RESEARCH ARTICLE

Different dynamics of IL-15R activation following IL-15 *cis*- or *trans*-presentation

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ABSTRACT. Interleukin (IL)-15 is a cytokine critical for the homeostasis and the function of NK cells, NK-T cells, and memory CD8$^+$ T cells. IL-15 signals are delivered through the IL-15Rβ and the common γ (γ_c) receptor chains. The third receptor chain, IL-15Rα, confers specificity and high affinity for the cytokine. While IL-15 can activate with high affinity the trimeric receptor expressed by a target cell (*cis*-presentation), IL-15Rα is also known to *trans*-present IL-15 with high affinity to target cells expressing the IL-15Rβ/γ_c complex. In order to compare the IL-15 *cis*- and *trans*-presentation processes, and using a T cell line expressing both IL-15Rα/β/γ_c and IL-15Rβ/γ_c, we analyzed cell surface receptor chain down-modulation, cytokine internalization and signaling responses induced either with IL-15 (*cis*-presentation) or with RLI, a protein resulting from fusion between IL-15 and an extended IL-15Rα sushi domain, that mimics *trans*-presentation. Whereas IL-15 bound with high affinity to IL-15Rα/β/γ_c, RLI bound with a similar high affinity to IL-15Rβ/γ_c. The kinetics of cell surface IL-15R down-modulation were slower following RLI treatment than after IL-15 treatment, as were the kinetics of RLI internalization, which was slower than that of IL-15. IL-15 and RLI dose-dependently induced the activation of similar signaling pathways. However, the kinetics and duration of these activations were markedly different, RLI-induced signaling, being slower, but more prolonged than that induced by IL-15, although the final proliferative responses at 48 h were similar. These findings collectively indicate that IL-15 *cis*- and *trans*-presentation mechanisms lead to different dynamics of receptor activation and signal transduction, with *cis*-presentation inducing fast and transient responses, and *trans*-presentation inducing slower, more persistent ones. They provide clues for a better understanding of how IL-15 action is controlled, and how it plays a key role in the coordination between innate and adaptative immunity.

Keywords: IL-15 receptor, *trans*-presentation, binding, internalization, cell signaling

Interleukin-15 (IL-15) is a cytokine that was originally described as a soluble factor mimicking IL-2 functions *in vitro* [1]. Despite a functional redundancy initially demonstrated *in vitro*, subsequent experiments have indicated that IL-2 and IL-15 exert complementary actions *in vivo*. Although both cytokines play pivotal roles in innate and adaptive immunity, the major role of IL-2 now appears one of limiting T cell responses and promoting the development of regulatory T cells, whereas IL-15 appears to be critical for the development of NK and NK-T cells, the initiation of T cell division, and the survival of memory T cells [2-4].

Both cytokines belong to the four-α-helix-bundle family, their membrane receptors sharing two subunits: the IL-15Rβ (CD122) and the common γ (γ_c or CD132) chains [5]. The IL-15Rβ/γ_c receptor, such as that expressed by most resting T and NK cells, is a common intermediate-affinity receptor that can be activated by nanomolar concentrations of IL-2 or IL-15. The high-affinity IL-2 and IL-15 receptors chains (IL-2Rα or CD25, and IL-15Rα) confer cytokine specificity and enhance affinity for cytokine binding. The trimeric, high-affinity receptors can be activated with picomolar concentrations of either cytokine [6], whereas the single chains IL-2Rα and IL-15Rα bind respectively IL-2 with a low affinity (K_d = 10 nM), and IL-15 with a high affinity (K_d = 0.100 nM) [7].

The IL-15Rβ and γ_c chains bind intracellular signaling complexes, and signal through three major pathways:

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Jak/STAT, MAPK, and PI3K/Akt [8-10]. The private α receptors are not thought to play a major role in cell signaling. However, the IL-15Rα cytoplasmic domain has been described as interacting with TRAF2 and Syk kinase signaling molecules [11, 12], although the role of these associations has not been well established for IL-15 function *in vivo*.

IL-15 signals can also be delivered through an original mechanism called *trans*-presentation, in which IL-15Rα, expressed at the surface of IL-15 producer cells (dendritic cells, macrophages and epithelial cells), presents IL-15 in *trans* to responder cells (NK or memory $CD8^+$ T cells) bearing the IL-15Rβ/γ_c receptor [13-15]. This specificity is due to the capacity of the α chain to bind IL-15 with a high affinity in the absence of the IL-15Rβ and γ_c chains. As a co-stimulatory event occurring at the immunological synapse, IL-15 *trans*-presentation now appears to be a dominant mechanism for IL-15 action *in vivo* [14], and seems to play a major role in tumor immunosurveillance [16].

A soluble form of the human IL-15Rα (sIL-15Rα) is naturally released from IL-15Rα-expressing cells by a shedding process involving matrix metalloproteinases. This sIL-15Rα is able to bind IL-15 with high affinity, and efficiently blocks the proliferation driven by the high-affinity IL-15Rα/β/γ_c signaling receptor *in vitro* [17]. Notably, sIL-15Rα preserves the capacity to *trans*-present IL-15, and high concentrations of soluble complex IL-15/IL-15Rα can support NK cell activation *in vitro* and *in vivo* [18, 19].

We previously engineered a fusion protein, RLI, comprising the IL-15Rα binding domain linked to IL-15 [20]. This molecule is able to bind the IL-15Rβ/γ_c receptor with a high affinity [20, 21]. RLI highly stimulated the mobilization of NK cells in a mouse model, deficient for the trafficking of these cells [22]. The highly agonistic activity of RLI on the development and the differentiation of NK cells was demonstrated *in vivo* in an HIS mouse model [23]. In the B16-F10 melanoma model, RLI inhibited the development of lung and liver metastases, and also reduced metastatic progression in a model of HCT-116 human colorectal cancer in the nude mouse. The antitumoral effect of RLI was abolished by *in vivo* depletion of NK cells [24].

In the present study, we used RLI as a tool for studying *trans*-presentation. In order to compare IL-15 *cis*- and *trans*-presentation modes, we analyzed cytokine receptor expression, cytokine binding, and signaling responses in a T cell line expressing both IL-15Rα/β/γ_c and IL-15Rβ/γ_c.

METHODS AND MATERIALS

Cytokines and reagents

Recombinant human IL-15 (rIL-15) was purchased from Peprotech, Inc. (Rocky Hill, NJ, USA), and recombinant human IL-2 (rIL-2) was purchased from Chiron (Emeryville, CA, USA). RLI fusion protein was produced in baculovirus-Sf9 cells using Bac-to-Bac expression system (Invitrogen), and was purified on an anti-FLAG-agarose affinity column (Sigma-Aldrich), essentially as described previously [21]. Monoclonal mouse anti-human IL-15 (MAB247), polyclonal goat anti-human IL-15Rα (AF247), polyclonal goat anti-human IL-2Rβ (AF224-NA), and PE-conjugated donkey anti-goat IgG (F0107) were obtained from R&D Systems (Abington, UK). The control isotype IgG goat was purchased from Santa-Cruz Biotechnology. Monoclonal mouse anti-FLAG M2 conjugated to peroxidase was purchased from Sigma-Aldrich (St Louis, MO, USA). Polyclonal rabbit and mouse antibodies anti-phospho-STAT5 (#9351), anti-phospho-STAT3 (#9131), anti-phospho-Akt (#9271), anti-Akt (#9272), anti-phospho-p44/42 MAPK (Erk 1/2) (#9106), and anti-p44/42 MAPK (Erk 1/2) (#9102) were obtained from Cell Signaling Technology. Monoclonal mouse antibodies anti-STAT5 (610191), and anti-STAT3 (610190) were purchased from BD Transduction Laboratories, and monoclonal mouse anti-actin antibody (MAB1501R) was acquired from Millipore.

Cells and media

The Kit225 T lymphoma human cell line [25] was cultured in RPMI-1640 medium (Sigma-Aldrich), containing 6% heat-inactivated FCS (Gibco), 2 mM glutamine, and 325 pM human rIL-2. This cell line was maintained at 37°C, in a humidified, 5% CO_2 atmosphere.

Proliferation assays

The proliferation-inducing activity of rIL-15 and RLI was assessed using [^{3}H]-thymidine incorporation by Kit225 cells as described previously [26]. To measure the residual proliferative response of Kit225 cells after rIL-15 or RLI treatment, Kit225 cells were maintained for three days with 500 pM rIL-15 or RLI. Cells were washed, starved for 24 h in cytokine-deprived medium, and plated at 10^4 cells in 100 µL of cytokine-deprived medium. After 48 h, residual radioactivity was measured by [^{3}H]-thymidine incorporation.

Western blot analysis

Exponentially growing Kit225 cells were washed and serum-starved to reduce basal phosphorylation (16 h in cytokine-deprived medium and 3 h in serum-free medium supplemented with 0.5% BSA). After stimulation with rIL-15 or RLI under various conditions at 37°C, cells were suspended in ice-cold, phosphate-buffered saline (PBS, pH 7.4). Cell pellets were lysed by addition of ice-cold lysis buffer (20 mM Tris, pH 7.4, 150 mM NaCl, 1% glycerol, 1% NP-40, 20 µM Na_3VO_4, 10 mM NaF, 1 mM EDTA, 0.4 mM Pefablock with aprotinin and leupeptin at 1 µg/mL). After incubation on ice for 20 min, samples were centrifuged (13,000 rpm, 15 min, 4°C), and protein concentration was determined with a BC Assay Kit (Uptima) using BSA as standard. Fifty µg of total protein cell lysates were analyzed on 10% SDS-PAGE and 4-12% Bis-Tris Gels (Invitrogen), and the resolved proteins were transferred to Immobilon-P PolyVinylidene DiFluoride (PVDF) membranes (Millipore, Bedford, MA, USA). Membranes were blocked with 5% milk, 0.05% Tween-20 in PBS for 1 h at room temperature. Subsequently, membranes were immunoblotted with specific antibodies according to their

technical data sheet. After incubation with secondary HRP-conjugated anti-mouse/anti-rabbit antibody (Roche, Mannheim, Germany) for 1 h at room temperature, visualization of specific proteins was conducted with a chemiluminescence system using BM Chemiluminescence Blotting Substrate (Roche), according to the manufacturer's instructions. Densitometric evaluation of the Western blot data was performed with ImageQuant Software.

Binding assays and internalization

rIL-15 and RLI were radiolabeled with [^{125}I]-labeled iodine, using a chloramine-T method, to a specific radioactivity of approximately 2000 cpm/fmol for IL-15 and 4000 cpm/fmol for RLI. Kit225 cells were used for binding assays, and these experiments were performed essentially as described previously [27]. Briefly, cells were incubated for 1 h at 4°C with increasing concentrations of labeled rIL-15 or RLI. Non-specific binding was determined in the presence of a 100-fold excess of unlabeled rIL-15 or RLI, and subtracted from total binding. Regression analysis of the binding data was accomplished using one-site and two-site equilibrium binding equations (GraphPad PRISM Software), and data were plotted in the coordinate system. For internalization analysis, Kit225 cells were treated mostly as described previously [20]. In short, cells were equilibrated at 4°C for 1 h with 1 nM labeled rIL-15 or RLI. The temperature was then switched to 37°C, and, at different time intervals, two samples were washed and treated for 8 min either with ice-cold glycine-HCl buffer (0.2 M, pH 2.5) or with ice-cold PBS. Total ligand binding was determined from the pellet of the cells treated with PBS, whereas the membrane-bound and internalized fractions were determined, respectively, from the supernatant and pellet of cells treated at low pH.

Flow cytometry analysis

Kit225 cells were maintained in culture medium for three days, washed, and starved for 24 h in cytokine-deprived medium. Cells were incubated with 500 pM rIL-15 or RLI for 0 to 48 h at 37°C. They were next plated at 0.2×10^6 cells in 100 µL, washed twice with PBS/0.1% BSA and incubated for 1 h at 4°C with 10 µg/mL anti-IL-15Rα, anti-IL-15Rβ or control isotype IgG antibody. Cells were then washed three times with PBS/0.1% BSA and incubated for 30 min in the dark at 4°C with 1.25 µg/mL of PE-anti-goat IgG. They were washed three times with PBS/0.1% BSA and analyzed on a FACScan fluorocytometer (BD Biosciences) using FlowJo Software.

RESULTS

IL-15 and RLI present different cell surface receptor binding characteristics

In order to compare IL-15 *cis*- and *trans*-presentation modes, we chose a human T lymphoma cell line, Kit225, expressing both hIL-15Rα/β/γ_c and hIL-15Rβ/γ_c. Low doses of IL-15 were used to stimulate the IL-15Rα/β/γ_c high affinity receptor (*cis*-presentation), whereas the RLI fusion protein previously described [20], was used to mimic *trans*-presentation. As expected, [^{125}I]-IL-15 displayed high and low affinity binding sites corresponding respectively to its binding to the trimeric IL-15Rα/β/γ_c receptors (K_d = 0.037 nM; B_{max} = 295 sites/cell), and dimeric IL-15Rβ/γ_c receptors (K_d = 19.6 nM; B_{max} = 2784 sites/cell) *(figure 1A)*. By contrast, [^{125}I]-RLI bound to a single class of high affinity binding sites (K_d = 0.186 nM; B_{max} = 3067 sites/cell) as expected for the dimeric IL-15Rβ/γ_c receptors (*figure 1B*).

IL-15 and RLI induce different kinetics of cell surface IL-15R down-modulation and display different kinetics of internalization

Cell surface expression of IL-15Rα and IL-15Rβ chains was monitored by flow cytometry following IL-15 or RLI treatment. As shown in *figure 2A*, IL-15 treatment induced a rapid reduction of IL-15Rα cell surface expression, detectable as early as 15 min, reaching an almost complete disappearance of expression at longer incubation periods (24 h). By contrast, upon RLI treatment, IL-15Rα cell surface expression was almost unchanged during the first hours, but then decreased slightly up to 24 h. IL-15 treatment did not significantly affect the IL-15Rβ pool during the 24 h incubation period, whereas RLI induced a late decrease *(figure 2B)*. These results show that IL-15 and RLI both induce the disappearance from the cell membrane of IL-15 receptor chains, but with different kinetics, as the effect of IL-15 on IL-15Rα is much faster than that of RLI on IL-15Rβ.
Cytokine internalization was then monitored after equilibration of Kit225 cells with radio-iodinated cytokines and temperature switching from 4°C to 37°C *(figure 3)*. [^{125}I]-IL-15 and [^{125}I]-RLI were both found to be efficiently internalized (between 40% and 50% maximal internalization) but [^{125}I]-IL-15 internalization was very quick (Int_{50} = 1.1 min) *(figure 3A)* compared to that of [^{125}I]-RLI (Int_{50} = 23.1 min) *(figure 3B)*. This difference between [^{125}I]-IL-15 and [^{125}I]-RLI as regards kinetics of internalization, was comparable to that observed for the down-regulation of cell surface IL-15R *(figure 2A)*. The maximal number of [^{125}I]-IL-15 molecules internalized per cell was five times lower than the maximal number of [^{125}I]-RLI molecules internalized per cell *(figure 3C)*, reflecting the fact that high affinity IL-15Rα/β/γ_c accounts for a small proportion of the total IL-15R. This probably explains why IL-15 induction of IL-15Rβ internalization through IL-15Rα/β/γ_c could not be detected *(figure 2B)*.

IL-15 and RLI activate similar signaling pathways, but with different dose- and time-dependent patterns

In order to compare the signal transductions induced by IL-15 and RLI, activation of the Jak/STAT, PI3K/Akt and MAPKs pathways was monitored by studying the phosphorylation of STAT5, STAT3, Akt, and p44/42 MAPK (Erk 1/2). Firstly, Kit225 cells were stimulated for 15 min with increasing concentrations of IL-15 and RLI, ranging from 0 to 1500 pM *(figure 4)*. Both cytokines were shown to stimulate the three signaling pathways in a dose-dependent manner, and, for each cytokine, the activation profiles were identical for all proteins of the signaling cascades. However, the efficiency of both molecules was somewhat different. The maximum signaling intensities for IL-15 were obtained at 50 pM, whereas

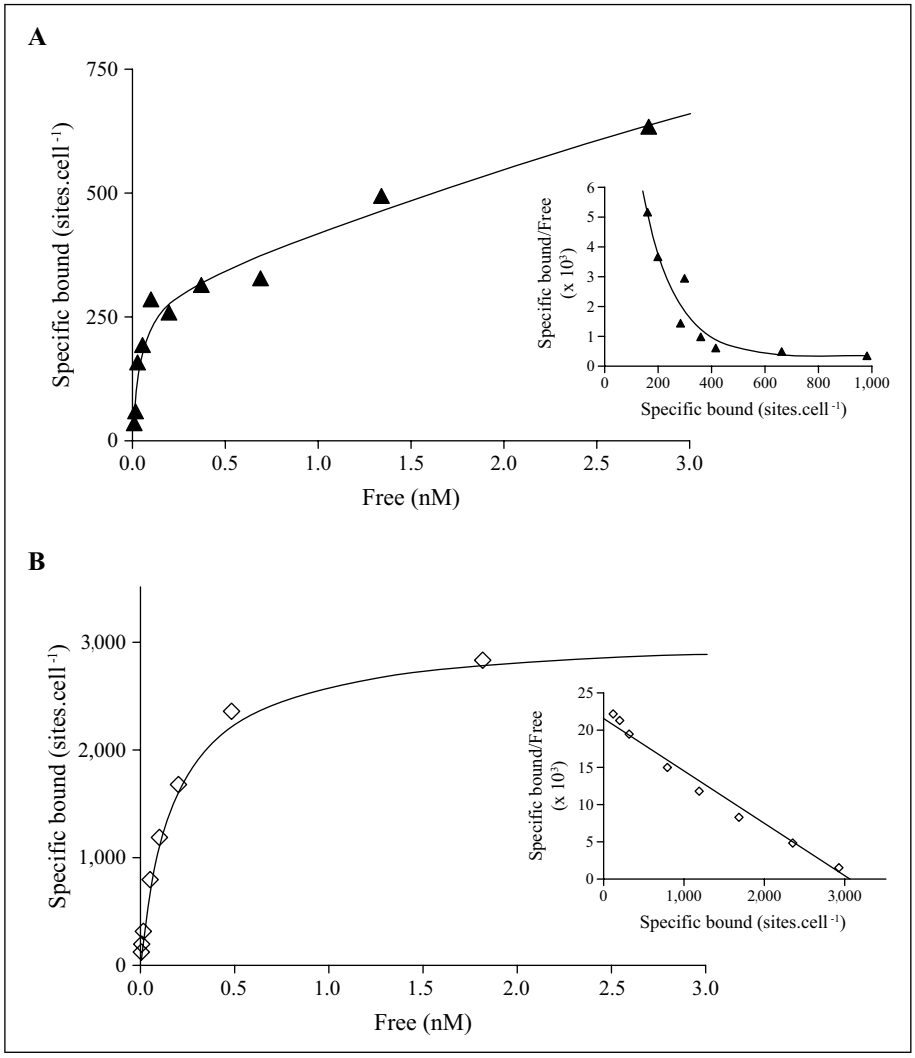

Figure 1

Binding properties of [^{125}I]-IL-15 and [^{125}I]-RLI on Kit225 cell receptors. Kit225 cells were incubated for 1 h at 4°C with increasing concentrations of labeled (**A**) [^{125}I]-IL-15 or (**B**) [^{125}I]-RLI. Specific binding was calculated by subtracting non-specific binding from total binding. Scatchard plots are shown in *insets*. Results are representative of three independent experiments.

at least 350 pM of RLI were necessary to reach similar intensities. IL-15 therefore seemed five to seven times more potent than RLI after a 15 min-incubation period.

We next compared the kinetic of the cell signaling induced by a maximal concentration of IL-15 (50 pM) or RLI (350 pM) at different time points between 0 and 48 h *(figure 5A, B)*. Here again, IL-15 and RLI induced the same activation profiles for all the signaling proteins: the quantification of densitometric scanning is shown only for STAT5. The signaling induced by IL-15 was strong, rapid (detectable as soon as 5 min, maximal by 15 min), and transient (strongly down-regulated after 1 h, and almost undetectable after 3-6 h). Signaling induced by RLI was as strong and rapid as for IL-15, but was far more persistent, being maintained at high levels until 16 h before then decreasing although still detectable at 48 h.

We have previously shown that IL-15 and RLI were able to induce identical proliferative responses by the Kit225 cell line over 48 h [20], with EC_{50} values in agreement with the activation of high-affinity receptors ($EC_{50} \sim 10^{-11}$ M). We therefore compared the signaling responses at identical (50 pM) concentrations of both molecules *(figure 5C, D)* over 48 h. The response to RLI was slower than that to IL-15 and was, again, more persistent. Calculation of the areas-under-curves (AUC) indicated that the integrated signals of STAT5 induction from 0 to 48 h were similar (11.4 and 16.2 units × hour after IL-15 and RLI treatments respectively), whether IL-15 or RLI was used as a stimulus, which is consistent with the similar 48 h proliferative responses induced by 50 pM IL-15 or RLI. When calculating the AUC at different IL-15 or RLI concentrations, they were found to be proportional to the proliferative responses (data not shown), suggesting that these responses are based on an integrative transmission mode rather than on the maximal intensity of signaling.

RLI induced a prolonged effect on cell proliferation after cytokine withdrawal

RLI, in contrast to IL-15, was still able to sustain cell signaling after 5 h of stimulation *(figure 5)*. In order to further document this persistence, we compared the residual proliferative responses of Kit225 cells after three days in the presence of 500 pM IL-15 or RLI, fol-

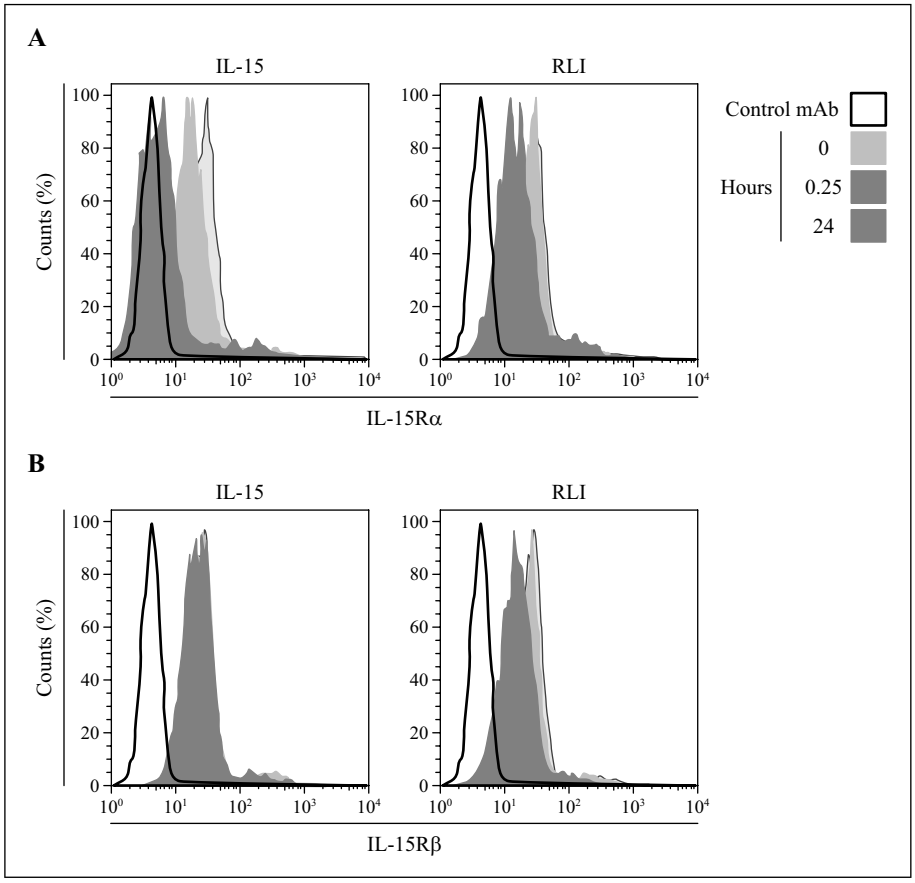

Figure 2
Cell surface down-modulation of IL-15Rα and IL-15Rβ after IL-15 or RLI treatment. Kit225 cells were treated with 500 pM IL-15 or RLI for 0 to 24 h at 37°C. They then were washed and incubated with anti-IL-15Rα and anti-IL-15Rβ antibodies. The expression levels of the receptors were analyzed by flow cytometry: (**A**) IL-15Rα expression after IL-15 or RLI stimulation, (**B**) IL-15Rβ expression after IL-15 or RLI stimulation. Data are representative of at least three experiments.

lowed by a 24-h starvation. We first verified that the specific activities of the cytokines were not affected during the three-day incubation period. As shown in *figure 6*, the residual proliferative response was significantly higher in the case of RLI, indicating a higher capacity of RLI versus IL-15, to induce long-term activation.

The sensitivity to RLI is more persistent than that to IL-15 upon re-stimulation

Having shown that IL-15 and RLI induced different kinetics of receptor chain down-modulation, cytokine down-regulation and signal transduction, we examined how this could impact the biological response upon cytokine re-stimulation. Kit225 cells, pre-treated or not with IL-15 for 24 h, were subsequently stimulated for 1 h with 50 pM IL-15 or RLI, and analyzed for their level of STAT5-phosphorylation *(figure 7)*. In the absence of IL-15 pre-treatment, and in agreement with *figure 5C*, pSTAT5 signals induced by IL-15 and RLI had similar, strong intensities. By contrast, cells having experienced a 24 h IL-15 pre-treatment had a markedly reduced secondary response to IL-15, while the secondary response to RLI was far less affected. This result is in agreement with a more pronounced down-regulation of IL-15Rα by IL-15 *(figure 2)* that mainly affects IL-15 high-affinity receptors and leaves most of the RLI receptors unaffected.

DISCUSSION

A number of reports have documented the existence of two modes of action of IL-15 (*cis*- and *trans*-presentation), and their relative importance in the context of immune activation has been widely discussed. In this study, we compared, on the same cell line, the effect of IL-15 *cis*-presentation on the high affinity IL-15Rα/β/γ_c to that of RLI, a protein resulting from the fusion between hIL-15 and hIL-15Rα that mimics the mechanism of IL-15 *trans*-presentation on the IL-15Rβ/γ_c complex. We provide evidence that these two modes of action of IL-15 are associated with different dynamics of receptor activation and signal transduction.

Kit225 cells expressed two classes of IL-15 binding sites: a majority (90%) of intermediate affinity ($K_d \sim 20$ nM) receptors corresponding to IL-15Rβ/γ_c, and a small proportion of high affinity ($K_d = 0.037$ nM) receptors corresponding to IL-15Rα/β/γ_c. On the other hand, the RLI fusion protein bound to a number of single class, high affinity ($K_d = 0.186$ nM) receptors, corresponding to the large pool of dimeric IL-15 receptors.

Analysis of IL-15 receptor chain internalization upon IL-15 *cis*- or *trans*-presentation, revealed different behavior. In the context of *cis*-presentation, IL-15 stimulation through IL-15Rα/β/γ_c induced a quick extinction of cell surface IL-15Rα, a result consistent with previous reports [28, 29], and reflecting a rapid IL-15Rα/β/γ_c internali-

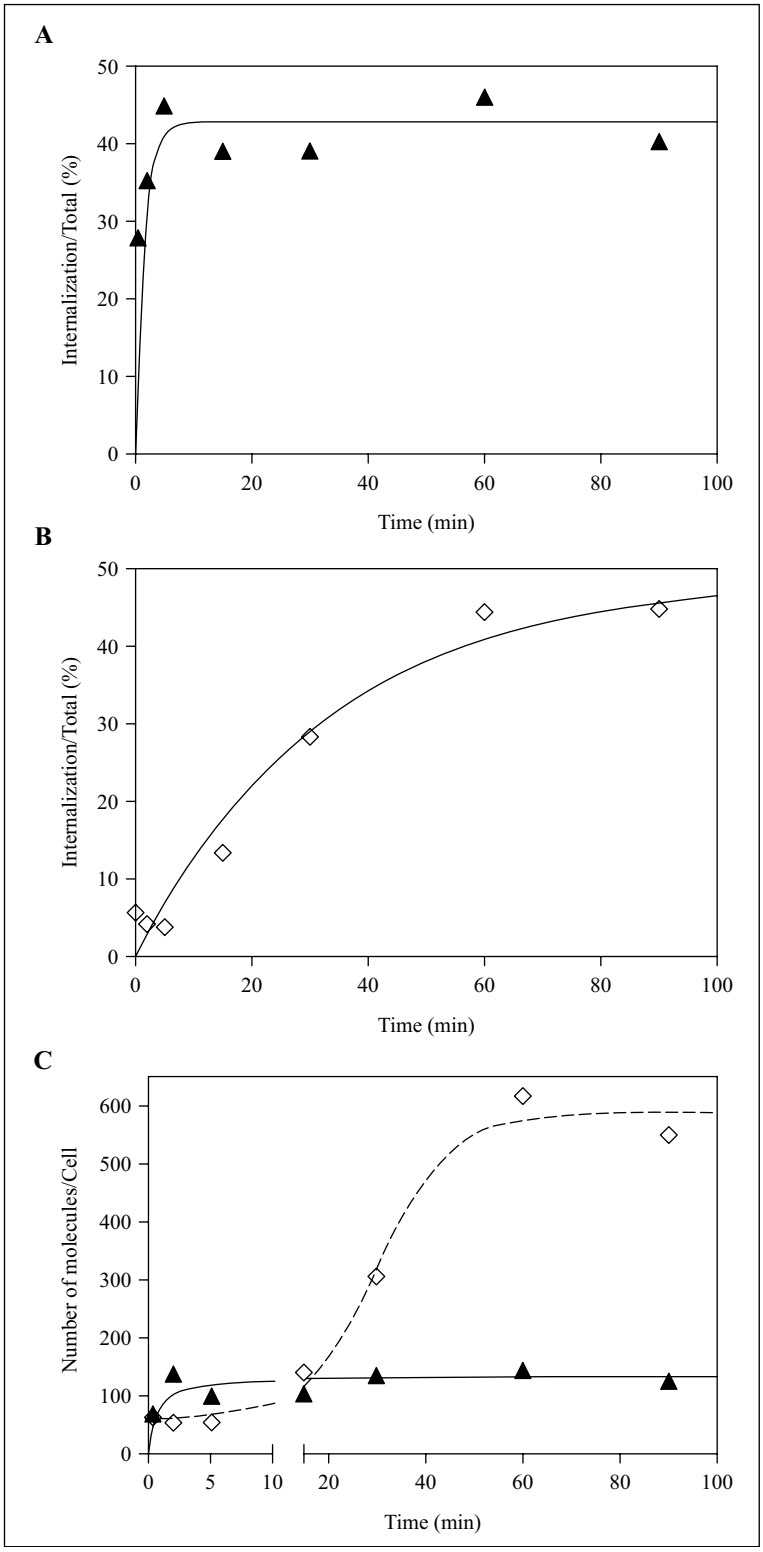

Figure 3
Characteristics of [^{125}I]-IL-15 and [^{125}I]-RLI internalizations. Kit225 cells were equilibrated for 1 h at 4°C with 1 nM labeled (**A**) IL-15 or (**B**) RLI. Temperature was then switched to 37°C and internalization was determined at different incubation times. (**C**) Results from (**A**) and (**B**) are shown in terms of number of internalized molecules per cell for [^{125}I]-IL-15 (▲) and [^{125}I]-RLI (◇). Results are representative of three separate experiments.

zation. A concomitant disappearance of cell surface IL-15Rβ chains could not be detected under our experimental conditions, as IL-15Rα/β/γ_c only accounts for 10% of IL-15Rβ-containing receptors. In the context of *trans*-presentation, RLI binding also led to the internalization of IL-15Rβ/γ_c, as revealed by the down-regulation of cell surface IL-15Rβ, but with kinetics that were far slower than those associated with the down-regulation of IL-15Rα by IL-15 (*cis*-presentation). Although RLI induced a slight decrease in cell surface IL-15Rα, the

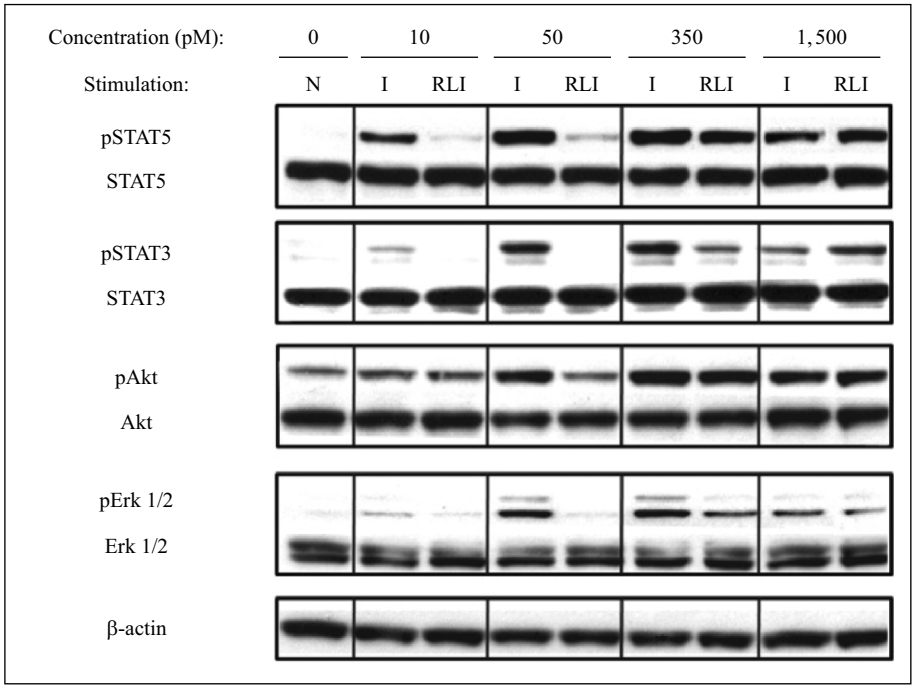

Figure 4

Dose-dependent induction of signal transduction by IL-15 and RLI. Kit225 cells were stimulated with IL-15 (I) or RLI at the indicated concentrations for 15 min at 37°C. Afterwards, cells were lysed, and STAT5, STAT3, Akt, and Erk 1/2 phosphorylations were analyzed by immunoblotting, β-actin was used as internal control. N: Not stimulated. Data are representative of three independent experiments.

fusion protein did not bind the IL-15Rα chain (G. B., data not shown). This finding is in agreement with the notion that IL-15Rα seems to be pre-associated or in close proximity with IL-15Rβ before cytokine binding. Such molecular proximity of IL-15Rα with the β and γ_c chains in lipid rafts on the surface of Kit225 cells has been suggested by flow cytometry and confocal microscopic FRET measurements [30].

Differences in the kinetics of cytokine internalization were also observed, and correlated well with those found for receptor internalization. RLI was indeed found to be internalized through IL-15Rβ/γ_c at a rate far lower than IL-15 through IL-15Rα/β/γ_c (half-time of maximal internalization for RLI > 20 times higher than for IL-15). The higher residual proliferative response found in the case of RLI, after cytokine withdrawal from the supernatant, was also consistent with a slower internalization rate for RLI. Together, these data showed that the kinetics of internalization of cytokine-receptor complexes were much slower in the context of *trans*-presentation than in the context of *cis*-presentation, suggesting differences in the molecular events involved. This could be linked to a specific role of IL-15Rα in contributing to a quick internalization of the IL-15Rα/β/γ_c complex in the context of *cis*-presentation. This could also be explained by the different interactions of receptor complexes with other membrane molecules, such as MHC I and II found in the molecular vicinity of IL-15R by FRET analysis [30], or with the adjacent cytoskeleton, as this was shown recently to be of major importance to the IL-7/IL-7R complex [31].

A number of studies have contributed to deciphering the signaling cascades associated with the activation of IL-15 receptors. The intracellular domains of the IL-15Rβ and γ_c chains are considered to be the major actors responsible for the initiation of signal transduction. They lead to the activation of multiple downstream pathways that include the Jak/STAT, Ras/MAPK/Erk, and PI3K/Akt pathways [32-34]. This study showed that these three main pathways were activated by both IL-15 and RLI; a more general analysis using a Phospho-Kinase array kit (R&D Systems), revealed no qualitative differences between IL-15- and RLI-induced protein phosphorylation (data not shown). These results therefore indicate that the same signaling pathways are activated in response to both *cis*- and *trans*-presented IL-15, which is in agreement with the known, common dependence of their biological responses on the IL-15Rβ/γ_c complex.

However, dose-dependent and kinetic analyses of signal transduction revealed major differences between IL-15 and RLI activation modes. The efficiency of IL-15 to stimulate the three signaling pathways after a 15 min-incubation period was found to be five to seven times greater than that of RLI. For instance, the maximal effect on STAT5 phosphorylation was observed with 50 pM and 350 pM of IL-15 and RLI respectively. This difference correlates with that found between the affinity constants of IL-15 for IL-15Rα/β/γ_c (K_d = 0.037 nM) and that of RLI for IL-15Rβ/γ_c (K_d = 0.186 nM). At these optimal concentrations (50 pM IL-15 and 350 pM RLI), the kinetics of induction of phosphorylation were rapid and very similar. Overall, these results suggest that the induction phase of the signaling response is dependent on the affinity of the IL-15R to both cytokines. At later time points, the duration of signal transduction was markedly different between IL-15 and RLI. IL-15-induced signaling disappeared quickly, whereas signaling persisted and decreased slowly in the case of RLI. These observations

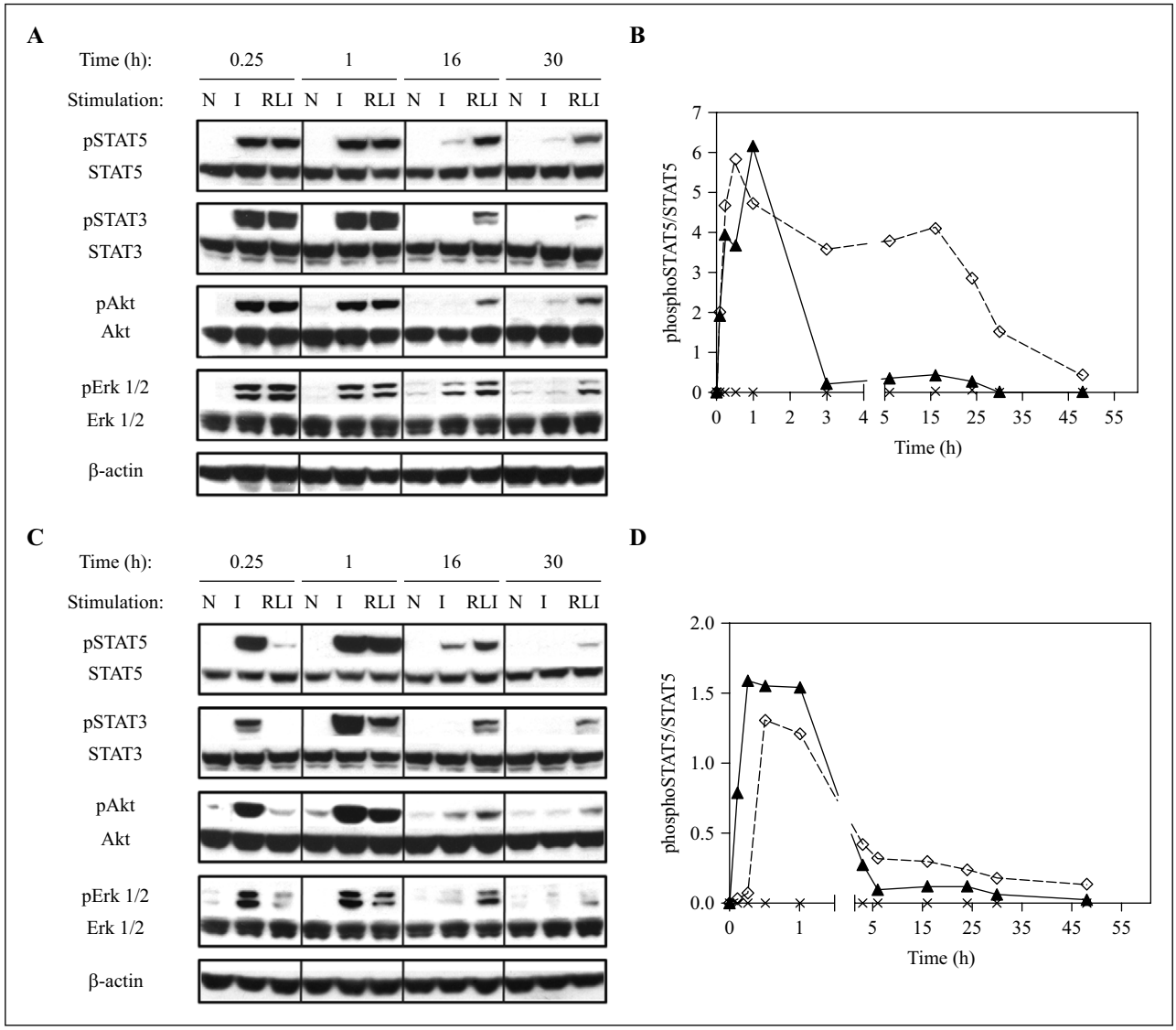

Figure 5

Kinetics of induction of signal transduction by IL-15 and RLI. Kit225 cells were stimulated with IL-15 (I) or RLI for 0 to 48 h at 37°C. Cells were treated as in *figure 4*, and intensities were measured by densitometric scanning of the spots with ImageQuant Software. N: Not stimulated. **(A)** Cells were stimulated with 50 pM IL-15 or 350 pM RLI, and **(B)** densitometric evaluation of STAT5 phosphorylation, upon stimulation with IL-15 (▲) or RLI (◇) or in the absence of stimulation (✕), was drawn (arbitrary units). **(C)** Cells were stimulated with 50 pM IL-15 or 50 pM RLI, and **(D)** STAT5 phosphorylation intensities were similarly drawn. All data are representative of three separate experiments.

are in agreement with those of Sato *et al.* [35], showing, *ex vivo*, that ribosomal S6 phosphorylation in $CD8^+$ T cells persisted longer following IL-15 *trans*-presentation by IL-15Rα$^+$-expressing DC cells than after stimulation by soluble IL-15 (120 h versus 24 h respectively). They also correlate with the kinetic differences that we observed for both ligand internalization (20 times faster for IL-15 than for RLI) and for down-regulation of cell surface receptor chains after cytokine stimulation (fast for IL-15 and slow for RLI), suggesting that the duration of signaling is related to the time of residence of the cytokine-receptor complex at the plasma membrane. These kinetic differences between IL-15 and RLI internalization and signaling were further analyzed for their impact on downstream biological effects. At IL-15 and RLI concentrations previously shown to induce similar proliferative dose-responses at 48 h, similar integrated signaling intensities over 48 hours were also found. More generally, the integrated intensities, as measured by the AUC, were found to be proportional to the concentration of the cytokine used. This suggests that the cellular proliferative response is based upon an integrative transmission mode (proportional to the AUC of signaling) rather than being based on the kinetics of signaling. However, these observations have to be confirmed in a more physiological context of *trans*-presentation involving an IL-15 *trans*-presenting cell and a responding cell.

Since free IL-15 cannot be detected in biological fluids under physiological conditions, it was suggested that IL-15 acts mainly as a membrane-associated protein bound to its high affinity IL-15Rα chain, IL-15Rα behaving as a necessary chaperone for the trafficking, production and secretion of IL-15 [36]. However, free-

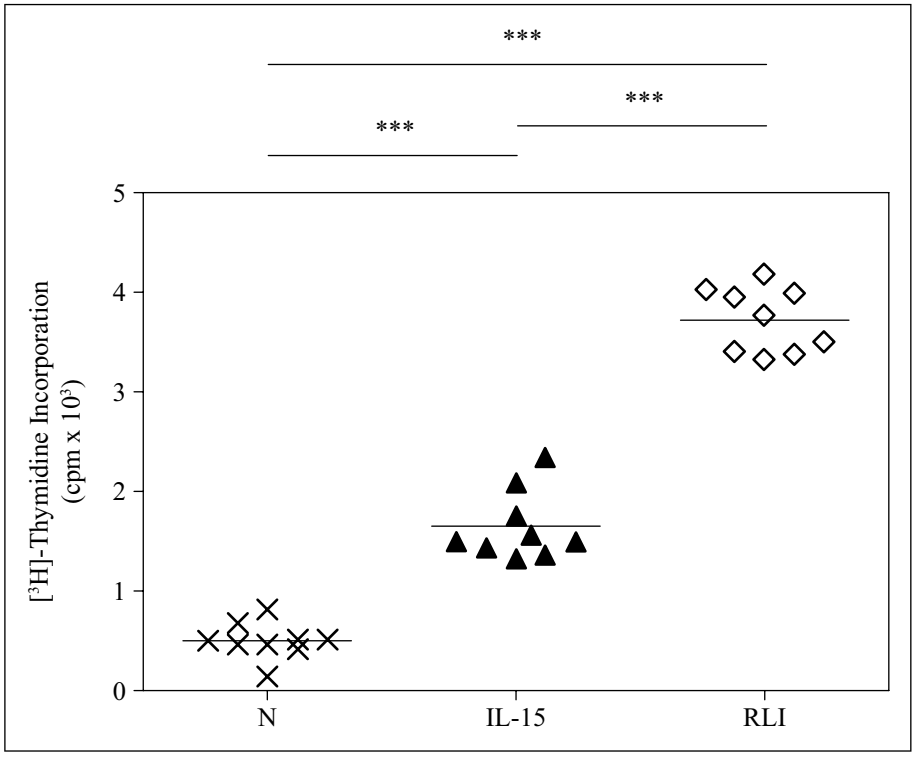

Figure 6
Residual proliferative responses of Kit225 cells after IL-15 or RLI stimulation. Kit225 cells were cultured for 72 h in the presence of 500 pM IL-15 (▲) or RLI (◇) at 37°C to achieve complete stimulation. Then, cells were washed and starved for a further 24 h. Their residual proliferative response was measured 48 h later by [^{3}H]-thymidine incorporation. N: Not stimulated (✕). Plots are representative of at least three separate experiments. *** $p < 0.001$.

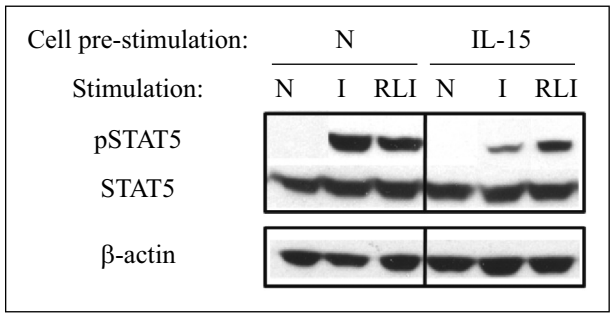

Figure 7
Induction of signal transduction upon cytokine re-stimulation. Kit225 cells were starved or pre-stimulated with 300 pM IL-15 for 24 h. Then, they were washed and stimulated with 50 pM IL-15 (I) or RLI for 1 h at 37°C. Cells were lysed and STAT5 phosphorylation was analyzed by immunoblotting, β-actin was used as internal control. N: Not stimulated. Data are representative of three independent experiments.

circulating IL-15 can be detected in the serum of patients with inflammatory and auto-immune diseases, or pathogen infections [37-39], suggesting that it may also function by *cis*-activation of the heterotrimeric IL-15R. A number of reports have documented this mode of action [30, 40, 41]. A structural model has been proposed [41] in which the highly flexible nature of the linker and/or proline-threonine-rich region of IL-15Rα allows the presentation of IL-15 in both *cis*- and *trans*-modes. Thus, IL-15 could be *cis*-presented, particularly in inflammatory situations. The early and rapid up-regulation of the expression of the IL-15Rα chain should allow a fast expansion of antigen-responsive T and NK cells and a strong immune response [42]. Afterwards, fast IL-15 down-regulation of its IL-15Rα chain, as shown in this study, would avoid harmful consequences due to excessive activation, including overproduction of inflammatory cytokines, extensive cell lysis, and incoherent adaptative cell responses. Pillet *et al.* [43] showed *in vitro* that human NK cell sensitivity to free IL-15 is increased in early activation stages, whereas their response is redirected at later stages toward IL-2 and *trans*-presented IL-15. The authors described a sequential expression of IL-15Rα and IL-2Rα, which may play a key role in coordinating the innate and adaptive branches of the immune system. The slower down-regulation of the IL-15Rβ/γ_c complex, as highlighted in our study, would then allow the cell to remain competent for IL-15 *trans*-presentation, a process that has been shown to be important for the long-term maintenance of antigen-memory cells.

We have recently shown that levels of a soluble form of IL-15Rα (sIL-15Rα) are elevated in the serum of head and neck cancer patients [44], and increased in the serum of patients with Crohn's disease that respond to infliximab treatment [39]. The sIL-15Rα protein, generated by proteolytic cleavage or through the expression of an alternative spliced variant of IL-15Rα [17, 45, 46], can act as a chaperone of IL-15, enhancing its biological activity. The soluble IL-15Rα could therefore *trans*-present IL-15 to responding cells without the need for cell-cell contact. In addition, sIL-15Rα has also been shown to increase the half-life of IL-15, and could therefore facilitate the diffusion of the cytokine and its action on remote cells and tissues expressing the IL-15Rβ/γ_c

receptor [19]. Similarly, IL-6 is known to bind a naturally occurring, soluble form of the IL-6R chain to form a complex that can stimulate cells expressing the signal transducing gp130 protein in the absence of IL-6R [47]. This mechanism, termed IL-6 "*trans*-signaling", is involved in the maintenance of the disease state of many chronic inflammatory diseases [48]. In view of our results, it would be interesting to explore whether IL-6 *cis*- and *trans*-signaling also involve different dynamics of receptor activation.

In summary, our present results demonstrate that IL-15 *cis*- and *trans*-presentation modes lead to different kinetics of receptor expression, cytokine internalization, and sequential cell signaling. This controlled distribution of IL-15, spatially and temporally, may constitute a program limiting the unwanted consequences of a powerful cytokine. *Cis*- and *trans*-presenting modes could equally play a key role in the coordination between innate and adaptative immunity. This work provides clues for a greater understanding of the IL-15 system, and consequently the design and optimization of immunotherapeutic treatments based on the use of cytokines as adjuvants.

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