Intracellular Interaction of Interleukin-15 with Its Receptor α during Production Leads to Mutual Stabilization and Increased Bioactivity*

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We show that co-expression of interleukin 15 (IL-15) and IL-15 receptor α (IL-15Rα) in the same cell allows for the intracellular interaction of the two proteins early after translation, resulting in increased stability and secretion of both molecules as a complex. In the absence of co-expressed IL-15Rα, a large portion of the produced IL-15 is rapidly degraded immediately after synthesis. Co-injection into mice of IL-15 and IL-15Rα expression plasmids led to significantly increased levels of the cytokine in serum as well as increased biological activity of IL-15. Examination of natural killer cells and T lymphocytes in mouse organs showed a great expansion of both cell types in the lung, liver, and spleen. The presence of IL-15Rα also increased the number of CD44high memory cells with effector phenotype (CD44highCD62L−). Thus, mutual stabilization of IL-15 and IL-15Rα leads to remarkable increases in production, stability, and tissue availability of bioactive IL-15 in vivo. The in vivo data show that the most potent form of IL-15 is as part of a complex with its receptor α either on the surface of the producing cells or as a soluble extracellular complex. These results explain the reason for coordinate expression of IL-15 and IL-15Rα in the same cell and suggest that the IL-15Rα is part of the active IL-15 cytokine rather than part of the receptor.

Interleukin-15 (IL-15) is a pleiotropic cytokine produced in many tissues. It is a member of the four α-helix bundle family of cytokines and was initially described as a T cell proliferation factor (1, 2). IL-15 shares with interleukin-2 (IL-2) a common receptor complex, consisting of the IL-2 receptors β and γ chains (3). Both IL-2 and IL-15 use an additional private receptor subunit responsible for the specificity of binding, the IL-2 receptor α (IL-2Rα) and IL-15 receptor α (IL-15Rα), respectively. Both molecules have a similar ligand-binding motif (sushi domain) as well as a relatively short intracellular tail (13 amino acids for human IL-2Rα and 41 amino acids for human IL-15Rα). In contrast to IL-2Rα, which displays a lower affinity for IL-2 (Kd ~ 10−8 M) and is expressed mainly on activated T cells, IL-15Rα has a high affinity for IL-15 (Kd ~ 10−11 M), and its mRNA has a wide tissue distribution (4). IL-15−/− and IL-15Rα−/− mice have profound defects in NK, NK-T, intraepithelial lymphocytes, and memory CD8+ T cells, indicating that IL-15 is essential for the homeostatic maintenance and function of these cells (5, 6). In contrast, IL-2−/− and IL-2Rα−/− mice develop autoimmune diseases with increased frequency of activated T and B cells (7, 8). Despite the clear results on the positive role of IL-15Rα for IL-15 function, several investigators have reported inhibitory effects of IL-15Rα on IL-15 function. Injection in mice of a soluble recombinant form of IL-15Rα protein (IL-15sRα) was reported to suppress natural killer (NK) cell proliferation and T-dependent immune responses in vivo (9). Addition of IL-15sRα in vitro was reported to block the response of cell lines to IL-15 (10, 11). Despite these findings, more recent reports show that a soluble sushi domain of IL-15Rα or IL-15sRα linked to an Fc fragment can enhance IL-15 activity both in vitro and in vivo (12–14).

IL-15 function is complex and depends on the presence of IL-15Rα. Trans-presentation of IL-15 by bone marrow-derived cells is thought to be the dominant mechanism for IL-15 action in vivo (15–21). In addition, it is possible that IL-15 functions in a soluble complex with IL-15Rα. It has been reported that IL-15Rα is cleaved by TACE/ADAM17 together with trans-presented IL-15 (22), and the soluble IL-15-IL-15sRα complexes may trigger signaling upon binding to target cells expressing intermediate/low affinity receptor. It was proposed that these soluble complexes may have inhibitory or stimulatory activities (23). Alternative splicing generating different forms of the IL-15Rα have been reported (4, 24–27), and mouse variants missing the transmembrane region have been proposed to form soluble complexes with IL-15 having inhibitory or stimulatory activity (25).

The low expression of IL-15 has been attributed to the complex regulation of the gene at the levels of transcription, translation, protein trafficking, and secretion (28–31). We have used optimized coding sequences for the human, rhesus macaque, and murine IL-15 and IL-15Rα to express the authentic pro-
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teins alone or together in tissue culture and in mice. Vectors for
the expression of IL-15 using the tissue plasminogen activator
(tPA) leader sequence and of the secreted extracellular portion
of IL-15Rα (IL-15sRα) were also produced. Our results show
that the co-expression of IL-15 and IL-15Rα leads to stabiliza-
tion of both molecules during production and to better secre-
tion. The levels of secreted cytokine are thus increased, and in
vivo experiments demonstrate that this leads to greatly
enhanced biological function. These results explain several
puzzling observations about IL-15 function and interactions
and suggest methods to use the improved IL-15 cytokine
expression plasmids for the optimal induction of the immune
system.

MATERIALS AND METHODS

DNA Plasmids—The backbone vector used for the genera-
tion of all the constructs, pCMVkan, contains the human cyto-
megalovirus promoter, the bovine growth hormone polyad-
enylation site, and the kanamycin resistance gene (32, 33). The
RNA-optimized expression vectors for the human IL-15, hav-
ing its own leader sequence (LSP) or substituted with the leader
sequence from tissue plasminogen activator (tPA) (plasmid
IL-15 tPA6, referred to as IL-15t herein), have been described
elsewhere (34). The human IL-15Rα sequence was RNA/codon-optimized by introducing multiple silent point muta-
tions that result in more stable mRNA. The amino acid
sequence of human IL-15Rα corresponds to GenBank™ acces-
sion number NP-002180. The plasmid expressing the soluble
form of human IL-15Rα (IL-15sRα, amino acids 1–205) was
generated by PCR. A dual promoter plasmid expressing IL-15t
from the simian CMV promoter and IL-15Rα from the human
CMV promoter was also used in some experiments. For the in
vivo studies, highly purified, endotoxin-free DNA plasmid
preparations were produced using Qiagen EndoFree Giga kit
(Hilden, Germany).

In Vitro Transient Transfection and Protein Expression—Hu-
man 293 cells were transfected by the calcium phosphate
coprecipitation technique using 0.1 μg of the IL-15-expressing
plasmid alone or in combination with 0.1 μg of either the
IL-15Rα or the IL-15sRα expression plasmids and superna-
tants, and cells were harvested after 24 or 48 h. Co-transfection
of 0.05 μg of the GFP expression vector pFRED143 (35) served
as internal control. GFP variation in the different samples
was less than 50%.

Human IL-15 levels were measured by ELISA (Quantikine
human IL-15 immunoassay; R & D Systems) or by Western
immunoblot (using the polyclonal goat anti-human IL-15 anti-
body AF315, R & D Systems). Human IL-15Rα expression was
analyzed by Western immunoblot using polyclonal goat anti-
human IL-15Rα antibody AF247 (R & D Systems). Protein
bands were visualized on immunoblots by enhanced chemilu-
minescence (GE Healthcare).

For co-immunoprecipitation studies, separate plates were
transfected with the FLAG-tagged IL-15 plasmid, the IL-15Rα
plasmid, or an equimolar mix of the two plasmids. Cells trans-
fected with the individual plasmids were harvested, washed at
4 °C, and split into two tubes. Recombinant human IL-15 (rhIL-
15) was added to one sample of the IL-15Rα transfected cells,
and the sample was mixed with one of the IL-15 transfected
cells and lysed. The other tubes were mixed and lysed in the
absence of rhIL-15. The co-transfected cells were lysed in the
presence or absence of rhIL-15. The cell lysates were processed
for immunoprecipitation using agarose-anti-FLAG antibody
(Sigma) for 1 h at 4 °C and were subsequently examined by
Western immunoblot analysis using an anti-IL-15Rα antibody.
Protein stability analysis was performed after treatment of
transfected cells with 25 μg/ml cycloheximide. Cells and media
were harvested 0–80 min after treatment, and the IL-15 levels
were quantified by ELISA.

RNA Analysis—After transfection of 293 cells, cytoplasmic
RNA was isolated using the PARIS kit (Ambion), according
to the company protocol. Poly(A) RNA was purified using Dyna-
beads® Oligo(dT)25 (Dynal®) (36), according to the protocol.
RNA was transferred using the standard capillary transfer
method onto Duralon-UV membranes (Stratagene) and
hybridized using the QuikHyb hybridization solution (Strat-
agene) as per the manufacturer’s instructions. A DNA probe for
the bovine growth hormone polyadenylation region was syn-
thesized using the Prime-It II random primer Labeling kit
(Stratagene) and was used to detect IL-15 and IL-15Rα. The
membrane was then stripped and rehybridized using a probe
specific for cellular GAPDH mRNA as internal control.

Localization of IL-15-IL-15Rα Complexes—Twenty four
hours after transfection, human 293 cells were harvested,
stained with phycoerythrin-conjugated anti-human IL-15
(IC247IP, R & D Systems), and analyzed by flow cytometry
using the LSR (BD Biosciences). For confocal microscopy, HeLa
cells were transfected by Superfect (Invitrogen) with 0.1 μg of
IL-15-FLAG plasmid alone or in combination with 0.1 μg of
the IL-15Rα or IL-15sRα plasmid. Twenty four hours later, the cells
were fixed with 3.7% paraformaldehyde and either permeabi-
liized or directly surface-stained using mouse anti-FLAG
(1:2000 dilution) and Alexa-488-labeled goat anti-mouse IgG
(1:500 dilution, Invitrogen).

In Vivo Hydrodynamic DNA Delivery—Six-week-old female
BALB/c mice were obtained from Charles River Laboratories,
Inc. (Frederick, MD). Hydrodynamic injection of the plasmid
DNA (37) encoding IL-15 and/or IL-15Rα was performed
essentially as described (38). Briefly, the IL-15 plasmid alone or
in combination with IL-15Rα plasmid in 1.6 ml of sterile 0.9%
NaCl were injected into mice through the tail vein within 7 s
using a 27.5-gauge needle. Mice were bled at day 1 and day 3
after injection, and the serum levels of IL-15 were measured
using human IL-15 chemiluminescent immunoassay (Quantii-
Glo, R & D Systems). Three days after injection, mice were
sacrificed, and liver, lungs, spleen, and mesenteric lymph nodes
were collected and analyzed.

Spleen, Lung, and Liver Cell Analysis—To make single cell
susensions, spleens were gently squeezed through a 100-μm
Cell Strainer (Thomas) and washed in RPMI 1640 medium
(Invitrogen) to remove any remaining organ stroma. The cells
were resuspended in RPMI 1640 medium containing 10% fetal
calf serum and counted using acridine orange (Molecular
Probes)/ethidium bromide (Fisher) dye. Lung and liver were
minced and incubated with 200 units/ml of collagenase (Sigma)
and 30 units/ml of DNase (Roche Applied Science) for 1 h at
37 °C, and single cells were then collected and resuspended in complete RPMI 1640 medium with 10% fetal calf serum. The bioactivity of IL-15 in vivo was monitored by analyzing the frequency of NK and T cells in liver, lung, and spleen using multicolor flow cytometry. Briefly, the cells were washed in FACS buffer containing 0.2% fetal calf serum and stained with the following panel of conjugated rat anti-mouse antibodies: CD3-APC-Cy7, CD4-PerCP, CD8-PECy7, CD44-APC, CD49b-FITC, and CD62L-PE (Pharmingen). Samples were acquired using FACSAria (BD Biosciences), and the data were analyzed by FlowJo software (Tree Star, San Carlos, CA).

**Statistical Analysis**—The \( p \) values for all the in vivo analyses were determined by one-way analysis of variance, Dunnett’s multiple comparison test, for comparisons of different experimental groups of mice with one control group (receiving IL-15t). Correlations of serum IL-15 level with an IL-15 effects were determined by one-way analysis of variance, Dunnett’s multiple comparison test, for comparisons of different experimental groups of mice with one control group (receiving IL-15t). Correlations of serum IL-15 level with an IL-15 effects were determined by one-way analysis of variance, Dunnett’s multiple comparison test, for comparisons of different experimental groups of mice with one control group (receiving IL-15t).

**RESULTS**

**IL-15Ra Co-expression Stabilizes IL-15**—The low expression of IL-15 has been attributed to the complex regulation of the gene at the levels of transcription, translation, protein trafficking, and secretion (28–31). IL-15 mRNA is unstable, and its expression is significantly improved by RNA/codon optimization (34), as shown previously for other mRNAs with low stability (33, 39, 40). It was also reported that IL-15 expression/secretion is decreased because of its native long signal peptide (LSP) (41). We therefore increased IL-15 production by replacing LSP with the secretory signal of the tPA, generating IL-15t (34). We found that the optimized IL-15t expression plasmid produces >100-fold more protein than the wild type IL-15 coding sequence. Similarly, we generated RNA/codon optimized expression vectors for the complete IL-15Ra as well as for IL-15sRα, the secreted extracellular part of the receptor, to study the effects of both cell-associated and non-cell-associated IL-15Ra. The synthetic coding regions were expressed using the human cytomegalovirus promoter (CMV) and bovine growth hormone poly(A) site, as described under “Material and Methods.”

We studied expression of these improved vectors after co-transfection in human 293 cells. Fig. 1 shows expression of human IL-15t alone or in combination with the IL-15Ra expression vectors. Interestingly, IL-15t co-expression with full-length IL-15Ra or the secreted IL-15sRα resulted in a great increase of cytokine production (~5- and ~7-fold, respectively, Fig. 1A). Approximately half of the produced IL-15 was cell-associated upon co-transfection with the complete IL-15Ra. In contrast, IL-15t co-expression with IL-15sRα resulted in secretion of more than 90% of IL-15 into the medium. The results of IL-15 quantification were also verified by Western immunoblot analysis (data not shown).

Flow cytometric analysis of cells transfected with the IL-15 expression vector in the presence or absence of IL-15Ra expression plasmid (Fig. 1B) revealed high levels of IL-15 at the cell surface only in the presence of full-length IL-15Ra. In contrast, co-expression of IL-15 with the soluble IL-15sRα resulted in rapid secretion of IL-15 to the medium and no retention of IL-15 at the cell surface (Fig. 1B).

Transfected cells were also examined for IL-15 localization by confocal microscopy (Fig. 1C). Cytoplasmic IL-15 was detected in permeabilized cells transfected with the IL-15 expression vector. High levels of surface IL-15 were detected only in the presence of full-length IL-15Ra. Co-expression of the soluble IL-15sRα did not show any surface staining (data not shown).

**IL-15 Stabilizes IL-15Ra**—We also examined the effect of IL-15 on IL-15Ra expression. Human 293 cells were transfected with IL-15Ra (Fig. 2A) or IL-15sRα (Fig. 2B), in the presence or absence of IL-15t. Culture supernatants and cell lysates were monitored for IL-15Ra expression by Western immunoblot. To quantify the results, serial dilutions of the samples were
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**FIGURE 2.** IL-15 stabilizes IL-15Rα. Human 293 cells were transfected with plasmids expressing 0.05 μg IL-15Rα (A) or IL-15sRα (B) alone or in combination with 0.05 μg of IL-15, as indicated. After 72 h, IL-15Rα production in the culture supernatants and cell extracts was analyzed by Western immunoblot using a goat anti-IL-15Rα antibody. Sample dilutions of 1:2 and 1:4 were loaded as indicated to quantify the produced IL-15Rα. Mock indicates transfection with a control plasmid only (Fig. 2B, top panel), whereas most of the 42-kDa form was secreted in the medium (Fig. 2B, bottom panel). In the presence of co-expressed IL-15, the intracellular nonglycosylated and partially glycosylated forms were drastically reduced, whereas the mature, fully glycosylated 42-kDa protein was greatly increased in the extracellular compartment. Immunofluorescence experiments using an anti-IL-15Rα antibody also showed that the complete receptor was localized at the cell surface, whereas the soluble receptor was not (data not shown). It is interesting that IL-15sRα, a secretable molecule, is found intracellularly at higher levels compared with IL-15Rα, when expressed alone. IL-15 co-expression promotes rapid modification to the fully glycosylated 42-kDa form and secretion of this molecule. These results suggest an early intracellular association of IL-15 with the IL-15Rα during the production of these two molecules, which results in more efficient secretion.

**IL-15 and IL-15Rα Co-expression Does Not Alter the mRNA Levels**—Co-expression of IL-15 and IL-15Rα after transfection is not expected to alter the expression of either of the two genes, because they are synthetic optimized constructs using the CMV promoter. To demonstrate this point directly, we compared mRNA expression of the two vectors after transfections either alone or in combination. mRNA was isolated and detected in Northern blots (Fig. 2C). As a loading control, levels of the cellular transcript for GAPDH were also probed on the same gel. There was no significant difference in the mRNA levels of IL-15 and IL-15Rα expressed alone or upon co-transfection. These results support the conclusion that co-expression affected the stability and not the production of the two proteins, because similar cytoplasmic mRNA levels were produced in both cases.

**Increased Stability of IL-15 Protein in the Presence of the Receptor**—To show that the stability of IL-15 is increased when co-expressed with the IL-15Rα, we transfected 293 cells with the vectors expressing these molecules alone and in combination, and after 24 h we inhibited protein synthesis by cycloheximide. Measurement of total IL-15 during the first 80 min after cycloheximide addition (Fig. 3) revealed that IL-15 was unstable with a half-life of ~70 min, which is in agreement with reported data (80 min in macaques after subcutaneous inoculation (42)). In contrast, IL-15 co-expressed with both IL-15Rα and IL-15sRα did not decrease during the same period, demonstrating the higher stability of the complex.

**IL-15 and IL-15Rα Interact at the Intracellular Level during Production**—The mutual stabilization of both cell-associated and secreted IL-15 and IL-15Rα suggested that association of these two molecules takes place early during production and intracellular trafficking. To confirm the intracellular IL-15-IL-15Rα complex formation, we performed co-immunoprecipitation experiments of cell extracts and culture supernatants from 293 cells transfected with the IL-15t plasmid in combination and culture supernatants loaded on the gel were 1:200 and 1:300, respectively.

An interesting finding is that expression of the membrane-associated receptor (Fig. 2A, top panel; ~3-fold) as well as the cleaved IL-15sRα in the medium (Fig. 2A, bottom panel). Similarly, co-expression of a plasmid producing exclusively the IL-15sRα with IL-15 led to increased levels of IL-15sRα in the medium (Fig. 2B, bottom panel; ~8-fold). Thus, increased in the steady-state level of IL-15Rα or IL-15sRα by IL-15 is similar to the increase in IL-15 upon IL-15Rα co-expression (Fig. 1).

We noted that expression of the membrane-associated full-length IL-15Rα (59 kDa), both in the presence and absence of co-expressed IL-15, resulted in substantial quantities of the soluble extracellular 42-kDa form found in the culture supernatants (Fig. 2A, bottom panel). This is consistent with the rapid cleavage of the receptor and the generation of the soluble form, which is secreted in the medium. Pulse-chase experiments showed that the cleavage of IL-15Rα from the membrane occurred within 2 h after translation, whereas the soluble receptor form produced from the IL-15sRα expression plasmid was detected in the medium within 30 min (data not shown). Expression of the soluble IL-15sRα showed that, in the absence of IL-15, most of the produced IL-15sRα remained cell-associated (Fig. 2B). Western immunoblot analysis revealed two predominant forms of IL-15sRα migrating as ~28 and ~30 kDa, respectively, in addition to the mature 42-kDa protein. They represent nonglycosylated and partially glycosylated forms of IL-15Rα. It has been reported previously that both the full-length and the soluble IL-15Rα are N- and O-glycosylated (27). Low levels of the mature IL-15sRα (42 kDa) were found cell-associated (Fig. 2B, top panel), whereas most of the 42-kDa form was secreted in the medium (Fig. 2B, bottom panel). In the presence of co-expressed IL-15, the intracellular nonglycosylated and partially glycosylated forms were drastically reduced, whereas the mature, fully glycosylated 42-kDa protein was greatly increased in the extracellular compartment. Immunofluorescence experiments using an anti-IL-15Rα antibody also showed that the complete receptor was localized at the cell surface, whereas the soluble receptor was not (data not shown). It is interesting that IL-15sRα, a secretable molecule, is found intracellularly at higher levels compared with IL-15Rα, when expressed alone. IL-15 co-expression promotes rapid modification to the fully glycosylated 42-kDa form and secretion of this molecule. These results suggest an early intracellular association of IL-15 with the IL-15Rα during the production of these two molecules, which results in more efficient secretion.
with the IL-15Rα or IL-15sRα plasmids using an anti-IL-15Rα antiserum. The precipitates were separated on a denaturing gel, transferred to nylon membranes, and probed with an anti-IL-15 antibody to detect any co-precipitated IL-15 (Fig. 4A). We found that anti-IL-15Rα can pull down IL-15-IL-15Rα complexes from cells co-transfected with the full-length as well as the soluble IL-15Rα. The IL-15-IL-15Rα complex is present in both the intracellular and extracellular compartment. The complexes contain the unglycosylated (13 kDa), the partially N-glycosylated (15 kDa), and the fully N-glycosylated (17 kDa) forms of IL-15.

To address the possibility that IL-15 interacts with IL-15Rα only after cell lysis, we performed co-transfection experiments using plasmids expressing IL-15Rα and IL-15 and lysed the cells in the presence or absence of 50-fold excess recombinant IL-15 protein (compared with IL-15 expressed by transfection). The IL-15 producing plasmid expressed IL-15 tagged with FLAG peptide at the C terminus, allowing IL-15 immunoprecipitation using anti-FLAG antibody. Potential complexes were immunoprecipitated using FLAG antibody and electrophoresed in a denaturing SDS-polyacrylamide gel, and the IL-15Rα was detected by Western immunoblot (Fig. 4B). We first transfected separate plates with either IL-15FLAG or IL-15Rα producing plasmids (Fig. 4B, left panel). Cells from the two different plates were mixed in the presence or absence of 50-fold excess recombinant IL-15 protein, lysed, and processed in parallel with the extracts of the co-transfected plasmids. This experiment verified that excess IL-15 recombinant protein was able to block the interaction of IL-15 to IL-15Rα after cell lysis. In contrast, exogenous IL-15 was not able to prevent complex formation in cells co-transfected with both plasmids (Fig. 4B, right panel), indicating complex formation prior to the emergence of these molecules at the cell surface. In addition to the mature IL-15Rα, an immature form having only N-glycosylation was detected in the complexes. This is additional evidence of early association of IL-15 to the receptor at the ER or Golgi compartment, prior to O-glycosylation, which takes place in the Golgi.

In summary, the presented results are consistent with an early association of IL-15 and IL-15Rα during production, while the two molecules are processed for secretion. Interaction leads to mutual stabilization intracellularly and results in complexes that are either cell-associated and retained in the plasma membrane or released into the medium after proteolytic cleavage.

Co-expression with IL-15Rα or IL-15sRα Increases IL-15 Serum Levels in Mice—To determine whether co-expression of IL-15 and IL-15Rα in vivo resulted in increased IL-15 levels or enhanced biological activity, we inoculated the human IL-15 vector together with either the IL-15Rα or IL-15sRα plasmids in mice by tail vein DNA injection (43), and we measured IL-15 levels in the serum at day 1 and 3 post-delivery (Fig. 5). Injection of 0.2 μg of IL-15t plasmid alone resulted in substantial levels of
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IL-15 in the serum (mean IL-15 was 5789 pg/ml, n = 6) as expected (34). Co-injection of IL-15t with 0.2 μg of IL-15Rα or IL-15sRα plasmid (approximate DNA molar ratio 1:1) resulted in an ~5- and ~50-fold increase in serum IL-15, respectively, measured at the peak at day 1 after injection. Similar results were obtained also with a single plasmid expressing both IL-15 and IL-15Rα (data not shown), indicating that the plasmids are expressed into the same cells in vivo. Titration experiments showed that IL-15 was detectable in the serum even after injection of as little as 0.003 μg of a plasmid producing both IL-15 and IL-15Rα (data not shown). Interestingly, when the IL-15Rα plasmid was injected at a higher molar ratio (IL-15:IL-15Rα 1:3), the IL-15 serum levels at both day 1 and day 3 were higher than in the group receiving 1:1 ratio of IL-15:IL-15Rα (Fig. 5, inverted triangles). This indicates that IL-15Rα is the limiting factor for efficient production of IL-15.

Co-expression with IL-15sRα led to the highest peak value of serum IL-15 (Fig. 5). However, IL-15 levels decreased more rapidly in these mice. Conversely, co-expression with the IL-15Rα led to more prolonged serum IL-15 levels, consistent with gradual cleavage from the cell surface and release in the bloodstream of the active IL-15:IL-15Rα complex.

The IL-15:IL-15Rα Complex Is a Potent Lymphocyte Growth and Mobilization Factor in Vivo—IL-15 plays a multifaceted role in the development and control of the immune system, supporting the expansion and migration of NK cells and the maintenance of T cells, with a strong effect on the memory CD8 T cell subset (44–47). To compare the effects of IL-15 in the absence or presence of co-expressed IL-15Rα, we studied different organs of mice 3 days after DNA injection. The strongest effect was seen in animals receiving the IL-15:IL-15sRα plasmids (using 0.2 μg of DNA each), where the spleen and mesenteric lymph nodes more than doubled in size within 3 days (Fig. 6A). There is a linear correlation between the peak IL-15 serum levels and the spleen weight ($r^2 = 0.88$) as well as the mesenteric lymph node weight ($r^2 = 0.77$) (Fig. 6B). These data demonstrate that the IL-15 bound to the soluble IL-15Rα and circulating in the serum is bioactive.

IL-15 in combination with IL-15sRα also affected the hematological profile of mice, resulting in leukocytosis, neutrophilia, monocytosis, and eosinophilia, as monitored 3 days after DNA injection (Table 1). There is a positive correlation between the peak IL-15 serum levels in all groups of mice and the white blood cell count ($r^2 = 0.71$), the neutrophil count ($r^2 = 0.67$), and the monocyte count ($r^2 = 0.89$). In contrast, no significant increase in blood lymphocytes was observed, despite great lymphocyte increases in several organs such as spleen, liver, and lung (see below), indicating that IL-15 also controls localization of lymphocytes.

To analyze the effects of systemic IL-15 and the IL-15:IL-15Rα complex on the distribution of different lymphocyte populations, lung, liver, and spleen were analyzed by multicolor flow cytometry 3 days after hydrodynamic injection of plasmid DNA. In these experiments we used a mixture of directly conjugated monoclonal antibodies to identify NK cells (CD3–CD49b+) and either CD4+ or CD8+ T cells with effector memory phenotype (CD3+CD44hiCD62L−). In general, the strongest effects were observed in mice receiving a combination of IL-15 and IL-15sRα expression plasmids (Fig. 7). The number of NK cells increased in the lung and liver by ~10-fold compared with mice receiving IL-15 plasmid alone, indicating rapid bone marrow-derived cell differentiation, expansion, and redistribution to the periphery. In the spleen, we found a significant increase in NK cells both in the presence of the full-length IL-15Rα and the soluble IL-15sRα (Fig. 7A). It should be noted that comparisons of IL-15:15sRα expressing mice to control mice receiving a GFP-expressing plasmid revealed increases in NK cells of ~30–40-fold. Increases in CD62L−NK cells in the
lung by IL-15-IL-15sRα were more than 100-fold compared with control mice, indicating a rapid increase of NK cells down-regulating this homing receptor.

Similarly, we found a significant increase in T cells in lung, liver, and spleen in the animals receiving IL-15 and the IL-15sRα plasmids. T cells were expanded by ~3-fold in lung and liver, and ~2-fold in spleen (Fig. 7B). Multicolor flow cytometric analysis also allowed the identification of different cell subsets contributing to the expansion of the T cell compartment. In lung, we found an increase in the number and the frequency of the CD8+ T cells with effector memory phenotype (CD44highCD62L−) by IL-15-IL-15sRα (Fig. 7C and Fig. 8). The great expansion of this subset of effector memory cells in the lung and liver indicates that high levels of IL-15 complexed with IL-15sRα induce redistribution of these cells from the secondary lymphoid organs. The frequency of effector memory

![TABLE 1](https://example.com/table1.png)

**TABLE 1**

Hematology profiles of mice 3 days after hydrodynamic injection of the indicated DNAs

| Cells × 10^3/μl | GFP | IL-15t | IL-15t + IL-15Rα | IL-15t + IL-15sRα |
|-----------------|-----|--------|------------------|------------------|
| White blood cells | 10.01 ± 2.67 | 10.24 ± 1.83 | 15.21 ± 2.49 | 18.57 ± 3.84* |
| Neutrophils | 1.47 ± 0.43 | 2.4 ± 1.21 | 5.38 ± 1.5 | 5.99 ± 3.16 |
| Monocytes | 0.69 ± 0.14 | 0.8 ± 0.27 | 1.38 ± 0.3 | 2.55 ± 1.14* |
| Eosinophils | 0.18 ± 0.021 | 0.13 ± 0.08 | 0.29 ± 0.06 | 0.61 ± 0.33 |
| Lymphocytes | 7.62 ± 2.14 | 6.88 ± 0.51 | 8.10 ± 1.02 | 9.26 ± 1.86 |

* Values indicate significant differences (p < 0.05) between animals receiving IL-15t and IL-15t + IL-15sRα.

**FIGURE 7.** The IL-15-IL-15Rα complexes are bioactive. Analysis of different lymphocyte subsets in mouse organs after hydrodynamic DNA injection using 0.1 μg (A) or 0.2 μg (B and C) of each of the indicated DNA plasmids. Three days after DNA injection, the mice were sacrificed, and lung, liver, and spleen were analyzed for the number of NK cells (A), T lymphocytes (B), and CD8+CD44highCD62L− lymphocytes (C) by flow cytometry. The results from lung and liver are expressed as NK, T, or CD8+CD44highCD62L− cells per 10^6 cells. Because the spleen size is dramatically increased by IL-15, the number of cells in the spleen panels are displayed after normalizing for spleen weight by multiplying the cell numbers with the spleen weight (in grams) and expressing the value per 10^6 cells. Braces indicate the groups having significant differences from IL-15t (p < 0.01, one-way analysis of variance, Dunnett’s multiple comparison test).
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CD8+ T cell is also significantly increased in the spleen of mice receiving IL-15:IL-15sRα (Fig. 7C), probably as a result of both local expansion and redistribution of the memory CD8+ T cell pool. We also observed a significant increase in CD4+ effector memory cells in all organs, whereas the total CD4 population was not significantly affected. The number of B cells in the spleen also increased rapidly, analogous to the enlargement of the organ observed 3 days post-DNA injection.

Results obtained by co-injection of the full-length IL-15R α paralleled those with IL-15sRα. Although it did not reach statistical significance within 3 days after hydrodynamic injection, there was a trend of expansion in both the NK and T cell populations in lungs and livers of mice receiving a combination of IL-15t and full-length IL-15R α. It should be noted that increasing the amount of injected DNA to 1 µg per mouse showed that IL-15t alone (34) or IL-15t plus IL-15R α significantly increased NK and T cells in the tissues examined. These data suggest that the increased production and stability of IL-15 co-expressed with its receptor α resulted in a potent biological effect, with an increased mobilization of T and NK cells and migration to non-lymphoid organs where they mediate surveillance and effector functions.

IL-15 Serum Levels Correlate with Bioactivity in Mouse Tissues—As shown above, the IL-15:IL-15R α complexes, either cleaved from the cell surface (after co-injection of IL-15Rα) or immediately secreted from the cells (after co-injection of IL-15sRα), are bioactive in vivo. To establish whether there is a correlation between the levels of IL-15:IL-15R α and the observed biological effects, we analyzed mice at day 3 post-injection with the indicated DNAs (Fig. 9). The serum levels of IL-15 were measured and correlated with the frequency of either NK or the CD8+ T cells with effector memory phenotype (CD44highCD62L−) in the lungs. Data were fitted to a sigmoidal dose-response curve (Fig. 9). There is a correlation between the peak IL-15 serum level and the number of NK cells (r² = 0.86) (Fig. 9A) or the number of CD8+CD44highCD62L− T cells (r² = 0.83) in the lung (Fig. 9B). Using the 3-day assay measuring the lung NK or T cell numbers, we estimated the ED₅₀ of IL-15 combining results of experiments injecting 0.1 to 1 µg of DNA plasmids (not shown). We concluded that the ED₅₀ of IL-15 for lung NK cells is ~10⁶-⁵ pg/ml. Interestingly, the ED₅₀ for CD8 memory cells was ~10-fold higher. These results correlate well with the expression levels of IL-2/IL-15 receptor β chain in NK and T cells, and the majority of NK cells constitutively express high levels of the β chain (CD122), whereas only a minor population of T cells express detectable levels of the receptor. In conclusion, peak levels of IL-15, regardless of the exact producing vector, correlate well with the observed biological effects.

DISCUSSION

We present data demonstrating that co-expression of IL-15 and IL-15Rα results in intracellular complex formation and sta-
bilation of both molecules. Stabilization of IL-15 in the presence of the IL-15Rα was demonstrated directly by measuring the decay of IL-15 after blocking protein synthesis by cycloheximide. The IL-15/IL-15R complex is transported to the cell surface where rapid cleavage of the receptor generates soluble IL-15/IL-15sRα complexes that enter the bloodstream and are bioactive independently of the producing cell. These combined processes result in the following two main active forms of IL-15 in vivo: membrane bound in complex with the full IL-15Rα molecule, and soluble IL-15/IL-15sRα. Our data demonstrate that IL-15 co-produced and complexed with soluble forms of IL-15Rα is functional and can lead to strong activation and tissue migration of NK and T cells, indicating that trans-presentation by membrane-bound IL-15 is not the only mechanism of function in vivo.

Our results have important implications for the understanding of IL-15 function, because they suggest that efficient IL-15 production requires simultaneous expression of IL-15Rα. It has been reported that IL-15 and IL-15Rα are co-expressed in several cell types, including dendritic cells and activated monocytes (4, 15, 36). The similarity between IL-15 and IL-15Rα promoters has been noted and is presumably the reason for such coordinate expression (15, 48, 49). IL-15 not bound to its IL-15Rα is unstable and probably unable to survive long enough for biological action at a distance. It remains to be determined whether IL-15 is expressed by any cell in the absence of the IL-15Rα and whether it can exert local effects. IL-15 could bind to and activate cells expressing IL-15Rα in addition to the β and γ receptor subunits. In fact, a minor fraction of circulating T cells expresses the high affinity IL-15 Rα, and it was shown that intratumoral CD8+ T cells expressing IL-15Rα could be activated by treatment with IL-15 in vitro (50). Although at present it cannot be excluded that IL-15 production alone has significant physiological role, one experiment utilizing bone marrow chimeras suggested that noncoordinate expression of IL-15 and IL-15Rα is not sufficient to promote the development of CD8 and NK cells (18). Bone marrow chimeras were produced using a mixture of IL-15−/− and IL-15Rα−/− bone marrow and used to reconstitute an irradiated host; these chimeras failed to develop or sustain memory CD8+ T cells and mature NK cells. These results support our hypothesis that stabilization during co-expression in the same cell is required for physiological levels of IL-15 production.

While these data were prepared for publication, we became aware of reports showing increased activity of IL-15 protein-Fc fusions complexed with IL-15 soluble receptor (12, 13). Our work is in agreement and expands these data, providing the molecular mechanisms underlying these results. We have used optimized DNA cassettes to express authentic and modified forms of IL-15 and IL-15Rα. In our experiments, co-expression of IL-15 and IL-15Rα or IL-15sRα in vitro and in vivo, rather than using modified cytokine-receptor protein complexes, allowed us to demonstrate the great stabilization upon IL-15/IL-15Rα co-expression and the rapid cleavage of the surface cytokine-receptor complex to generate soluble, bioactive complexes. Our in vivo expression demonstrates that these soluble complexes enter and can survive for a long time in the bloodstream and also have strong biological activity in the absence of plasma membrane association through the IL-15Rα.

This is not possible for IL-15 alone, because of its instability. The complexes are also predicted to bind and activate all cells expressing IL-2/IL-15 β and γ receptors, whereas IL-15 alone might require the presence of IL-15Rα for efficient binding and function. It should be noted that we obtained similar stabilization upon co-expression of plasmids producing IL-15Rα and different forms of IL-15, including those having the LSP and the short signal leader peptide sequences.3

Using acrid treatment of cells to dissociate the IL-15/IL-15Rα complexes, it was concluded that the complexes can recycle between endosomes and cell surface and thus can trans-present IL-15 to neighboring cells for long periods (15). Our data cannot be explained by recycling, because we achieved even higher stabilization with the IL-15/IL-15sRα complexes, which cannot be anchored to the cell membrane and recycle back to intracellular endosomes. In addition, our transfection experiments are performed in cells lacking the IL-2/IL-15 common β and γ receptors, and therefore binding of IL-15 to the βγ receptor complex does not occur. Thus, the observed stabilization is independent of recycling. A recent report (51) has demonstrated stabilization of IL-15 protein delivered in mice by intraperitoneal injections. Although exogenous delivery of high dose IL-15 clearly results in cell-associated stabilization, this process may not be sufficient for physiological function in vivo if IL-15 and IL-15Rα are expressed endogenously by different cells (16, 18, 52).

The IL-15Rα is both N- and O-glycosylated (27). This provides reliable biochemical markers to follow the intracellular trafficking of the molecule, because N-glycosylation takes place in the ER and O-glycosylation in the Golgi (53, 54). The model that fits all our data suggests that co-expressed IL-15 and IL-15Rα meet in the ER lumen, and the resulting complex travels through the Golgi and is rapidly O-glycosylated and secreted. Soluble IL-15sRα appears more stable intracellularly and is found at higher levels as nonfully glycosylated form in the ER. Membrane anchoring may facilitate the rapid movement of any receptor molecule not associated with IL-15 to the ER-associated degradation pathway. Further experiments are required to verify this model and to assess the role of ER chaperones in this process.

The results obtained in vivo indicate that soluble IL-15/IL-15sRα is a potent growth and mobilization factor for all lymphocytes. Three days after DNA injection, the spleen size of animals receiving IL-15/IL-15sRα doubles or triples in size, with all types of lymphocytes expanding, including T and NK cells. The frequency of NK cells in the periphery (such as lungs) is increased by at least 1 log, indicating rapid cell mobilization from the bone marrow, expansion, and migration. In fact, NK cells lacking the lymphoid tissue homing receptor CD62L increase by more than 100-fold within 3 days. The soluble form of IL-15/IL-15sRα was the most potent inducer of both NK and T cells. Both CD4 and CD8 cells expanded in the presence of IL-15/IL-15Rα, although CD8 cells appear to increase more. CD4 effects were observed only on effector memory CD4 cells in the tissues. These data are in agreement with the CD4 effector memory expansion and tissue migration observed in macaques after administration of IL-15 protein (55). It has been

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3 C. Bergamaschi, R. Jalal, M. Rosati, B. K. Felber, and G. N. Pavlakis, unpublished data.
recently proposed that IL-15 trans-presented by dendritic cells is essential for NK cell activation and tissue migration (56). Our data using DNA expression vectors producing non-cell-associated forms of IL-15 and IL-15 receptor show that this cytokine can act in a non-cell-associated form. The use of Fc fusion molecules in previous experiments (12, 13) did not allow the conclusion that IL-15 and IL-15 receptor can function on both NK and T cells in the absence of cell association. Quantification of circulating levels of IL-15 under different conditions in the presence or absence of co-delivered IL-15 receptor and comparison of biological effects showed a strong correlation between IL-15 levels and bioactivity for all groups of mice, suggesting that receptor-bound IL-15 is equally bioactive in vivo. The described biology of IL-15 and IL-15 receptor and the complexes resulting from coordinate expression, protein processing, and cleavage indicate that IL-15 receptor should be viewed not only as part of the receptor but also as an integral part of the active cytokine.

Our results with optimized DNA constructs also indicate that in vivo delivery produced enough cytokine for measurable biological activity at the local and systemic levels. We have verified this in both mice (Figs. 6–9) and macaques. Delivery of small amounts of optimized DNA expression vectors in vivo can achieve high levels of IL-15 in mouse serum (up to 1 μg/ml of serum) without any apparent toxic effects from IL-15 overproduction. In fact, DNA delivery compares favorably with IL-15 protein. It has been reported that intraperitoneal delivery of 5 μg of recombinant IL-15 in wild type mice results in serum levels of around 103 pg/ml 1 day later (51). In our experiments injection of 0.2 μg of IL-15 plasmid produced ~6 × 103 pg/ml, whereas combination of 0.2 μg of IL-15 and IL-15 receptor plasmids gave serum levels of 106 to 107 pg/ml. We found that as little as 0.003 μg of DNA of a plasmid producing IL-15 and IL-15 receptor resulted in detectable serum expression of IL-15 (~200 pg/ml). Monitoring the biological effects of IL-15 after 3 days, we could detect increased levels of NK cells after delivery of 0.03 μg of IL-15 and IL-15 receptor DNA, which resulted in ~103 pg/ml peak serum levels of IL-15. Hydrodynamic delivery of 1–2 μg of vector DNA resulted in increasing peak levels of serum IL-15, but the number of lung NK cells did not increase further, approaching saturation at about 1 μg/ml peak IL-15 serum levels. Using data from experiments such as shown in Fig. 9, we concluded that the ED50 of IL-15 for lung NK cells is 10−4.5 pg/ml and the ED50 for CD8 memory cells is ~10-fold higher. This difference may reflect the levels of IL-2/IL-15 receptor β and γ subunit expression. Indeed, all NK cells express high levels of IL-2 receptor β, whereas only a minor fraction of circulating CD8 lymphocytes expresses high levels of this subunit. ED50 concentrations of IL-15 were only achieved after co-expression with the IL-15 receptor and have not been detected naturally. The basal levels of IL-15 are usually undetectable in humans. In Indian rhesus macaques, the basal levels of IL-15 are 5–9 pg/ml of plasma. After partial lympho-ablation, the serum IL-15 levels in humans increase to 50–60 pg/ml. Such IL-15 levels are probably not sufficient to maximally and rapidly activate all potential targets. Our mouse data suggest that pharmacologic doses of more than 10 ng/ml of IL-15 in the serum are required for the rapid mobilization (3-day assay) and activation of NK cells in peripheral non-lymphoid organs such as lung. Therefore, IL-15 availability is an important factor regulating NK and T cell frequencies and function in the periphery. It would be of interest to explore the possibility that pharmacological concentrations of IL-15 may increase the number of T cells in lymphopenic conditions such as after bone marrow transplantation. Such levels of systemic IL-15 are achieved only after co-expression or co-delivery with the IL-15 receptor.

The expression of high levels of IL-15 from optimized vectors appears to be safe and effective also after intramuscular DNA delivery in mice and macaques. These potent vector combinations are therefore able to achieve systemically active cytokine levels and may be used for efficient IL-15 delivery in vivo.

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