

Naive CD8⁺ T cells differentiate into protective memory-like cells after IL-2–anti-IL-2 complex treatment in vivo

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An optimal CD8⁺ T cell response requires signals from the T cell receptor (TCR), co-stimulatory molecules, and cytokines. In most cases, the relative contribution of these signals to CD8⁺ T cell proliferation, accumulation, effector function, and differentiation to memory is unknown. Recent work (Boyman, O., M. Kovar, M.P. Rubinstein, C.D. Surh, and J. Sprent. 2006. *Science*. 311:1924–1927; Kamimura, D., Y. Sawa, M. Sato, E. Agung, T. Hirano, and M. Murakami. 2006. *J. Immunol.* 177:306–314) has shown that anti-interleukin (IL) 2 monoclonal antibodies that are neutralizing in vitro enhance the potency of IL-2 in vivo. We investigated the role of IL-2 signals in driving CD8⁺ T cell proliferation in the absence of TCR stimulation by foreign antigen. IL-2 signals induced rapid activation of signal transducer and activator of transcription 5 in all CD8⁺ T cells, both naive and memory phenotype, and promoted the differentiation of naive CD8⁺ T cells into effector cells. IL-2–anti-IL-2 complexes induced proliferation of naive CD8⁺ T cells in an environment with limited access to self-major histocompatibility complex (MHC) and when competition for self-MHC ligands was severe. After transfer into wild-type animals, IL-2–activated CD8⁺ T cells attained and maintained a central memory phenotype and protected against lethal bacterial infection. IL-2–anti-IL-2 complex–driven memory-like CD8⁺ T cells had incomplete cellular fitness compared with antigen–driven memory cells regarding homeostatic turnover and cytokine production. These results suggest that intense IL-2 signals, with limited contribution from the TCR, program the differentiation of protective memory-like CD8⁺ cells but are insufficient to guarantee overall cellular fitness.

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Abbreviations used: C57BL/6, B6; HP, homeostatic proliferation; LM-OVA, *Listeria monocytogenes*–expressing soluble OVA.

The CD8⁺ T cell response to an acute systemic infection entails vigorous expansion of antigen-specific cells, followed by a contraction phase in which 90–95% of the cells die by apoptosis. The cells remaining after the contraction phase become memory T cells, which possess properties distinct from the naive population, such as rapid acquisition of effector function on reencounter with pathogen. Accumulating evidence suggests that naive CD8⁺ T cells are programmed to become memory cells in the early phase of the CD8⁺ T cell response, when appropriate signals from the TCR, co-stimulatory molecules, and cytokines associated with inflammation are thought to be required (1, 2).

IL-2, which is produced by activated T cells, is one of the cytokines involved in the CD8⁺ T cell response. IL-2 induces intracellular signals through the IL-2 receptor complex, consisting of CD25 (IL-2R α), CD122 (IL-2/IL-15R β),

and CD132 (common γ chain). Stimulation of the receptor complex by IL-2 induces several signal transduction pathways, including the activation of STAT5 (3). Recent studies have demonstrated that IL-2 supports the maintenance of Foxp3⁺ CD4⁺ regulatory T cells, which bear the IL-2R α chain (CD25) (4–6). The requirement for IL-2 signals to maintain regulatory T cells limits the use of cytokine or cytokine receptor knockout mice to study other in vivo roles of IL-2. Instead, the effect of IL-2 signals on CD8⁺ T cells during an immune response has been investigated by creating situations in which CD25, CD122, or IL-2 are selectively deficient in CD8⁺ T cells. These studies revealed a modest role for IL-2 in the primary expansion and differentiation of CTL (7–12), whereas IL-2 appears to support expansion of primary CTL in nonlymphoid organs (13). Very recently, another role for IL-2 signals in antigen-stimulated CD8⁺ T cells was revealed using mixed BM chimeric mice containing both wild-type

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and CD25^{-/-} cells. In these mice a complete compartment of regulatory T cells is reconstituted, and the mice remain healthy (11, 12). Upon acute infection, effector and memory CD8⁺ T cells lacking CD25 were generated and normally maintained, but their secondary expansion after pathogen rechallenge was severely compromised compared with that of CD25-sufficient memory cells (11, 12). Intriguingly, replenishment of IL-2 signals to CD25^{-/-} CD8⁺ T cells during the primary infection, but not during the secondary challenge, restored their ability to expand in a recall response (11). This result clearly indicates a programming effect of IL-2 signaling during the primary response in driving the complete differentiation of memory CD8⁺ T cells.

Recent data suggest that the anti-IL-2 mAb S4B6, which has been widely used as a neutralizing antibody *in vitro*, enhances the bioactivity of IL-2 *in vivo* (14, 15). Administration of rIL-2 mixed with the anti-IL-2 mAb (IL-2-anti-IL-2 complexes) or the concurrent treatment with plasmid DNA expressing mouse IL-2 and the antibody substantially and preferentially increased the proliferation of CD44^{hi} CD122^{hi} memory-phenotype CD8⁺ T cells and NK cells (14, 15). Injection of anti-IL-2 mAb alone had a similar effect because of the capture of endogenously secreted IL-2 by the mAb, although the efficacy of this treatment is much weaker than the cotreatment with IL-2-anti-IL-2 complexes (14, 15). The precise mechanism of the enhanced potency of the immune complex remains unclear, although the presentation of IL-2-anti-IL-2 complexes via the Fc portion of the mAb has been suggested (14).

We report that the administration of IL-2-anti-IL-2 complexes stimulated all CD8⁺ T cells, both naive and memory phenotype, *in vivo*. The naive CD8⁺ T cells proliferated, became effector cells, and differentiated to memory-phenotype cells capable of providing protection against pathogen challenge. Remarkably, proliferation of naive CD8⁺ T cells by treatment with IL-2-anti-IL-2 complexes was induced in the absence of foreign antigen and when competition for self-MHC class I ligands was intense. Because most experimental systems used for the study of CD8⁺ T cell responses are associated with strong TCR stimulation, our approach using IL-2-anti-IL-2 complexes allowed us to dissect and examine the relative contribution of IL-2 and antigen signals to CD8⁺ T cell responses, revealing that intense IL-2 signals coupled with weak TCR ligation have the potential to program the differentiation of protective memory-like CD8⁺ T cells *in vivo*.

RESULTS

IL-2-anti-IL-2 complexes activate STAT5 in all CD8⁺ T cells CD44^{hi} CD122^{hi} memory-phenotype CD8⁺ T cells and NK cells express high levels of CD122 (IL-2/IL-15R β), providing an explanation for the preferential expansion of these populations after treatment with complexes of rIL-2 and anti-IL-2 mAb (IL-2-anti-IL-2 complexes) (14, 15). However, CD44^{lo} naive CD8⁺ T cells also express detectable levels of CD122, intermediate between those of memory-phenotype CD8⁺ T cells and naive CD4⁺ T cells (unpublished data) (16).

In addition, after treatment with IL-2-anti-IL-2 complexes, almost the entire CD8⁺ population adopts a CD44^{hi} CD122^{hi} phenotype (unpublished data) (14). We therefore asked whether CD44^{lo} naive CD8⁺ T cells could respond to IL-2-anti-IL-2 complexes injected *in vivo*. IL-2 signal transduction is known to involve the activation of STAT5, which is important for the biological activity of IL-2 (3, 4). Therefore, we used the phosphorylation status of STAT5 as a readout of induction of an IL-2 signal *in vivo*. C57BL/6 (B6) mice were given a single injection of IL-2-anti-IL-2 complexes, and at the indicated time points, splenocytes were fixed without restimulation *in vitro*, and activated (tyrosine-phosphorylated) STAT5 was detected by flow cytometry. As shown in Fig. 1, CD44^{hi} memory-phenotype CD8⁺ T cells responded rapidly after the treatment: the phosphorylation of STAT5 was induced in ~50% of the population at 15 min and increased to >90% by 60 min. Surprisingly, the rapid activation of STAT5 was also observed in CD44^{lo} naive CD8⁺ T cells with slightly delayed kinetics. At 60 min, >80% of the CD44^{lo} naive CD8⁺ population contained the activated form of STAT5. NK cells (NK1.1⁺ TCR β ^{NEG}) also showed STAT5 activation, whereas B cells (B220⁺ TCR β ^{NEG}) showed no detectable activation of STAT5 at 60 min (unpublished data). The rapid activation of STAT5 suggests that CD44^{lo} naive CD8⁺ T cells, as well as memory-phenotype CD8⁺ T cells, receive signals directly from IL-2-anti-IL-2 complexes.

IL-2-anti-IL-2 complexes induce proliferation of naive CD8⁺ T cells

These results prompted us to examine the physiological consequence of treating naive CD8⁺ T cells with IL-2 signals *in vivo*. Congenically marked CD44^{lo} CD122^{lo} naive OT-I cells were isolated, labeled with CFSE, and transferred into nonirradiated syngeneic B6 mice (Fig. S1 A, available at <http://www.jem.org/cgi/content/full/jem.20070543/DC1>). After treatment of the host animals with IL-2-anti-IL-2 complexes, the naive OT-I cells divided extensively (Fig. 2 A) and accumulated (Fig. 2 B) in the absence of any other stimulation. The expansion of naive OT-I cells was almost equivalent to that of the host CD8⁺ T cell population (Fig. 2 B). Surface markers associated with activation/memory, including

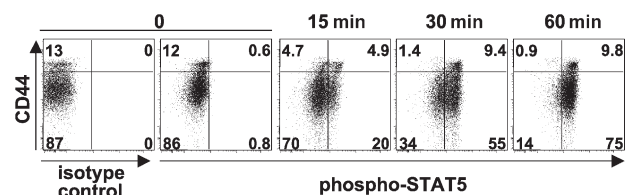


Figure 1. IL-2-anti-IL-2 complex treatment induces rapid activation of STAT5 in naive CD8⁺ T cells. Mice were given a single injection of IL-2-anti-IL-2 complexes. At the indicated time points, splenocytes were fixed and permeabilized for intracellular staining of the tyrosine-phosphorylated form of STAT5. Mice for the zero time point received no injection. The data are gated on the CD8⁺ population, and the numbers represent the percentage of gated cells in each quadrant.

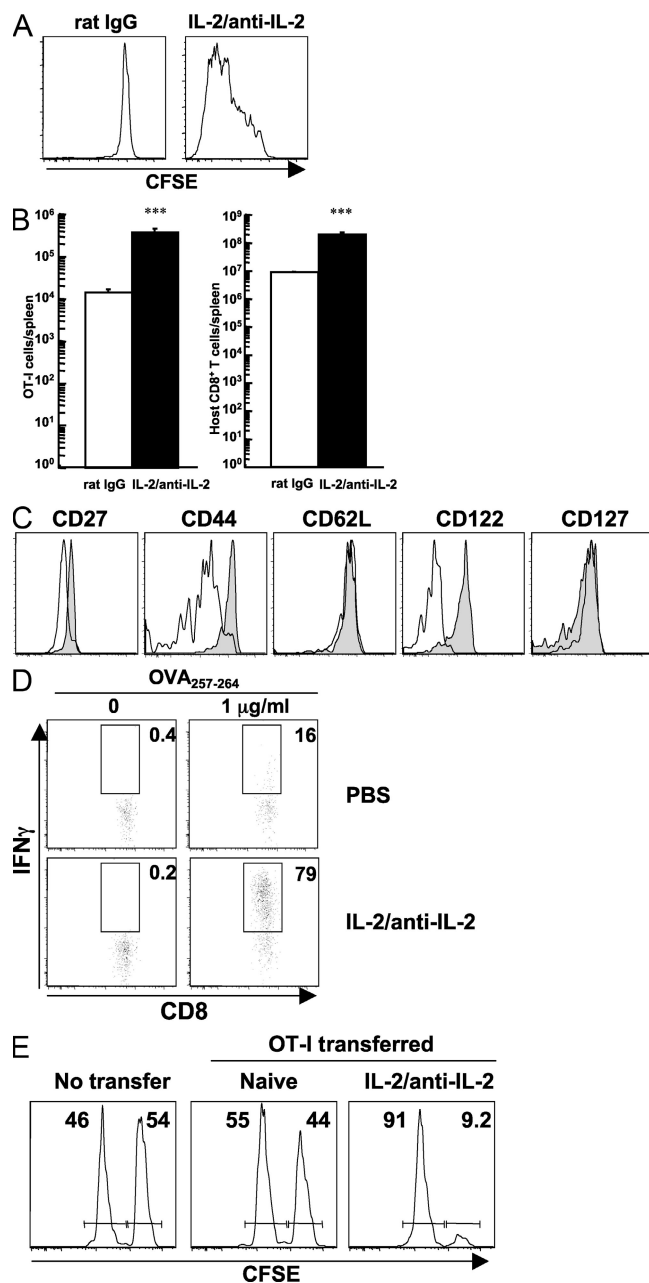


Figure 2. IL-2-anti-IL-2 complexes induce proliferation of naive antigen-specific CD8⁺ T cells. 0.5×10^6 purified CD44^{lo} CD122^{lo} naive OT-I cells were transferred into nonirradiated mice on day -1. The mice were given IL-2-anti-IL-2 complexes or control (rat IgG) on days 0 and 2, and the assays were performed on day 6. (A) Proliferation of donor cells was evaluated by CFSE dilution. (B) Absolute number of donor cells (left) and host CD8⁺ T cells (right) in the spleen. The data represent the mean + SEM ($n = 3$). ***, $P < 0.001$ versus rat IgG treatment. (C) Phenotypic changes of the donor cells. Rat IgG and IL-2-anti-IL-2 complex treatment are indicated with open and shaded histograms, respectively. (D) IFN- γ production by donor OT-I cells was assessed after 4 h of stimulation in vitro. Numbers represent the percentage of gated OT-I cells making IFN- γ . (E) Equal numbers (1.1×10^6 cells) of naive OT-I cells and IL-2-anti-IL-2 complex-activated OT-I cells were transferred to naive animals. An in vivo CTL assay with OVA peptide-pulsed (CFSE^{hi}) and control (CFSE^{lo}) targets was performed the next day. The killing activity was examined 20 h later.

CD27, CD44, and CD122, were up-regulated on OT-I cells that received IL-2 signals, whereas CD62L and CD127 levels remained unchanged as compared with OT-I cells in animals receiving control injections (Fig. 2 C). This phenotype resembles a central memory phenotype (17). IL-2-anti-IL-2 complex-mediated proliferation in a lympho-replete environment was not unique to OT-I cells or mediated by a second TCR possibly expressed on TCR-transgenic cells, because other naive TCR-transgenic CD8⁺ T cells (from P14 or V β 5 TCR-transgenic mice) and OT-I cells on a RAG-1^{-/-} background also proliferated (Fig. S2). In contrast, proliferation of naive polyclonal CD8⁺ T cells was not as vigorous (unpublished data), as previously reported (14). Functionally, OT-I cells that had received IL-2 signals were able to produce IFN- γ after a 4-h stimulation with their cognate peptide in vitro (Fig. 2 D) and killed target cells in an antigen-specific manner in vivo (Fig. 2 E), both of which are typical characteristics of effector cells. These results indicate that signals provided by IL-2-anti-IL-2 complexes induce the proliferation and activation of naive antigen-specific CD8⁺ T cells in nonlymphopenic environments without cognate antigen stimulation.

Fc γ receptors are dispensable for the action of IL-2-anti-IL-2 complexes

Why IL-2-anti-IL-2 complexes are so potent in vivo is still unclear. One possible mechanism that has been proposed is the presentation of IL-2-anti-IL-2 complexes by Fc γ receptor-bearing cells to cells expressing CD122/CD132 (14). Thus, IL-2 complexed to F(ab')₂ fragments of the anti-IL-2 mAb had only a modest effect in vivo (14). To investigate the role of Fc γ receptors, mice deficient in both FcR γ chain and Fc γ RII (FcR γ ^{-/-}Fc γ RII^{-/-}) were used. Because the FcR γ chain is necessary for surface expression of the activating IgG Fc receptors, including Fc γ RI, III, and IV, whereas the inhibitory receptor Fc γ RII does not share the FcR γ chain (18, 19), FcR γ ^{-/-}Fc γ RII^{-/-} mice should be devoid of known functional Fc γ receptors on hematopoietic cells. In fact, Fc γ RII and Fc γ RIII expression was undetectable on B cells from FcR γ ^{-/-}Fc γ RII^{-/-} mice by staining with anti-CD16/32 (Fc γ RIII/II) mAb (Fig. S3 A, available at <http://www.jem.org/cgi/content/full/jem.20070543/DC1>). In addition, anti-CD3-induced cytokine release in vivo, which requires presentation and cross-linking via Fc γ receptors (20, 21), was severely impaired in FcR γ ^{-/-}Fc γ RII^{-/-} mice, indicating a gross functional defect of Fc γ receptors in these mice (Fig. S3 B). Because FcR γ ^{-/-}Fc γ RII^{-/-} mice are on a mixed genetic background, we tested the efficacy of IL-2-anti-IL-2 complexes by sampling PBLs before and after treatment rather than using an adoptive transfer system. Both control and FcR γ ^{-/-}Fc γ RII^{-/-} mice displayed a similar small population of CD44^{hi} CD122^{hi} memory-phenotype CD8⁺ T cells before treatment (Fig. 3 A). By day 5 after treatment with

Numbers represent the percentage of CFSE^{hi} and CFSE^{lo} targets remaining. The donor cells (A–D) and target cells (E) were distinguished from host populations by congenic markers.

IL-2–anti-IL-2 complexes, CD44^{hi} CD122^{hi} memory-phenotype CD8⁺ T cells increased dramatically in control and FcR γ ^{-/-}Fc γ RII^{-/-} mice (Fig. 3 A). Total numbers of PBLs increased significantly after treatment in both strains and correlated with vigorous expansion of CD8⁺ T cells and NK cells (Fig. 3 B). These results suggest that Fc γ receptor-mediated presentation is dispensable for the action of IL-2–anti-IL-2 complexes in vivo.

IL-2–anti-IL-2 complexes stimulate naive CD8⁺ T cells with limited TCR stimulation by self-ligands

IL-2 signals induced the proliferation of naive TCR-transgenic CD8⁺ T cells in a complete lymphoid compartment and in the absence of cognate antigen stimulation (Fig. 2 A and Fig. S2). However, it was possible that TCR stimulation by self-ligands had an important role, as is the case for homeostatic proliferation (HP) of naive CD8⁺ T cells in a lymphopenic environment (22–24). To explore the dependency on MHC class I ligands, we chose MHC class I K^b^{-/-}D^b^{-/-} mice as host animals, because the homeostasis of IgG is impaired in β_2 microglobulin-deficient mice (25), which might affect the half-life of IL-2–anti-IL-2 complexes. Because CD8⁺ T cells transferred into MHC class Ia-deficient mice were almost completely rejected after IL-2–anti-IL-2 complex treatment (unpublished data), MHC class Ia-deficient mice were irradiated with 10 Gy and transplanted with syngeneic BM cells to prevent rejection. Irradiated, BM-reconstituted mice were injected with CFSE-labeled naive OT-I cells and IL-2–anti-IL-2 complexes. As controls, B6 mice were also irradiated and

reconstituted with syngeneic BM. As shown in Fig. 4 A, naive OT-I cells underwent three to four divisions in lethally irradiated WT hosts after rat IgG treatment, because of lymphopenia-induced HP. Proliferation was delayed in MHC class Ia-deficient hosts, confirming that HP of naive CD8⁺ T cells is MHC class I-dependent (22–24). In contrast, IL-2 signals provided by injected IL-2–anti-IL-2 complexes stimulated extensive division of naive OT-I cells in the MHC class Ia-deficient and –sufficient host animals. The recovery of donor OT-I cells in the spleen after the complex treatment was also similar in both groups (Fig. 4 B).

These results suggest a minimal requirement for TCR ligation by self-MHC for the proliferation of naive CD8⁺ T cells stimulated by IL-2–anti-IL-2 complexes, but it does not rule out the possibility of stimulation by MHC class I molecules on the donor cells themselves or by irradiation-induced cytokines. In this regard, a recent report has strongly suggested the requirement for TCR recognition of self-MHC ligands to observe the accumulation of IL-2-driven CD8⁺ cells (26). We took a second approach to examine the dependency on TCR stimulation based on the finding that adoptively transferred TCR-transgenic CD8⁺ T cells do not undergo HP in hosts with the identical TCR clonotype because of clonal competition for MHC class I/peptide ligands (27, 28). Accordingly, naive OT-I cells were labeled with CFSE and transferred into nonirradiated OT-I/RAG-1^{-/-} hosts. As shown in Fig. 4 C, signals provided by IL-2–anti-IL-2 complexes induced several divisions of naive OT-I cells even in this environment, where competition for self-MHC ligands is intense.

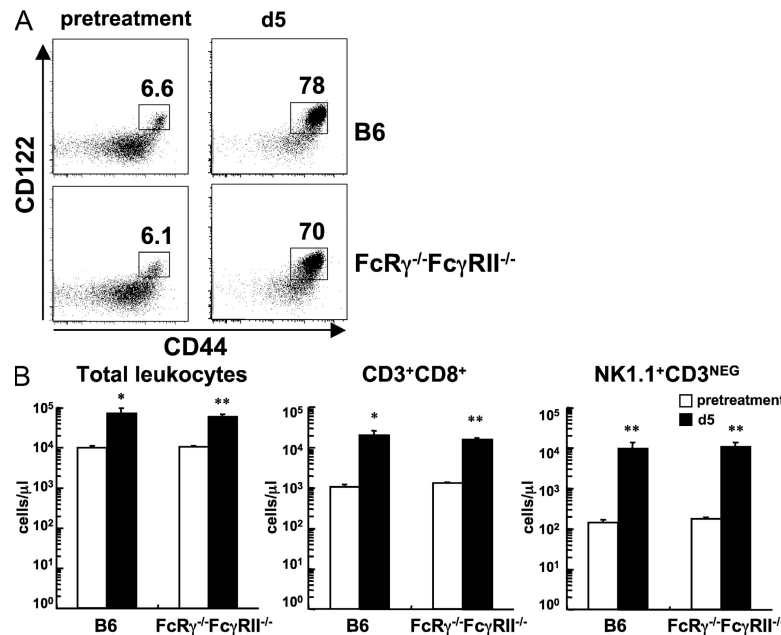


Figure 3. Fc γ receptors are dispensable for the action of IL-2–anti-IL-2 complexes. B6 mice and FcR γ ^{-/-}Fc γ RII^{-/-} mice were treated with IL-2–anti-IL-2 complexes on days 0 and 2. The mice were bled before starting the treatment (pretreatment) and on day 5. (A) Phenotype of the CD8⁺ T cell population of the same animal in each group is shown. The data are gated on CD3⁺ CD8⁺ cells. Numbers represent the percentage of gated cells within the box. (B) Absolute numbers of total leukocytes, CD8⁺ T cells (CD3⁺ CD8⁺), and NK cells (NK1.1⁺ CD3^{NEG}) in the peripheral blood before and after (day 5) the treatment. Each column represents the mean + SEM ($n = 3$). *, $P < 0.05$; or **, $P < 0.01$ versus pretreatment.

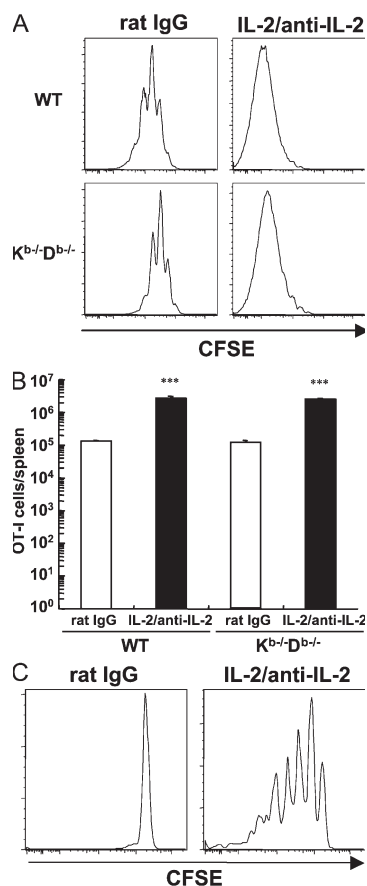


Figure 4. IL-2-anti-IL-2 complexes stimulate naive CD8⁺ T cells with limited TCR stimulation. Host mice that received CFSE-labeled naive OT-I cells 1 d earlier were treated with IL-2-anti-IL-2 complexes on days 0 and 2. CFSE levels of donor cells from the spleen were examined on day 6. The data are gated on CD3⁺ CD8⁺ congenically marked donor cells. (A) WT and MHC class I K^{b-/-}D^{b-/-} mice that were irradiated with 10 Gy and transplanted with syngeneic BM cells were used as host animals. (B) Absolute number of OT-I cells recovered from the spleen of animals in A is shown. The data represent the mean + SEM ($n = 2$). ***, $P < 0.001$ versus rat IgG treatment. (C) Nonirradiated OT-I/RAG-1^{-/-} mice were used as hosts.

This result suggested that robust expansion of naive CD8⁺ T cells in response to potent IL-2 signals requires only minimal TCR stimulation.

IL-2-anti-IL-2 complexes generate memory cells with a central memory phenotype

We next addressed whether naive CD8⁺ T cells activated by IL-2-anti-IL-2 complexes could differentiate to memory cells. OT-I mice were injected with IL-2-anti-IL-2 complexes, resulting in the conversion of almost all the CD8⁺ T cells to a CD44^{hi} CD122^{hi} activated phenotype (Fig. S1 B). 15×10^6 IL-2-activated OT-I cells were then transferred at day 12 to normal B6 hosts. At 1 d after transfer, the OT-I cells exhibited rapid IFN- γ production in response to peptide stimulation in vitro (unpublished data). For comparison, conventional, antigen-driven OT-I cells were generated by transfer of 10^4 naive

OT-I cells into B6 recipients, followed by immunization with *Listeria monocytogenes*—expressing soluble OVA (LM-OVA; Fig. S1 C). More than 75 d after transfer of the IL-2-anti-IL-2 complex-driven or antigen-driven memory cells, the OT-I cells accounted for $\sim 3\%$ of the CD8⁺ population in the spleen (Fig. 5). At this point, the antigen-driven memory OT-I cells were CD44^{hi} CD122^{hi} and contained CD62L^{hi} central and CD62L^{lo} effector memory subsets. In contrast, all of the IL-2-anti-IL-2 complex-driven cells had the phenotype of central memory cells. These results suggest that naive CD8⁺ T cells activated by IL-2-anti-IL-2 complex treatment revert to stable central memory-phenotype cells.

IL-2-anti-IL-2 complex memory CD8⁺ T cells protect against bacterial infection

Functional memory CD8⁺ T cells rapidly expand in numbers and effectively control a challenge with pathogen (1, 2). We therefore assessed the ability of IL-2-anti-IL-2 complex memory OT-I cells to mount a protective recall response to a lethal dose of LM-OVA. Mice bearing IL-2-anti-IL-2 complex memory OT-I/RAG-1^{-/-} cells (91 d after transfer) were infected with a lethal dose of LM-OVA. As shown in Fig. 6 (A and B), IL-2-anti-IL-2 complex memory OT-I/RAG-1^{-/-} cells expanded over 30-fold within 3 d after the LM-OVA challenge. To examine the protective ability of IL-2-driven memory cells, a control group of mice received 1.5×10^6 untreated OT-I/RAG-1^{-/-} cells to constitute a population of these cells at an equivalent level to that of mice containing IL-2-anti-IL-2 complex memory OT-I/RAG-1^{-/-} cells (132 d after transfer). Before challenge with LM-OVA, the OT-I donor cell populations in peripheral blood were $1.3 \pm 0.11\%$ and $1 \pm 0.04\%$ of CD8⁺ T cells in control and IL-2-anti-IL-2 complex memory groups, respectively. At 3 d after challenge, mice containing IL-2-anti-IL-2 complex memory OT-I/RAG-1^{-/-} cells were able to significantly decrease LM-OVA burden in both the spleen and liver compared with mice containing untreated OT-I/RAG-1^{-/-} cells and mice without cell transfer (Fig. 6 C). Furthermore, adoptive transfer of IL-2-anti-IL-2 complex memory OT-I cells provided significant protection to naive animals, whereas an equal number of naive OT-I cells did not (Fig. S4, available at <http://www.jem.org/cgi/content/full/jem.20070543/DC1>). These results demonstrate that IL-2-anti-IL-2 complex-driven memory CD8⁺ T cells are able to mount a rapid and robust response to antigen challenge and afford protection.

IL-2-anti-IL-2 complex memory CD8⁺ T cells display incomplete cellular fitness

We further characterized IL-2-anti-IL-2 complex memory OT-I cells, and compared them to LM-OVA memory OT-I cells and with spontaneously arising memory-phenotype CD8⁺ T cells. Memory CD8⁺ T cells proliferate slowly to maintain a stable pool (6, 24), and this homeostatic turnover can be measured by the uptake of BrdU. LM-OVA antigen-driven memory OT-I cells displayed a rate of homeostatic turnover that was similar to that of memory-phenotype

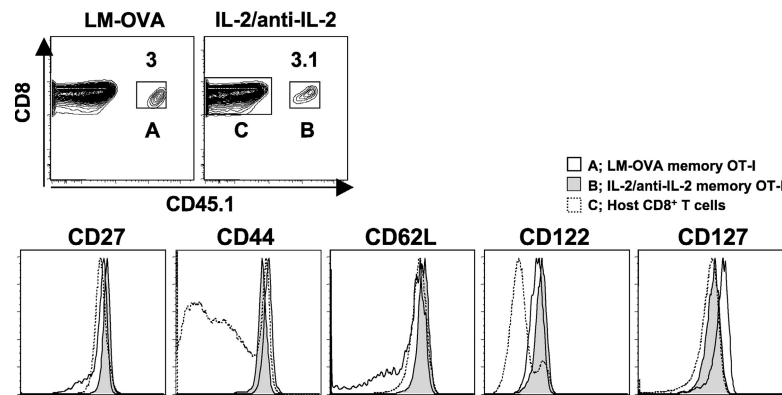


Figure 5. IL-2–anti-IL-2 complexes generate memory cells with a central memory phenotype. Memory OT-I cells generated by LM-OVA infection (75 d after infection; population A), IL-2–anti-IL-2 complex memory OT-I cells (12 d of treatment plus 76 d after transfer; population B), and host CD8⁺ T cells (population C) were examined for the expression of cell-surface markers. Contour plots are gated on CD8⁺ cells. Numbers represent the percentage of CD8⁺ cells within the box.

CD8⁺ T cells in the same mouse (Fig. 7 A). In contrast, although IL-2–anti-IL-2 complex memory OT-I cells also exhibited homeostatic turnover, the rate was significantly less than that of antigen-driven memory cells or CD44^{hi} CD122^{hi} memory-phenotype CD8⁺ T cells in the same mouse (Fig. 7, A and B).

Another cardinal feature of memory CD8⁺ T cells is the rapid production of cytokines after reencounter with antigen (1, 2). Splenocytes containing IL-2–anti-IL-2 complex memory OT-I cells or LM-OVA memory OT-I cells were stimulated with OVA peptide or PMA plus ionomycin for 4 h in vitro, and cytokine production was assessed by flow cytometry. Upon antigenic stimulation, almost all (>95%) antigen-driven memory cells produced IFN- γ ; most of the IFN- γ -producing population also produced TNF- α , and ~40% of them were IL-2 positive (Fig. 7 C). In comparison, a reduced fraction of memory cells generated by IL-2–anti-IL-2 complexes were positive for the cytokines tested (Fig. 7 C). In addition, the amounts of IFN- γ , TNF- α , and IL-2 produced on a per-cell basis, as judged by the mean fluorescence intensity, were also decreased in IL-2–anti-IL-2 complex memory OT-I cells compared with antigen-driven memory cells (Fig. 7 D). Differences in cytokine production were evident when IL-2 complex-driven or antigen-driven memory cells were stimulated with PMA plus ionomycin (Figs. 7, E and F), implying that alterations in signal transduction proximal to the TCR do not account for the changes. The cytokine production levels of IL-2–anti-IL-2 complex memory OT-I cells resembled those of the host memory-phenotype CD8⁺ T cells upon PMA/ionomycin stimulation, although a marked difference was observed in IFN- γ levels (Fig. 7 F). Similar results were obtained when activated OT-I cells were prepared from OT-I/RAG-1^{-/-} mice that had been treated with IL-2–anti-IL-2 complexes (Fig. 7 B and not depicted). These results suggest that strong IL-2 signals coupled with weak TCR signals are not sufficient to guarantee the overall cellular fitness of memory CD8⁺ T cells.

DISCUSSION

The recent realization that complexing IL-2 with anti-IL-2 mAbs leads to a dramatic increase in the potency of signaling via the intermediate affinity CD122/CD132 receptor in vivo (14, 15) allowed us to examine the consequences of such signals to CD8⁺ T cells in normal, nonlymphopenic animals in the absence of foreign antigen stimulation. Injected IL-2–anti-IL-2 complexes caused the rapid activation of STAT5 in naive as well as memory-phenotype CD8⁺ T cells (Fig. 1). This was the case even though CD122 levels are much higher on memory than on naive CD8⁺ T cells (unpublished data) (16) and the IL-2–anti-IL-2 complex signaling depends on CD122 (29). Under such conditions, potent IL-2 signals stimulated naive CD8⁺ T cells to proliferate and differentiate into functional memory cells. Such IL-2–anti-IL-2 complex-driven memory cells had a conventional central memory phenotype (Fig. 5) and were able to control a bacterial challenge in both lymphoid and nonlymphoid organs (Fig. 6 C and Fig. S4). The downstream molecular basis for the IL-2–anti-IL-2 complex-driven programming of memory CD8⁺ T cells awaits further investigation. Target genes of STAT5, an important component of IL-2 signaling (3, 4), are likely to be a key component of this process, because overexpression of STAT5 in T cells results in the selective expansion of memory-phenotype CD8⁺ T cells (30, 31).

Although IL-2–anti-IL-2 complex-generated memory CD8⁺ T cells were able to expand, accumulate, and provide protection after challenge with a high dose *Listeria* infection (Fig. 6 and Fig. S4), we found that they differed from conventional antigen-driven memory cells. In comparison with conventional memory cells, they had a slower rate of homeostatic turnover and reduced cytokine production after short-term restimulation (Fig. 7). This slow turnover may result in disappearance of the complex-driven memory cells in the long term, although a clear correlation between the reduced BrdU uptake and loss of donor cells was not consistently observed up to 80 d after transfer (unpublished data). The turnover of

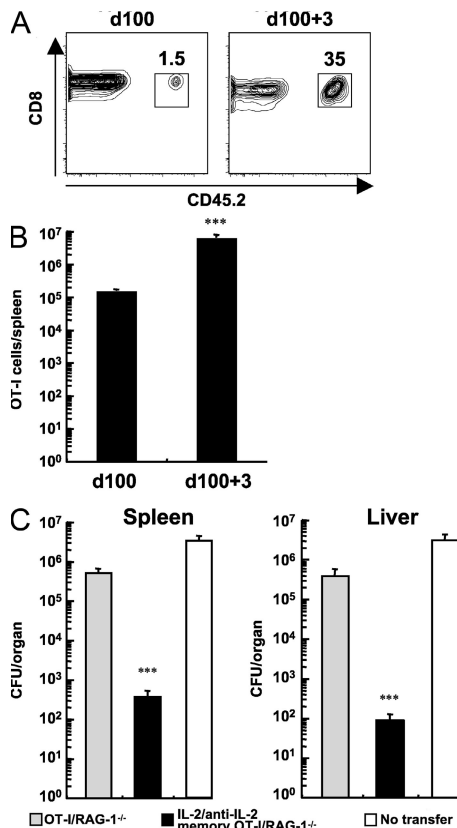


Figure 6. IL-2-anti-IL-2 complex memory CD8⁺ T cells protect against bacterial infection. (A) Enumeration of IL-2-anti-IL-2 complex memory OT-I/RAG-1^{-/-} cells (9 d of treatment and 91 d after transfer; CD45.2⁺) in the spleen before and 3 d after challenge with 10⁵ CFU of LM-OVA. The data are gated on CD8⁺ cells. Numbers represent the percentage of CD8⁺ cells within the box. (B) Absolute numbers of IL-2-anti-IL-2 complex memory OT-I/RAG-1^{-/-} cells in the spleen before and after LM-OVA challenge. Data represent the mean + SEM ($n = 3-4$). ***, $P < 0.001$. (C) CFU of LM-OVA in the spleen and liver at 3 d after challenge of mice containing equal numbers of naive OT-I/RAG-1^{-/-} or IL-2-anti-IL-2 memory OT-I/RAG-1^{-/-} cells. Data represent the mean + SEM ($n = 4-6$). ***, $P < 0.001$ versus the OT-I/RAG-1^{-/-} group.

memory CD8⁺ T cells is regulated by cytokines such as IL-7 and IL-15 (6). The expression levels of the receptors for these cytokines, namely CD127 (IL-7R α) and CD122 (IL-2/IL-15R β) and CD132, did not noticeably differ between complex-driven memory cells, antigen-driven memory cells, and memory-phenotype cells (Fig. 5 and not depicted). In addition, treatment with PMA plus ionomycin did not override the defect in cytokine production of IL-2-anti-IL-2 complex memory CD8⁺ T cells (Fig. 7, E and F), implying that the reduced fitness of IL-2-anti-IL-2 complex memory CD8⁺ T cells might be caused by intracellular differences, such as in the epigenetic remodeling of genes involved in hallmark characteristics of memory cells. We speculate that more potent signals via the TCR may be required to operate in conjunction with IL-2 signals to promote the complete programming of memory CD8⁺ T cell differentiation. In this context, it is

interesting to note that naive CD8⁺ T cells that receive strong TCR signals after pathogen infection, but weak or no IL-2 signals (i.e., IL-2R α ^{-/-} cells), develop into memory cells that show normal levels of homeostatic turnover and cytokine production but fail to accumulate in response to secondary antigenic challenge (11). Thus, predominant TCR signals without IL-2 give rise to memory CD8⁺ T cells that fail to accumulate in response to a secondary challenge, whereas predominant IL-2 signals with weak TCR stimulation result in memory cells with reduced turnover and cytokine production.

IL-2-anti-IL-2 complexes dramatically enhance IL-2 activity in vivo (14, 15), but the mechanism of this enhancement is still unclear. Because IL-2-anti-IL-2 complexes lacking the Fc region (i.e., IL-2 plus the F(ab')₂ portion of the anti-IL-2 mAb) had less activity than complexes with the whole IgG, it was suggested that Fc γ receptor-positive cells in lymphoid organs presented the IL-2 to T cells (14). However, we show that IL-2-anti-IL-2 complexes showed similar potent activity in FcR γ ^{-/-}Fc γ RII^{-/-} mice as in B6 control mice (Fig. 3). It is possible that other nonclassical Fc receptor-like molecules (32) or leaky expression of Fc γ RI without the FcR γ subunit (33) may mediate the activity of IL-2-anti-IL-2 complexes in vivo. However, FcR γ ^{-/-}Fc γ RII^{-/-} mice showed a gross functional defect in the stimulatory presentation of anti-CD3 IgG (Fig. S3 B), leading us to conclude that presentation of IL-2-anti-IL-2 complexes via Fc γ receptors may not be responsible for the enhancing effect. The half-life of IgG in vivo is dependent on the presence of the Fc region (25, 34), and this may explain the weak activity of IL-2-anti-IL-2 immune complexes lacking Fc. Moreover, there are many studies documenting the fact that the in vivo half-life of various cytokines is prolonged by the formation of immune complexes. These include human IL-2-anti-IL-2 (35), IL-4-anti-IL-4 (36), IL-6-anti-IL-6 (37), and soluble IL-15-IL-15R α -Fc (38) complexes. Therefore, we favor the idea that IL-2-anti-IL-2 complexes extend the half-life of IL-2 in vivo by preventing renal excretion, the major pathway of IL-2 clearance in mice (39).

Other recent reports have demonstrated the development of memory CD8⁺ T cells from naive cells without cognate antigen stimulation. Stimulation of naive CD8⁺ T cells with soluble IL-15-IL-15R α complexes, which act as superagonists (40, 41), induced proliferation and converted the cells to memory-phenotype CD8⁺ T cells (38). Because both soluble IL-15-IL-15R α complexes and IL-2-anti-IL-2 immune complexes signal through the CD122/CD132 receptor complex on CD8⁺ T cells (6, 42), these two types of complex may result in similar consequences, although the function of the memory-phenotype CD8⁺ T cells generated by soluble IL-15-IL-15R α complexes was not addressed in detail (38). In another report, Hamilton et al. demonstrated that lymphopenia-induced HP resulted in the generation of protective memory-like CD8⁺ T cells (43). Similar to IL-2-anti-IL-2 complex-mediated proliferation, HP is induced in the absence of cognate antigen (22-24), and similar phenotypic changes occur after proliferation induced by IL-2 signals (Fig. 2 C and Fig. 5) and by HP (43).

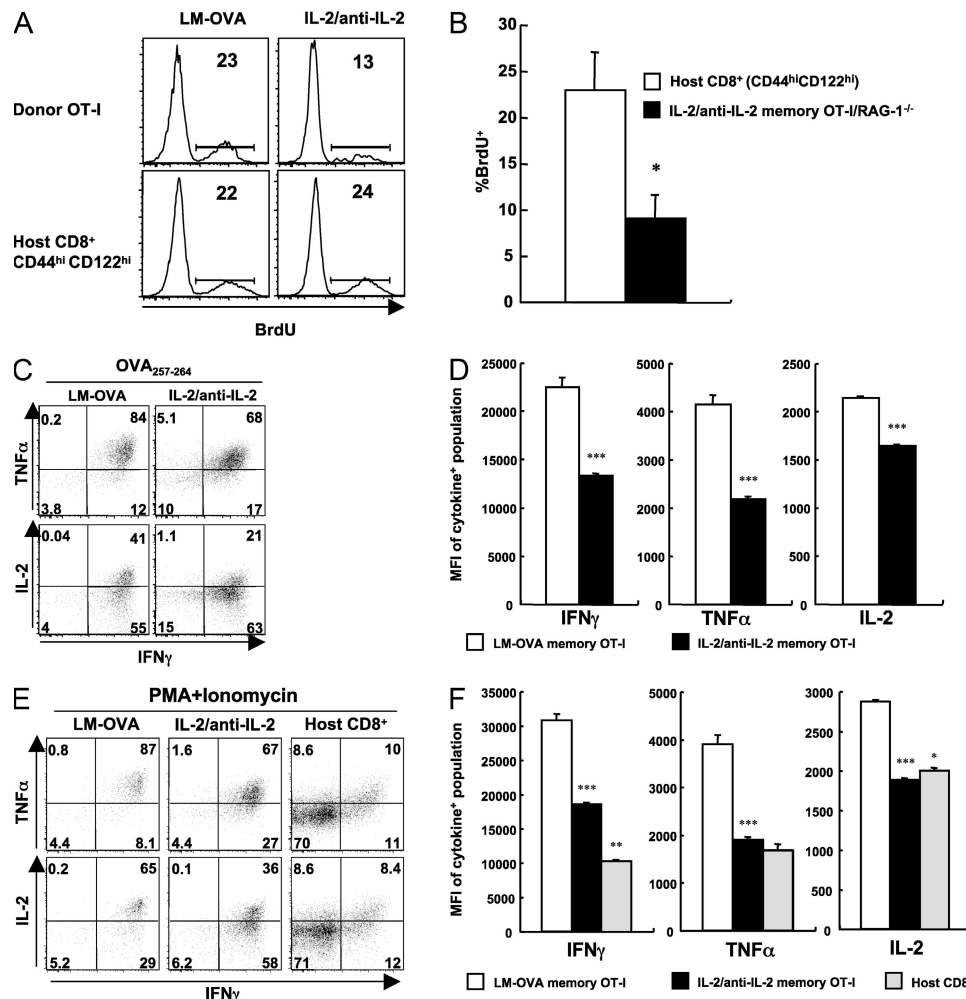


Figure 7. IL-2-anti-IL-2 complex memory CD8⁺ T cells display incomplete cellular fitness. (A) Mice bearing LM-OVA antigen-driven memory OT-I cells at 68 d after infection or IL-2-anti-IL-2 complex memory OT-I cells after 81 d (12 d of treatment plus 69 d after transfer) were given BrdU in drinking water for 7 d. BrdU incorporation by the indicated subsets of memory CD8⁺ T cells in the spleen is shown. Numbers represent the percentage of gated cells that are BrdU positive. (B) BrdU-positive population of host CD44^{hi} CD122^{hi} CD8⁺ T cells and IL-2-anti-IL-2 complex memory OT-I/RAG-1^{-/-} cells after 61 d (9 d of treatment plus 52 d after transfer). The data are the mean ± SEM (*n* = 3). *, *P* < 0.05. (C–F) Splenocytes containing memory OT-I cells generated by LM-OVA infection (62 d after infection) or IL-2 signals (12 d of treatment plus 63 d after transfer) were stimulated *in vitro* with OVA peptide (C and D) or PMA plus ionomycin (E and F). Intracellular cytokine production was examined 4 h later. Numbers in C and E represent the percentage of gated cells in each quadrant. (D and F) The mean fluorescent intensity (MFI) of cytokine staining is shown (mean ± SEM; *n* = 3). The MFI was calculated within the population positive for the respective cytokine. * and **, *P* < 0.05 and 0.001, respectively, versus the complex-driven memory cells; ***, *P* < 0.001 versus LM-OVA memory cells.

HP of naive CD8⁺ T cells in a lymphopenic environment requires both TCR stimulation through MHC class I-peptide complexes, in addition to cytokines such as IL-7 (22–24). Proliferation of naive CD8⁺ cells driven by IL-2-anti-IL-2 complexes in irradiated mice may also require TCR signals from self-MHC ligands (26). IL-7-dependent HP is enhanced by IL-12, but no substantial involvement of IL-2 has been reported (44–46). Given that stimulation by foreign antigen plus cytokines (such as pathogen infections), self-antigen plus IL-7 (HP), or intense IL-2 signals (IL-2-anti-IL-2 complexes) all give rise to protective memory CD8⁺ T cells, key components of

programming memory CD8⁺ T cell differentiation may reside within pathways common to these stimulations.

MATERIALS AND METHODS

Mice. B6 mice were obtained from the Jackson Laboratory. B6.SJL, MHC class I K^b-/-D^b-/-, OT-I/RAG-1^{-/-}, and FcγR^{-/-}FcγRII^{-/-} mice were purchased from Taconic. These animals were backcrossed to a B6 genetic background, except for FcγR^{-/-}FcγRII^{-/-} mice, which were on a 129 × B6 mixed background. Animals were housed in specific pathogen-free conditions in the animal facilities at the University of Washington. OT-I TCR-transgenic mice (47) congenic for Thy1.1 and CD45.1 were bred and maintained in the same facilities. All experiments were performed in compliance

with the University of Washington Institutional Animal Care and Use Committee regulations.

Preparation of IL-2–anti-IL-2 complexes. Carrier-free recombinant mouse IL-2 was obtained from eBioscience. Hybridoma of anti-IL-2 mAb (S4B6) was grown in HB basal medium with HB101 supplement (Irvine-Scientific). The anti-IL-2 mAb was purified from the culture medium with a protein G column. IL-2–anti-IL-2 complexes were prepared by mixing 1.5 μ g rIL-2 and 50 μ g anti-IL-2 mAb per mouse. Rat IgG (Sigma-Aldrich) plus PBS was used as a control treatment, as previously described (15, 48). The dose of IL-2–anti-IL-2 complexes has been shown to be effective in vivo in previous studies (11, 14).

Detection of phospho-STAT5 in vivo. Mice were killed at the time points indicated in the figures after a single i.p. injection of IL-2–anti-IL-2 complexes. The intracellular phospho-STAT5 staining was performed as previously described (49, 50). In brief, splenocytes were fixed in 1.6% formaldehyde/PBS for 30 min at room temperature, followed by permeabilization with ice-cold methanol. The fixed and permeabilized cells were stained in 2% FCS/PBS with PECy7-labeled anti-CD44, PerCP-labeled anti-CD8 α , and Alexa Fluor 647-labeled anti-phospho-STAT5 (Y694; Phosflow, BD Biosciences) mAbs. Alexa Fluor 647-labeled mouse IgG1 mAb (Phosflow; BD Biosciences) was used as the isotype control.

Adoptive transfer of donor CD8⁺ T cells. CD44^{lo} naive CD8⁺ T cells were purified by magnetic cell sorting using the CD8a⁺ T cell isolation kit (Miltenyi Biotec) supplemented with 0.003 μ g per million cells of biotinylated anti-CD44 mAb (BD Biosciences) to deplete CD44^{hi} CD8⁺ T cells (46). After sorting, >98% of CD8⁺ T cells were CD44^{lo} CD122^{lo} naive phenotype. 0.5–10⁶ donor cells per mouse were transferred i.v. into nonirradiated hosts. In some experiments, the donor cells were labeled with 1.6 μ M CFSE (Invitrogen) for 10 min at 37°C before transfer. Host animals in Fig. 4 (A and B) received 10 Gy total body irradiation and were reconstituted with syngeneic BM transplantation. For assays of IL-2–anti-IL-2 complex memory cells, OT-I or OT-I/RAG-1^{−/−} mice were injected with IL-2–anti-IL-2 complexes five to six times every other day until most OT-I CD8⁺ T cells acquired the CD44^{hi} CD122^{hi} phenotype. These cells were isolated by the magnetic cell sorting system without biotinylated anti-CD44 mAb. 7–17 \times 10⁶ of these activated OT-I cells per mouse were injected i.v. into host mice. The analysis of memory OT-I cells was performed at least 50 d after transfer. Engraftment of the donor cells at the time of assay was between 1 and 10% of total CD8⁺ T cells.

Intracellular cytokine staining. Splenocytes were incubated with 1 μ g/ml OVA_{257–264} (SIINFEKL) or PMA plus ionomycin in the presence of Brefeldin A (GolgiPlug; BD Biosciences) for 4 h at 37°C. After staining of cell-surface molecules, the cells were fixed and permeabilized with the Cytofix/Cytoperm kit (BD Biosciences). The intracellular cytokine staining was performed with FITC-labeled anti-TNF- α , PE-labeled anti-IL-2, and PECy7-labeled IFN- γ mAbs (eBioscience).

In vivo CTL assay. To prepare target cells, splenocytes were incubated with or without 10 μ g/ml OVA_{257–264} peptide for 30 min at 37°C. These peptide-pulsed target and nontarget populations were labeled with 1.6 and 0.16 μ M CFSE, respectively, mixed at a 1:1 ratio, and injected i.v. into mice. After 20 h, the target cell killing activity was evaluated using the ratio of CFSE/positive populations in the spleen. The CFSE-labeled populations were distinguished from host populations by congenic markers.

BrdU labeling in vivo. Mice were given 0.8 mg/ml BrdU (Sigma-Aldrich) in their drinking water for 7 d. Intracellular BrdU staining was performed by following the instructions of the BrdU Flow Kit (BD Biosciences).

LM-OVA infection, generation of LM-OVA-induced memory OT-I cells, and protection assay. An erythromycin-resistant recombinant strain of *L. monocytogenes* that expresses a secreted form of OVA (LM-OVA) was

provided by H. Shen (University of Pennsylvania School of Medicine, Philadelphia, PA) (51). LM-OVA was grown in Brain-Heart Infusion broth (BD Biosciences). At a midlog growth phase, culture samples were measured by OD and diluted in PBS for the desired titers. For preparation of LM-OVA-induced OT-I memory cells, mice that received 10⁴ CD44^{lo} CD122^{lo} naive OT-I cells per mouse 1 or 2 d earlier were infected i.v. with 3,000 CFU of LM-OVA. These mice were used at least 50 d later. Engraftment of the donor cells at the time of assay was between 1 and 10% of total CD8⁺ T cells. For lethal infection, mice were inoculated i.v. with 10⁵ CFU. For protection assay, organ suspension was prepared in 5 ml PBS, and 10-fold serial dilutions were made in PBS containing 0.1% NP-40. These 100- μ l dilutions were plated onto the Brain-Heart Infusion plates containing 5 μ g/ml erythromycin. The limit of detection was 50 CFU per organ.

Statistical analysis. Data with logarithmic presentation were transformed to log₁₀ (43). Statistical differences between groups were examined by a paired, two-tailed (for Fig. 3 B) or an unpaired, two-tailed Student's *t* test using Prism software (GraphPad). *P* < 0.05 was considered statistically significant.

Online supplemental material. Fig. S1 shows the experimental protocols used for studying IL-2–anti-IL-2 complex-induced proliferation and for the generation of IL-2–anti-IL-2 complex memory and antigen-driven memory cells. Fig. S2 shows the proliferation of different kinds of TCR-transgenic CD8⁺ T cells induced by IL-2–anti-IL-2 complexes. Fig. S3 shows the surface staining and the functional defect in Fc γ R^{−/−} Fc γ R^{II}^{−/−} mice. Fig. S4 shows the protection against bacterial challenge afforded by an equal number of naive OT-I, IL-2–anti-IL-2 complex memory OT-I, or LM-OVA-induced memory OT-I. Online supplemental material is available at <http://www.jem.org/cgi/content/full/jem.20070543/DC1>.

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