

The IL-15/IL-15R α on cell surfaces enables sustained IL-15 activity and contributes to the long survival of CD8 memory T cells

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We previously described unique features of the IL-15 receptor (IL-15R) α . IL-15R α by itself forms stable complexes with IL-15 on cell surfaces and presents IL-15 *in trans* to neighboring natural killer/T cells. Moreover, the membrane IL-15/IL-15R α complexes (membIL-15) undergo endosomal internalization but survive lysosomal degradation, allowing the complexes to recycle back to the cell surface. Here, we show that membIL-15⁺ cells act as a persistent source of IL-15 for the surrounding microenvironment (intercellular reservoir effect). Additionally, membIL-15⁺ cells give rise to augmented retention of IL-15 in the circulation as well as in tissues. Curiously, IL-15 retention was particularly associated with lungs, rather than with lymph nodes, in normal unstimulated mice, which correlated with the preferential homing of antigen-specific CD8 T cells to lungs during their contraction phase in an IL-15R α -dependent manner. Furthermore, membIL-15, unlike soluble IL-15, caused sustained IL-15 signal transduction in the target cells. Collectively, these characteristics define IL-15 as a unique cytokine with prolonged *in vivo* survival and sustained biological action on the target cells, which may account for the proposed persistent action of IL-15 that helps the long-term survival of functional CD8 memory T cells *in vivo*.

cytokine retention *in vivo* | memory CD8 homing

Despite the sharing of critical signaling components between IL-2 and IL-15 (1, 2), these two cytokines exert fundamentally distinct functions (reviewed by Waldmann and Tagaya in ref. 3). Mice deficient in these cytokines or their private receptor components show nonoverlapping phenotypes (3–9). In our previous study (10) we demonstrated the fundamental differences in the way these two cytokines act. Briefly, IL-15 exists as a membrane-associated cytokine acting on cell-to-cell contact as part of an immunological synapse. In contrast, IL-2 remains a soluble factor. Other groups have provided compelling evidence that the transpresentation may be the major style of IL-15 action *in vivo* (11–18). IL-15 is produced by activated dendritic cells (DCs), monocytes, and stromal cells (3), whereas IL-2 is produced by antigen (Ag)-stimulated T cells. Thus, it is reasonable that IL-15 that bridges accessory and T cells requires presentation *in trans* whereas IL-2 activates T cells in an autonomous manner.

IL-15 may be persistently needed *in vivo*, especially in the context of the IL-15-dependent prolonged survival of functional memory CD8 T cells (19, 20) as well as in the developmental requirement for IL-15 in the ontogeny of natural killer cells (7, 21) and $\gamma\delta$ T cells (22–24). Based on RT-PCR, IL-15 expression was considered rather constitutive. However, IL-15 protein is not easily detectable by biochemical means in normal humans and mice (3). Thus, the mechanism underlying such persistent IL-15 action *in vivo* remains elusive. In our previous study (10) we demonstrated that a cytokine-dependent cell line, CTLL-2, when cultured with IL-15, became resistant to apoptotic death upon IL-15 withdrawal (remained viable >72 h), whereas the same cells cultured with IL-2 underwent prompt apoptosis within 12 h of IL-2 withdrawal (10). Because IL-15/IL-15R α complexes recycled after endosomal internaliza-

tion, cells could continue to use IL-15 after the removal of soluble IL-15 from the culture media (10). Thus, the IL-15/IL-15R α system has an intrinsic characteristic that makes this cytokine act stably *in vivo*. We tried to exploit this persistence of IL-15 in the context of biological function and found that indeed IL-15 is a well retained cytokine *in vivo* because of recycling. In addition, we show here that IL-15R α -expressing cells are constantly present in select tissues in normal mice, thereby contributing to the retention and persistent presence of IL-15 in these tissues that maintain IL-15 activity in the absence of constitutive production of the IL-15 protein. We also show evidence that links this observation with the IL-15-mediated homing of Ag-primed CD8 T cells in the contraction phase.

Furthermore, we demonstrate the unique nature of growth signals provided by the cell-surface membrane-associated IL-15/IL-15R α complex (membIL-15). In short, membIL-15 presented *in trans* to target cells transduces signals that are qualitatively distinct from those provided by soluble IL-15, which lead to more prolonged and persistent activation of the target cells. These distinct characteristics of IL-15 may provide a mechanistic explanation for the persistent action of IL-15 *in vivo* and makes this cytokine a special factor among those γ c-using cytokines (IL-15, IL-2, and IL-7) (25, 26) that are known to contribute to the generation and survival of the Ag-specific memory CD8 T cells.

Results

IL-15R α -Positive Cells Can Provide a Persistent IL-15 Reservoir to the Surrounding Microenvironment. We previously reported that IL-15/IL-15R α complexes formed on the cell surfaces undergo internalization but survive this intracellular endosomal processing to recycle back to the cell surface as a biologically active cytokine (10). To determine whether the recycled IL-15/IL-15R α complexes release free IL-15 into the environment, we transfected 293T cells with an IL-15R α expression construct and added recombinant human (rh) IL-15 to the culture for a 6-h incubation. The cells were then washed with acidic PBS (pH 3.5) to completely strip the cell-bound IL-15 (completion of which was confirmed by flow cytometry; data not shown). These cells containing intracellular IL-15/IL-15R α complexes were transferred to a cytokine-free environment to determine whether the intracellularly stored IL-15 was released after recycling. An ELISA (Fig. 1A) and a biological CTLL-2 assay (Fig. 1B) allowed us to confirm that biologically active IL-15 was released

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Abbreviations: rh, recombinant human; membIL-15, cell-surface membrane-associated IL-15/IL-15R α complex; poly I:C, poly inosinic poly cytidylic acid; DC, dendritic cell; Tg, transgenic; LN, lymph node; Ag, antigen; OVA, ovalbumin; TCR, T cell receptor.

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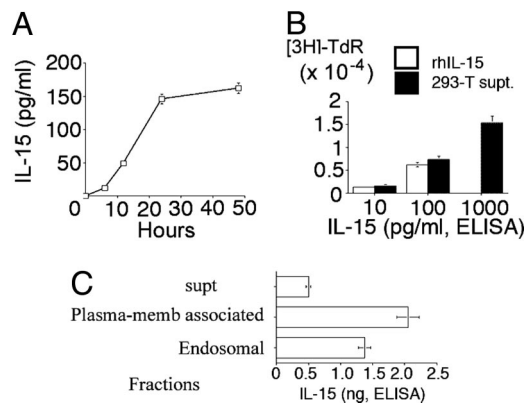


Fig. 1. IL-15R α -positive cells provide a persistent IL-15 reservoir. (A) Progressive release of the soluble IL-15 after recycling of the IL-15/IL-15R α complexes. Human 293T cells (negative for IL-15 and IL-15R α expression) were transfected with a plasmid encoding IL-15R α (10) and incubated with 1 nM rhIL-15 (PeproTech) for 6 h to facilitate the recycling of IL-15/IL-15R α complexes. Cells were then stripped of IL-15 (10) from the surface IL-15/IL-15R α complexes and transferred to a cytokine-free culture. Aliquots were removed from the supernatant at the indicated time points, and the amounts of soluble IL-15 were determined by a specific ELISA. (B) Biological activities of the released IL-15 and rhIL-15 (matched in concentrations) were compared by using a CTLL-2 proliferation assay. (C) The intercellular reservoir effect. The majority of the recycled IL-15 molecules remain associated with the plasma membrane fraction while a small fraction of the recycled IL-15 is released. Forty-eight hours after the initiation of the recycling experiment, 2 ml of the 293T culture was separated into supernatant and cellular pellets. Cells were homogenized and fractionated over a continuous sucrose-gradient by ultracentrifugation. The plasma-membrane fractions were identified by the migration of biotinylated cell-surface proteins, and the endosomal fractions were identified by the distribution of early endosomal protein 1 (10). Shown is the amount of IL-15 released back into the culture supernatant and those of IL-15 recovered in membrane-associated and endosomal fractions. The IL-15 in the plasma-membrane fractions was considered as cell-retained.

in a time-dependent manner. During the recycling of IL-15/IL-15R α , cell surface of these cells become positive for membIL-15 (10). Even after the release of some IL-15 molecules into the supernatant, the cell surfaces of the treated 293T cells remained positive for recycled IL-15/IL-15R α complexes (data not shown). At this time, the majority of the IL-15 was still associated with cells because IL-15 recovered from plasma-membrane-associated fractions after sucrose-gradient ultracentrifugation contained five times more IL-15 protein than was released (Fig. 1C). These results suggest that the IL-15R α -positive cells (note that these are not necessarily IL-15-producing cells) can capture and stably store IL-15 molecules as intracellular reservoir through recycling, which then provides an environment with released biologically active IL-15. Nevertheless, the majority of the IL-15 was retained in association with the plasma membrane, presumably as IL-15/IL-15R α complexes because of the high binding affinity between these two molecules, thus allowing these cells to behave as an IL-15 reservoir for a prolonged time. We propose to call this function an “intercellular reservoir effect.”

IL-15 Is an Effectively Retained Cytokine *in Vivo*. Levels of a cytokine in *in vivo* environments are determined by various factors including their production, consumption, and excretion. We postulated that the intercellular reservoir effect might prolong the biological availability of IL-15 *in vivo*. To test this hypothesis, we administered the γ -cytokines IL-2, IL-7, and IL-15 into mice and examined the rate of the clearance of these proteins from the circulation. Because of the lack of a sensitive ELISA for mouse IL-15, we injected (i.p.) 5

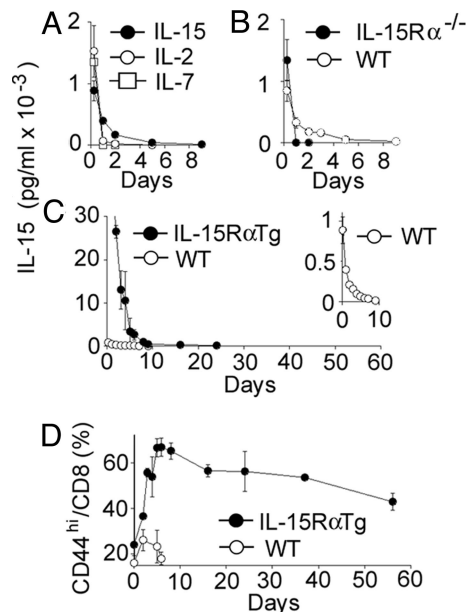


Fig. 2. IL-15 retention in the circulation of WT, IL-15R α ^{-/-}, and IL-15R α Tg mice. (A) Unique prolonged retention of IL-15 in the circulation of WT mice. Mice were injected with 5 μ g of rhIL-15, rhIL-2, and rhIL-7 (i.p.). Serum samples were collected at the indicated timings, and their cytokine concentrations were determined by specific ELISAs (R & D Systems). (B) Rapid clearance of administered IL-15 from IL-15R α ^{-/-} mice. Five micrograms of rhIL-15 was injected (i.p.) into WT or IL-15R α ^{-/-} mice. (C) Augmented IL-15 retention in IL-15R α Tg mice. Five micrograms of rhIL-15 was injected (i.p.) into IL-15R α Tg mice or WT mice. Because the serum IL-15 concentrations from WT mice were too compressed to visualize at the given scale, an expanded graph with a readable scale is inserted on the right. (D) Concomitant prolonged expansion of CD44^{hi}CD8 T cells in IL-15-injected IL-15R α Tg mice. Peripheral blood mononuclear cells from IL-15-injected WT or IL-15R α Tg mice were analyzed by flow cytometry, and percentages of CD44^{hi} population in the entire CD8 subset are shown.

μ g of human cytokines into mice (27). As described, IL-2 and IL-7 levels declined to undetectable levels by 48 h after the injection (28). However, the serum levels of IL-15 showed different kinetics. There was a rapid decay during the first 24 h (28), followed by a second retention phase, and IL-15 levels remain detectable (>10 pg/ml) even 120 h after the injection, demonstrating that the administered IL-15 was retained longer than other γ -cytokines in WT mice (Fig. 2A).

IL-15R α Mediates the Retention of IL-15 in Mice. To determine whether the IL-15 retention was mediated by a specific mechanism involving IL-15R α , 5 μ g of human cytokines was injected into IL-15R α ^{-/-} mice. IL-15 failed to display the retention phase in IL-15R α ^{-/-} mice (Fig. 2B). Notably, the rapid clearance pattern of IL-15 in IL-15R α ^{-/-} mice resembled that of IL-2 or IL-7 in WT mice (Fig. 2A and B), suggesting that IL-15 behaved like other γ -cytokines in the absence of IL-15R α .

Augmentation of IL-15R α Expression Led to Enhanced Retention of IL-15 *in Vivo*. In general, the internalization-mediated processing facilitates the catabolism of the soluble ligands, contributing to terminating the signal initiated by the ligands. Because of the unique intercellular reservoir effect, we speculated that IL-15 might have an intrinsically accumulating nature. To test this possibility, we administered rhIL-15 (5 μ g, i.p.) into human IL-15R α transgenic (IL-15R α Tg) mice, in which the promoter of the elongation factor 1 α drove the ubiquitous expression of the human IL-15R α molecule. At 24 h after injection, we observed \approx 100-fold more IL-15 retention in the sera of the IL-15R α Tg mice than the WT mice. The

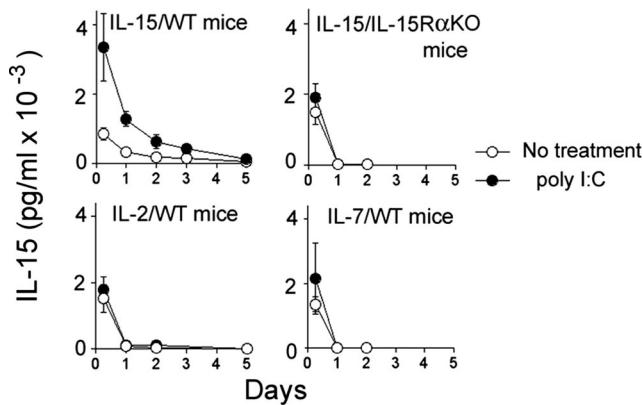


Fig. 3. Increased IL-15 retention in mice challenged by poly I:C. Mice were injected with 5 μ g of rhIL-15 (i.p.) after treatment with or without poly I:C (100 μ g, i.p. injected 24 h before the IL-15 injection). The IL-15 concentrations in serum samples were determined by a specific ELISA ($n = 5$).

serum IL-15 stayed above detectable levels (>10 pg/ml) for \approx 16–20 days (Fig. 2C). No such prolonged retention was observed with IL-2 and IL-7 in any mice (data not shown). Concomitantly, we observed \approx 50-day prolonged expansion of CD44^{hi}CD8 T cells (Fig. 2D), a hallmark of augmented *in vivo* IL-15 action that was also seen in IL-15 Tg mice (29) or in WT mice repeatedly infused with IL-15 (27) for 24–30 consecutive days.

Systemic Challenge of Mice with Toll-Like Receptor Ligands That Stimulate IL-15R α Expression Resulted in Augmented Retention of IL-15. Microbial invasion induces IL-15R α on accessory cells. To test whether IL-15R α induction on these cells augments the IL-15 retention *in vivo*, we challenged mice with Toll-like receptor ligands such as poly inosinic poly cytidylic acid (poly I:C) or LPS to induce IL-15R α expression (30) and observed a prolonged IL-15 retention in WT, but not in IL-15R α ^{-/-}, mice (Fig. 3). No such prolonged retention was observed with IL-2 or IL-7 under the same circumstances. These data collectively demonstrate that the expression of IL-15R α regulates the extent of IL-15 retention in mice.

Tissue-Specific Retention of IL-15 in Steady-State WT Mice. The IL-15 retention in the circulation seems to be a consequence of the intercellular reservoir effect caused by IL-15R α -positive cells, which prompted us to examine whether IL-15 is found associated with some tissues in mice. Because of the lack of a reliable antibody to detect mouse IL-15, we again injected rhIL-15 into mice and homogenized their tissues to detect associated human IL-15. Among the tissues examined [the liver, spleen, kidney, thymus,

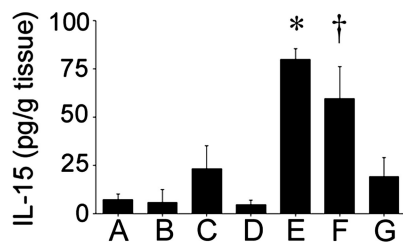


Fig. 4. Tissue distribution of injected IL-15. Bars are as follows: A, serum; B, liver; C, kidney; D, spleen; E, lung; F, LN; G, thymus. WT mice were injected with 5 μ g of rhIL-15 (i.p.). Nine days after the administration, various tissues were collected from these mice and homogenized (except blood samples), and the IL-15 associated with each tissue was determined by a specific ELISA. Serum, liver, and spleen showed no meaningful levels of IL-15 (<10 pg/ml) whereas values from lungs and LNs appeared significantly positive ($n = 4$). *, $P < 0.0001$; †, $P = 0.0001$ compared with serum level.

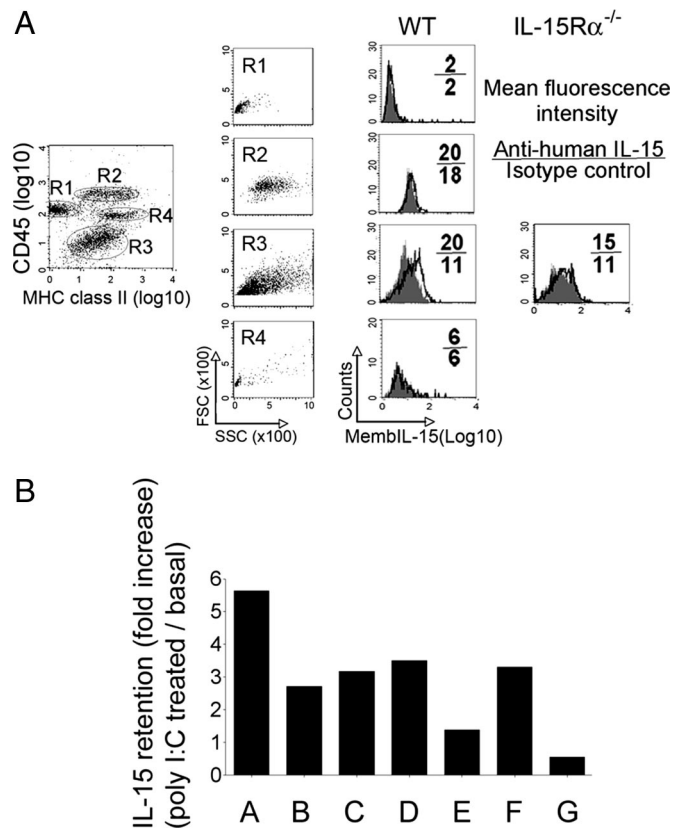


Fig. 5. Detection of nonhematopoietic IL-15R α positive cells in lung tissue. (A) IL-15R α expression in lung cell subsets demonstrated by membIL-15 staining. For the detection of IL-15R α cells from the homogenized lung tissue were incubated with 1 nM rhIL-15 for 30 min on ice, followed by staining with anti-IL-15 (10) (R & D Systems), antiCD45, and anti-I-A^b antibodies for 30 min on ice. Four major populations were defined and analyzed by separate gates as shown (Left). From each gate, the morphological information (forward light scatter vs. side light scatter) and IL-15 staining are shown (Right). In addition, mean fluorescence intensity of the surface IL-15 staining is shown for each cell population. Cells from R3 (CD45⁻) showed significant surface IL-15 staining. (B) Increase of IL-15 retention after poly I:C injection into WT mice. Bars are as follows: A, serum; B, liver; C, kidney; D, spleen; E, lung; F, LN; G, thymus. After systemic poly I:C and 5 μ g of rhIL-15 injections, tissue samples were collected and homogenized from mice. The amounts of tissue-associated IL-15 were determined by a specific ELISA, and the fold increase after the poly I:C challenge is shown.

lymph nodes (LNs), and lungs], only the lungs and LNs gave a significant signal when assessed with a specific ELISA at 9 days after the initial injection of rhIL-15 (Fig. 4). In the spleen, IL-15 was barely detectable as early as at day 3. It is noteworthy that IL-15 was no longer detectable in the circulation of these mice, suggesting that the rhIL-15 in tissues did not reflect that left in the circulation, but rather IL-15 was retained by a specific mechanism.

Demonstration of IL-15R α -Expressing Cells in Select Tissues in Mice. Based on the previous results, we postulated a constitutive existence of IL-15R α -positive cells in some tissues such as the lungs and LNs. Unfortunately, we found the commercial anti-IL-15R α antibodies not reliable for tissue staining, because they nonspecifically stained cells from IL-15R α ^{-/-} mice. Therefore, we chose to first incubate the tissue cells with rhIL-15, followed by a staining using anti-IL-15 Abs. In fact, we could detect membIL-15⁺ cells in the lungs (Fig. 5A). To confirm the specificity of such a staining scheme, similar experiments were performed by using IL-15R α ^{-/-} mice with which we detected no meaningful staining for membIL-15 (Fig. 5A, R3) despite the presence of a similar cell population in the CD45/class

LN, and peripheral blood of IL-15R $\alpha^{-/-}$ mice suggests that various factors other than IL-15 may also contribute to the survival of memory CD8 T cells in these tissues. Interestingly, these other factors appear to play relatively limited roles in the lungs, emphasizing the relevance of the unexpected connection between the pulmonary CD8 memory homing and IL-15 retention.

The degradation-resistant nature of IL-15/IL-15R α complexes during endosomal recycling (10) gave rise to another aspect of IL-15 characteristics that makes it a persistent *in vivo* factor through the intercellular reservoir effect. Small molecules like cytokines are rapidly lost from the body by the efficient renal filtration mechanism. IL-15 appears to be an exception. The clearance of IL-15 has two phases. In the first rapid clearance phase, IL-15 behaves like other cytokines (28), but detectable levels of IL-15 remain in circulation or in association with select tissues for more than a week after a bolus injection. This retention of IL-15 is mediated by IL-15R α , because IL-15 disappears from IL-15R $\alpha^{-/-}$ mice without retention. Thus, we speculate that endogenous IL-15 can be captured and retained by IL-15R α^{+} cells long after the cessation of its transient production. The rather exaggerated expansion of CD44^{hi}CD8 T cells in IL-15-injected IL-15R α Tg mice would be a proof of such an endogenous mechanism. Oh *et al.* (19) demonstrated a similar prolonged IL-15 action *in vivo*. They transiently produced IL-15 in mice by transducing a vaccinia–IL-15 construct. Nevertheless, the IL-15 effect was “imprinted” for several months after the cessation of IL-15 production. IL-2–vaccinia, on the other hand, failed to have such a sustained effect (19). Our observation may shed light on the mechanism underlying this intriguing observation.

It is plausible that in humans or mice kept in a nonclean facility, the survival of memory CD8 T populations might depend on minor bystander microbial infections. Periodical exposure to such pathogens would enable accessory cells to produce IL-15, which then would be captured by nonhematopoietic IL-15R α^{+} cells in immunologic portals to constitute an intercellular reservoir maintained for an extended time thereafter. Thus, the turnover (intermittent division) of memory CD8 T cells may be maintained by preserving minute quantities of IL-15 produced upon periodic minor infections until the original pathogen invades the body to recall the memorized immune response.

Most extracellular ligands undergo receptor-mediated internalization (33–35). Ligands are then degraded whereas the receptor components are recycled back to the cell surface for another round of signal transduction. Failure in this process could lead to pathogenic conditions in humans. A mutation in the low-density lipoprotein receptor makes cells incapable of degrading the ligand through endosomal internalization, leading to the accumulation of low-density lipoprotein in the circulation, which eventually causes hypercholesterolemia (36), which appears similar to the remarkable IL-15 retention observed in IL-15R α Tg mice. It would be interesting to design an IL-15 mutant that would retain full binding capability to IL-15R α under neutral pH conditions but would sharply lose binding affinity as the pH shifts to acidic, because such an IL-15 would help distinguish long-term vs. short-term IL-15 effects.

Does soluble IL-15 act *in vivo*? Although naïve and activated T cells and natural killer cells do not express IL-15R α , differentiated memory CD8 T cells may express IL-15R α (19, 37) and respond to soluble IL-15. Thus, it becomes important to determine whether soluble IL-15 and membIL-15 could transduce qualitatively different signals. Our data suggest that the signal mediated by membIL-15 persists longer than that generated by soluble IL-15 at doses generating similar impacts on β/γ -expressing CD8 T cells standardized by short-term DNA synthesis. Interestingly, Cornish *et al.* (31) recently demonstrated that IL-2 and soluble IL-15 transduce qualitatively different

signals, in that IL-2 signals are more persistent and result in proliferation and protein synthesis, thus making IL-2 act as a growth factor, whereas signals by soluble IL-15 decay quicker and primarily induce cellular proliferation, thus IL-15 acting as a pure mitogen. Our data suggest that membIL-15 may be a more comprehensive growth factor than soluble IL-15. It is possible that IL-2, soluble IL-15, and membIL-15 all have unique roles *in vivo*. We want to point out that IL-15 can still be presented to IL-15R α^{+} target cells (10). How do soluble and membrane IL-15 transduce different signals? This distinction may arise from the spatial distribution and temporal nature of receptor engagements across the cellular surface. Alternatively, longer and intimate occupation of the receptor by membIL-15 enabled by persistent cell-to-cell contact could be responsible.

Two recent publications (14, 18) have proposed that IL-15 and IL-15R α need to be expressed by the same cell for the transpresentation to occur, which may seem to contradict our proposal in this article. However, the assessment by those articles was limited to homeostatic maintenance of natural killer cells and CD44^{hi}CD8 T cells, in which IL-15 levels remain relatively low. After a dynamic production of IL-15 triggered by microbial intrusion, more IL-15 may be produced than just captured by the producer cells. It is also possible that these nonhematopoietic cells found in the lungs and other tissues may produce their own IL-15.

In this article we demonstrate unique features of IL-15 that are found with no other typical cytokine. These features enable a persistent *in vivo* activity of this cytokine as well as a prolonged signaling in CD8 T cells. These features may contribute to the long-term maintenance of memory CD8 T cells and control the homing of these cells to certain immunological portals.

Materials and Methods

Antibodies, Cytokines, and Related Reagents. Human and mouse recombinant cytokines were purchased from PeproTech (Rocky Hill, NJ). Cytokine ELISAs were from R & D Systems (Minneapolis, MN). Antibodies for flow cytometry were purchased from eBioscience (San Diego, CA). Anti-mouse IL-15R α polyclonal antibody (AF551) and anti-human IL-15 antibody (mAb 247) were from R & D Systems. AF551 was conjugated with fluorescein isothiocyanate (Sigma, St. Louis, MO) in house. Alexa Fluor 488 anti-phosphor S6 antibody was from Cell Signaling Technology (Danvers, MA). OVA (257–264; SIIN-FEKL)-tetramer was from Beckman-Coulter (Fullerton, CA). Poly I:C and LPS were from Sigma.

Cells. Cells were maintained in 10% FCS/RPMI medium 1640 (Invitrogen, Carlsbad, CA) supplemented with 2 mM glutamine, 5×10^{-5} M 2-mercaptoethanol, and appropriate antibiotics. *Ex vivo* DCs were derived by culturing bone marrow precursor cells with 20 ng/ml mouse granulocyte–macrophage colony-stimulating factor for 8–10 days. For the proliferation assay, cells were seeded in the culture medium at 25,000 cells per well, cultured with the cytokine for 24 h (CTLL-2) or 72 h (*ex vivo* CD8 T cells), and pulsed with 1 μ Ci (1 Ci = 37 GBq) of [³H]thymidine during the last 4 h.

Mice. Human IL-15R α Tg mice were generated in our facility by microinjecting a linearized plasmid fragment consisting of cDNAs encoding human IL-15R α , a bovine preprolactin signal peptide (10), SV40 polyA signal, and a human EF-1 α promoter into fertilized eggs of C57BL/6 mice.

IL-15R $\alpha^{-/-}$ mice (The Jackson Laboratory, Bar Harbor, ME) were backcrossed to the C57B6 strain for 10 additional generations in our facility. F₅ TCR Tg mice (expressing TCR specific for influenza NP68 peptide) were from the Taconic Farm (Hudson, NY). All of the animal experiment protocols were

