High antitumor activity of RLI, an interleukin-15 (IL-15)–IL-15 receptor α fusion protein, in metastatic melanoma and colorectal cancer

Anne Bessard,[1,2] Véronique Solé,[1,2] Grégory Bouchaud,[1,2] Agnès Quéméner,[1,2] and Yannick Jacques[1,2]

[1]Institut National de la Santé et de la Recherche Médicale U892, Centre de Recherche en Cancérologie Nantes/Angers, Groupe Cytokines et Récepteurs; [2]Université de Nantes, IFR26, Nantes, France

Abstract

Interleukin (IL)-15 has an important role in tumor immunosurveillance and has a contemplated use in tumor immunotherapy. We have previously engineered the fusion protein RLI, composed of the NH_2-terminal (amino acids 1–77, sushi+) domain of IL-15 receptor α coupled via a linker to IL-15, and shown that it displayed far better efficacy than IL-15 *in vitro*. In this report, we investigated *in vivo* whether RLI would be a better alternative than IL-15 and IL-2 for cancer treatment using two distinct animal models. B16F10 mouse melanoma cells were injected in C57BL/6 mice either i.v. or intrasplenically for lung or liver metastasis, respectively. HCT-116 human colorectal cancer cells were injected in the cecum of nude mice. We show that RLI has a higher efficiency than IL-15 or IL-2 to reduce lung and liver metastasis and enhance survival in the mouse B16F10 melanoma model, a result that was associated with a higher half-life *in vivo*. We also found that the antitumoral effect of RLI was completely abolished by *in vivo* depletion of natural killer cells using anti-asialoGM1 antibody. Moreover, RLI was also efficient to reduce by 50% tumor growth and the progression of metastasis of human colon carcinoma cells in an orthotopic nude mouse model. The fusion protein RLI has revealed strong anticancer effect in two different cancer models overcoming the limited effect of IL-15 by increasing its bioavailability and efficiency. These findings hold significant importance for the use of RLI as a potential adjuvant/therapeutic. [Mol Cancer Ther 2009;8(9):2736–45]

Received 3/25/09; revised 7/9/09; accepted 7/28/09; published OnlineFirst 9/1/09.

Grant support: Institut National de la Santé et de la Recherche Médicale, Centre National de la Recherche Scientifique, Association de la Recherche Contre le Cancer, and Région Pays de Loire (mAbImpact and CIMATH networks; A. Bessard and V. Solé). G. Bouchaud is a recipient of a fellowship from the Ministère de la Recherche et des Nouvelles Technologies and the Association de la Recherche Contre le Cancer.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Note: A. Quéméner and Y. Jacques contributed equally to this work.

Requests for reprints: Anne Bessard, Institut National de la Sante et de la Recherche Medicale U892, Institut de Recherche Thérapeutique, 8 quai Moncousu, BP 70721, 44007 Nantes Cedex 1, France. Phone: 33-2-28-08-03-05; Fax: 33-2-28-08-02-04. E-mail: anne.bessard@univ-nantes.fr

doi:10.1158/1535-7163.MCT-09-0275

Introduction

Interleukin (IL)-15 is a cytokine of the four α-helix bundle family, structurally related to IL-2 (1, 2). It shares with IL-2 similar *in vitro* biological properties due to the common usage by the two cytokines of the IL-2/IL-15Rβ and γc receptor chains (3). In addition, IL-2 and IL-15 each uses a private α chain (IL-2Rα and IL-15Rα) that confers cytokine specificity and enhances the affinity of cytokine binding (4, 5). Although both cytokines play pivotal roles in innate and adaptive immunity, the major role of IL-2 is to limit continuous expansion of activated T cells, whereas IL-15 seems to be crucial for initiation of T-cell division, development of natural killer (NK) cells, and generation and maintenance of $CD8^+$ memory T cells (6–9).

The antitumor roles of IL-15 have been shown in several experimental models using IL-15 or *IL-15* gene as an adjuvant for vaccination, in conjunction with adoptive T-cell immunotherapy or in systemic cytokine therapy. Ugen et al. (10) showed the therapeutic antitumor potential of IL-15–expressing plasmid when delivered intratumorally into established s.c. B16 melanoma tumors in C57BL/6 mice. Intratumoral injection of IL-15 strongly increased efficacy of vaccines secreting IL-12, granulocyte macrophage colony-stimulating factor, tumor necrosis factor α (TNFα), and IL-6/soluble IL (sIL)-6R, otherwise being frequently inefficient (11, 12). *IL-15* gene transfer suppressed tumor metastasis in a murine metastatic lymphoma model, and the cotransfection by *IL-15* and *IL-21* genes was shown to protect completely from lethal i.v. challenge with the lymphoma cells, which was not achieved by administration of either cytokine gene alone (13). The antitumor effects of *IL-15* gene transfer were also evidenced in the human N592 MHC class I–negative small cell lung cancer cell line xenotransplanted in nude mice, with a complete rejection of N592 cells observed on cotransfection of the *IL-15* and *IL-12* genes (14). Daily administration of IL-15 prolonged the period of remission induced by cyclophosphamide in mice bearing rhabdomyosarcoma (15). In a 4T1 mammary carcinoma mouse model, coadministration of intralesional IL-7 and IL-15 following radiofrequency thermal ablation of tumor inhibited tumor development and lung metastasis (16). IL-15 protected against toxicity

and potentiated the antitumor activity of 5-fluorouracil and irinotecan in rats bearing colorectal cancer (17, 18). Moreover, IL-15 improved the *in vivo* antitumor activity of adoptively transferred $CD8^+$ T cells (19).

Recently, IL-15 has been ranked as the best agent with high potential for use in treating cancer (20). However, high doses of IL-15 are necessary to achieve biological response *in vivo* (21, 22). Several studies have shown that the dominant mode of action of IL-15 *in vivo* is not through activation by sIL-15 of the trimeric IL-15Rα/β/γ receptor (*cis*-activation) but rather through a transpresentation mode in which IL-15 bound to IL-15Rα expressed by antigen-presenting cells activates the IL-15Rβ/γ complex expressed by neighboring naive T or NK cells (23). Moreover, recent studies have shown that IL-15 secretion is highly dependent on its intracellular preassociation with IL-15Rα, suggesting that the IL-15/IL-15Rα complex is the biologically dominant-active form (24). Accordingly, several studies revealed that the biological activity of IL-15 could be enhanced ~50-fold *in vivo* by coadministration with sIL-15Rα-Fc (25–28). Binding of sIL-15Rα-Fc to IL-15 also extended the half-life of IL-15 *in vivo*. Compared with IL-15, IL-15/IL-15Rα-Fc complexes induced robust proliferation of memory $CD8^+$ T cells, NK cells, and NK T cells *in vitro* and *in vivo*; increased cytotoxicity of NK cells; and dramatically reduced tumor burden in B16 melanoma mouse model (25–28).

In an attempt to mimic the IL-15/IL-15Rα complex, we have previously engineered a fusion protein (RLI) that consists of the NH_2-terminal (amino acids 1–77, sushi+) domain of IL-15Rα coupled via a 20–amino acid linker to IL-15. This fusion protein was shown to exert higher biological activities than IL-15 or even the noncovalent association of IL-15 with the sIL-15Rα *in vitro* to drive cell proliferation through the IL-15Rβ/γ receptor (29) and *in vivo* to promote mobilization and expansion of NK cells (30). In the present study, we further investigated the interest of RLI as an antitumoral agent in two mouse models: a syngenic model (lung and liver metastasis of the B16F10 mouse melanoma cells in C57BL/6 mice) and a xenogenic model (tumor growth and metastasis of HCT-116 human colon cancer cells in nude mice).

Materials and Methods

Cells and Culture Conditions

The Mo7e myeloid leukemia human cell line was cultured in RPMI 1640 (Sigma) containing 10% heat-inactivated FCS, 2 mmol/L glutamine, and 10 ng/mL granulocyte macrophage-colony stimulating factor (R&D Systems). The Kit225 T lymphoma human cell line was cultured in RPMI 1640 containing 6% FCS, 2 mmol/L glutamine, and 10 ng/mL human recombinant IL-2 (Chiron BV). Murine B16F10 melanoma cells and human HCT-116 colorectal cancer cells were maintained in RPMI 1640 supplemented with penicillin (100 IU/mL), streptomycin (100 μg/mL), and 10% FCS. All cells were maintained at 37°C under a humidified 5% CO_2 atmosphere. Cells were harvested using trypsin/EDTA, and for *in vivo* experiments, cells were resuspended in RPMI 1640 alone.

Cytokines and Reagents

Recombinant human IL-15 was purchased from PeproTech, Inc. or from CellGenix. ILR, RLI, and sushi+ were produced as described previously (29, 31). ILR and RLI are two fusion proteins linking IL-15 and the sushi+ domain. For the two constructions, a flexible linker was introduced between the COOH terminus of IL-15 and the NH_2 terminus of the sushi domain (ILR) or vice versa (RLI). Proleukin (recombinant IL-2, Aldesleukine, 18×10^6 IU) was purchased from Chiron BV.

Rabbit polyclonal anti-asialoGM1 antibody (Wako Chemicals GmbH) was reconstituted in 1 mL sterile water according to the manufacturer's instructions. Control rat IgG was purchased from Indicia Biotechnology.

Proliferation Assays

The proliferative responses of Mo7e or Kit225 cells to IL-15, RLI, or ILR were measured by [^{3}H]thymidine incorporation, as described (32), in the presence of anti–IL-15Rβ A41 antibody (66 nmol/L) for Kit225.

Mice

Seven- to 8-wk-old male C57BL/6 and athymic female nude mice (NMRI *nu/nu*) were purchased from Janvier. Mice were maintained under pathogen-free conditions. Experiments were carried out in accordance with French laws and regulations.

Lung and Liver Metastasis in the B16F10 Melanoma Model

C57BL/6 mice were inoculated with 1×10^5 B16F10 cells via tail vein on day 0. Treatments were done at days 1, 2, and 10 by i.p. injections of saline, 2 μg RLI, 2 μg ILR, 0.8 μg sushi+, 1.2 μg IL-15, or the association of sushi+ and IL-15. For dose-response experiments, mice were treated with 1 to 10,000 pmol of either RLI (0.05–8 μg), IL-15 (0.03–4.8 μg), or IL-2 (0.2–80 μg). When necessary, rabbit polyclonal anti-asialoGM1 antibody or control rat IgG was injected i.p. into mice (30 μL of antibody in 70 μL of saline) on days −2, 1, 5, 12, and 19. In a first set of experiments, mice were sacrificed on day 21, lungs were removed, and metastatic tumors (black nodules) were counted under a binocular microscope. In another set of experiments, mice were monitored for survival on a daily basis for up to 90 d after inoculation.

For induction of liver metastasis, C57BL/6 mice were injected intrasplenically with 0.6×10^6 B16F10 cells, and 5 min after the injection, the spleen was removed. On days 1, 3, 7, and 10, the animals were treated with RLI (2 μg, i.p.) or vehicle. Thirteen days after tumor cell inoculation, the mice were sacrificed and the liver weight was recorded for evaluation of tumor metastasis.

Flow Cytometric Analysis of *In vivo* NK Depletion

Selective NK depletion was confirmed by using a FACSCalibur flow cytometer (Becton Dickinson) on day 21. The mouse splenocytes were incubated with saturating amounts (1 μg/10^6 cells) of phycoerythrin-conjugated anti-mouse NK1.1 monoclonal antibody (mAb) and FITC-conjugated anti-mouse CD3e mAb (BD Pharmingen). Data were processed using FlowJo software (BD Biosciences).

Pharmacokinetic Experiments

The pharmacokinetic behavior of RLI, IL-2, and IL-15 following single i.p. doses was evaluated in C57BL/6 mice. The blood was collected and immediately centrifuged, and the plasma was frozen at −20°C until analysis by ELISA assays. Three samples for plasma drug concentrations were obtained at each time points, ranging from 0 to 72 h. Pharmacokinetic parameters were calculated using a one-compartment model with GraphPad Prism software.

ELISA for Detection of IL-2, IL-15, RLI, TNFα, and Prostaglandin E_2

Concentration of human IL-2 and mouse TNFα in mouse sera was evaluated by eBioscience ELISA kits according to the manufacturer's recommendations (CliniSciences). The concentration of the fusion protein RLI was measured by ELISA with the anti–IL-15 mAb 247 (R&D Systems) as the capture antibody and the anti-FLAG-M2-peroxidase conjugate (Sigma) as the revealing antibody. For IL-15 detection, the same capture antibody as RLI was used but the revealing antibody was a specific anti–IL-15 biotinylated antibody kindly provided by Dr. H. Gascan (Institut National de la Santé et de la Recherche Médicale U564, Angers, France) followed by incubation with streptavidin-peroxidase. Prostaglandin E_2 levels in serum were measured by ELISA kit according to the manufacturer's instructions (R&D Systems).

Orthotopic Implantation of HCT-116, a Human Colorectal Cancer Cell Line

Nude mice were anesthetized with ketamine and xylazine. After exteriorizing the cecum, 1×10^6 HCT-116 cells/100 μL RPMI 1640 were injected slowly into the cecal wall. The cecum was returned to the abdominal cavity and the wound was closed with surgical grapes. One day after the cecal injection, groups of mice were randomly assigned to receive saline (control) or RLI treatments (2 μg, i.p. thrice per week) for 4 wk. The body weight was recorded throughout the treatment. The mice were killed on day 28, and the presence of local tumor at the injection site and in other organs (mesenteric lymph nodes, peritoneum, liver, spleen, and lungs) was investigated. Scoring of tumor development was achieved using the four-stage system developed by Cespedes et al. (33) and by a new system that we defined by scoring each organ for the presence of tumor or foci, the final stage corresponding to the sum of all organ scores.

Statistical Analysis

The data are presented as mean ± SEM. The animal survival data were analyzed using Kaplan-Meier survival analysis. Statistical analysis used the two-tailed unpaired Student's *t* test for comparison of two experimental groups (HCT-116 experiments) and one-way ANOVA followed by Dunnett's multiple comparison's post-test for melanoma experiments. *P* values of <0.05 were considered significant.

Results

RLI Is More Efficient than ILR and IL-15 for Activating IL-15Rβ/γ and IL-15Rα/β/γ *In vitro*

We first compared the effects of the two fusion proteins RLI and ILR with that of IL-15 for activating the human IL-15Rβ/γ and IL-15Rα/β/γ receptors. On the Mo7e cell line that expresses human IL-15Rβ and IL-15Rγ but not IL-15Rα (Fig. 1A), the three proteins at saturation induced

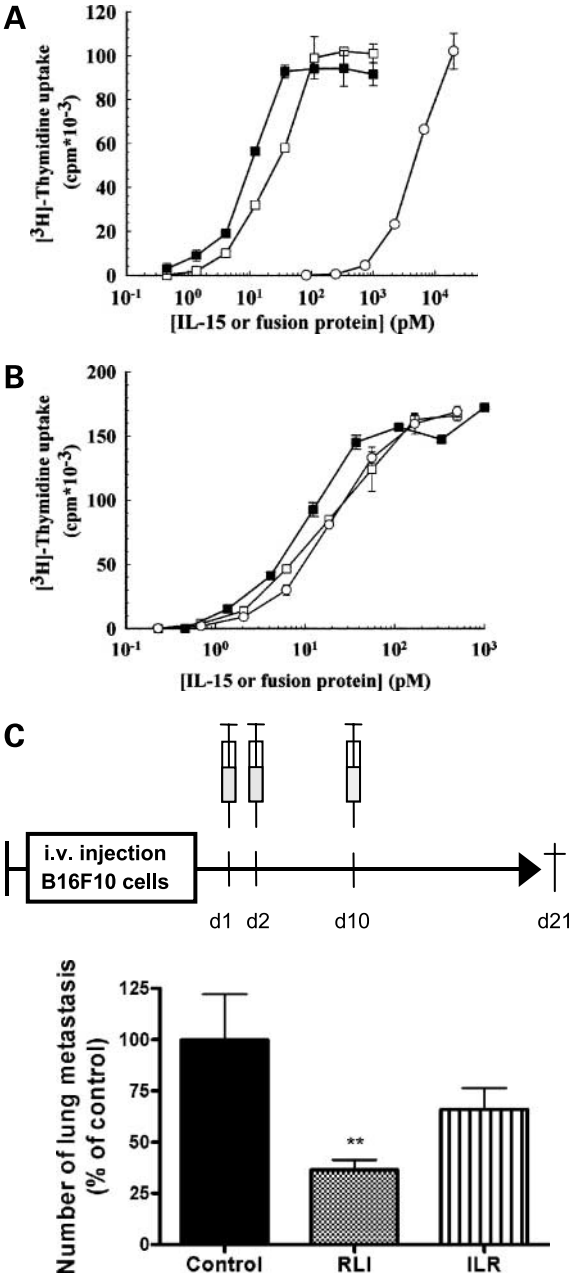


Figure 1. Comparison of RLI and ILR. Cell proliferation was evaluated by incorporation of [^{3}H]thymidine. **A,** Mo7e cells were cultured with increasing concentrations of human recombinant IL-15 (○), RLI (■), or ILR (□). **B,** Kit225 cells were incubated with increasing concentrations of IL-15 (○), RLI (■), or ILR (□) in the presence of a fixed concentration of A41 (66 nmol/L). *Points,* mean of triplicate wells and are representative of two independent experiments; *bars,* SD. **C,** *top,* schematic of the regimen in mice bearing i.v. B16F10 melanoma cells (1×10^5). Mice were given three doses of either saline (*Control*; $n = 22$), RLI (2 μg; $n = 22$), or ILR (2 μg; $n = 26$) i.p. At day 21, B16 nodules on the lungs were counted. *Columns,* mean; *bars,* SEM. **, $P < 0.01$ versus control, Dunnett's multiple comparison test.

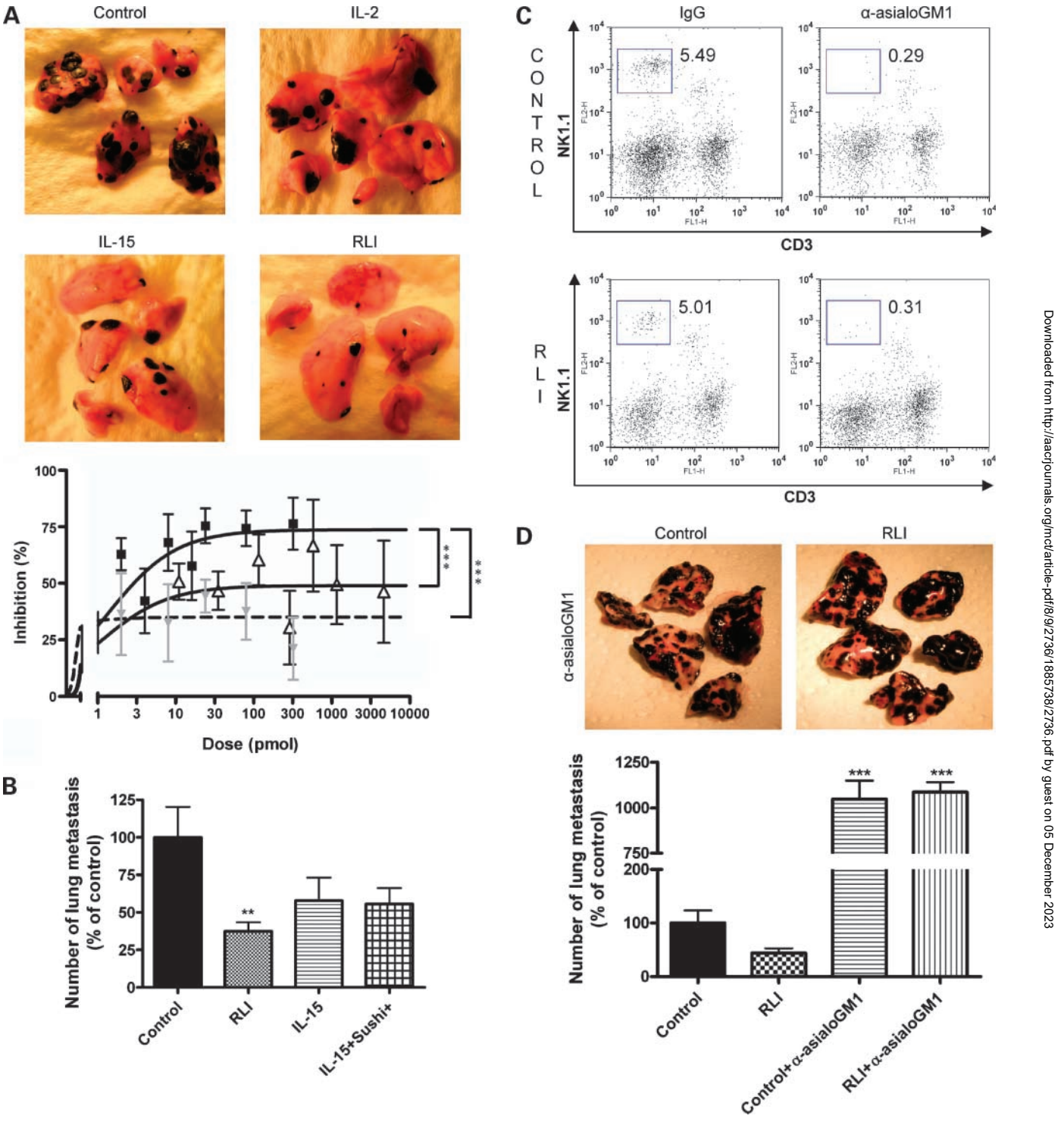


Figure 2. Effect of IL-2, IL-15, and RLI in the B16F10 mouse melanoma model. **A,** mice were treated with RLI, IL-15, IL-2, or saline on days 1, 2, and 10. On day 21, mice were sacrificed and tumors (black nodules) were counted. *Top,* representative pictures of lungs; *bottom,* dose-response curves of RLI (■), IL-15 (▼), and IL-2 (Δ) on lung metastasis. Data are expressed as percentage of inhibition versus control (saline). Maximal effects are statistically different. ***, $P < 0.001$, F test. **B,** RLI (2 μg) or IL-15 (1.2 μg) alone or preassociated with sushi+ (0.8 μg) was administered i.p. on days 1, 2, and 10. *Columns,* mean of each group ($n = 18$); *bars,* SEM. **, $P < 0.01$ versus control, Dunnett's multiple comparison test. **C** and **D,** *in vivo* NK cell depletion with α-asialoGM1 on days −2, 1, 5, 12, and 19 after i.v. injection of B16F10 cells. Mice were treated with RLI or saline on days 1, 2, and 10. On day 21, mice were sacrificed. **C,** the isolated splenic lymphocytes of mice were stained using FITC-conjugated anti-mouse CD3e and phycoerythrin-conjugated anti-mouse NK1.1 mAbs and then analyzed using flow cytometry. **D,** *top,* representative imaging of lung metastasis in the presence of α-asialoGM1 antibody; *bottom,* lung metastasis was counted. ***, $P < 0.001$ versus control, Newman-Keuls multiple comparison test.

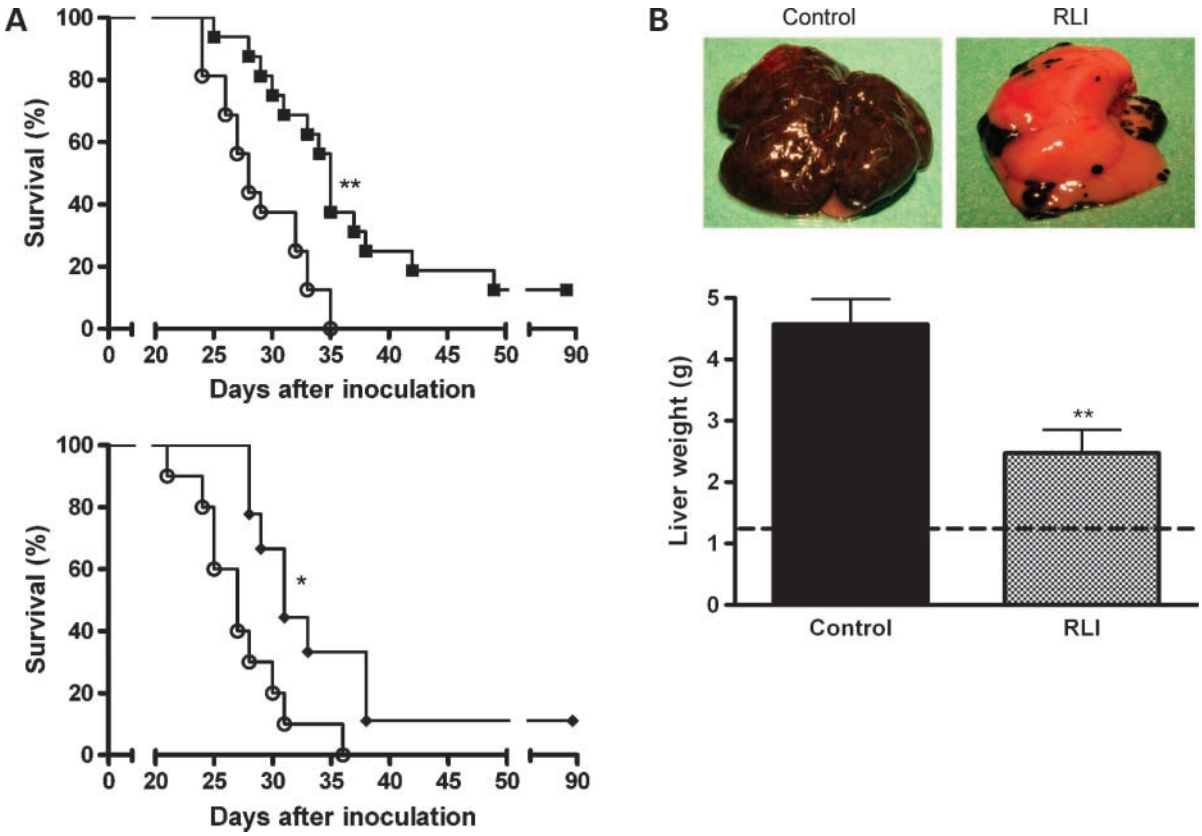


Figure 3. **A,** Kaplan-Meier survival analysis of mice i.v. injected with B16F10 cells. *Top,* mice were treated with RLI (■; 2 μg) or saline (○) on days 1, 2, and 10 ($n = 16$); *bottom,* mice received 12 i.p. injections (twice per week) of either RLI (♦; 2 μg) or saline (○) for long-term treatment ($n = 10$). *, $P < 0.05$; **, $P < 0.01$ versus control in log-rank test for prolonged survival. **B,** mice were injected intrasplenically with 0.6×10^6 B16F10 cells and treated i.p. with RLI (2 μg) or saline on days 1, 3, 7, and 10. On day 13, mice were sacrificed. *Top,* representative metastasized liver of RLI-treated groups (*right*) and saline-treated groups (*Control, left*); *bottom,* in addition, the liver weight was measured. The weight average of normal livers is 1.2 g and is represented with the dotted line. **, $P < 0.01$ versus control, Student's *t* test.

similar maximal levels of cell proliferation. However, based on the doses necessary to induce half-maximal proliferation (EC_{50}), RLI was found 2-fold more active than ILR and ~500-fold more efficient than IL-15. We also analyzed the proliferation of the human lymphoma cell line Kit225 that expresses endogenous IL-15Rα, IL-15Rβ, and IL-15Rγ (Fig. 1B). This study was conducted in the presence of a saturating concentration of A41, an anti–IL-15Rβ mAb that we previously shown to inhibit IL-15 activation of the IL-15Rβ/γ receptor without affecting that of the IL-15Rα/β/γ receptor (34). Under these conditions, RLI was found 2-fold more efficient than ILR or IL-15 in inducing cell proliferation through IL-15Rα/β/γ (Fig. 1B).

RLI Is More Efficient than ILR, IL-15, IL-15 plus sIL-15Rα, or IL-2 to Reduce the Metastatic Potential of B16F10 Mouse Melanoma

RLI and ILR were first compared *in vivo* for their ability to inhibit the development of lung metastasis in a B16F10 melanoma model. Flow cytometry showed that B16F10 cells did not express any of the IL-15Rα, IL-15Rβ, or IL-15Rγ chains, therefore ruling out any potential direct effect of IL-15, RLI, or ILR on the tumor cells (data not shown). This was confirmed by the absence of inhibitory effect of RLI on B16F10 proliferation *in vitro* (data not shown). C57BL/6 mice were inoculated i.v. with 1×10^5 B16F10 melanoma cells on day 0 and treated i.p. with RLI (2 μg) or ILR (2 μg) at days 1, 2, and 10 (Fig. 1C). At day 21, mice were sacrificed and the lung metastatic melanoma nodules visible as black spots (see Fig. 2A for examples) were counted under a binocular microscope. Both proteins reduced lung metastasis but the effect of RLI was superior to that of ILR, and only the effect of RLI was found significantly different from that of control (**, $P < 0.01$).

Using the same experimental design (Fig. 1C), we then compared the efficiency of RLI with that of IL-2, a cytokine that was approved by the U.S. Food and Drug Administration in 1998 for treatments of adults with advanced metastatic melanoma but that can induce, at high doses, severe toxicities such as hemodynamic complications (35, 36). After inoculation of B16F10 melanoma cells, mice were treated with increasing doses of IL-2, IL-15, or RLI (Fig. 2A). Administration of RLI had a marked inhibitory effect on the number of lung metastasis, with a maximal plateau effect reaching 75% of inhibition, whereas the maximum

inhibitory effects induced by IL-2 and IL-15 were 45% and 35%, respectively (Fig. 2A, *bottom*). In addition, the mean size of the tumor metastasis on the lung surface was found smaller in the RLI group than in the IL-2, IL-15, and control groups (Fig. 2A, *top*).

The noncovalent association of sIL-15Rα with IL-15 has been shown to enhance the activity of IL-15 *in vitro* (29, 31) and *in vivo* (26, 27), and we have recently shown that RLI was more active than the noncovalent association of its two linked moieties (i.e., IL-15 and IL-15Rα-sushi+ domain) to activate *in vitro* the IL-15Rβ/γ and IL-15Rα/β/γ receptors (29). We therefore compared the maximal activity of RLI with that of a noncovalent association of IL-15 plus IL-15Rα-sushi+ domain in the B16F10 melanoma model (Fig. 2B). The association of IL-15 with sushi+ did not induce a better decrease of metastasis than IL-15 alone. Only RLI induced a significant reduction versus control of the number of lung metastasis.

To investigate the importance of NK cells in mediating the antitumoral effects, *in vivo* antibody depletion experiments were done in the B16F10 melanoma model. Flow cytometry analysis revealed that NK cells were almost totally depleted (~95%) in the spleen of saline- or RLI-treated mice on day 21 (Fig. 2C). Anti-asialoGM1 treatment rendered mice treated with saline or RLI highly susceptible to B16F10 tumor cell growth (Fig. 2D), with several lung metastasis that was strongly increased and the appearance of metastatic nodules in the peritoneum and liver. The antitumoral activity of RLI was completely abolished after *in vivo* depletion of NK cells.

The effect of RLI was then tested on the survival of C57BL/6 mice (Fig. 3A). Control mice that were treated with saline died with a median survival of 28 days. Treatments with RLI significantly increased the median survival to 35 days, and 2 of 16 (12.5%) of the mice survived until at least day 90, with body weights that were identical to those of control mice (data not shown). Increasing the duration of treatment with RLI (twice per week over 6 weeks) did not change the median survival that was 31 days compared with 27 days for control mice (Fig. 3A, *bottom*).

We also analyzed the development of liver metastasis after intrasplenic injection of B16F10 cells in C57BL/6 mice (Fig. 3B). This injection induced liver metastasis resulting in a 3-fold increase of liver weight in mice treated with saline. The i.p. administration of RLI (twice per week over 2 weeks) strongly prevented liver metastasis of B16F10 melanoma, resulting in a significant 65% inhibition of the liver weight increase.

RLI Has a Better Bioavailability *In vivo* than IL-15 or IL-2

To determine whether RLI had a favorable pharmacokinetic profile compared with IL-2 and IL-15, C57BL/6 mice were injected i.p. with a single dose of RLI (4 or 48 μg), IL-2 (2.5 or 30 μg), or IL-15 (2.4 or 28.8 μg), equivalent to 0.16 nmol (low dose) and 1.92 nmol (high dose) for each drug. The profiles of IL-2 and IL-15 were similar as reflected by their calculated pharmacokinetic parameters, whereas that of RLI showed a markedly increased bioavailability (Fig. 4; Table 1). The half-lives of IL-2 and IL-15 were 20 and 30 minutes, respectively, whereas RLI half-life was 3 hours. For each injected dose, the maximal plasma

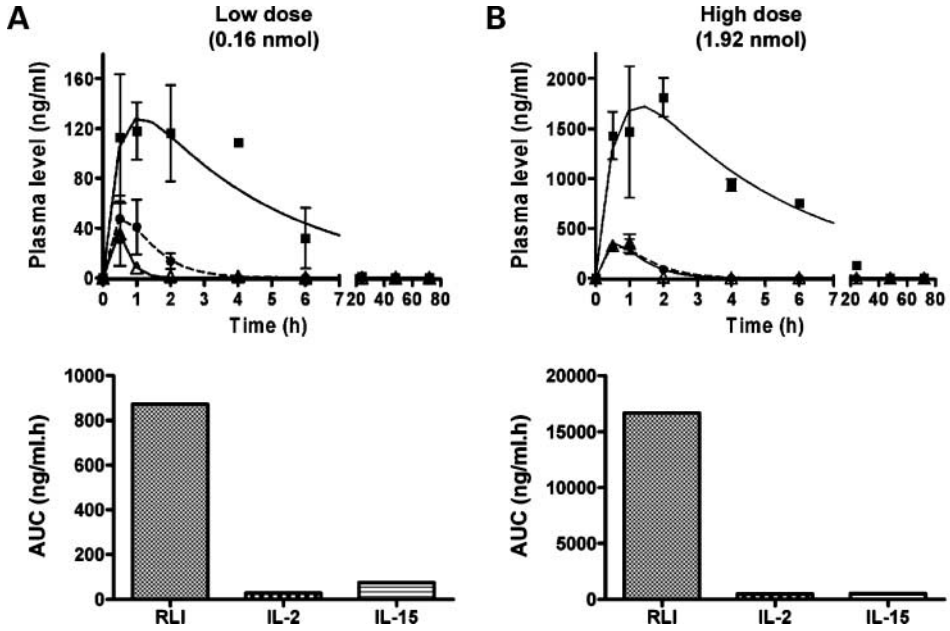


Figure 4. Pharmacokinetic profiles of RLI (■), IL-2 (Δ), or IL-15 (●) following a single i.p. administration of equivalent low (**A**; 0.16 nmol) or high (**B**; 1.92 nmol) dose of these compounds: RLI, 4 or 48 μg; IL-2, 2.5 or 30 μg; and IL-15, 2.4 or 28.8 μg. Male C57BL/6 mice were injected i.p. and plasma concentrations were determined at the indicated points. *Bottom,* AUC of RLI, IL-2, and IL-15.

Table 1. Summary of the pharmacokinetic parameters of RLI, IL-2, and IL-15 in male C57BL/6 mice following a single i.p. administration

	RLI		IL-2		IL-15	
Dose (μg)	4	48	2.5	30	2.4	28.8
Dose (nmol)	0.16	1.92	0.16	1.92	0.16	1.92
C_{max} (ng/mL)	118	1,810	35	350	47	319
T_{max} (h)	1	2	0.5	1	0.5	1
$T_{1/2}$ (h)	3	3	0.17	0.42	0.5	0.5
AUC (ng/mL.h)	872	16,638	28	484	75	524

Abbreviations: C_{max}, maximum plasma concentration; T_{max}, time to reach maximum plasma concentration; $T_{1/2}$, half-life.

concentration of RLI was found to be 3- to 5-fold higher than that of IL-2 and IL-15, and its area under the plasma concentration curve (AUC) was 12- to 34-fold greater than those of IL-2 and IL-15 (Fig. 4, *bottom*). As a control for non–side effect (36), the serum levels of TNFα and prostaglandin E_2 were not affected by the injection of low or high doses of RLI, IL-15, or IL-2 (data not shown).

RLI Treatment of Human Colon Cancer Cells HCT-116 Growing in the Cecum of Nude Mice

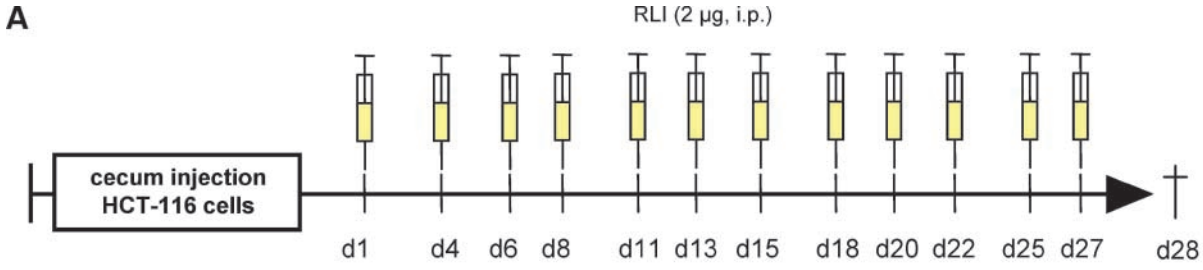
The antitumor effect of RLI was evaluated on another cancer model: the colorectal cancer. This is the second most frequent cause of cancer-related death worldwide, and patients with metastatic colorectal cancer have a median of 8.5 months without chemotherapy (37). We used a human colorectal cancer HCT-116 cell line, described as the most aggressive human colorectal cancer cell line by Cespedes et al. (33). These cells were preliminary checked for the absence of any IL-15R chain expression (data not shown).

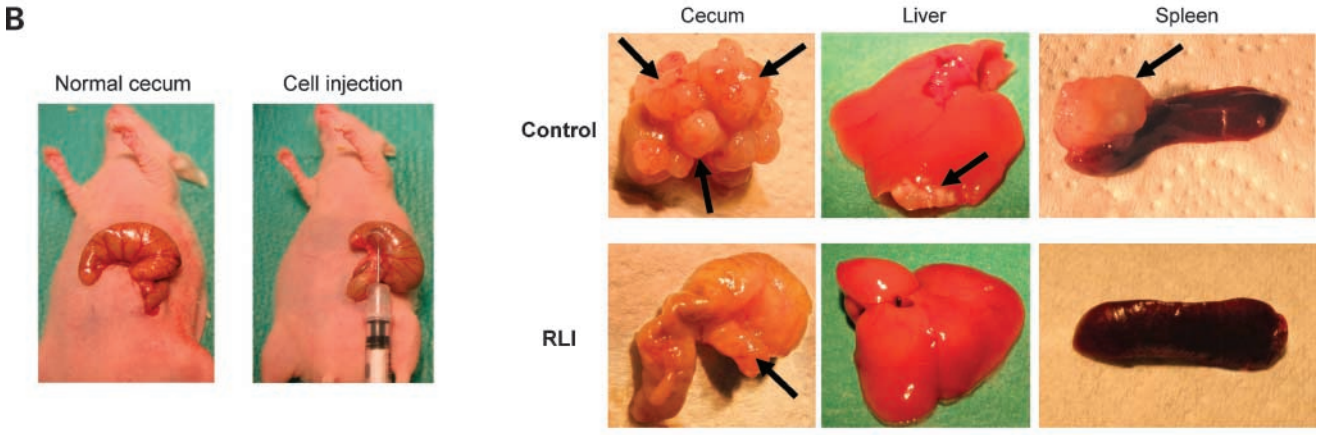
HCT-116 cells (1×10^6) were injected into the cecal wall of female nude mice (Fig. 5B, *left*). One day after, mice were treated or not with one i.p. injection of RLI (2 μg) thrice per week for 4 weeks and tumor progression was evaluated at day 28 (Fig. 5A). None of the treatment produced loss of body weight and tumor incidence was 100%. Mice treated with saline (control) had large primary tumors in the cecum and metastatic nodules in the peritoneum and other organs such as liver and spleen. Mice receiving RLI had smaller primary tumors and a reduced incidence of metastasis (Fig. 5B, *right*). To quantify the results, two related scoring systems were used: first, the system of Cespedes et al. that takes into account four main tumor development stages (Fig. 5C, *column 2*), and second, a system that we defined by dividing each main stage (I, II, III, and IV) in substages (Fig. 5C, *column 1*) and attributing relative scores to each of them, with a maximal score of 4.5 (Fig. 5C, *column 3*). With the first staging system, it was found that RLI treatment induced a strong inhibition of tumor growth in the cecum and metastasis dissemination in peritoneal cavity and other organs. In control mice, most of the tumors were at stage IV (59.3%; stage I: 3.7%, stage II: 29.6%, and stage III: 7.4%), whereas in RLI-treated mice tumors were mainly at stage I (55.6%; stage II: 11.1%, stage III: 14.8%, and stage IV: 18.5%; Fig. 5D, *top*). Using the second scoring system, it was found that the overall effect of RLI was to reduce by 50% tumor growth and metastasis (***, $P < 0.001$; Fig. 5D, *bottom*).

Discussion

IL-2 is Food and Drug Administration approved for the treatment of metastatic renal cancer and malignant melanoma since 1992 (35). However, its toxicity at high doses as well as its ability to promote activation-induced cell death and expansion of T regulatory cells have limited its contemplated use in cancer treatment (38). In contrast, IL-15 is less toxic than IL-2, inhibits activation-induced cell death, and stimulates the persistence of memory $CD8^+$ T cells, appearing therefore as a better alternative than IL-2 in the treatment of cancer and as a component of vaccines directed to cancer (20, 39). Its potential use as a cancer immunotherapeutic agent has already been suggested in several mouse cancer models (10–19). Its interest as adjuvant for enhancing the effect of chemotherapeutic agents and adoptive cell therapy on transplanted tumors has been widely established, but its therapeutic potential on its own was found limited, one of the reason being, as described for IL-2, its poor pharmacokinetics *in vivo* (21, 22). Considering the IL-15 transpresentation model, we previously developed the fusion protein RLI, linking human sIL-15Rα-sushi+ domain to human IL-15, and showed its better biological activities than IL-15 *in vitro* (29, 31). The present studies were therefore conducted to evaluate whether RLI would be a better alternative than IL-15 and IL-2 for cancer treatment using two distinct animal models and to measure its bioavailability *in vivo*.

In the B16F10 mouse metastatic melanoma model, RLI was shown here to markedly reduce the number of lung metastasis after i.v. injection of tumor cells as well as liver metastasis after intrasplenic injection. The maximal effect on lung metastasis (75% of inhibition) was significantly higher than those induced by IL-2 (45%) or IL-15 (35%). The better *in vivo* efficiency of RLI versus IL-15 is likely related to its better *in vitro* efficiency to stimulate IL-15Rα/β/γ–bearing and/or IL-15Rβ/γ–bearing cells as previously reported (29, 31). Our pharmacokinetic experiments also indicate that the better efficiency of RLI over IL-15 or IL-2 may also be related to a better serum distribution profile. The AUC and serum half-life of RLI were indeed approximately 30 times and 6 times higher, respectively, than those of IL-15 or IL-2. A previous study has shown a marked (20-fold) improvement of IL-15 serum half-life when associating with sIL-15Rα-Fc, an effect that could be attributed to the Fc part fused to IL-15Rα (26). Our results show that the IL-15Rα-sushi+ domain by itself increases the half-life of IL-15 *in vivo*. sIL-15Rα-IgG1-Fc has been shown in a similar B16 model to be more potent than sIL-15Rα to increase the antitumor activity of IL-15, again stressing the roles of both Fc and sIL-15Rα (27). Comparison of these data with our present data indicated that RLI (2 μg, three injections) favorably compared with the IL-15/IL-15Rα-Fc complex even used at much higher doses (10 μg, nine injections) to increase mice survival. Both IL-15/IL-15Rα-Fc and RLI induce a





C

Organ	Cespedes's Stage	Stage
Cecum	I	0.5
or Before cecum		0.25
or After cecum		0.25
Peritoneal cavity	II	0.4
or Diaphragm		0.3
or Mesenteric lymph nodes		0.3
Pancreas	III	0.5
or Spleen		0.5
Liver	IV	1
or Lung		0.5
Sum		**4.5**

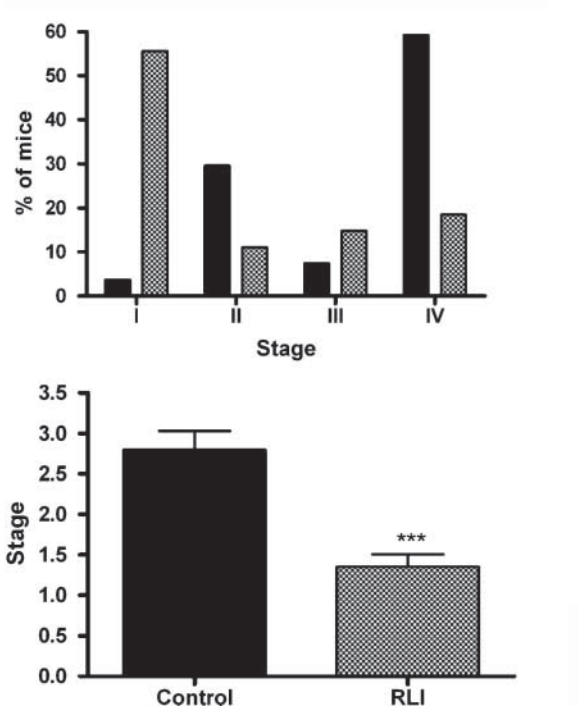


Figure 5. Therapy of HCT-116 human colon cancer cells implanted in the cecum of nude mice. **A,** schematic of the RLI treatment in mice injected with 1×10^6 HCT-116 cells into the cecal wall. Nude mice received one i.p. injection of RLI (2 μg) or saline (control) at day 1 and thrice per week for 28 d. **B,** *left,* representative pictures of cecum before or during HCT-116 cell injection in the cecum of nude mice; *right,* metastasis (*arrows*) in cecum, liver, or spleen of control and RLI-treated mice. **C,** definition of tumor staging 28 d after HCT-116 cell injection: *column 2,* according to Cespedes et al.; *column 3,* according to our tumor staging. **D,** development stages of HCT-116 tumors after treatment with RLI (*checkered columns*) or vehicle (*black columns*; $n = 27$ for each group). *Top,* tumor staging (% of mice) according to Cespedes et al.; *bottom,* quantification of local tumor and dissemination pattern. ***, $P < 0.001$ versus control, Dunnett's multiple comparison test.

similar 7-day extension of the median survival compared with PBS-treated mice (27). However, although no mice survived under IL-15/IL-15Rα-Fc treatment after 36 days, there was a significant number (10–15%) of mice with long-term survival (>90 days) under RLI treatment. In addition, in our B16F10 experiments, RLI was also found to be more active than the IL-15/IL-15Rα-sushi+ complex. These results agree with our previous results showing a better activity of RLI versus the noncovalent IL-15/IL-15Rα-sushi+ complex *in vitro* (29). In these studies, RLI was shown to activate with similar high affinity both the IL-15Rα/β/γ and the IL-15Rβ/γ complexes, whereas IL-15 alone was 50-fold less efficient on the IL-15Rβ/γ complexes, and the noncovalent association of IL-15 and IL-15Rα-sushi+ was 3-fold less effective than RLI on IL-15Rβ/γ and 10-fold less effective than RLI or IL-15 on IL-15Rα/β/γ. We have further shown an enhanced activity of RLI over the IL-15/IL-15Rα-Fc complex to increase the number of human NK cells in mice reconstituted with a human immune system (30). Our present data extend these observations by indicating a better antitumoral therapeutic efficiency of RLI over noncovalent IL-15/sIL-15R complexes.

The antitumoral effects of RLI were not due to a direct action on the tumor cells that do not express any of the IL-15R chains and probably reflect an action via tumor-associated $CD8^+$ T cells (28) and/or NK cells. Rowley et al. (40) have recently shown that IL-15 linked to IL-15Rα expressed on tumor cells induced a strong inhibition of tumor growth in an NK1.1+ cell–dependent and $CD8^+$ T-cell–dependent manner in tumor-challenged mice. In this study, depletion of NK cells completely abrogated the antitumoral activity of RLI against the growth of B16F10 cells, indicating that NK cells represent a major effector subset involved in the antitumoral effects of RLI.

Interestingly, ILR, another fusion protein that we previously described (31) and differing from RLI by inverse positions of IL-15 and IL-15Rα-sushi+ with regard to the central flexible linker, displayed a weaker antitumoral effect, comparable with that of IL-15. This difference may in part be due to the 2-fold lower activity of ILR versus RLI to induce proliferation of IL-15–dependent cells *in vitro*. However, a better *in vivo* stability of IL-15/IL-15Rα-sushi+ complex in the frame of RLI fusion protein than in the frame of ILR may not be excluded.

The HCT-116 human colon cell line was also used to investigate the anticancer therapeutic properties of RLI. Colorectal cancer is one of the highest incidence of cancers worldwide (41). Whereas early-stage colorectal cancer is frequently curable with surgery, unresectable metastatic disease is uniformly fatal (42). Therefore, there is a clear need for new treatment strategies to improve the prognosis of colorectal cancer patients. The orthotopic microinjection of HCT-116 cells allows a dissemination pattern closely replicating all relevant metastatic sites observed in humans. In this model, RLI induced a strong antitumoral effect by reducing the primary tumor growth in the cecum as well as decreasing metastatic progression in organs of the peritoneal cavity. Animals treated with RLI developed mainly stage I tumors (55.6%), whereas 59.3% of control mice presented stage IV tumors. Based on the study of Cespedes et al., as tumor staging closely correlated with animal survival, a survival time around 110 days, 2- to 3-fold longer than that of control animals, could be expected for RLI-treated mice.

Adjuvant and palliative treatments for colorectal cancer mainly involve fluorouracil-based chemotherapy and irinotecan, and more recently oxaliplatin (43, 44). However, when patients become refractory to these cytotoxic agents, there are essentially no established treatment options with shown efficacy. The last few years have witnessed growing interest and significant advances in the use of targeted therapy for colorectal cancer. For example, in 1998, Cao et al. (17, 18) had already shown the ability of IL-15 to provide significant protection from fluorouracil- and irinotecan-induced intestinal toxicity with maintenance of antitumor activity, resulting in an increase in the therapeutic index of the cytotoxic agents. It will therefore be of interest to evaluate the advantage of RLI over IL-15 in such combination strategies.

In conclusion, RLI has revealed strong anticancer therapeutic effect in two different cancer models: the B16F10 mouse lung and liver metastatic melanoma models and the HCT-116 human colon tumor mouse model. This fusion protein overcomes the limited effect of IL-15 by increasing its bioavailability and efficiency in stimulating IL-15Rα/β/γ–bearing and/or IL-15Rβ/γ–bearing cells. These findings hold significant importance for the use of RLI as a potential adjuvant/therapeutic.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

We thank Isabelle Barbieux and Sandrine Minault for technical assistance with fluorescence-activated cell sorting and animals, respectively.

References

1. Grabstein KH, Eisenman J, Shanebeck K, et al. Cloning of a T cell growth factor that interacts with the β chain of the interleukin-2 receptor. Science 1994;264:965–8.

2. Burton JD, Bamford RN, Peters C, et al. A lymphokine, provisionally designated interleukin T and produced by a human adult T-cell leukemia line, stimulates T-cell proliferation and the induction of lymphokine-activated killer cells. Proc Natl Acad Sci U S A 1994;91:4935–9.

3. Giri JG, Anderson DM, Kumaki S, Park LS, Grabstein KH, Cosman D. IL-15, a novel T cell growth factor that shares activities and receptor components with IL-2. J Leukoc Biol 1995;57:763–6.

4. Anderson DM, Kumaki S, Ahdieh M, et al. Functional characterization of the human interleukin-15 receptor α chain and close linkage of IL15RA and IL2RA genes. J Biol Chem 1995;270:29862–9.

5. Giri JG, Kumaki S, Ahdieh M, et al. Identification and cloning of a novel IL-15 binding protein that is structurally related to the α chain of the IL-2 receptor. Embo J 1995;14:3654–63.

6. Kennedy MK, Glaccum M, Brown SN, et al. Reversible defects in natural killer and memory CD8 T cell lineages in interleukin 15-deficient mice. J Exp Med 2000;191:771–80.

7. Lodolce JP, Burkett PR, Boone DL, Chien M, Ma A. T cell-independent interleukin 15Rα signals are required for bystander proliferation. J Exp Med 2001;194:1187–94.

8. Li XC, Demirci G, Ferrari-Lacraz S, et al. IL-15 and IL-2: a matter of life and death for T cells *in vivo*. Nat Med 2001;7:114–8.

9. Waldmann TA. Effective cancer therapy through immunomodulation. Annu Rev Med 2006;57:65–81.

10. Ugen KE, Kutzler MA, Marrero B, et al. Regression of subcutaneous B16 melanoma tumors after intratumoral delivery of an IL-15-expressing plasmid followed by *in vivo* electroporation. Cancer Gene Ther 2006;13: 969–74.

11. Lasek W, Basak G, Switaj T, et al. Complete tumour regressions induced by vaccination with IL-12 gene-transduced tumour cells in combination with IL-15 in a melanoma model in mice. Cancer Immunol Immunother 2004;53:363–72.

12. Basak GW, Zapala L, Wysocki PJ, Mackiewicz A, Jakobisiak M, Lasek W. Interleukin 15 augments antitumor activity of cytokine gene-modified melanoma cell vaccines in a murine model. Oncol Rep 2008;19:1173–9.

13. Kishida T, Asada H, Itokawa Y, et al. Interleukin (IL)-21 and IL-15 genetic transfer synergistically augments therapeutic antitumor immunity and promotes regression of metastatic lymphoma. Mol Ther 2003;8: 552–8.

14. Di Carlo E, Comes A, Basso S, et al. The combined action of IL-15 and IL-12 gene transfer can induce tumor cell rejection without T and NK cell involvement. J Immunol 2000;165:3111–8.

15. Evans R, Fuller JA, Christianson G, Krupke DM, Troutt AB. IL-15 mediates anti-tumor effects after cyclophosphamide injection of tumor-bearing mice and enhances adoptive immunotherapy: the potential role of NK cell subpopulations. Cell Immunol 1997;179:66–73.

16. Habibi M, Kmieciak M, Graham L, Morales JK, Bear HD, Manjili MH. Radiofrequency thermal ablation of breast tumors combined with intralesional administration of IL-7 and IL-15 augments anti-tumor immune responses and inhibits tumor development and metastasis. Breast Cancer Res Treat 2009;114:423–31.

17. Cao S, Black JD, Troutt AB, Rustum YM. Interleukin 15 offers selective protection from irinotecan-induced intestinal toxicity in a preclinical animal model. Cancer Res 1998;58:3270–4.

18. Cao S, Troutt AB, Rustum YM. Interleukin 15 protects against toxicity and potentiates antitumor activity of 5-fluorouracil alone and in combination with leucovorin in rats bearing colorectal cancer. Cancer Res 1998; 58:1695–9.

19. Klebanoff CA, Finkelstein SE, Surman DR, et al. IL-15 enhances the *in vivo* antitumor activity of tumor-reactive $CD8^+$ T cells. Proc Natl Acad Sci U S A 2004;101:1969–74.

20. Cheever MA. Twelve immunotherapy drugs that could cure cancers. Immunol Rev 2008;222:357–68.

21. Kobayashi H, Carrasquillo JA, Paik CH, Waldmann TA, Tagaya Y. Differences of biodistribution, pharmacokinetics, and tumor targeting between interleukins 2 and 15. Cancer Res 2000;60:3577–83.

22. Zamai L, Ponti C, Mirandola P, et al. NK cells and cancer. J Immunol 2007;178:4011–6.

23. Dubois S, Mariner J, Waldmann TA, Tagaya Y. IL-15Rα recycles and presents IL-15 in *trans* to neighboring cells. Immunity 2002;17:537–47.

24. Mortier E, Woo T, Advincula R, Gozalo S, Ma A. IL-15Rα chaperones IL-15 to stable dendritic cell membrane complexes that activate NK cells via trans presentation. J Exp Med 2008;205:1213–25.

25. Rubinstein MP, Kovar M, Purton JF, et al. Converting IL-15 to a superagonist by binding to soluble IL-15Rα. Proc Natl Acad Sci U S A 2006; 103:9166–71.

26. Stoklasek TA, Schluns KS, Lefrancois L. Combined IL-15/IL-15Rα immunotherapy maximizes IL-15 activity *in vivo*. J Immunol 2006;177: 6072–80.

27. Dubois S, Patel HJ, Zhang M, Waldmann TA, Muller JR. Preassociation of IL-15 with IL-15R α-IgG1-Fc enhances its activity on proliferation of NK and $CD8^+$/$CD44^{high}$ T cells and its antitumor action. J Immunol 2008; 180:2099–106.

28. Epardaud M, Elpek KG, Rubinstein MP, et al. Interleukin-15/interleukin-15Rα complexes promote destruction of established tumors by reviving tumor-resident $CD8^+$ T cells. Cancer Res 2008;68:2972–83.

29. Bouchaud G, Garrigue-Antar L, Sole V, et al. The exon-3-encoded domain of IL-15Rα contributes to IL-15 high-affinity binding and is crucial for the IL-15 antagonistic effect of soluble IL-15Rα. J Mol Biol 2008; 382:1–12.

30. Huntington ND, Legrand N, Alves NL, et al. IL-15 trans-presentation promotes human NK cell development and differentiation *in vivo*. J Exp Med 2009;206:25–34.

31. Mortier E, Quemener A, Vusio P, et al. Soluble interleukin-15 receptor α (IL-15Rα)-sushi as a selective and potent agonist of IL-15 action through IL-15Rβ/γ. Hyperagonist IL-15 × IL-15Rα fusion proteins. J Biol Chem 2006;281:1612–9.

32. Bernard J, Harb C, Mortier E, et al. Identification of an interleukin-15α receptor-binding site on human interleukin-15. J Biol Chem 2004;279: 24313–22.

33. Cespedes MV, Espina C, Garcia-Cabezas MA, et al. Orthotopic microinjection of human colon cancer cells in nude mice induces tumor foci in all clinically relevant metastatic sites. Am J Pathol 2007;170:1077–85.

34. Lehours P, Raher S, Dubois S, Guo J, Godard A, Jacques Y. Subunit structure of the high and low affinity human interleukin-15 receptors. Eur Cytokine Netw 2000;11:207–15.

35. Rosenberg SA, Yang JC, Topalian SL, et al. Treatment of 283 consecutive patients with metastatic melanoma or renal cell cancer using high-dose bolus interleukin 2. JAMA 1994;271:907–13.

36. Baluna R, Vitetta ES. Vascular leak syndrome: a side effect of immunotherapy. Immunopharmacology 1997;37:117–32.

37. Simmonds PC. Palliative chemotherapy for advanced colorectal cancer: systematic review and meta-analysis. Colorectal Cancer Collaborative Group. BMJ 2000;321:531–5.

38. Schwartz RN, Stover L, Dutcher J. Managing toxicities of high-dose interleukin-2. Oncology (Williston Park) 2002;16:11–20.

39. Waldmann TA, Dubois S, Tagaya Y. Contrasting roles of IL-2 and IL-15 in the life and death of lymphocytes: implications for immunotherapy. Immunity 2001;14:105–10.

40. Rowley J, Monie A, Hung CF, Wu TC. Inhibition of tumor growth by $NK1.1^+$ cells and $CD8^+$ T cells activated by IL-15 through receptor β/common γ signaling in *trans*. J Immunol 2008;181:8237–47.

41. Jemal A, Siegel R, Ward E, et al. Cancer statistics, 2008. CA Cancer J Clin 2008;58:71–96.

42. Wolpin BM, Mayer RJ. Systemic treatment of colorectal cancer. Gastroenterology 2008;134:1296–310.

43. Saltz LB, Cox JV, Blanke C, et al. Irinotecan plus fluorouracil and leucovorin for metastatic colorectal cancer. Irinotecan Study Group. N Engl J Med 2000;343:905–14.

44. Goldberg RM, Sargent DJ, Morton RF, et al. A randomized controlled trial of fluorouracil plus leucovorin, irinotecan, and oxaliplatin combinations in patients with previously untreated metastatic colorectal cancer. J Clin Oncol 2004;22:23–30.