition of 4-1BB induction and there was little or no effect of the PI3K inhibitor Ly294002 (Fig. 3*A*). Fig. 3*B* shows that a similar percentage of cells remain viable by forward scatter and side scatter in the presence of the ERK inhibitor U0126 at 5 μM compared with vehicle control, whereas the proportion of blasting cells was greatly reduced.

Generation of memory-like $CD8^+$ T cells in vitro

To analyze the role of 4-1BB in maintenance of memory $CD8^+$ T cell survival, we adapted an in vitro model in which TCR transgenic naive T cells are treated with Ag and then with IL-15 to generate memory

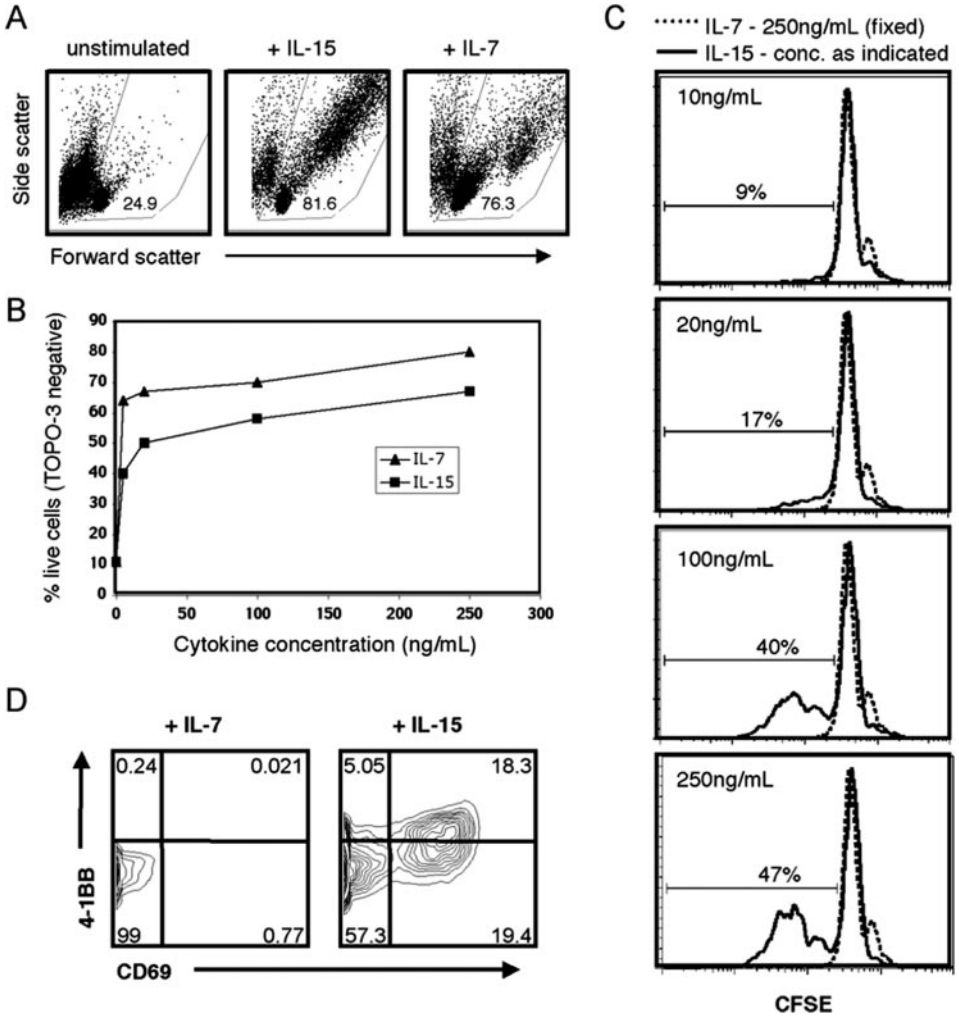

FIGURE 2. IL-7 is a survival factor for $CD8^+$ T cells, while IL-15 activates $CD8^+$ T cells and induces proliferation. Purified splenic murine T cells were cultured at 10^6 cells/ml for 48 h with various doses (10–250 ng/ml) of recombinant human IL-15 or IL-7. *A*, Cell size and granularity were assessed by forward scatter/side scatter using flow cytometry. *B*, TO-PRO-3-negative staining was used to measure the proportion of live cells as a function of cytokine concentration. *C*, CFSE staining was used to measure cell division at different doses of IL-15 (solid line) and IL-7 at 250 ng/ml (dashed line). The gate defines the percentage of events that indicate at least one cell division, as indicated on histogram plots. IL-7 did not result in cell division at doses from 10 to 250 ng/ml. *D*, Dual staining for 4-1BB and CD69 was measured at 48 h following treatment with 100 ng/ml IL-7 or IL-15. Plots in *C* and *D* are gated on live $CD8^+$ T cells. Data are representative of three separate experiments.

phenotype T cells (27, 28). This approach allows us to track a homogenous population of T cells after transfer without the complexity of an ongoing immune response and ensures that effects of 4-1BB are Ag independent once transferred into WT or 4-1BBL-deficient naive hosts. OT-I splenocytes were incubated with $OVA_{257–264}$ peptide overnight, followed by washing out of Ag and further stimulation for 24 h (Fig. 4, *middle column*; D2-activated T cells). Nonadherent cells were transferred to tissue-culture flasks for an additional 24 h and then viable lymphocytes were isolated and incubated with IL-15 for another 5–6 days (Fig. 4, *right column*; D8, IL-15-treated).

More than 90% of the unstimulated splenic $CD8^+$ T cells were naive: $CD69^{neg}$, $CD44^{low/int}$, $CD62L^{high}$, and $4\text{-}1BB^{neg}$ (Fig. 4, *left column*). The day 2 Ag-activated OT-I T cells exhibited the expected induction of the early activation marker CD69, high levels of CD44, and some down-regulation of CD62L (Fig. 4, *middle column*). Following culture of the Ag-experienced $CD8^+$ T cells for an additional 5 days in IL-15, the day 8 OT-I T cells exhibited a central memory-like phenotype: $CD69^{low}CD44^{high}CD62L^{high}$ (Fig. 4, *right column*). OT-I T cells expanded 5- to 8-fold with addition of 20 ng/ml IL-15 every 2 days. Naive OT-I $CD8^+$ T cells did not express 4-1BB, but once 4-1BB was up-regulated (Fig. 4, *middle column*) this expression was maintained at intermediate levels following IL-15 stimulation expression (Fig. 4, *bottom right*).

Decreased survival of memory-like cells in 4-1BBL-deficient hosts

To test whether 4-1BB interaction with its ligand is required for memory cell survival, the in vitro-generated CD45.1 OT-I memory-like cells were adoptively transferred i.v. into naive syngeneic CD45.2 WT or $4\text{-}1BBL^{-/-}$ hosts without immunization. Initial time-course experiments showed a progressive loss of donor cells in 4-1BBL-deficient mice (Fig. 5*A*). At 6 wk, very few cells could be recovered from the spleen so we focused on the 3-wk time point. Fig. 5*B* illustrates that although the percentages recovered in 4-1BBL-deficient mice were small, it is a distinct population. In four experiments with a total of 15 mice per group we saw a modest but consistent defect in the number of surviving $CD8^+$ T cells in $4\text{-}1BBL^{-/-}$ compared with WT mice. On average, by 3 wk posttransfer, ~40–50% fewer transferred cells were recovered from $4\text{-}1BBL^{-/-}$ mice compared with WT mice (Fig. 5*C*). In additional experiments, we transferred a higher starting population of OT-I memory cells (3×10^6 per mouse). As an additional genetic control, we also compared transfer into mice lacking another TNF family member, CD40L (Fig. 5*D*). Transfer of twice the number of memory cells resulted in two times greater recovery of WT cells and again showed a substantial (70%) defect in $4\text{-}1BBL^{-/-}$ mice. Furthermore, transfer of OT-I memory cells into WT or CD40L-deficient mice showed similar recovery of T cells, illustrating the specificity of the effect of 4-1BBL deficiency.

To determine whether decreased recovery of memory T cells from 4-1BBL-deficient mice was due to decreased proliferation, OT-I memory-like T cells were stained with CFSE to monitor cell division in vivo. As shown in Fig. 5*E*, donor cells had not divided by 1 wk, and one to two divisions were observed by 3 wk. The rate of division and proportion of divided cells was similar in both WT and $4\text{-}1BBL^{-/-}$ hosts, suggesting that effects on T cell numbers in

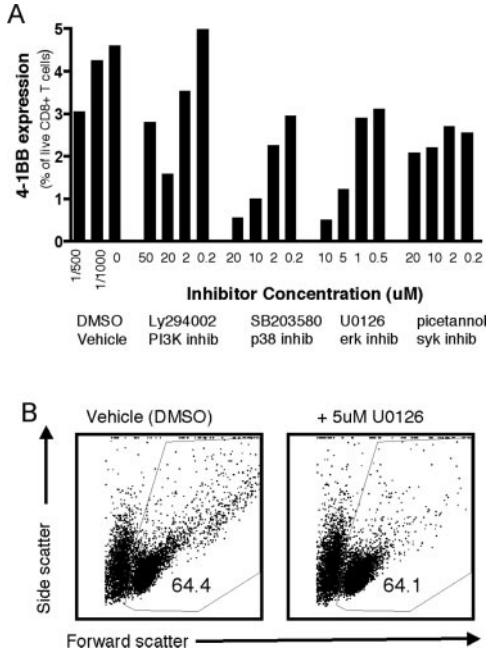

FIGURE 3. IL-15-induced expression of 4-1BB is ERK- and p38-dependent. Purified murine splenic T cells were cultured at 10^6 cells/ml for 24 h with 100 ng/ml recombinant human IL-15 with or without inhibitors of different signaling pathway components: Ly294002 (0.25–50 μM), SB203580 (0.25–20 μM), U0126 (0.5–10 μM), and piceatannol (0.2–20 μM). These concentration ranges were based on recommended IC_{50} values from the literature. Cells were preincubated for 1 h with inhibitors before addition of IL-15. Inhibitors were reconstituted in DMSO and added at a 1/500 or 1/1000 dilution to the cultures. *A*, 4-1BB expression on live $CD8^+$ T cells with vehicle or inhibitors. *B*, Inhibitor toxicity was assessed by comparing percentages of live cells, using cell size as an indicator of surviving cells. Representative FACS plots are shown for DMSO vehicle and most effective inhibitor 5 μM U0126. Toxicity was not observed for any other inhibitors at the doses used (data not shown). These data are representative of three separate experiments.

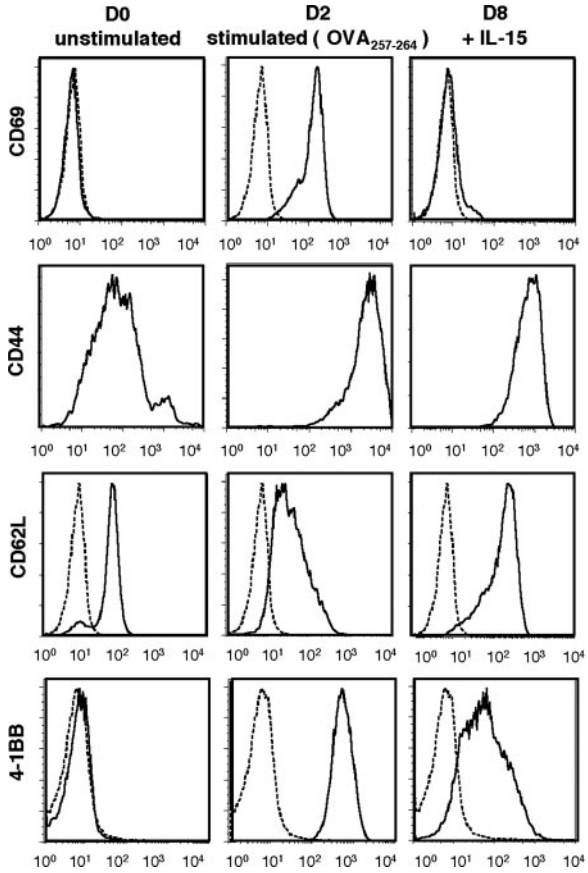

FIGURE 4. Phenotype of in vitro-generated memory-like OT-I cells. OT-I cells were analyzed for activation/memory markers (CD69, CD44, CD62L) and 4-1BB expression during in vitro generation of memory-like cells. *Left column*, Starting population of unstimulated naive MHC-I-restricted transgenic OT-I murine splenocytes. *Middle column*, OT-I cells (5×10^6 cells/ml) at 48 h following stimulation with 10 μg/ml $OVA_{257–264}$ (SIINFEKL). *Right column*, Poststimulation, nonadherent cells were rested for an additional 24 h, and then viable OT-I cells were cultured with 20 ng/ml IL-15 for 5 additional days. Media with IL-15 was changed every 2 days. Memory-like cells were assessed at end of culture (day 8). At this point, cells were used for adoptive transfer experiments described in Fig. 5. All histogram plots are gated on live $CD8^+$ T cells. Isotype controls are shown as dotted lines. These data are representative of five separate experiments.

WT vs 4-1BBL-deficient hosts are likely due to differences in survival rather than division. These results establish an Ag-independent role for 4-1BB/4-1BBL interaction in maintaining survival of "memory phenotype" Ag-experienced $CD8^+$ T cells in vivo.

Decreased numbers of memory-like cells in 4-1BBL-deficient hosts following in vivo challenge

To determine whether the increased survival of memory OT-I cells in WT hosts also translated into increased recall immunity, mice were challenged with OVA/LPS and the T cell response was analyzed 5 days later (Fig. 6). Mice were challenged either 24 h or 3 wk after transfer of memory OT-I cells to distinguish between effects of 4-1BBL on secondary expansion vs effects on maintenance of memory cells (Fig. 6, *A* and *C*). 4-1BBL-deficient hosts showed a slightly reduced expansion of transferred cells following immediate OVA/LPS stimulation in vivo (Fig. 6*B*), although this modest defect was not statistically significant. In contrast, when mice were challenged 3 wk posttransfer of OT-I memory cells, the OT-I T cells in the 4-1BBL-deficient hosts again showed a substantial defect in cell numbers (Fig. 6*D*) with a concomitant reduction in the number of IFN-γ-producing cells detected following a 5 h restimulation with peptide (Fig. 6*E*). When sufficient cells could be recovered from 4-$1BBL^{-/-}$ hosts, they were clearly capable of making IFN-γ upon restimulation. Thus, 4-1BB-4-1BBL interaction is critical for maintaining CD8 T cell numbers, but is dispensable for maintenance of effector function of the recovered T cells.

4-1BB-4-1BBL interactions maintain $CD8^+$ memory cell numbers in the bone marrow

Fig. 1 shows that IL-15 induces 4-1BB on $CD44^{high/int}$ bone marrow $CD8^+$ T cells. Therefore, we also analyzed bone marrow from WT and 4-1BBL-deficient mice for the presence of transferred OT-I memory T cells, at 3 wk posttransfer. There were significantly less transferred cells in the bone marrow of 4-1BBL-deficient hosts (Fig. 7*A*), confirming the defect observed in the spleen.

If indeed IL-15 in the bone marrow is important in inducing 4-1BB and allowing maintenance of CD8 T cell survival through 4-1BB/4-1BBL interactions, a prediction of the model is that unmanipulated, unimmunized 4-1BBL-deficient mice should also have a lower frequency of CD8 memory cells in the bone marrow compared with WT mice. Indeed, comparison of the $CD8^+$ $CD44^{high}$ populations in the bone marrow of 13 WT and 13

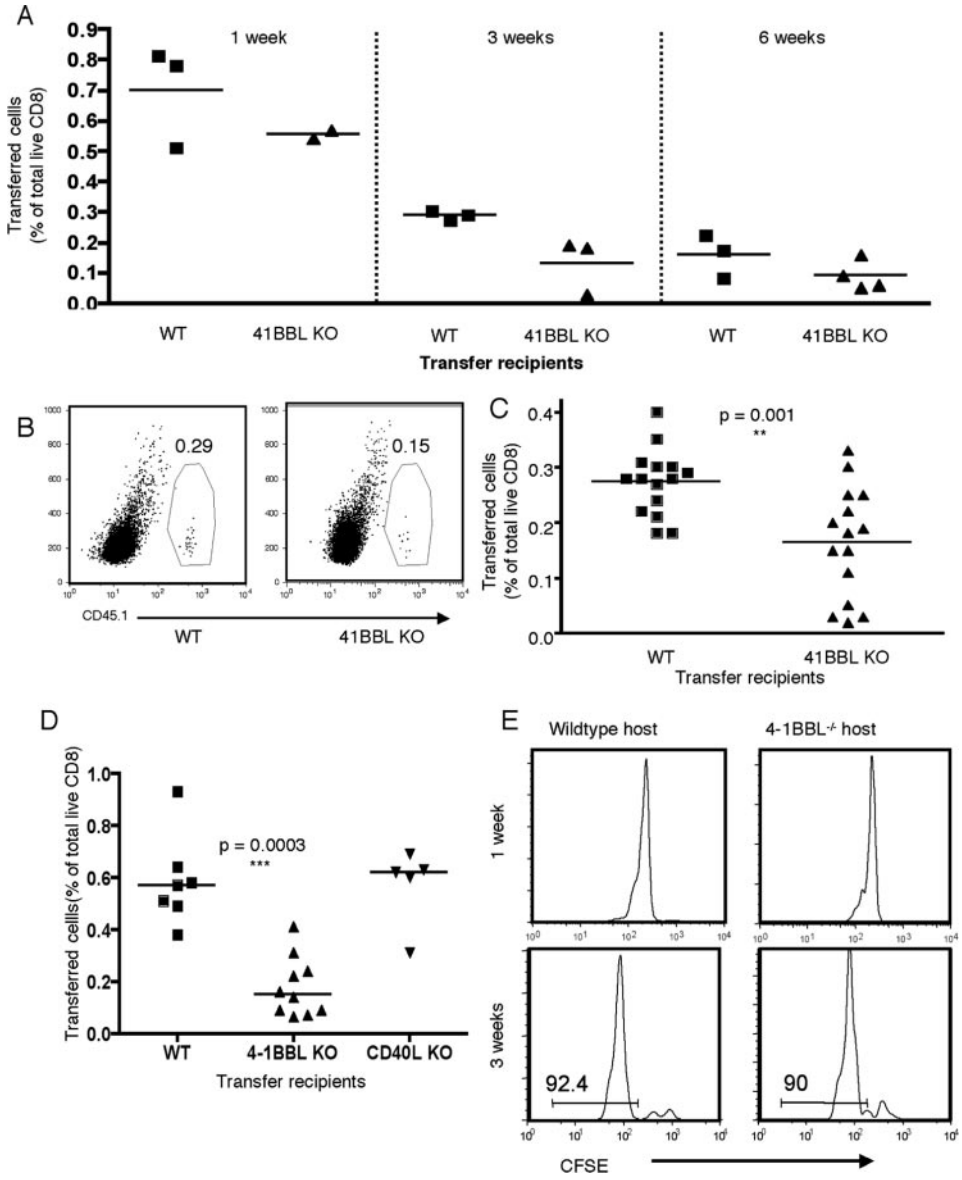

FIGURE 5. Survival of OT-I memory-like cells is reduced in 41BBL$^{-/-}$ mice compared with WT mice. CD45.1^{+} OT-I memory-like cells (1×10^6), as generated in Fig. 4, were transferred i.v. into congenically distinct (CD45.2^{+}) WT or 4-1BBL$^{-/-}$ hosts. *A*, Transferred cells were recovered from mice spleens at 1, 3, and 6 wk posttransfer. One- and 6-wk time points are representative of two separate experiments with at least three mice per group. *B*, Representative FACS plots showing population of CD45.1^{+}-transferred cells in WT and 4-1BBL$^{-/-}$ hosts. *C*, Summary of four experiments at 3 wk posttransfer. *D*, A total of 3×10^6 OT-1 memory cells was transferred into WT, 4-1BBL$^{-/-}$, and CD40L$^{-/-}$ hosts, and splenic cells were stained for CD45.1^{+}-transferred cells 3 wk posttransfer. Data are pooled from two separate experiments. *E*, OT-I memory-like cells were stained with CFSE before transfer and analyzed at 1 and 3 wk posttransfer. All plots are gated on live CD8^{+} cells and expressed as a percentage of CD8^{+} cells. CFSE staining was gated on CD8^{+}CD45.1^{+} cells.

4-1BBL$^{-/-}$ mice, analyzed over three experiments, showed a significant defect in the proportion of CD8^{+} memory phenotype cells in the bone marrow (Fig. 7*B*). In contrast, no such defect was seen in the CD44^{high}CD4^{+} T cell population (Fig. 7*C*).

CD62L and IL-7Rα have been used as markers of central vs effector memory (29). However, the proportion of CD62L^{high} (~60%) and IL-7Rα-expressing memory CD8 T cells (~55%) was unaltered between WT and 4-1BBL$^{-/-}$ mice (data not shown). These data suggest that 4-1BB-4-1BBL interactions contribute to the homeostasis of normal memory CD8^{+} T cells.

Discussion

In this report, we show that IL-15 induces 4-1BB surface expression selectively on CD44^{high}CD8^{+} T cells (Fig. 1). 4-1BB induction by IL-15 was dependent on activation (Fig. 2) and used p38 and ERK signals (Fig. 3). When in vitro-generated memory OT-I T cells were transferred into naive WT or 4-1BBL-deficient hosts, the absence of 4-1BBL resulted in a 2- to 3-fold cell recovery defect by 3 wk posttransfer, without impacting cell division (Fig. 5). This decreased memory T cell recovery was observed in both the bone marrow and spleen of 4-1BBL-deficient mice (Figs. 5 and 7). Restimulation of T cells following transfer into 4-1BBL-deficient or WT mice revealed that the main effect of 4-1BBL was on maintenance of cell numbers rather than on effector function (Fig. 6). Furthermore, the absence of 4-1BBL in an unmanipulated mouse also resulted in a generalized defect in the number of CD44^{high}CD8^{+} memory T cells with no effect on the CD4^{+} memory T cell population (Fig. 7). These data demonstrate a role for 4-1BBL in maintenance of CD8^{+} T cells, independently of the persistence of Ag.

4-1BB is expressed on the CD44^{high} CD8^{+} memory T cell population and not on the CD44^{low} population following IL-15 treatment in the absence of Ag (Fig. 1). Because the starting population includes a mixed population of CD44^{high} and CD44^{low} cells, it was not clear whether IL-15 was acting on naive cells. A previous study indicated that IL-15 does not induce CD44 up-regulation on the CD44^{low} cells (30). Furthermore, in our experiments with bone marrow cells, one can see that the proportion of CD44^{high} cells does not change with IL-15 stimulation, rather the 4-1BB^{+} population appears to be derived from the CD44$^{int/high}$ population (Fig. 1*D*). These effects of IL-15 mainly on the memory phenotype

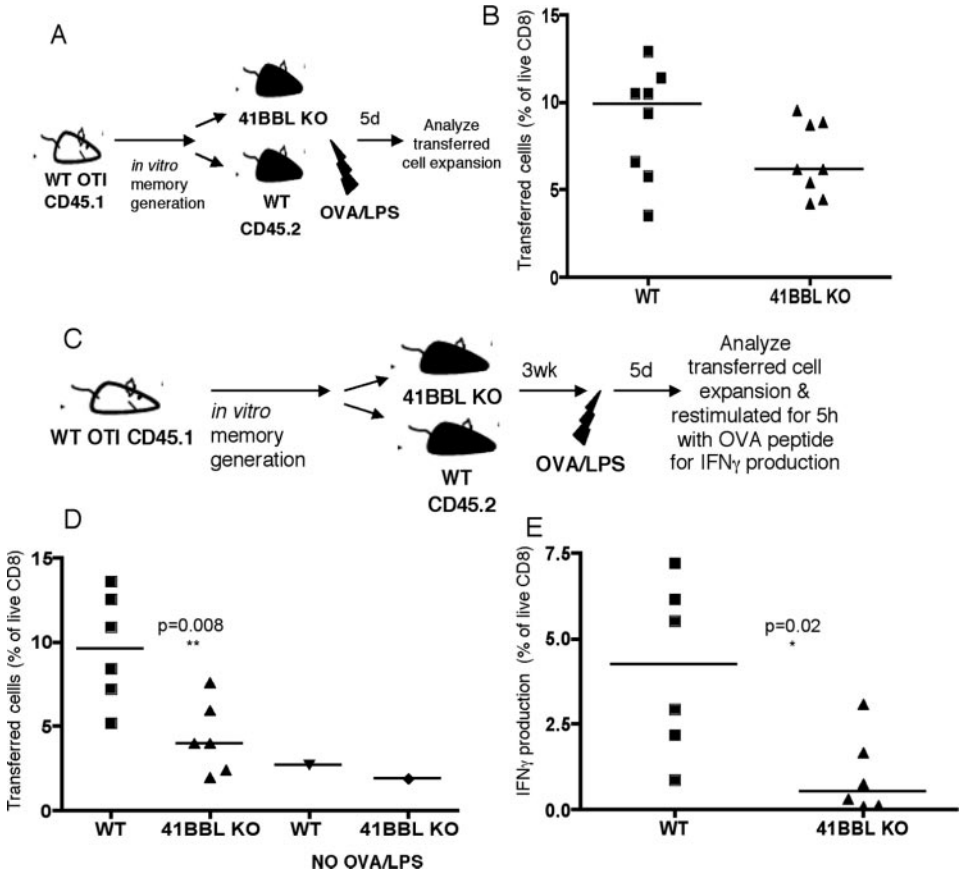

FIGURE 6. Response to Ag rechallenge in vivo is reduced in 4-1BBL$^{-/-}$ mice compared with WT mice. CD45.1$^+$ OT-I memory-like cells (3×10^6) were transferred i.v. into WT or 4-1BBL$^{-/-}$ hosts as described in Fig. 5 and were rechallenged with 2 mg of OVA Ag and 50 μg of LPS s.c. *A*, Schematic representation of rechallenge immediately (24 h) after transfer. *B*, Donor cell expansion at peak of response (day 5) as measured by CD45.1 CD8$^+$ staining. These data are pooled from two separate experiments. *C*, Schematic representation of rechallenge 3 wk posttransfer. *D*, Donor cell expansion as well as controls without OVA/LPS challenge. *E*, IFN-γ production after 5-h restimulation with 1 μM SIINFEKL and Golgi Stop. These data are representative of two separate experiments. All plots are gated on live CD8$^+$ cells and expressed as a percentage of CD8$^+$ cells.

cells could be due to higher expression of IL-15Rα on memory vs naive CD8$^+$ T cells (20). Human umbilical cord blood T cells, a source of naive CD8$^+$ T cells, show expression of 4-1BB when treated with a combination of IL-12 and IL-15 (31). In that study, the effects of IL-15 were not tested in isolation, suggesting that IL-12 functioned in cooperation with IL-15 to induce 4-1BB on the naive T cells.

During Ag-specific T cell activation in vivo, 4-1BB can be induced on both CD4$^+$ and CD8$^+$ T cells (15). However, 4-1BB influences CD8$^+$ but not CD4$^+$ T cell recall responses to viruses (8, 32). The differential effects of IL-15 on CD8$^+$ vs CD4$^+$ T cells with respect to 4-1BB induction are consistent with a preferential role for 4-1BB in maintenance of CD8$^+$ rather than CD4$^+$ memory T cell responses.

Cell activation may be essential for 4-1BB induction, as IL-15 treatment resulted in coexpression of CD69 and 4-1BB, whereas IL-7 failed to activate cells and did not induce 4-1BB on CD8$^+$ or CD4$^+$ T cells (Figs. 1 and 2). The finding that IL-7 was very effective at increasing cell survival without affecting proliferation (Fig. 2), agrees with a previous report showing that IL-7 acts mainly as a survival factor for memory CD8$^+$ T cells in vivo (33). The coexpression of 4-1BB and CD69 is also observed during primary activation of OT-I T cells with Ag in vivo (15). Microarray analysis shows remarkably similar gene expression and effector function between IL-15 and anti-CD3 stimulation (34), suggesting that 4-1BB expression may be a general consequence of T cell activation via either Ag or IL-15.

Why would a memory CD8$^+$ T cell require a 4-1BB/4-1BBL-induced survival signal, if it had already received a cytokine-mediated signal? Because IL-7 is critical for survival of naive T cells (35), it is possible that competition for this cytokine limits its availability to memory T cells, whereas IL-15 alone may not induce sufficient survival signals. Our data suggest that the induction of 4-1BB by IL-15 signaling may allow a feedback survival loop, to enhance or sustain survival signaling in memory T cells.

Inhibition of various IL-15R signaling pathways identified that the MAPK pathways ERK and p38 are required for IL-15-mediated 4-1BB expression (Fig. 3). ERK and its target transcription factor c-Jun have been shown to be involved in TCR stimulation-dependent transcriptional regulation of the 4-1BB promoter (36). It is also of interest that IL-7 does not appear to activate ERK in T cells (37), which may explain the differential effects of these two cytokines on Ag-independent 4-1BB induction.

The magnitude of the defect in memory T cell survival in this study (Fig. 5) is similar to the defect observed in the spleen in the maintenance of influenza-specific CD8$^+$ T cells at 3 wk postinfection of 4-1BBL-deficient mice (7). This defect was significant enough to confer a substantial decrease in the number of IFN-γ-producing cells in response to Ag rechallenge (Fig. 6). The effects of 4-1BBL during the secondary response to Ag are quite small (Fig. 6*B*), thus the majority of the recall response defect appears to be due to the lack of maintenance of the memory CD8 T cells in the weeks posttransfer (Fig. 6*D*).

4-1BBL only confers a 2- to 3-fold effect on maintenance of memory CD8$^+$ T cells over 3 wk, thus, it is possible that other TNFR family members also contribute to memory CD8$^+$ T cell survival. Our laboratory recently showed in a similar adoptive transfer model, that OT-I T cells lacking TRAF1, an adaptor molecule downstream of several TNFR family members, have approximately a 7- to 8-fold survival defect in the absence of Ag over 3 wk (C. Srokowski, G. Pulle, L. Snell, L. Sabbagh, E. Tsitsikov, and T. H. Watts, submitted for publication). Therefore, 4-1BBL may represent only one of several molecules regulating memory

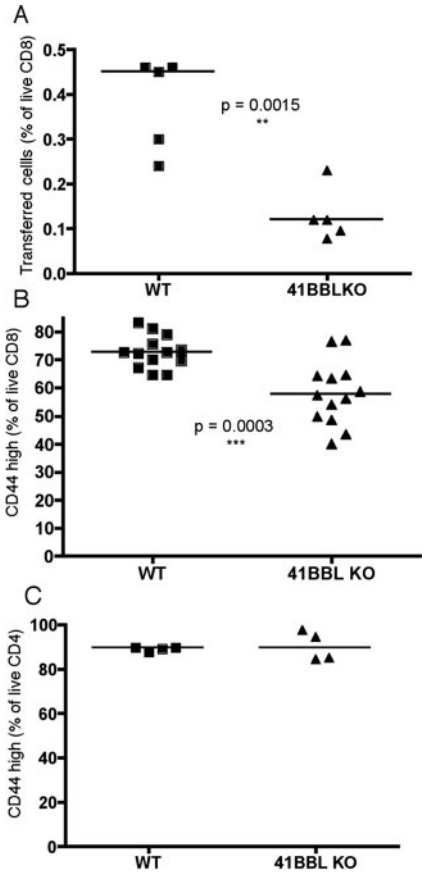

FIGURE 7. Maintenance of memory $CD8^+$ T cells is impaired in bone marrow of 4-$1BBL^{-/-}$ mice. Femur bone marrow was isolated from 11- to 14-wk-old WT and 4-$1BBL^{-/-}$ mice. *A*, Transferred OT-I memory-like cells 3 wk posttransfer. These data are pooled from two separate experiments. *B*, Memory phenotype $CD8^+$ T cells in bone marrow of naive, unimmunized mice. These data are pooled from three separate experiments. *C*, Memory phenotype $CD4^+$ T cells in bone marrow of naive, unimmunized mice.

$CD8^+$ T cell survival. Because TRAF1 associates with the cytoplasmic tail of 4-1BB (38) and has been shown to play a role in survival signaling (39–41), it is likely that TRAF1 plays a role in the 4-1BB-mediated survival signaling. Although we have not identified the 4-1BBL-induced signals leading to memory CD8 T cell survival, based on the literature, it is likely that the TRAF-dependent induction of NF-κB which in turn leads to up-regulation of Bcl-x_L and Bfl-1 in response to 4-1BB signaling (13) contributes to the memory CD8 T cell survival.

Memory $CD8^+$ T cells can persist indefinitely in MHC-I-deficient mice (42). Maintenance of these cells is instead supported by IL-15 (43), but the mechanism by which IL-15 supports $CD8^+$ T cells has been unclear. The present report suggests that IL-15 may promote Ag-independent memory $CD8^+$ T cell maintenance at least in part through 4-1BB. This likely affects survival rather than homeostatic proliferation, as CFSE staining of transferred cells showed minimal and similar division in 4-$1BBL^{-/-}$ vs WT hosts. In our study, the transferred T cells did not persist indefinitely, and, in fact, very few cells were left after 6 wk. It may be that too few cells were transferred to establish a strong memory pool. Alternatively, although the T cells exhibit the CD69, CD44, and CD62L phenotype associated with central memory cells, the short in vitro culture may be insufficient for full development into long-lived memory cells (30). Indeed, the phenotype of in vitro-generated memory cells is similar but not identical to persistent memory cells that develop in vivo in response to viral infection (44, 45). An additional consideration is that the present studies were done in the absence of $CD4^+$ T cell help, as T cells were stimulated with only a $CD8^+$ epitope peptide, and then transferred into naive mice. As $CD4^+$ help has been shown to contribute to the maintenance of $CD8^+$ memory T cells (46), direct effects of 4-1BBL and other TNF family ligands on $CD8^+$ T cells, may be insufficient for long-lived memory in the absence of $CD4^+$ help.

The bone marrow has been reported to be a reservoir and site of homeostatic proliferation and activation for memory $CD8^+$ cells (23, 24, 47). Many cell types express IL-15 mRNA (48), but the protein is difficult to detect, possibly due to poor translation, short half-life, and sequestration by the IL15Rα (49). Thus, it is not clear what levels of IL-15 are present in vivo. Transpresentation of IL-15 by bone marrow-derived cells requires that IL-15 is produced by the same cell that expresses its high-affinity receptor IL-15Rα (50). Thus, the receptor is the limiting factor, and cells expressing IL-15Rα may trap its cytokine, forming IL-15-rich niches in the bone marrow. Although there was no evidence of extensive expression of 4-1BB on memory $CD8^+$ T cells in the bone marrow, addition of IL-15 to the bone marrow cells clearly induced 4-1BB on the memory population (Fig. 1). The failure to find a constitutive population of 4-1BB in the bone marrow may be due to the transient nature of its expression in vivo (10, 15).

If 4-1BBL is indeed important in memory T cell survival, then a prediction of our model was that there should be a generalized defect in the number of memory cells in 4-1BBL-deficient mice. Examination of the bone marrow of unmanipulated 4-1BBL-deficient mice supports this hypothesis and demonstrates that the effect is specific to the memory $CD8^+$ and not the $CD4^+$ T cell subset (Fig. 7). In contrast, we did not observe a statistically significant defect in memory $CD8^+$ T cell numbers in the spleen of unmanipulated mice (data not shown). Because the bone marrow provides an enriched source of memory cells, it may be that it is easier to detect this subtle defect in the bone marrow compartment. Furthermore, it is likely that compensatory homeostatic effects may serve to minimize the overall defect in 4-1BBL-deficient mice.

Defects in memory $CD8^+$ T cell recovery in spleen and bone marrow in 4-1BBL, but not CD40L-deficient, hosts clearly indicates that 4-1BBL plays a role in Ag-independent T cell survival. However, the location of the 4-1BBL in the unimmunized mouse remains unknown. Although 4-1BBL has been reported on APC following activation with LPS or anti-CD40 (51–55), it has been difficult to detect on in vivo-activated APC following influenza or lymphocytic choriomeningitis virus infection (M. A. DeBenedette and T. H. Watts, unpublished results). To test whether IL-15 might induce 4-1BBL as well as its receptor, we added IL-15 to collagenase-treated splenocytes and bone marrow-derived immature dendritic cells, but 4-1BBL was not detected (data not shown). We also analyzed isolated bone marrow cells by flow cytometry and again detected no 4-1BBL either with or without IL-15 stimulation (data not shown). Thus, it is possible that 4-1BBL is expressed on a small subset of cells or that its induction depends on the appropriate cellular environment or architecture. 4-1BBL could be expressed on mesenchymal cells, another source of IL-15, but these cells are very difficult to analyze by flow cytometry. It is also possible that 4-1BBL is constitutively present on a rare cell type, perhaps in the bone marrow, that interacts with $CD8^+$ memory T cells that have been activated by IL-15. Further resolution of this issue will likely require more sensitive analysis such as enhanced GFP reporter mice for 4-1BBL.

An analogous model by Lane and colleagues (56, 57) describes the impact of TNFR family members on the survival of $CD4^+$ memory T cells. TCR transgenic OT-II $CD4^+$ T cells were found

to interact with a $CD4^+CD3^-OX40L^+CD30L^+$ accessory cell, thought to be the adult equivalent of the lymphoid inducer cell. This interaction correlated with the increased survival of OX40 and CD30-expressing $CD4^+$ T cells several weeks following immunization of mice (57, 58). Furthermore, OX40 expression on memory $CD4^+$ T cells was induced by IL-7 treatment (57). Thus, OX40 induction by IL-7 is important for sustaining $CD4^+$ T cell memory, and 4-1BB induction by IL-15 is important for sustaining $CD8^+$ T cell memory cell survival.

In summary, our data support a role for 4-1BBL in the Ag-independent survival of $CD8^+$ T cells weeks after transfer into naive hosts. The data suggest a model whereby memory $CD8^+$ T cells, perhaps in the bone marrow, encounter an IL-15-rich niche that induces expression of 4-1BB. Encounter with 4-1BBL then allows the T cells to obtain additional survival signals through 4-1BB/4-1BBL interaction.

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Disclosures

The authors have no financial conflict of interest.

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