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Preassociation of IL-15 with IL-15Rα-IgG1-Fc Enhances Its Activity on Proliferation of NK and CD8$^+$/CD44^{high} T Cells and Its Antitumor Action[1]

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In the induction of an immune response, IL-15Rα on APCs transpresents IL-15 to NK and CD8$^+$/CD44^{high} T cells that express the IL-2/15Rβ and γc subunits only. In this study, we show data mimicking this transpresentation by using IL-15 preassociated with a chimeric protein that is comprised of the extracellular domain of murine IL-15Rα and the Fc portion of human IgG1. When tested in vitro, IL-15Rα-IgG1-Fc strongly increased the IL-15-mediated proliferation of murine NK and CD8$^+$/CD44^{high} T cells. The effect of IL-15Rα-IgG1-Fc was dependent on the presence of both IgG1-Fc and IL-15Rα. When injected into mice, IL-15Rα-IgG1-Fc enhanced the capacity of IL-15 to expand the number of NK and CD8$^+$/CD44^{high} T cells. The effect on cell numbers in vivo also depended on Fc receptor binding because reduced expansion was observed in *FcRγ*$^{-/-}$ mice. NK cells cultured in IL-15/IL-15Rα-IgG1-Fc complex gained cytotoxic activity toward a number of NK-sensitive targets. When mice bearing the NK-sensitive syngeneic tumor B16 were treated, the presence of IL-15Rα-IgG1-Fc increased the antitumor activity of IL-15. Thus, a preassociation with IL-15Rα-IgG1-Fc enhances the activities of IL-15 in vivo and in vitro that may be useful in the treatment of tumors. ***The Journal of Immunology,*** **2008, 180: 2099–2106.**

Interleukin-15 plays roles in both innate and adaptive immunity via facilitating the survival and proliferation of distinct sets of lymphocytes (1, 2). Mice with genetic deletions of *IL15* or its private receptor chain, *IL15Ra* are characterized by decreased numbers of NK, NKT, CD8$^+$/CD44^{high} T, TCRγ$^+$/δ$^+$ T, and intraintestinal CD8α$^+$/β$^-$ T cells (3, 4). NK cells are an important part of innate immunity. IL-15 mediates their survival and differentiation into fully functional NK cells capable of lysing virally infected cells and tumor cells (5).

The study of viral infections in *IL15*-deficient mice revealed that IL-15 participates in maintaining the pool of CD8$^+$ memory T cells, part of the adaptive arm of immunity (6, 7). The induction of proliferation of CD8$^+$ memory phenotype T cells by IL-15 suggests that the activity on memory cells is mediated by a direct effect of IL-15 (8, 9). We have shown an anti-apoptotic activity of autocrine IL-15 on mature dendritic cells (DCs)[3] that may also contribute to maintaining CD8 memory (10).

Transfer experiments showed that both IL-15 and IL-15Rα have to be coexpressed on nonlymphoid cells to support NK and CD8$^+$/CD44^{high} T cells (11–13). The induction of both IL-15 and IL-15Rα can be found on the surface of DCs and monocytes in response to IFN-γ and TLR stimulation or CD40 ligation (10, 14, 15). In this context, IL-15Rα retains IL-15 via a high-affinity interaction on the cell surface and trans-presents it to neighboring NK and CD8$^+$/CD44^{high} T cells that express IL-2/15Rβ and γc but not IL-15Rα, thus inducing the proliferation of responsive cells (16).

IL-15 has been shown to inhibit tumor growth in various mouse models (17, 18). Depending on the tumor, the antitumor effect was mediated by either NK, CD8$^+$ T cells, or by both. IL-15 has also been used to support the growth of NK cells and CD8$^+$ T cells in vitro in cellular therapies of neoplastic diseases (17).

In this study, we show data using a soluble IL-15/IL-15Rα-IgG1-Fc complex (19). We show that a preassociation of IL-15 to IL-15Rα-IgG1-Fc enhances its activity to induce proliferations of NK and CD8$^+$/CD44^{high} T cells both in vitro and in vivo. We also show a beneficial effect of IL-15Rα-IgG1-Fc on the antitumor action of IL-15.

Materials and Methods

Plasmids

Plasmids were constructed using PCR-amplified cDNA fragments from spleen cells and standard cloning techniques. All coding sequences were inserted downstream of a CMV promoter (pcDNA3.1, Invitrogen Life Technologies), a murine Ig Kozak sequence and sequence encoding the murine Ig leader peptide "MAVLVLFLCLVAFPSCVLS". Sequence encoding the following proteins were cloned downstream of the leader peptide: murine IL-15 aa 30–162; murine IL-15 aa 30–162 followed by human IgG1 aa 239–469 or followed by the human Ig κ-L chain aa 129–236; murine IL-15Rα aa 33–205 followed by an artificial stop codon; murine IL-15Rα aa 33–205 followed by human IgG1 aa 239–469, human IgG2 aa 243–468, human IgG3 aa 292–521, or followed by human IgG4 aa 233–473. A mutation that decreases the binding affinity of human IgG1 to Fc receptors (D265A, Ref. 20) was introduced using the QuikChangeII kit (Stratagene). No plasmid encoded unrelated amino acids. Plasmids were expressed in 293HEK cells (American Type Culture Collection CRL-1573) after transfection with Lipofectamine 2000 (Invitrogen Life Technologies) that resulted in greater than 90% transfection efficiency. Supernatants were collected 48 h later.

To verify the binding of IL-15 to IL-15Rα, 1 μg of soluble IL-15/IL-15Rα-IgG1-Fc (sIL-15) complex in 1% FBS was immunoprecipitated with protein A/G agarose (Pierce). Proteins from the protein A/G-immunodepleted supernatant as well as from an additional 1 μg of sIL-15 complex were precipitated in 20% trichloroacetic acid, washed in cold acetone,

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[3] Abbreviations used in this paper: DC, dendritic cell; sIL-15 complex, soluble IL-15/IL-15Rα-IgG1-Fc complex; WT, wild type.

dried, and rehydrated in Laemmli buffer. Both immuno- and protein-precipitated samples were subjected to SDS-PAGE and immunoblotted with Abs against IL-15 and IL-15Rα (R&D Systems). To verify the generation of sIL-15 complex by 293HEK cells, 2 ml of supernatants were immunoprecipitated with protein A/G agarose that was followed by SDS-PAGE and immunoblotting against IL-15 and IL-15Rα.

Mice

C57BL/6 mice were purchased from The Frederick Research Facility, C57BL/6-*IL15*$^{-/-}$ and C57BL/6-*FcRγ*$^{-/-}$ mice were provided by Taconic Farms. C57BL/6-*IL15Ra*$^{-/-}$, C57BL/6-*Rag1*$^{-/-}$, and C57BL/6-CD11c-DTR mice were from The Jackson Laboratory. All mice used were females between 8 and 12 wk of age. All treatments were done by i.p. injection. To specifically inhibit FcRII/III interactions in vivo, mice received 100 μg of the mAb 2.4G2 (BD Pharmingen). To remove DCs, C57BL/6^{-}CD11c^{-} DTR mice were treated with 6 ng diphtheria toxin (Sigma-Aldrich)/g body weight 24 h before injections of sIL-15 complex. All mice were cared for in accordance with National Institutes of Health guidelines, and the studies were approved by the Animal Care and Use Committee.

Cytometry and cell sorting

Abs that were used for cytometry were from BD Biosciences. For cytometry analyses, cells were blocked with a mixture of rat IgG1, IgG2a, IgG2b, mouse IgG1, and hamster IgG1 for 15 min on ice that was followed by a 30-min incubation on ice with the specific Ab. For biotinylated Abs, an additional 15-min incubation on ice was done with streptavidin-PE-CY5 (BD Biosciences). BrdU stains were done 12 h after the i.p. injections of 1 mg BrdU using the BrdU Flow Kit (BD Biosciences). NK cells were sorted from spleens using negative isolation microbeads, and CD8$^+$ cells were sorted from spleens using CD8α microbeads (positive sorting) or the CD8$^+$ T cell isolation kit (negative sorting, Miltenyi Biotec).

Cell culture

All cells were cultured in RPMI 1640 supplemented with 10% FBS, 50 μM β–mercaptoethanol and antibiotics. Blood cells were cultured after removing erythrocytes via Ficoll centrifugation. Erythrocytes were removed from spleen cell suspensions by lysis in 0.15 M NH_4Cl, 10 mM $KHCO_3$, and 0.1 mM EDTA (pH 7.2). Blood and spleen cells were cultured in 1 nM murine sIL-15 complex (provided by R&D Systems). All cytokines were used at concentrations that were indicated by the suppliers. For reasons of simplification, molarities refer to the number of IL-15 molecules even though more than one IL-15 molecule may be part of the protein complexes.

Proliferation assays

Cells that had been cultured for 7–14 days in 1 nM sIL-15 complex were washed three times and plated into 96-well plates at 5×10^4 per well. Cells were incubated for 48 h. [^{3}H]Thymidine (1 μCi; PerkinElmer) was present during the final 12 h of the assay. Additional FcR signaling was induced by coating plates with human IgG1 (daclizumab, 10 μg/ml in PBS, 4°C, 12 h) before adding cells.

To determine whether cell concentrations affected proliferation in vitro, NK and CD8$^+$ T cells were sorted from spleens of untreated mice, stained with CFSE (Molecular Probes; 2.5 μM, 10 min, 37°C), and cultured in 1 nM sIL-15 complex at various cell concentrations. The dilution of CFSE as a measure of proliferation was determined 3 days later by FACS.

Lysis assay

For NK cell-mediated cytotoxicity, we used sorted NK cells that had been cultured in 1 nM sIL-15 complex. In addition, we determined the cytotoxic activity of freshly isolated NK cells with or without prior injections of sIL-15 complex (10 μg 7 and 4 days before isolation). As target cells, we used YAC-1 (ATCC TIB-160), MC38, and B16 (provided by Drs. Tagaya and Restifo, National Institutes of Health, Bethesda, MD) as well as EL-4 (ATCC TIB-39). Target cells (2.5×10^6) were labeled with 1 mCi Chromium-51 (sodium chromate, Amersham Biosciences) for 1 h at 37°C in 100% FBS and incubated for 4 h with effector cells at various E:T ratios. Supernatants were transferred into 96-well plates (Wallac) and the radioactivity in the liquid phase was measured. Specific lysis was determined by using the formula: % lysis = 100 × [(mean experimental cpm − mean spontaneous cpm)/(mean maximum cpm − mean spontaneous cpm)]. The maximum release value was determined from target cells treated with 1% (v/v) Triton X-100 (Sigma-Aldrich).

B16 tumor protocol

Mice (12 wk, female) were randomly distributed into three groups of ten animals. One million B16 cells in 0.2 ml PBS were injected i.v. Treatments were done at days 3, 5, 7, 10, 12, 14, 17, 19, and 21 by i.p. injections of PBS, 2 μg murine IL-15 or 10 μg murine sIL-15 complex (R&D Systems). The survival of mice was monitored. The presence of melanoma cells in the lungs after death was confirmed for all animals. Statistical significance was determined with the log rank test using GraphPad Prism (GraphPad Software).

Results

IL-15Rα-IgG1-Fc enhances the IL-15-induced proliferation in vitro

Both IL-15 and IL-15Rα have to be coexpressed on the surface of APCs to induce proliferation in lymphocytes (11–13). An expansion of CD8$^+$/CD44^{high} T cells can be achieved in vivo by injecting mature DCs that express both proteins (10). Our goal was to mimic this cellular IL-15 trans-presentation with a soluble protein complex. Murine IL-15 was preassociated with the extracellular portion of murine IL-15Rα via its high-affinity interaction (Fig. 1*A*). The IL-15Rα portion was genetically attached to part of the human IgG1-Fc region to facilitate the isolation of the complex, its dimerization as well as to extend its half-life in vivo. The association of IL-15 and IL-15Rα-IgG1-Fc was verified by immunoprecipitating IgG1-Fc and immunoblotting IL-15 (Fig. 1*B*, *left panels*). This sIL-15 complex was first tested in vitro.

The effects of IL-15 are best described for NK and CD8$^+$/CD44^{high} T cells. Both lymphocyte populations were sorted from spleens of wild-type (WT) mice and cultured. The presence of 1 nM sIL-15 complex resulted in an expansion of NK and CD8$^+$/CD44^{high} T cells without the need for additional stimulation. This NK and CD8$^+$/CD44^{high} T cell culture proved very robust with cell number doubling times of <24 h over a period of at least 28 days (data not shown). When NK and CD8$^+$/CD44^{high} T cells that had been cultured in sIL-15 complex for 7 days were used to measure thymidine uptake, sIL-15 complex was at least 10-fold more active compared with IL-15 alone in inducing proliferation (Fig. 1*C*). IL-15Rα-IgG1-Fc did not cause proliferation in the absence of IL-15 (data not shown).

To investigate whether the Fc portion of the IL-15Rα-IgG1-Fc fusion protein is required for its IL-15-enhancing effect, various soluble IL-15Rα constructs were coexpressed with IL-15 in 293HEK cells. Expression levels were monitored by immunoprecipitating 293HEK cell supernatants with protein A/G and immunoblotting against IL-15Rα and IL-15 (Fig. 1*B*, right panels). Supernatants from 293HEK cells were used to induce the proliferation of sIL-15 complex-cultured NK cells. Fig. 1*D* shows that IL-15 itself, IL-15Rα-IgG1-Fc itself, or supernatants from mock-transfected 293HEK cells caused little proliferation. Coexpressing the extracellular portion of IL-15Rα without an Ig Fc portion induced a slight proliferation in the presence of IL-15. Supernatants containing IL-15 and chimeric proteins that comprised the extracellular portion of IL-15Rα and the Fc portions of human IgG1, IgG2, or IgG3 caused robust NK cell proliferation. Similar proliferation rates were achieved with a mutated IgG1-Fc portion that is unable to bind FcRs (Fc1M in Fig. 1*D*). In contrast, the presence of the Fc portion of human IgG4 failed to increase proliferation if compared with proliferation in response to IL-15/IL-15Rα. These data show that the Fc portions of human IgG1, IgG2, or IgG3 are able to enhance IL-15/IL-15Rα-induced proliferation of NK cells. Similar data were obtained with CD8$^+$/CD44^{high} T cells (data not shown).

To test whether the IL-15Rα portion is necessary within the sIL-15 complex, we fused IL-15 directly to IgG1-Fc or to the C-terminal portion of the human κ-L chain. Neither chimeric IL-15-Ig

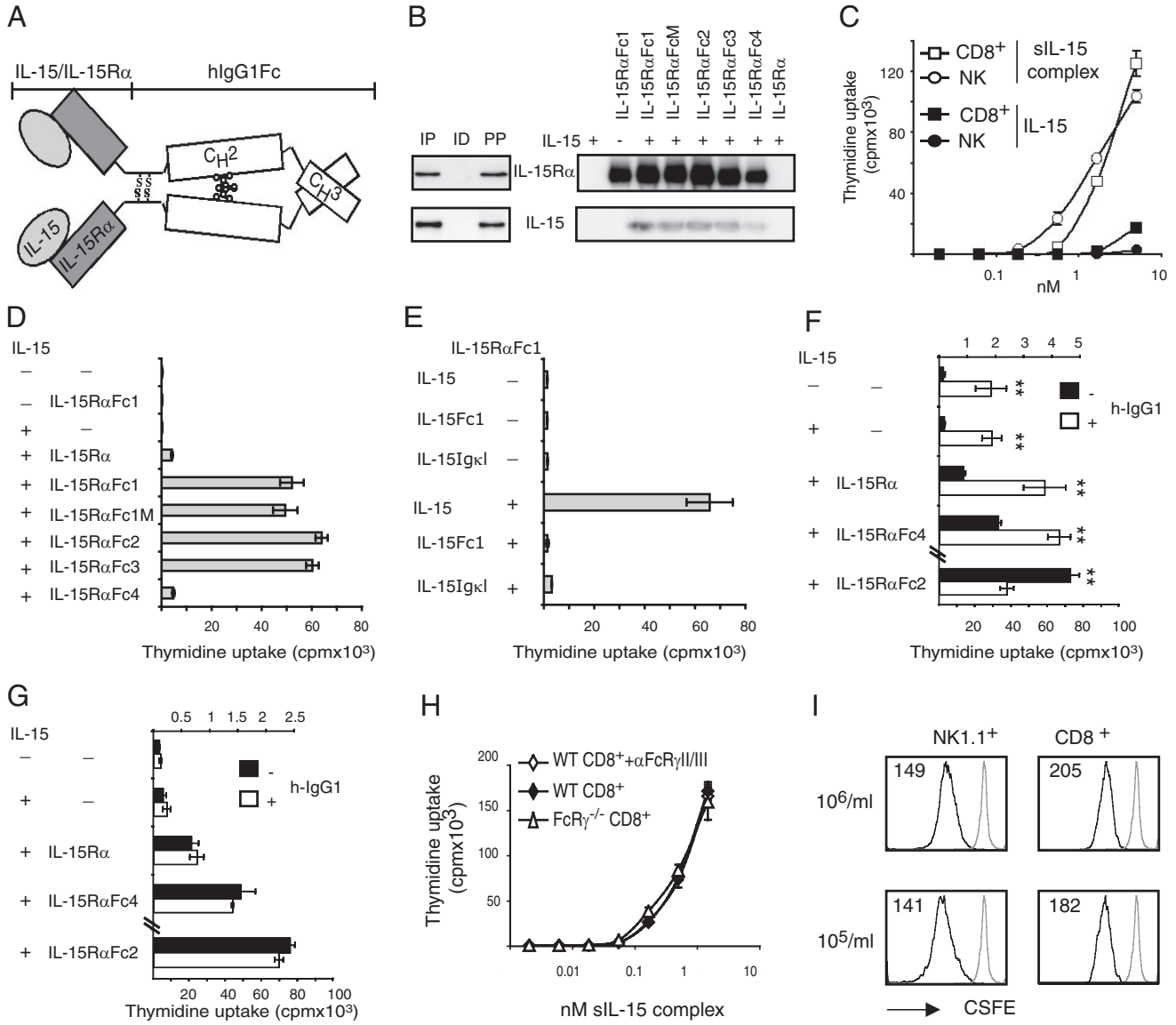

FIGURE 1. Proliferation responses of NK and CD8$^+$/CD44^{high} T cells to sIL-15 complex in vitro. *A*, Schematic depiction of the sIL-15 complex. *B*, Biochemical characterization of the sIL-15 complex. *Left panels*, 1 μg of sIL-15 complex was immunoprecipitated with protein A/G (IP), immunodepleted with protein A/G and protein-precipitated (ID), or protein-precipitated (PP). The resulting precipitates were subjected to SDS-PAGE and immunoblotted against IL-15 and IL-15Rα. *Right panels*, 293HEK cell-generated supernatants were immunoprecipitated with protein A/G and analyzed by SDS-PAGE and immunoblotting. *C*, Splenic NK and CD8$^+$ T cells that had been cultured in sIL-15 complex were incubated for an additional 48 h in various concentrations of sIL-15 complex or of IL-15 alone. Proliferation was measured by the incorporation of [^{3}H]thymidine that was present during the final 12 h. Values shown are averages $\pm$ SD from three experiments. Although NK (○) or CD8$^+$ T cells (□) proliferated in the presence of nanomolar concentrations of sIL-15 complex, little proliferation was observed in the presence of IL-15 alone. *D*, Supernatants from 293HEK cells that had been transfected with IL-15 and IL-15Rα constructs were used for NK cell proliferation. These supernatants represented 25% of the total volume. Although the presence of IL-15 and of the extracellular domain of IL-15Rα caused a slight proliferation, fusing IL-15Rα to Fc portions of IgG1, IgG2, or IgG3, but not of IgG4, substantially increased proliferation rates. Mutating the FcR binding site in IgG1-Fc (Fc1M) had little effect on NK cell proliferation. *E*, Proliferation assays done as described in *D* revealed that supernatants containing IL-15 directly fused to IgG1-Fc or to the C-terminal portion of the Ig κ-L chain were unable to induce NK cell proliferation in the absence or presence of IL-15Rα-IgG1-Fc. *F*, Effect of additional FcR signaling on sIL-15 complex-induced proliferation of WT NK cells. Human IgG1 was bound to 96-well plates. Proliferation assays were done in 25% of 293HEK cell-generated supernatants. Although additional signaling with plate-bound IgG1 increased proliferation rates induced by control supernatants, IL-15, IL-15/IL-15Rα, or IL-15/IL-15Rα-IgG4-Fc, it decreased proliferation in response to IL-15/IL-15Rα-IgG2-Fc. **, $p < 0.0005$. Note two different *x*-axis scales. *G*, The same proliferation assay as described in *F* done with *FcR*$\gamma^{-/-}$ NK cells revealed little effect of plate-bound IgG1. *H*, CD8$^+$/CD44^{high} T cells that had been derived from WT or *FcR*$\gamma^{-/-}$ mice responded equally to sIL-15 complex. Neutralizing FcRII/III did not affect in vitro proliferations. *I*, Freshly isolated NK and CD8$^+$/CD44^{high} T cells were labeled with CFSE (gray) and cultured in sIL-15 complex for 3 days (black). Cell concentrations had little effect on proliferation rates. Numbers represent mean fluorescence intensities of cultured cells.

molecule induced significant NK cell proliferation either in the presence or absence of IL-15Rα-IgG1-Fc (Fig. 1*E*). This indicates the necessity of IL-15Rα for sIL-15 complex-induced proliferation.

NK cells can be activated via FcR signaling during Ab-dependent cellular cytotoxicity (21). We tested whether signaling via FcRs would affect sIL-15 complex-induced proliferation. Additional FcR

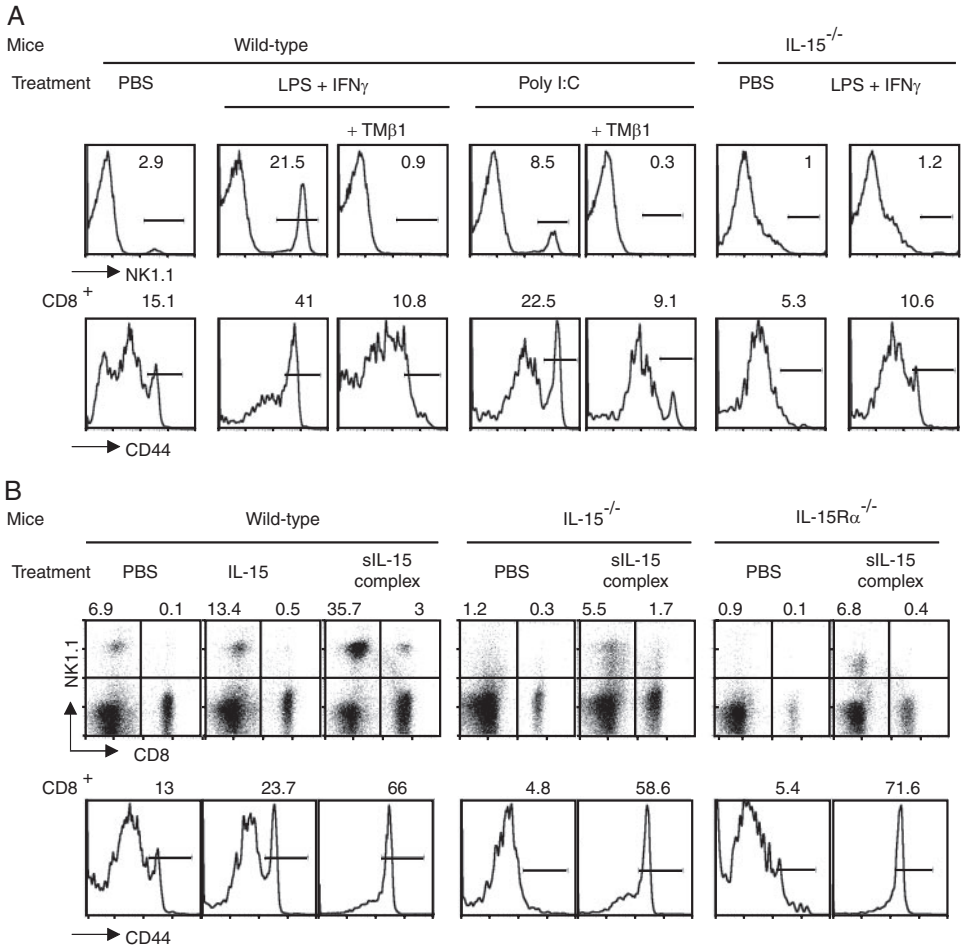

FIGURE 2. Expansions of NK and CD8$^+$/CD44^{high} T cells by sIL-15 complex in vivo. *A*, Acute expansions of NK and CD8$^+$/CD44^{high} T cells depend on IL-15. Mice were injected with 5 μg LPS and 1 μg IFN-γ or with 150 μg poly I:C. Cytometry analyses of blood cells 3 days after treatment revealed substantial increases in the number of NK cells (*upper graphs*) and of CD8$^+$/CD44^{high} T cells (*lower graphs*) after treatment with LPS and IFN-γ or poly I:C. The expansion of both cell types was prevented by a simultaneous injection of the Ab Tmβ1 that inhibits the binding of IL-15 to IL-2/15Rβ (CD122). No expansions were observed in *IL15*$^{-/-}$ (*right graphs*) and in *IL15Ra*$^{-/-}$ mice (data not shown). *B*, Injections of sIL-15 complex (10 μg at days 0 and 3, analyses at day 7) were sufficient to result in NK and CD8$^+$/CD44^{high} T cell expansions similar to LPS/IFN-γ treatment. sIL-15 complex was more efficient than IL-15 alone (2 times 2.5 μg) to expand NK and CD8$^+$/CD44^{high} T cells in vivo. Significant expansions were also observed if sIL-15 complex was injected into *IL15*$^{-/-}$ or *IL15Ra*$^{-/-}$ mice. The cell expansions were similar in blood (shown here) and in spleen (data not shown). Treatment with sIL-15 complex also caused the appearance of CD8/NK1.1 double-positive cells that were absent in untreated mice (upper right quadrants in dot blots).

signaling induced via plate-bound human IgG1 slightly increased NK cell proliferation in the presence of 293 cell-generated IL-15, IL-15/IL-15Rα, IL-15/IL-15Rα-IgG4-Fc, and even of control supernatants, but this increase stayed well below levels that were observed with IL-15/IL-15Rα-IgG2-Fc (Fig. 1*F*, note two different *x*-axis scales). The same FcR cross-linking actually inhibited IL-15/IL-15Rα-IgG2-Fc-mediated proliferation of NK cells. These data suggest that either FcR signaling inhibits sIL-15 complex-induced proliferation, or that signals coming from IL-15/IL-15Rα and from IgG2-Fc have to originate from the same molecule for maximum NK cell proliferation in vitro.

To test whether FcR binding and/or signaling is required for the sIL-15 complex to induce proliferation in vitro, we analyzed *FcR*$\gamma^{-/-}$ NK cells (22). The FcRγ-chain is necessary for signaling via FcRs but does not by itself bind immunoglobulins. *FcR*$\gamma^{-/-}$ mice lack FcRIII expression, and several other FcRs do not bind their cognate immunoglobulins. NK cells with a deletion of the *FcR*γ-chain have reduced surface FcR expression (data not shown). Fig. 1*G* shows that in contrast to WT NK cells, FcR cross-linking had little effect on the proliferation of *FcR*$\gamma^{-/-}$ NK cells. However, sIL-15 complex induced similar proliferations when cultured WT cells were compared with *FcR*$\gamma^{-/-}$ cells (shown in Fig. 1*H* for CD8 cells, similar results were obtained for NK cells that are not shown). In addition, neutralizing FcRII/III with a mAb did not affect the proliferation of WT CD8$^+$/CD44^{high} T cells (Fig. 1*H*) or of NK cells (data not shown). Therefore, the sIL-15 complex does not appear to require FcR signaling to induce proliferation in vitro.

To test whether the activity of the sIL-15 complex requires trans-presentation from neighboring cells, we cultured freshly isolated NK and CD8$^+$/CD44^{high} T cells at various cell concentrations, and their proliferation was monitored by CFSE dilution (Fig. 1*I*). If trans-presentation were required, higher cell concentrations should support proliferation. However, little differences were observed. This result is more consistent with the hypothesis that the IL-15/IL-15Rα and the IgG1-Fc portions of the sIL-15 complex induce NK or CD8$^+$/CD44^{high} T cell proliferation via a mechanism other than trans-presentation. In summary, a preassociation with IL-15Rα-IgG1-Fc increases the proliferative activity of IL-15 in vitro. This in vitro effect requires the Fc portion but not signaling via FcRs.

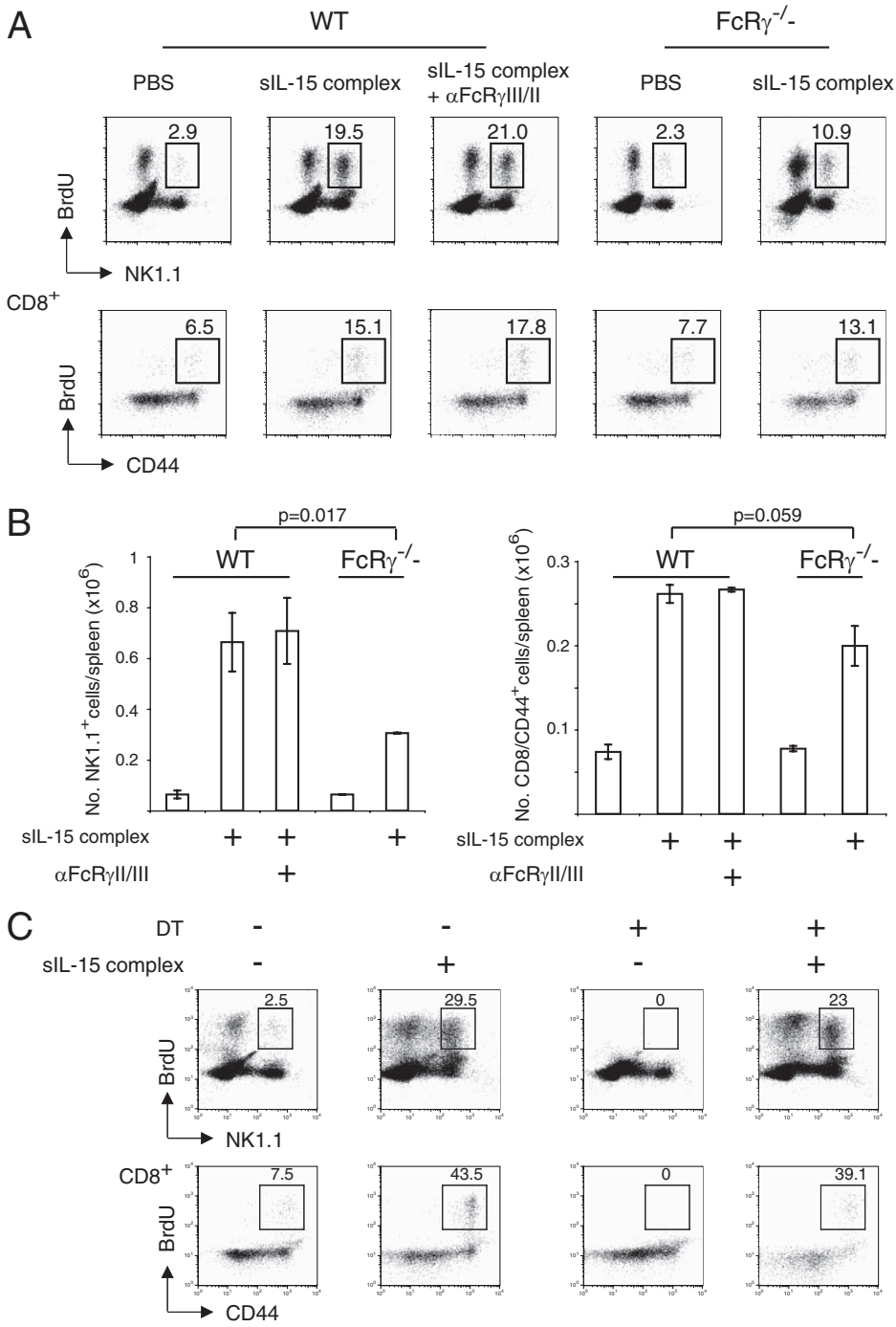

FIGURE 3. sIL-15 complex-induced cell expansions are caused by proliferation. *A*, Mice were injected with 1 μg sIL-15 complex and 1 mg BrdU 60 h later. The analyses of NK (*upper graphs*) and CD8$^+$/CD44^{high} T cells (lower graphs) 12 h after BrdU treatment in blood revealed that spontaneous proliferation rates of $<$10% were increased to 20 and 15%, respectively. Numbers represent the percentage of BrdU$^+$ cells among NK and CD8$^+$/CD44^{high} T cells. Neutralizing FcRII/III did not affect BrdU incorporation. Comparisons between WT and *FcR*$\gamma^{-/-}$ mice revealed similar spontaneous but decreased sIL-15 complex-induced cell proliferation in the absence of FcRγ. *B*, Total numbers of BrdU$^+$ splenic NK (*left*) and CD8$^+$/CD44^{high} T cells (*right*) 3 days after treatments with 1 μg sIL-15 complex are shown ($n = 4$). *C*, DCs are necessary for spontaneous but are dispensable for sIL-15 complex-induced NK (*upper graphs*) and CD8$^+$/CD44^{high} T cell (*lower graphs*) proliferations. DCs were removed in CD11c-DTR mice by treatments with diphtheria toxin (DT) that was followed by sIL-15 complex injections 24 h and by BrdU injections 60 h later. Although DT treatment prevented spontaneous cell proliferations, significant sIL-15 complex-induced BrdU incorporations were observed for both NK and CD8$^+$/CD44^{high} T cells even in the absence of DCs.

sIL-15 complex causes acute expansions of NK and CD8$^+$/CD44^{high} T cells in vivo

The number of NK and CD8$^+$/CD44^{high} T cells in vivo depends on the trans-presentation of IL-15 by IL-15Rα. To investigate whether the sIL-15 complex could mimic IL-15 trans-presentation in vivo we studied acute expansions of both lymphocyte populations. Expansions of NK and CD8$^+$/CD44^{high} T cells are observed during the acute phase of viral infections and in response to injections of TLR activators and IFN-γ (10, 23). As previously shown by Judge and coworkers (24), poly I:C or LPS/IFN-γ increased the number of NK and CD8$^+$/CD44^{high} T cells 3 days after injection (Fig. 2*A*). The Ab Tmβ1 neutralizes IL-15 activity at the IL-2/15 receptor β-chain (CD122). When this Ab was coinjected with poly I:C or LPS/IFN-γ, no increases in the number of either cell population were detected. Injecting IFN-γ and LPS into *IL15*-deficient mice also had little effect on the number of NK and CD8$^+$/CD44^{high} T cells (Fig. 2*A*). This shows that IL-15 is necessary for IFN-γ/LPS-induced expansions of NK and CD8$^+$/CD44^{high} T cells in vivo.

We studied whether sIL-15 complex is sufficient to induce the proliferation of NK and CD8$^+$/CD44^{high} T cells in vivo. When between 1 and 10 μg of this complex were injected into WT mice, a robust expansion of NK and CD8$^+$/CD44^{high} T cells was observed in blood and spleen. Fig. 2*B* shows an example in which two 10-μg injections of sIL-15 complex over a 7-day period increased the number of NK cells to 35% among leukocytes, and nearly two-thirds of all CD8$^+$ T cells expressed high levels of CD44. When we injected equimolar doses of IL-15, smaller

increases in both cell populations were observed. Treatments with sIL-15 complex also led to the appearance of CD8/NK1.1 double-positive cells that were not observed in untreated mice. In contrast to LPS/IFN-γ, treatment with sIL-15 complex also increased the number of NK and $CD8^+/CD44^{high}$ T cells in *IL15*$^{-/-}$ and in *IL15Ra*$^{-/-}$ mice although NK cell numbers did not reach the levels seen in WT mice. Thus, treatment with the sIL-15 complex is sufficient to induce acute expansions of NK and $CD8^+/CD44^{high}$ T cells in both WT mice and in mice deficient in *IL15* or *IL15Ra*.

To determine whether the observed cell expansions had resulted from proliferation, we measured BrdU incorporation rates (Fig. 3*A*). Although spontaneous BrdU incorporation was below 10% during a 12-h period, nearly 20% of NK cells and 15% of $CD8^+/CD44^{high}$ T cells in blood had divided in WT mice between 60 and 72 h after injections of 1 μg sIL-15 complex.

We further analyzed the involvement of FcR signaling in sIL-15 complex-mediated cell expansions. Neutralizing FcRII/III with injecting 2.4G2 did not affect sIL-15 complex-induced NK and $CD8^+/CD44^{high}$ T cell proliferations (Fig. 3*A*). In contrast, BrdU incorporations were reduced for both cell types if *FcRγ*$^{-/-}$ mice were compared with WT mice after sIL-15 complex treatments (Fig. 3*A*). In addition, total splenic numbers of $BrdU^+$ NK and $CD8^+/CD44^{high}$ T cells were also reduced in *FcRγ*$^{-/-}$ mice three days after sIL-15 complex injections (Fig. 3*B*). Thus, FcR binding and/or signaling appears to be important for maximum sIL-15 complex-induced cell expansion in vivo.

DCs have been reported to mediate NK cell activation via IL-15 trans-presentation (25). To test whether DCs participate in sIL-15 complex-induced cell proliferations, DCs were removed in mice that express a CD11c promoter-driven diphtheria toxin receptor by injections of diphtheria toxin that was followed by treatments with sIL-15 complex 24 h later. The absence of $CD11c^{bright}$ cells within the spleen 24 h after diphtheria toxin treatments was confirmed by cytometry (data not shown). Analyses of BrdU incorporations between 36 and 48 h later revealed that a lack of DCs decreased spontaneous proliferations of NK and $CD8^+/CD44^{high}$ T cells (Fig. 3*C*). In contrast, sIL-15 complex-induced BrdU incorporations were less affected by diphtheria toxin treatments suggesting that DCs are dispensable for sIL-15 complex-induced cell proliferations. In summary, a preassociation with IL-15Rα-IgG1-Fc enhances the effect of IL-15 to expand NK and $CD8^+/CD44^{high}$ T cells in vivo.

Lysis activity of sIL-15 complex-cultured NK cells

Treatments with IL-15 show some efficiency against tumors in mice (17). To investigate the feasibility of using sIL-15 complex against tumors, we initially determined whether NK cells cultured with sIL-15 complex retained their cytotoxic activity for tumor cells. NK cells were sorted from spleens and grown for 7 to 14 days in the presence of sIL-15 complex. As lysis target cells, we used YAC-1, MC38, and B16. We had previously shown that the effect of IL-15 in inhibiting the pulmonary metastasis following administration of MC38 colon carcinoma cells depended on NK cells (18). Fig. 4*A* shows that NK cells efficiently lysed both YAC-1 and MC38 but did not target EL4 cells that we used as a non-NK target control. In contrast, little lysis activity was detected in sIL-15-cultured $CD8^+/CD44^{high}$ T cells (data not shown). A preincubation of the NK-sensitive melanoma line B16 with IFN-γ increased the surface expression of MHC I resulting in a loss of NK lysis activity (Fig. 4*B*, *inset* shows the induction of MHC I). sIL-15 complex-cultured $CD8^+/CD44^{high}$ T cells were unable to lyse B16 regardless of the presence of IFN-γ (data not shown). We also investigated the lysis activity of *IL15*$^{-/-}$ NK cells and found no significant difference when compared with WT cells after culturing the cells for 7 days in sIL-15 complex (Fig. 4*C*). When

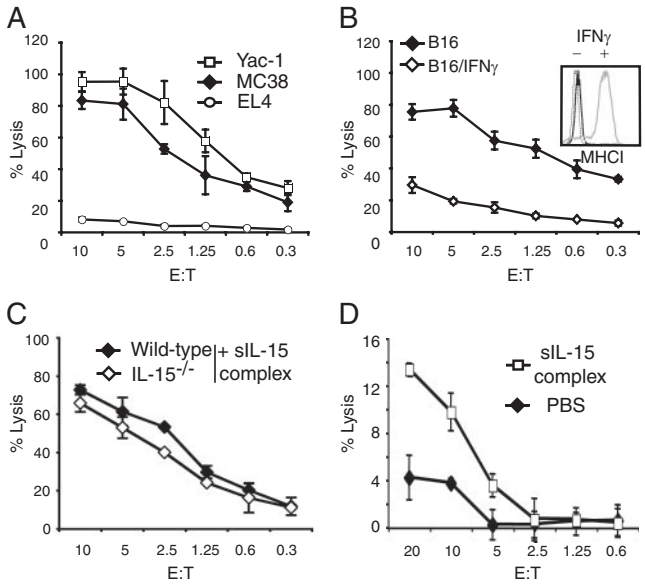

FIGURE 4. Lysis activity of sIL-15 complex-expanded NK cells. ^{51}Cr-labeled target cells were coincubated with NK cells for 4 h at various E:T ratios. Lysis activity was assessed by the amount of radioactivity in the supernatant. Values shown are averages ± SD from three experiments. *A*, NK cells lyse MC38 and Yac-1 but not EL4 cells. *B*, A 24-h pretreatment of B16 melanoma cells with IFN-γ inhibited NK cell-mediated lysis. The insert depicts the levels of MHC I that were expressed by the same B16 cells. *C*, sIL-15 complex-cultured NK cells that were derived from *IL15*$^{-/-}$ mice showed similar lysis activity toward MC38 when compared with WT cells. *D*, Freshly isolated NK cells were tested for their ability to lyse MC38. Prior injections of sIL-15 complex (10 μg 7 and 4 days before isolation) increased their lysis activity. However, levels stayed below those of cultured NK cells (compare with *A*).

freshly isolated NK cells were analyzed, prior injections of sIL-15 complex into mice also increased their cytotoxic activity (Fig. 4*D*). However, these levels remained well below the cytotoxicity of sIL-15 complex-cultured NK cells. Thus, sIL-15 complex increased the ability of NK cells to lyse target cells.

Increased antitumor effect of sIL-15 complex

IL-15 has been shown to inhibit tumor growth in various mouse models (17, 18). We tested whether pre-associating IL-15 with IL-15Rα-IgG1-Fc would enhance this activity. As a tumor model, we choose the melanoma cell line B16 that was efficiently lysed by sIL-15 complex-cultured NK cells (Fig. 4*B*). When injected i.v., mice largely develop tumors in the lungs causing death 3–4 wk after tumor injection.

Three groups of ten WT mice were injected i.v. with 10^6 B16 cells. Starting 3 days after tumor injections, mice received nine i.p. injections over 3 wk of either PBS, or of approximately equimolar doses of murine IL-15 (2 μg) or murine sIL-15 complex (10 μg). The survival was monitored, and the presence of large pulmonary melanoma masses was confirmed in all mice after death. As shown in Fig. 5*A*, control mice that were mock-treated with PBS died with a median survival of 23 days. Treatments with IL-15 alone increased the median survival to 26 days ($p = 0.0192$). The longest median survival was observed in mice that had been injected with sIL-15 complex (30 days). This survival proved significantly different from both PBS-treated mice ($p = 0.0003$) and from IL-15-treated mice ($p = 0.0369$).

To test whether NK cells mediated the antitumor activity of sIL-15 complex, we repeated the same tumor protocol in *Rag1*$^{-/-}$ mice that lack B or T lymphocytes. Fig. 5*B* shows a similar outcome in that the median survival was increased from 23 days in

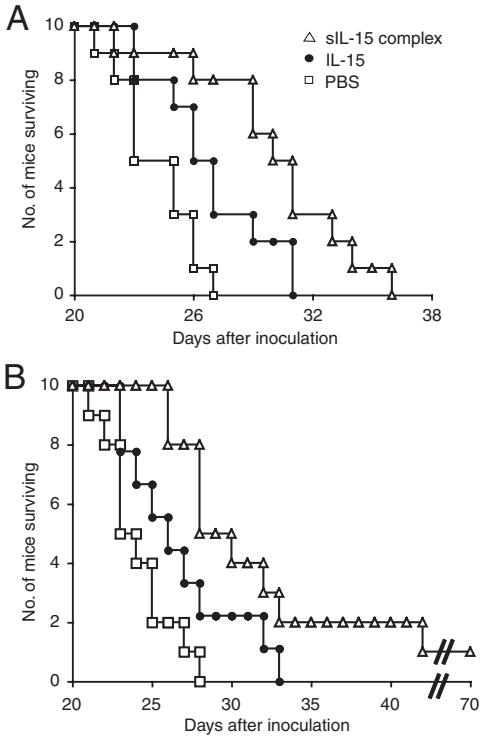

FIGURE 5. Survival increase of B16-bearing mice by sIL-15 complex administration. *A*, Three groups of ten WT mice each were injected with 10^6 B16 melanoma cells. Mice were treated with PBS or with nine doses of 2 μg IL-15 or 10 μg sIL-15 complex. Treatments with sIL-15 complex increased the survival that was significantly longer ($p < 0.05$) than treatments with PBS or IL-15. *B*, Treatments with IL-15 or sIL-15 complex resulted in similar survival increases if *Rag1*$^{-/-}$ mice were used.

PBS-treated mice to 26 days after IL-15 treatment ($p = 0.018$) to 28 days in sIL-15 complex-treated mice ($p = 0.002$). Thus, sIL-15 complex treatment proved efficient in extending the survival of B16-bearing mice in a T or B cell-independent manner.

Discussion

IL-15 is presented by IL-15Rα on the surface of nonlymphoid cells including monocytes and DCs to induce the proliferation of neighboring lymphocytes that only express the β and γ-chains (CD122/132) of the IL-15 receptor (16). In this study, we describe the use of a soluble protein complex able to replicate the cell surface IL-15/IL-15Rα activity. This complex comprises a fusion protein of the extracellular domain of IL-15Rα and the C-terminal two constant regions of human IgG1 preassociated with IL-15. An injection of this complex into mice resulted in a rapid expansion of NK and CD8$^+$/CD44^{high} T cells that could be sustained for weeks. When we used this complex in *IL15*$^{-/-}$ or *IL15Ra*$^{-/-}$ mice, we were able to correct their phenotypical lymphocyte defects. Both strains responded to complex treatment with expansions of NK and CD8$^+$/CD44^{high} T cells. We also showed that the acute response of NK and CD8$^+$/CD44^{high} T cells to injections of poly I:C or LPS plus IFN-γ strictly depended on IL-15 because it could not be found in either *IL15*$^{-/-}$ or *IL15Ra*$^{-/-}$ mice. Neutralizing IL-15 binding to IL-2/15Rβ with a mAb (Tmβ1) in WT mice also inhibited this acute proliferation. Thus, an injection of sIL-15 complex appeared to mimic this acute response of NK and CD8$^+$/CD44^{high} T cells. Its activity in vivo was substantially more effective than injections of IL-15 alone.

The soluble IL-15 complex also proved efficient in expanding IL-15-responsive cells in tissue culture using peripheral tissues as source. A distinct set of lymphocytes is reported to depend on IL-15 based on their lack in *IL15*$^{-/-}$ and in *IL15Ra*$^{-/-}$ mice (3, 4). Among these, we were able to expand NK and CD8$^+$/CD44^{high} T cells in vitro from various peripheral tissue sources. NK cells that were expanded in vitro with sIL-15 complex gained cytotoxic activity toward a number of tumor targets if compared with NK cells that were used directly ex vivo without prior culturing.

The sIL-15 complex proved again more efficient than IL-15 alone when the proliferation of NK and CD8$^+$/CD44^{high} T cells was assessed in vitro. The sensitivity of cells to the sIL-15 complex correlated with their CD122 expression. NK cells expressed the highest levels of CD122 and responded to the lowest doses of the complex (data not shown). We also observed that all three subunits of the complex, IL-15, IL-15Rα, and IgG1-Fc, were necessary for its effect in vitro: 1) IL-15 is necessary because no proliferations were caused by IL-15Rα-IgG1-Fc without IL-15 (Fig. 1). 2) IL-15Rα cannot be omitted in vitro because FcR cross-linking in the presence of IL-15 induced only weak proliferation responses. In addition, the presence of IL-15Rα caused a slight proliferation if IL-15 was present, and a direct fusion of IL-15 to IgG1-Fc was without activity (Fig. 1). 3) The Fc portion of IgG1, IgG2, or IgG3 is necessary for maximum proliferation in vitro as shown by the comparison of IL-15Rα molecules that had been fused to various Fc portions (Fig. 1). The activities of the Fc portions appear to be complex. In addition to Fc portions of IgG1 and IgG3, proliferation was also enhanced by IgG2-Fc and by mutated IgG1-Fc that prevents binding to FcRs. Also, while WT and *FcR*$\gamma^{-/-}$ NK and CD8$^+$/CD44^{high} T cells responded to sIL-15 complex with similar proliferation rates in vitro, they differed in their response to cross-linking via plate-bound human IgG1 (Fig. 1, *F–H*). It is currently unclear whether the proliferation-enhancing effect of various Fc portions of the sIL-15 complex is mediated by binding to FcRs or to an unidentified membrane protein. It is possible that the Fc portion has to be physically linked to IL-15Rα because FcR cross-linking via plate-bound IgG1 only weakly supported the proliferation response to IgG4-Fc-containing complex, and it actually inhibited the response to complex with IgG2-Fc (Fig. 1) or IgG1-Fc (data not shown). Contact between NK cells or between CD8$^+$/CD44^{high} T cells does nor appear to be required for sIL-15 complex-induced proliferation in vitro as similar proliferation rates were observed for various cell concentrations (Fig. 1). Together, these in vitro data are most consistent with the hypothesis that binding and signaling of the IL-15/IL-15Rα portion via CD122/CD132 plus an additional activity of the IgG-Fc portion are necessary to yield maximum proliferation.

Testing the activity of sIL-15 complex to expand NK and CD8$^+$/CD44^{high} T cells in vivo also showed a requirement for IL-15 because no expansions were seen with IL-15Rα-IgG1-Fc alone (data not shown). In contrast to sIL-15 complex-induced proliferations in vitro however, FcR binding and/or signaling appears to play a role in vivo because less NK or CD8$^+$/CD44^{high} T cell proliferations were observed in *FcR*$\gamma^{-/-}$ mice (Fig. 3). This shows that sIL-15 complex-induced in vitro proliferations only partially reflect its activities in vivo. The need for FcR signaling appears to be similar to recently reported IL-2-supporting activities of Abs in vivo (26). Boyman et al. (26) reported that two anti-IL-2 mAbs inhibited T cell proliferation in vitro but caused IL-2-dependent T cell expansions in vivo. The most consistent explanation for the data of Boyman et al. and the data shown in this article appears to be that both the sIL-15 complex and an IL-2/anti-IL-2 complex bind simultaneously to a FcR-positive accessory cell via the Fc portions and to responder cells via IL-15 or IL-2. Signaling via the FcR could induce the expression of additional activators on accessory cells that could further support the proliferation of NK

or $CD8^+/CD44^{high}$ T cells in the case of sIL-15 complex. We tested whether $CD11c^+$ DCs would mediate such a step, but our data do not support this hypothesis (Fig. 3*C*). Additional experiments are necessary to delineate the precise in vivo mechanisms of sIL-15 complex action.

IL-15-supporting in vitro effects of IL-15Rα-IgG1-Fc have been reported previously (27). A recent study suggests a hyperagonist activity of the IL-15-binding sushi domain of IL-15Rα if in vitro proliferations were analyzed (28). Rubinstein et al. reported increased expansions of NK and memory phenotype $CD8^+$ T cells in vivo after preassociating IL-15 with IL-15Rα (19). In addition, Mortier et al. and Budagian et al. (29, 30) described metalloproteinase-mediated cleavage of IL-15Rα that would generate an IL-15-inhibitory molecule. In our hands, the extracellular portion of IL-15Rα has a slight supporting effect on IL-15-induced in vitro proliferations of NK or $CD8^+/CD44^{high}$ T cells. The observed differences may be explained by the precise amino acid sequences, the source of the proteins as well as the target cells used for proliferation. We have also attempted to detect soluble endogenous IL-15/IL-15Rα heterodimers. We analyzed the serum of LPS/IFN-γ-treated mice by immunoprecipitation/immunoblot and were unable to detect endogenous IL-15/IL-15Rα complex at concentrations that are necessary for cell expansions (>500 pM, data not shown). Therefore, an involvement of such soluble, endogenous complexes in expansions of NK and $CD8^+/CD44^{high}$ cells in vivo appears unlikely.

Our data show that sIL-15 complex increases the survival of tumor-bearing mice. The survival proved significantly longer compared with IL-15 alone if treatments were done with equimolar doses. We choose as a tumor model B16 melanoma cells that are efficiently lysed by sIL-15 complex-cultured NK but not by $CD8^+/CD44^{high}$ T cells. The antitumor effect of sIL-15 complex in $Rag1^{-/-}$ mice shows that B or T lymphocytes are not necessary for its survival-enhancing effect. Therefore, NK cells are most likely mediating the antitumor effect of sIL-15 complex described in this article.

The precise mechanisms that underlie sIL-15 complex-mediated inhibition of tumor growth remain to be determined. Part of its activity may be the effect of IL-15Rα-IgG1-Fc on the half-life of IL-15. A preassociation with IL-15Rα-IgG1-Fc increases the time that is necessary for an initial 50% decline of serum levels for injected radiolabeled IL-15 from 30 to 60 min, and its half-life increases from 50 to >120 min in the subsequent period (data not shown). In addition to its effect on half-life, our in vitro data suggest that the Fc portion of the sIL-15 complex has a unique function that results in both expansion and activation of NK cells.

In conclusion, an association with IL-15Rα-IgG1-Fc enhances activities of IL-15 both in vitro and in vivo. The use of sIL-15 complex in vivo causes the expansion of distinct sets of responsive lymphocytes that may have a potential application in the treatment of tumors.

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Disclosures

All authors have a pending patent: Expansion of Natural killer and CD8 T cells with IL-15R/Ligand activator complexes. U.S. Provisional Patent Application No. 60/750,639.

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