Evidence of a Novel IL-2/15Rβ-Targeted Cytokine Involved in Homeostatic Proliferation of Memory CD8+ T Cells

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The homeostasis of memory CD8+ T cells is regulated by cytokines. IL-15 is shown to promote the proliferation of memory CD8+ T cells, while IL-2 suppresses their division in vivo. This inhibitory effect of IL-2 appears to occur indirectly, through other cell populations including CD25+CD4+ T cells; however, the details of this mechanism remain unclear. In this study, we show that 1) both Ag-experienced and memory phenotype CD8+ T cells divided after the depletion of IL-2 in vivo; 2) this division occurred normally and CD44highIL-2/15Rβhigh CD8+ T cells generated after IL-2 depletion in IL-15 knockout (KO) and in IL-7-depleted IL-15 KO mice; 3) surprisingly, the blockade of IL-2/15Rβ signaling in IL-2-depleted IL-15 KO mice completely abolished the division of memory CD8+ T cells, although the only cytokines known to act through IL-2/15Rβ are IL-2 and IL-15; and 4) the expression of IL-2/15Rβ molecules on memory CD8+ T cells was required for their division induced by IL-2 depletion. These results demonstrate that the depletion of IL-2 in vivo induced memory CD8+ T cell division by an IL-15-independent but by an IL-2/15Rβ-dependent mechanism, suggesting the existence of a novel IL-2/15Rβ-utilizing cytokine that acts directly on memory CD8+ T cells to promote cell division.

The Journal of Immunology, 2004, 173: 6041–6049.

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Received for publication May 17, 2004. Accepted for publication September 3, 2004.

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injection in vivo requires the expression of IL-15Rα molecules on non-memory CD8+ T cells but requires that of IL-2/15Rβ molecules by the memory CD8+ T cells (23), suggesting that the transpresentation of IL-15 to memory CD8+ T cells could occur in vivo. Another possibility that could account for the dispensability of IL-15Rα expression on CD8+ T cells is that the proliferation of memory CD8+ T cells may be mediated by an undefined growth factor released from IL-15Rα-bearing non-T cells, as proposed by Ma and colleagues (27).

We herein report that the MHC class I-independent division of memory CD8+ T cells induced by IL-2 depletion occurred normally even in the absence of IL-15. In addition, we provide evidence that this process was mediated by a putative novel IL-2/15Rα-utilizing cytokine, since treatment with anti-IL-2/15Rβ Ab completely abrogated the anti-IL-2-mediated division of memory CD8+ T cells in IL-15 KO mice. Furthermore, we show that the expression of IL-2/15Rβ molecules on memory CD8+ T cells was essential for action of the putative cytokine. These results demonstrate the existence of a previously undefined mechanism for the homeostasis of memory CD8+ T cells in vivo.

Materials and Methods

Mice

Retired C57BL/6 (B6) mice — 5-7 mo of age were obtained from CLEA Japan (Osaka, Japan) or SLC (Shizuoka, Japan). IL-15 KO, KTC+ double KO, and B6.SJL (CD45.1)-congenic mice were obtained from Taconic Farms (Germantown, NY). IL-2 KO and B6.PL (Thy-1.1)-congenic mice were obtained from Taconic. Retired C57BL/6 (B6) mice, IL-2 KO and B6.PL (Thy-1.1)-congenic mice were purchased from The Jackson Laboratory (Bar Harbor, ME). OT-1 TCR-transgenic and IL-7Rα KO mice were kindly provided by Drs. W. R. Heath (Walter and Eliza Hall Institute of Medical Research, Melbourne, Australia) and K. Ikuta (Kyoto University, Kyoto, Japan), respectively. All strains used in this study were a B6 background. All of these mice except IL-15 KO mice were kept at the Institute of Experimental Animal Sciences at Osaka University Medical School (Osaka, Japan). IL-2/15Rβ KO mice were kept at the Institute of Experimental Animal Sciences at Nagoya University Medical School (Nagoya, Japan).

mAb for flow cytometry and cell sorting

The mAbs used for flow cytometry and cell sorting were: PE-, PE-carboxy cyanin (Cy5)-, or APC-labeled anti-CD4 mAb (RM4-5); PE-Cy5- or APC-labeled anti-CD8α mAb (53-6.7); APC-labeled anti-CD25 mAb (PC61); FITC-, PE-Cy5-, or APC-labeled anti-CD44 mAb (IM7); biotinylated or APC-labeled anti-CD62L mAb (MEL-14); biotinylated anti-CD69 mAb (H1.2F3); APC-labeled Thy-1.1 (CD90.1) mAb (G7); PE-Cy5-labeled Thy-1.2 (CD90.2) mAb (53-2.1); biotinylated or PE-labeled anti-IL-2/15Rβ (CD122) mAb (TM-1 and 5H4); biotinylated anti-IL-7Rα mAb (TM-34); and anti-Vε2 TCR mAb (B20.1). PE-Cy5- or APC-conjugated streptavidin was used for the biotinylated mAbs. These mAbs and streptavadin reagents were obtained from BD Biosciences (Mountain View, CA), eBioscience (San Diego, CA), BioLegend (San Diego, CA), and Caltag Laboratories (Burlingame, CA).

Adoptive transfer of donor T cells

Spleen and lymph nodes were harvested and homogenated by cell strainers (BD Biosciences, Costa Mesa, CA). Western blot analysis under nonreducing conditions using anti-rat IgG (H + L)-HRP (Zymed Laboratories, San Francisco, CA) was performed. The intensity of the band corresponding to whole IgG (150 kDa) in the (F(ab’)2) preparation was below the detection limit by ELISA using an anti-IgG (H + L)-HRP (Zymed Laboratories, San Francisco, CA) and anti-rat IgG (H + L)-HRP (Zymed Laboratories, San Francisco, CA) was performed. The intensity of the band corresponding to whole IgG (150 kDa) in the (F(ab’)2) preparation was equivalent to a 1/1000 dilution of the original anti-IL-2/15Rβ mAb. Polyclonal (Amersham Biosciences, Piscataway, NJ) was injected at 50–100 μg/mouse i.p. after donor T cell transfer.

In vivo BrdU labeling

Mice were given 0.8 mg/ml BrdU (Sigma-Aldrich) in their drinking water or it was administered i.p. at 1 mg/mouse during the anti-IL-2 treatment. BrdU staining for flow cytometry was performed using the BrdU Flow kit (BD Biosciences).

In vitro survival assay

CD44hiCD60hiIL-2/15Rβhigh CD8+ T cells were sorted and cultured at 1–2 × 10^5 cells/well in RPMI 1640 medium plus 10% heat-inactivated FCS with or without murine IL-7 or IL-15 (100 ng/ml; PeproTech, Rocky Hill, NJ). In some wells, anti-IL-2/15Rβ mAb (TM-1) or anti-IL-2 mAb (S4B6) was added at 1 µg/ml. After 4 days, the cells were stained with 7-aminoactinomycin D (7AAD, Via-Probe; BD Biosciences) and assayed for flow cytometry.

Results

In vivo depletion of IL-2 selectively induced the homeostatic division of CD44hiIL-2/15Rβhigh memory phenotype CD8+ T cells

Our previous reports revealed that IL-2 depletion induced the division of CD44hi memory phenotype CD8+ T cells, but not that of CD44lo naive CD8+ T cells (9, 10). To extend this finding, we first examined the effect of IL-2 depletion on the CD4+ T cell compartments. Memory CD8+ T cells can be distinguished from naive CD8+ T cells by the expression levels of several surface markers, such as CD44, IL-2/15Rβ, and Ly6C (28). Similarly, the expression level of CD44 defines the naive and memory phenotype CD4+ T cell subsets. Naive CD4+ (CD44lo), memory phenotype CD4+ (CD44hi), naive CD8+ (CD44loIL-2/15Rβlo), or memory phenotype CD8+ (CD44hiIL-2/15Rβhi) T cells were sorted from the splenocytes and lymph node cells of retired mice, in which a large number of memory phenotype T cells accumulates (10, 29). These T cell populations bearing Thy-1.2 molecules were labeled with CFSE and transferred into nonirradiated Thy-1.1-congenic mice. The recipient mice were then given anti-IL-2-neutralizing mAb (S4B6). The Ab treatment in this experiment was designed to be a longer injection...
CD8 H11001
CD44highIL-2/15R memory CD8ferred into Ag-inexperienced recipient mice. Similar to the Materials and Methods generated (see We also examined the effect of anti-IL-2R CD44highIL-2/15R cells. In addition, no change was observed for the expression levels of poly(I:C) injections did not associate with the up-regulation of an ory phenotype CD8BrdU incorporation of CD44high memory phenotype CD4 T cells were not affected by the anti-IL-2 treatment protocols tested here (Fig. 1, A and B). Furthermore, the division of CD44highIL-2/15Rβhigh memory phenotype CD8+ T cells was also induced in IL-2 KO mice even without Ab treatment (data not shown).

We next examined changes in the expression levels of cell surface markers upon cell division induced by anti-IL-2 mAb and compared them with those induced by poly(I:C), a well-known inducer of bystander proliferation of memory CD8+ T cells (30). As shown in Fig. 1C, the division of CD44highIL-2/15Rβhigh memory phenotype CD8+ T cells induced by anti-IL-2 treatment or by poly(I:C) injections did not associate with the up-regulation of an activation marker, IL-2Rα (CD25), as reported previously (9, 30). In addition, no change was observed for the expression levels of TCRβ, CD8α, CD44, CD45, CD62L, CD69, Thy-1, IL-2/15Rβ, and IL-7Rα by anti-IL-2 treatment (Fig. 1C and data not shown). We also examined the effect of anti-IL-2 treatment, which led to a blockade of IL-2 signaling and depletion of IL-2Rαhigh cells like CD25+CD4+ regulatory T cells on the division of CD44highIL-2/15Rβhigh memory phenotype CD8+ T cells. The host mice that had received CFSE-labeled memory phenotype CD8+ T cells were treated with rat IgG, anti-IL-2 mAb, anti-IL-2Rα mAb (PC61), or a combination of anti-IL-2 and anti-IL-2Rα mAbs. In contrast to IL-2 depletion by anti-IL-2 mAb, anti-IL-2Rα treatment rarely promoted the division of CD44highIL-2/15Rβhigh memory phenotype CD8+ T cells compared with rat IgG treatment (data not shown), suggesting neutralization of IL-2 molecules themselves may be critical for induction of memory CD8+ T cell division by this short-term experiment. As reported previously (9), however, treatment with the combination of anti-IL-2 and anti-IL-2Rα mAbs resulted in more enhanced division of these cells than anti-IL-2 treatment alone.

Taken together, these results suggest that the endogenous level of IL-2 selectively suppresses the homeostatic division of CD44highIL-2/15Rβhigh memory phenotype CD8+ T cells in vivo.

**FIGURE 1.** IL-2 depletion selectively induces the division of CD44high IL-2/15Rβhigh memory phenotype CD8+ T cells in vivo. A, CD44low or CD44highCD8+ T cells and CD44lowIL-2/15Rβlow or CD44highIL-2/15Rβhigh CD8+ T cells from mice expressing Thy-1.2 molecules were sorted and labeled with CFSE. The CFSE-labeled donor cells were transferred into nonirradiated syngeneic Thy-1.1 mice on day 0. Anti-IL-2 mAb ($\beta$B6, 1 mg) or control Ab (rat IgG, 1 mg) was given i.p. on days 1, 3, 4, 5, 6, 7, and 8. CFSE levels were examined on day 11. Histograms shown are gated on the Thy-1.2-expressing donor population. B, Mice were given BrdU in their drinking water for 8 days. The anti-IL-2 treatment was performed for the first 6 days. The BrdU-positive population in the T cell compartments was examined. C, CFSE-labeled CD44highIL-2/15Rβhigh CD8+ T cells were transferred into nonirradiated hosts on day 0. The anti-IL-2 treatment was conducted on days 1–5. Poly(I:C) was injected i.p. at 100 μg on days 1, 3, and 5. The CFSE levels and IL-2Rα, CD8α, and TCRβ levels were examined on day 7. The plot of CFSE vs IL-2Rα was gated on lymphocyte (low side scatter but not dead cells) and TCRβ population and those of the others were gated on the lymphocyte population. The CFSE-negative population is derived from the host mouse. The results are representative of more than two to three independent experiments.

IL-2 depletion induced the division of Ag-experienced memory as well as CD44highIL-2/15Rβhigh memory phenotype CD8+ T cells

CD44highIL-2/15Rβhigh memory-phenotype CD8+ T cells have many properties of memory cells (5, 31), but it is uncertain whether they have experienced Ag stimulation or not. To examine whether IL-2 also controls Ag-experienced memory CD8+ T cells, OVA-specific TCR-transgenic (OT-1) memory CD8+ T cells were generated (see Materials and Methods, CFSE-labeled, and transferred into Ag-inexperienced recipient mice. Similar to the CD44highIL-2/15Rβhigh memory phenotype CD8+ T cells, the Ag-experienced OT-1 memory CD8+ T cells underwent several cell divisions after the anti-IL-2 treatment (Fig. 2). Given that both CD44highIL-2/15Rβhigh memory phenotype and Ag-experienced memory CD8+ T cells were found to be regulated by IL-2 in a period (11 days) compared with our standard protocol (6–7 days) to perform a detailed examination whether CD4+ T cells have an ability to divide after anti-IL-2 treatment. Eleven days after the transfer, the division of donor T cells was detected by the dilution of CFSE. As shown in Fig. 1A, IL-2 depletion did not affect the division of CD44low naive CD4 or CD44low naive CD8 T cells. In contrast, the division of CD44highIL-2/15Rβhigh memory phenotype CD8+ T cells was significantly reduced, as reported previously (9, 10). Consistent with this result, DNA synthesis, as judged by BrdU incorporation, was selectively increased by the anti-IL-2 treatment in the CD44high fraction of CD8+ T cells compared with control IgG (rat IgG) treatment (Fig. 1B). The cell division and BrdU incorporation of CD44high memory phenotype CD4+ T cells were not affected by the anti-IL-2 treatment protocols tested here (Fig. 1, A and B). Therefore, the division of CD44highIL-2/15Rβhigh memory phenotype CD8+ T cells was also induced in IL-2 KO mice even without Ab treatment (data not shown).
similar way, we used CD4^hi^IL-2/15R^hi^ memory phenotype CD8^+^ T cells as donor cells in the following experiments.

**IL-2 depletion induced the division of memory CD8^+^ T cells in the absence of IL-15**

It has been demonstrated that the maintenance of memory CD8^+^ T cells can be established independent of MHC class I molecules (3). To examine whether the division of memory CD8^+^ T cells induced by IL-2 depletion requires MHC class I molecules, CD4^hi^IL-2/15R^hi^ memory phenotype CD8^+^ T cells were transferred into MHC class I KO (K^b^Db^d^ double KO) mice. Consistent with our previous report, in which memory phenotype CD8^+^ T cells lacking B2-microglobulin molecules could divide upon IL-2 depletion in B2-microglobulin KO mice (9), donor CD4^hi^IL-2/15R^hi^ memory phenotype CD8^+^ T cells exhibited multiple divisions after IL-2 depletion in the recipients lacking MHC class I molecules (Fig. 3A). We also previously reported evidence suggesting that the inhibitory effect of IL-2 in vitro does not appear to be due to a direct action on memory phenotype CD8^+^ T cells (10). These observations led us to hypothesize that endogenous IL-2 suppresses the activity of a soluble factor, such as a cytokine, that triggers memory phenotype CD8^+^ T cell-specific proliferation in vivo. The most likely candidate for this agent was IL-15, because IL-15 is a potent growth factor for memory CD8^+^ T cells and currently known mechanisms for the Ag-independent division of these cells in vivo are largely dependent on IL-15 (13–23, 27, 31). To test this hypothesis, CFSE-labeled CD4^hi^IL-2/15R^hi^ memory phenotype CD8^+^ T cells isolated from WT mice were transferred into IL-15 KO mice, and the host mice were then treated with anti-IL-2 mAb. Surprisingly, the division of CD4^hi^IL-2/15R^hi^ memory phenotype CD8^+^ T cells was induced normally in the IL-15 KO hosts (Fig. 3B), even though we could not see any division of CD4^hi^IL-2/15R^hi^ memory phenotype CD8^+^ T cells in IL-15 KO mice after poly(I:C) treatment as described previously (Fig. 3B) (25).

IL-15 is known to be produced by a variety of cell types and T cells are thought not to be a major IL-15 producer (32, 33). However, to rule out the possibility that IL-15 derived from WT donor T cells mediated the proliferation after IL-2 depletion shown in Fig. 3B, WT or IL-15 KO mice were given BrdU during the anti-IL-2 treatment and endogenous BrdU-positive proliferating cells were observed. IL-15 KO mice contained only a marginal population of CD4^hi^IL-2/15R^hi^ memory phenotype CD8^+^ T cells, as reported previously (18, 25), and they were mostly BrdU negative (Fig. 3C, IL-15 KO treated with rat IgG). IL-2 depletion in IL-15 KO mice increased the BrdU-positive CD8^+^ T cells bearing the CD4^hi^ phenotype in the spleen compared with IL-15 KO mice treated with the control Ab (Fig. 3C, top). This increased population of CD4^hi^CD8^+^ T cells in the IL-2-depleted IL-15 KO mice expressed a high level of IL-2/15R^β^ molecules (Fig. 3C, bottom). The increase in the CD4^hi^IL-2/15R^β^CD8^+^ T cell population in the IL-2-depleted IL-15 KO mice was also observed in nonlymphoid organs such as the liver and lungs (data not shown). It is intriguing that IL-2 depletion could restore the phenotype of IL-15 KO mice, which usually show a marked reduction in CD4^hi^IL-2/15R^β^ memory phenotype CD8^+^ T cells in both lymphoid and nonlymphoid organs.

We next examined the involvement of IL-7 in this process, because the overexpression of IL-7 in mice overcomes the IL-15 dependency of CD4^hi^ memory phenotype CD8^+^ T cells (34), and the irradiation-induced acute homeostatic proliferation of memory CD8^+^ T cells is halted when both IL-7 and IL-15 are suppressed (20, 35). Therefore, if IL-2 depletion up-regulates the production of IL-7, then CD4^hi^IL-2/15R^β^ memory phenotype CD8^+^ T cells could proliferate even in the absence of IL-15. To examine this possibility, CFSE-labeled CD4^hi^IL-2/15R^β^ memory phenotype CD8^+^ T cells were transferred into IL-15 KO mice, and the mice were then treated with the combination of anti-IL-7 and anti-IL-7Rα mAbs to remove both IL-7 and IL-15 at the same time. They were then treated with anti-IL-2 mAb. As shown in Fig. 3D, inhibition of IL-7 and its signaling in IL-15 KO mice had no effect on the division of donor cells. B lymphopoiesis in the bone marrow was impaired in the anti-IL-7- plus anti-IL-7Rα mAb-treated mice, verifying the efficacy of these Abs (data not shown). In addition, the CD4^hi^ memory phenotype CD8^+^ T cells from IL-7Rα KO mice divided in IL-15 KO mice after the anti-IL-2 treatment (data not shown). These results suggested that IL-2 negatively controlled the homeostasis of CD4^hi^IL-2/15R^β^ memory phenotype CD8^+^ T cells in vivo in an IL-15-independent fashion by a mechanism that is distinct from MHC class I- or IL-7-dependent processes.

A putative novel IL-2/15Rβ-utilizing cytokine mediates the division of memory CD8^+^ T cells

We demonstrated previously that anti-IL-2/15Rβ treatment, which is known to block both IL-2 and IL-15 receptor-mediated signaling, diminishes the basal homeostatic division of memory phenotypetype CD8^+^ T cells in vivo (9). However, in the present study we found that CD4^hi^IL-2/15R^β^ memory phenotype CD8^+^ T cells divided normally under the IL-2-depleted and IL-15-null condition (Fig. 3, B–D). Only IL-2 and IL-15 have been identified as IL-2/15Rβ-utilizing cytokines. However, if there were a third IL-2/15Rβ-utilizing cytokine or factor that induces the division of memory CD8^+^ T cells, it would explain the discrepancy noted above. Namely, under the IL-2-depleted, IL-15-null condition created by the anti-IL-2 treatment in IL-15 KO mice, the hypothesized third IL-2/15Rβ-utilizing cytokine could induce the division of memory CD8^+^ T cells. To examine this hypothesis, IL-2-depleted IL-15 KO mice received CFSE-labeled CD4^hi^IL-2/15R^β^ memory phenotype CD8^+^ T cells and were simultaneously treated with anti-IL-2/15Rβ mAb. As expected, this treatment resulted in complete inhibition of the anti-IL-2-induced division of

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**Figure 2.** IL-2 depletion induces Ag-experienced memory CD8^+^ T cells in vivo. OVA-specific OT-1 memory CD8^+^ T cells were generated in mice that received OT-1 CD8^+^ T cells and were subsequently immunized with OVA-loaded bone marrow-derived dendritic cells. CD4^hi^ IL-1 memory or CD4^hi^IL-2/15R^β^ memory phenotype CD8^+^ T cells were labeled with CFSE and transferred into nonirradiated mice on day 0. The Ab treatment was performed on days 2–6. CFSE levels were examined on day 7. The CFSE-negative population is derived from the host mouse. The results are representative of three independent experiments.
CD44^highIL-2/15R^high memory phenotype CD8^+ T cells in IL-15 KO mice (Fig. 4A). In contrast to anti-IL-2/15R, treatment with anti-IL-2α mAb had no inhibitory effect, but rather intensified the efficacy of anti-IL-2 mAb alone in IL-15 KO mice (Fig. 4, A and C), indicating that the effect of the anti-IL-2/15R treatment was not simply due to a blockade of signaling from the remaining IL-2 molecules in the IL-2-depleted IL-15 KO mice. The first peak of CFSE signals from the donor IL-2/15R^high CD8^+ T cells, which might be depleted by an anti-IL-2/15R complement reaction, was clearly detectable in the anti-IL-2/15R-treated mice (Fig. 4A), and the F(ab')2 of anti-IL-2/15R mAb was also effective (Fig. 4B), excluding the possibility of the Fc-mediated depletion of donor cells. The F(ab')2 of anti-IL-2/15R mAb was less efficacious than the intact one in terms of blocking anti-IL-2-induced division of memory CD8^+ T cells (Fig. 4B). It was reported that a half-life of F(ab')2, in vivo is generally shorter than that of whole IgG (36, 37). We think this could be the case. In addition, when compared with the intact anti-IL-2/15R mAb, at least five times more F(ab')2 was needed to block the binding of a biotinylated anti-IL-2/15R mAb on CD44^highIL-2/15R^high CD8^+ T cells (data not shown), suggesting that removal of the Fc portion of Abs might also affect their avidity to Ags. As shown in Fig. 4C, the anti-IL-2/15R treatment also negated the emergence of the CD44^highIL-2/15R^high CD8^+ T cell population induced by IL-2 depletion in IL-15 KO mice.

TM-β1 has been used as a blocking mAb for IL-2/15R-mediated signaling in vivo (9, 25). But if this anti-IL-2/15R mAb induced an unexpected inhibitory signal in memory CD8^+ T cells, their division must be stopped. We also suggested that the endogenous level of IL-2 may induce apoptosis of memory phenotype CD8^+ T cells (9). We therefore examined the effect of the mAb on cell survival. With a 4-day culture in vitro, only <5% of CD44^highIL-2/15R^high memory phenotype CD8^+ T cells were alive without cytokines, as judged by the 7AAD-negative population (Fig. 5). IL-7, which belongs to the IL-2-family cytokine but does not utilize IL-2/15R (7), rescued ~50% of these cells from apoptosis. The addition of control IgG (rat IgG) to the culture had no effect (data not shown); however, anti-IL-2/15R mAb (TM-β1) only slightly inhibited IL-7-mediated survival of CD44^highIL-2/15R^high memory phenotype CD8^+ T cells as shown in Fig. 5 (11.3% inhibition compared with IL-7 alone). A similar degree of inhibition was observed by the addition of anti-IL-2 mAb (S4B6; 11.2% inhibition compared with IL-7 alone), suggesting that IL-2 has some role in IL-7-mediated survival of CD44^highIL-2/15R^high memory phenotype CD8^+ T cells in vitro and that the slight inhibition of cell survival by anti-IL-2/15R mAb (TM-β1) may be due to an indirect secondary effect (i.e., a blockade of autocrine IL-2) but not as a result of an
inhibitory signaling through the IL-2/15Rβ molecules. In contrast, anti-IL-2/15Rβ mAb (TM-β1), but not anti-IL-2 mAb (S4B6), completely suppressed IL-15-mediated survival of CD44^high/IL-2/15Rβ^high memory phenotype CD8^+ T cells as expected (Fig. 5). These results indicate that anti-IL-2/15Rβ mAb (TM-β1) acts as a specific blocking mAb and does not transduce an unexpected inhibitory signal that suppresses cell survival. These data strongly suggest the existence of a novel IL-2/15Rβ-utilizing cytokine distinct from IL-2 and IL-15 that mediates the division of memory CD8^+ T cells in vivo.

Expression of IL-2/15Rβ molecules on memory CD8^+ T cells is essential for their division after IL-2 depletion

The results using anti-IL-2/15Rβ mAb suggested the presence of the novel IL-2/15Rβ-utilizing cytokine (Fig. 4), but they did not reveal which cell type(s) can respond to the putative cytokine. To investigate whether the putative IL-2/15Rβ-utilizing cytokine acts directly on memory CD8^+ T cells, CD44^low naive or CD44^high memory phenotype CD8^+ T cells were cultured for 4 days without any cytokines (medium only) or in the presence of IL-7 or IL-15 (100 ng/ml). Anti-IL-2/15Rβ mAb (TM-β1) or anti-IL-2 mAb (S4B6) was added at 100 μg/ml in some cultures. Living cells were detected as a 7AAD-negative population. The data represent the mean ± SD. Similar experiments were performed twice.

The putative IL-2/15Rβ-targeting factor is a member of the IL-2 family

It is well known that every member of the IL-2-family cytokines shares the γc molecule as a receptor component (7, 8). Therefore, we examined whether the division of memory CD8^+ T cell induced by IL-2 depletion could be inhibited by anti-γc blocking mAb. CFSE-labeled CD44^high/IL-2/15Rβ^high memory phenotype CD8^+ T cells isolated from WT mice were transferred into IL-15KO hosts. The host mice were then treated with the combination...
of anti-γc blocking mAbs (4G3 and 3E12) during the anti-IL-2 treatment. As shown in Fig. 7, the division of donor cells in response to IL-2 depletion was totally abrogated when γc signaling was blocked. This result suggests that the putative IL-2/15Rβ-utilizing cytokine shares the γc and belongs to the IL-2 family of cytokines.

Discussion

Previously described mechanisms for the positive regulation of memory CD8+ T cells in homeostasis are largely dependent on IL-15 (13–23, 27, 31). In contrast to those observations, our present study shows that anti-IL-2 treatment induced the division of CD44highIL-2/15Rβhigh memory phenotype CD8+ T cells, even in IL-15 KO mice and in IL-7-depleted IL-15 KO mice (Fig. 3, B–D). These findings imply a novel process for the Ag-independent homeostasis of memory CD8+ T cells in vivo. We found that the treatment of IL-2-depleted IL-15 KO mice with anti-IL-2/15Rβ mAb, but not with anti-IL-2Rα mAb, abrogated the division of the CD44highIL-2/15Rβhigh memory-phenotype CD8+ T cells (Fig. 4A). In addition, we showed that memory phenotype CD8+ T cells from IL-2/15Rβ KO mice failed to divide in response to IL-2 depletion (Fig. 6A). Only IL-2 and IL-15 have been identified as IL-2/15Rβ-utilizing cytokines, suggesting the existence of a putative third IL-2/15Rβ-utilizing cytokine that acts on memory CD8+ T cells to induce IL-15-independent division of these cells in vivo. Since a blockade of γc signaling inhibited the division of memory CD8+ T cells induced by IL-2 depletion (Fig. 6B), we suggest that the putative IL-2/15Rβ-utilizing cytokine is a member of the IL-2-family of cytokines.

Several lines of evidence suggest that the putative factor is not IL-2 itself. First, it is reasonable that there is almost no IL-2 in the mice treated with anti-IL-2 mAb, because we hardly saw CD25highCD4+ regulatory T cells and clearly detected an excess amount of the Ab molecules in serum in the mice (data not shown). Second, a blockade of IL-2/15Rβ or γc signaling was inhibitory, whereas that of IL-2Rα signaling was stimulatory for the cell division induced by anti-IL-2 treatment (Figs. 1D, 4, and 7). Third, there is a direct evidence that autocrine IL-2 is not required for the memory CD8+ T cell division by an adoptive transfer of the cells from IL-2KO mice (Fig. 6B). Finally, if IL-2 acts in a paracrine manner even after anti-IL-2 treatment, memory CD4+ T cell division must have been observed (Fig. 1).

Theze and colleagues (39) reported that a chemically synthesized 30-aa fragment of the N-terminal human IL-2 (p1–30) acts as an agonist for the IL-2/15Rβ signaling). In addition, several alternative forms of mouse IL-2 were listed in the expressed sequence tags databases, although there were no N-terminal short fragments similar to the p1–30 in the databases and S4B6, the anti-IL-2 mAb we used, recognizes an N-terminal portion of IL-2 molecules (p26–45) that overlaps with the p1–30 (40). However, it might still be possible to speculate that in vivo IL-2 depletion by S4B6 increases the activity of the alternative forms that do not contain the S4B6 determinant (e.g., p1–26). We hypothesize that such natural variants might act as agonists for IL-2/15Rβ-induced signaling on memory CD8+ T cells, especially if the concentration of such variants increases after IL-2 depletion in vivo. To examine this possibility, we performed RT-PCR experiments using splenic...
cDNA with or without the anti-IL-2 treatment by several primer sets for the murine IL-2 gene. However, we did not observe a consistent result showing that the transcripts for the alternative forms significantly increased after anti-IL-2 treatment. Additionally, we have not seen the existence of the short N-terminal fragments of the IL-2 mRNA (data not shown). Furthermore, we observed that the blockade of γc signaling abrogated the division of memory CD8+ T cells after IL-2 depletion in IL-15 KO mice (Fig. 6B). This result shows a sharp contrast to the report by Eckenberg et al. (39), since they demonstrated that IL-2/15Rβ-mediated, but not γc-derived, signaling is critical for the action of p1–30. Therefore, we suggest that the putative cytokine described here is not the natural variant(s) of IL-2 molecules that are agonistic to the IL-2/15Rβ signaling, similar to p1–30.

Since a deficiency of IL-15 or IL-15Rα in mice leads to significant impairment of the maintenance of CD44highIL-2/15Rβ memory CD8+ T cells (17–19), we hypothesize that the putative IL-2/15Rβ-utilizing factor would act downstream of or in parallel to IL-15. In this regard, it is intriguing that the expression of IL-15Rα on memory CD8+ T cells is dispensable, whereas its expression on the other cells is shown to be important for their IL-15-mediated division in vivo (22–24). It has also been reported that IL-2/15Rβ, another component for IL-15R complex, is required to be expressed on memory CD8+ T cells to respond to IL-15 injection (23). Transpresentation of IL-15 by the IL-15Rα-bearing cells to other IL-2/15Rβ- and γc-expressing cells such as memory CD8+ T cells (IL-15Rα-mm or IL-15Rα-CD44high) has been reported in vitro (22, 26), which could explain the dispensability of IL-15Rα and the necessity of IL-2/15Rβ expression on memory CD8+ T cells in vivo. Additionally, another possibility proposed by Ma and colleagues (27) is that the proliferation of memory CD8+ T cells may be induced by an undefined growth factor secreted from IL-15Rα-bearing non-T cells after IL-15 stimulation. The in vivo function of the putative IL-2/15Rβ-utilizing cytokine suggested here might be consistent with their idea, if IL-15 induces and IFN-γ suppresses secretion of the cytokine or expression of its receptor component(s).

Blattman et al. (41) recently reported that the injection of rIL-2 increases the proliferation of virus-specific resting memory CD8+ T cells in mice. The administration of exogenous IL-2 is also beneficial for the proliferation of activated CD8+ T cells in vivo (42). One interpretation for the inconsistency between our results (i.e., IL-2 depletion induces the division of CD44highIL-2/15Rβhigh memory CD8+ T cells shown in this study and in Refs. 9 and 10) and theirs is that IL-2 acts as a two-edged sword: the endogenous, relatively low concentration of IL-2 is inhibitory, but the exogenous IL-2 supplement could raise the concentration to levels that are much higher than in the normal in vivo state, thereby promoting the division of memory CD8+ T cells. In fact, a high concentration of IL-2 stimulates the proliferation of CD44high memory phenotype CD8+ T cell in vitro (10). It is likely that the rIL-2 treatment would have a direct action on memory CD8+ T cells even in vivo. In contrast, our previous data strongly suggest that the inhibitory effect of IL-2 on the division of memory phenotype CD8+ T cells is the result of an indirect action through other cell populations, including CD25+CD4+ T cells (10). Therefore, these distinct mechanisms of IL-2 action, i.e., direct vs indirect, on memory CD8+ T cells could cause the different outcomes.

In summary, our present study demonstrates that IL-2 negatively regulates the homeostatic division of memory CD8+ T cells through an IL-15-independent mechanism. In addition, we provide evidence that this process is completely dependent on IL-2/15Rβ molecules expressed on memory CD8+ T cells, suggesting the existence of a third IL-2/15Rβ-utilizing cytokine that regulates the homeostasis of memory CD8+ T cells in vivo, although we understand the possibility that differential efficacies of different Abs that may influence the results might be retained along with multiple indirect effects of Abs on various cell types in vivo. Molecular identification of the putative IL-2/15Rβ-utilizing cytokine, one of our current research efforts, is thus an issue of great importance for understanding the regulation of memory CD8+ T cell homeostasis.

**Acknowledgments**

We appreciate Dr. Phillipa Marrack (National Jewish Medical and Research Center, Denver, CO) for providing the hybridomas and for a critical reading of this manuscript. We also thank Dr. Brian C. Schaefer (Uniformed Services University of the Health Sciences, Bethesda, MD) for a critical discussion and a critical reading of this manuscript. We are grateful to Drs. William R. Heath (Walter and Eliza Hall Institute of Medical Research) and Koichi Ikuta (Kyoto University) for providing OT-1 TCR-transgenic mice and IL-7Rα KO mice, respectively. We also thank Ryoko Masuda and Ayako Kubota for their excellent secretarial assistance.

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