Supporting Information

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SI Materials and Methods

Cytokines, Abs, and Reagents. Recombinant human IL-15 (rIL-15) was purchased from Peprotech, and recombinant human IL-2 (rIL-2) was purchased from Chiron. Polyclonal goat anti-human IL-15Rα (AF247), PE conjugated donkey anti-goat IgG (F0107), PE and FITC-conjugated mouse anti-human IL-15 (IC2471F and IC2471P), purified mouse IgG1 anti-human IL-15 (MAB247 and MAB647), and isotype control IgG1 were obtained from R&D Systems, and purified anti-human IL-15 (B-E29) was purchased from Diaclone. Alexa-647–conjugated anti-CD4 was obtained from BD Biosciences. Anti–IL-15Rα M161 mAb was provided by Amgen. Mouse IgG1 anti-human CD122, Clone A41, (Diaclone) was reported (1).

Cell Culture. The human adherent epithelial HeLa cell line and the Human Embryonic Kidney 293 cells (HEK-293) were cultured in DMEM containing 10% of heat-inactivated FCS (Gibco), 2 mM glutamine and 1 mg/mL glucose. The Kit225 human T lymphoma cell line was cultured in RPMI-1640 medium (Sigma-Aldrich), containing 6% heat-inactivated FCS (Gibco), 2 mM glutamine, and 5 ng/mL human rIL-2. NK92 cell line, kindly provided by Henri Vié (Inserm U892, Nantes, France) was cultured in RPMI-1640 medium (Gibco-Invitrogen) containing 10% heat-inactivated human serum, 2 mM glutamine, and 5 ng/mL human rIL-2. Transwell membranes were obtained from BD Falcon. Monocytes were obtained from the DTC Facility (CIC Biotherapy 0503) from Nantes. Cells were cultured in flasks at 2×10^6 cells per mL in RPMI 1% glutamin supplemented with 2% human albumin (Laboratoire Français de Fractionnement et de Biotechnologies, Les Ulis, France), 1,000 U/mL recombinant human GM-CSF and 200 U/mL recombinant human IL-4 (CellGenix). Immature DCs were harvested at day 5 and cultured in fresh medium with cytokines in 24-well plates. DC maturation was induced by the addition of 1 mg/mL of LPS (Sigma-Aldrich). All cell lines were maintained at 37 °C, in a humidified, 5% CO_2 atmosphere.

Molecular Constructs and RT-PCR. The plasmid corresponding to the full length of IL-15Rα was generated by PCR as previously described (2). For mutagenesis, full length IL-15Rα sequence was subcloned in pNo plasmid using Xho and Xba sites. Deletions were realized using sequences containing deletion and Quick Change Site-directed Mutagenesis kit (Stratagene) protocol. After amplification, the sequences were ligated at the Xho and Xba sites of the pcDNA3.1/*myc*-His mammalian expression vector (Invitrogen) and sequenced. For the generation of IL-15Rα variants, the 5′ and 3′ sequences framing each part of the deletion on exon 6 were amplified separately and ligated in pcDNA3.1/*myc*-His mammalian expression vector (Invitrogen). For ΔI178 deletion, the sense primer 5′-CACCACTGTGGCTTCCACGTCCACTG-3′ and the antisense primer 5′-CAGTGGACGTGGAAGCCACTGTGGTG-3′ were used. For ΔS179 deletion, the sense primer 5′-CCACTGTGGCTATCAGGTCCACTGTCC-3′ and the antisense primer 5′-GGACAGTGGACGTGATAGCCACAGTGG-3′ were used. For ΔT180 deletion, the sense primer 5′-CTGTGGCTATCTCCTCCACTGTCCTGC-3′ and the antisense primer 5′-GCAGGACAGTGGAGGAGATAGCCACAG-3′ were used. For ΔS181 deletion, the sense primer 5′-GGCTATCTCCACGACTGTCCTGCTGTG-3′ and the antisense primer 5′-CACAGCAGGACAGTCGTGGAGATAGCC-3′ were used. Membrane anchored ILR is a fusion molecule in which a flexible linker was introduced between the C-terminus of IL-15 and the N-terminus of the sushi domain (3). Briefly, a first fragment composed of BamHI IL-15-Linker 26-Pst was generated by PCR using following primers: sense primer 5′-TTGGATCCGCCGCCACCATGGACAG-3′ and antisense primer 5′-GTAGCTCTTGTCCCAGATGTC-3′. The second fragment composed of Pst-IL-15Rα-stop-Xho was generated by PCR using following primers: sense primer 5′-AACTGCAGATCACATGCCCTCCCCCCAT-3′ and antisense primer 5′-GACTCTCGAGCTATAGGTGGTGAGAGCAGTTTTCC-3′. After amplification, each fragment was digested with specific enzymes, ligated and inserted between the BamHI and Xho sites of pcDNA3.1/*myc*-His mammalian expression vector (Invitrogen). Soluble sILR by which the human IL-15Rα sushi domain (amino acids 1–77) and human IL-15 were separated by a linker 26 was produced as described (3).

Total RNA were isolated using the RNA isolation kit (Macherey-Nagel) according to the manufacturer's instructions. One μg of RNA was subsequently transcribed into cDNA using the Maxima First Strand cDNA Synthesis kit for RT-qPCR (Thermo Scientific). The following PCR was performed using the Thermo Scientific Phusion Hot Start II High-Fidelity DNA Polymerase (Thermo Scientific). Primers used for the PCR are the following: sense primer: 5′-CATTTTGGGCTGTTTCAGTG-3′ and antisense primer: 5′-GGTTTACAAGTAGTTGTGAAGAACT-3′.

Transfections and NK92 Cell Isolation. For transfection, 1×10^6 HEK-293 or HeLa cells were cultured overnight and transfected with 10 μg of plasmid following a standard PEI Polyplus transfection protocol. After 24 h, the medium was replaced and transfection efficiency verified by flow cytometry. For stable transfections, cells were cultured for 2 wk in 1 mg/mL Geneticin, G418 (Invitrogen) for positive selection and subsequent cloning was done using a FACS Aria III cytometer (Becton Dickinson). For NK92 cells isolation, cells cocultured for 24 h with IL-15 presenting cells, were purified by positive selection with magnetic beads using CD56 microbeads (Miltenyi Biotech) according to the manufacturer's instructions. For alamarBlue proliferation assays, 2×10^4 purified NK92 cells were plated in a 96-well plate in 100 μL of assay medium [1:9 ratio of alamarBlue (AbD Serotec) to cytokine-deprived medium] for 24 h. Metabolic activity was quantified by measuring the fluorescence at 540/590 nm.

RIA. The quantification of sIL-15Rα and IL-15.sIL-15Rα complex was determined by Sandwich Radio-Immuno-Assay as described (2). Briefly, goat anti-human IL-15Rα polyclonal antibody (AF247) was used as capture antibody and radio-iodinated neutralizing monoclonal anti-human IL-15Rα antibody M161 or radio-iodinated mouse monoclonal anti-human IL-15 (BE-29) were used as tracer. The M161 and BE-29 monoclonal antibodies were iodinated using the iodination reagent method. AF247 capture antibody was coated at 5 μg/mL to high-adsorption wells (breakable strips). Wells were washed and saturated with PBS containing 0.5% of BSA for 15 min at room temperature. A purified recombinant sIL-15Rα protein (2) and recombinant IL-15 (Peprotech) were used as standards. Samples and standards were incubated for 1 h at 4 °C. Radio-iodinated M161 or BE-29 antibodies were then added at 500 pM for 1 h at 4 °C. Supernatants of each well were collected and the wells were washed twice with PBS containing 0.5% of BSA. The radioactivity associated with the wells (bound fraction) and contained in the supernatants and washes (unbound fraction) was determined.

For radioactive ELISA detection of intracellular endogenous IL-15.IL-15Rα complexes, 5×10^6 HEK-293 or purified NK92

cells were washed in PBS and cell pellets were lysed using ice-cold RIPA lysis buffer [20 mM Tris (pH 7.5), 150 mM NaCl, 0.1% deoxycholic acid, 1% Nonidet P-40, 20 μM NaVO4, 10 mM NaF, 0.4 mM Pefablock, aprotinin and leupeptin at 1 μg/mL and protease inhibitors (Roche)]. After incubation on ice for 20 min, samples were centrifuged (15,000 × *g* for 15 min at 4 °C) and protein concentration was determined with a BC Assay Kit (Uptima) using BSA as standard.

Flow Cytometry. Cell surface expression of IL-15 and IL-15Rα were performed by incubating the cells for 30 min at 4 °C in the dark at optimal concentrations of antibodies. Intracellular detection of IL-15 was performed using BD Cytofix/Cytoperm Plus Fixation/Permeabilization kit (BD Biosciences) according to manufacturer instructions. For p-Stat5 staining, 5×10^5 Kit225 cells were fixed 10 min at 37 °C using Fix Buffer I (BD Biosciences) and permeabilized in Perm Buffer III (BD Biosciences) 30 min at 4 °C. Cells were then labeled with PE-conjugated mouse anti-human phospho-Stat5 (Y694) or PE-conjugated control isotype (BD Biosciences) for 1 h at room temperature. Annexin V staining was performed according to the protocol of the manufacturer (BD Biosciences). Cells were analyzed with a Calibur cytometer (Becton Dickinson) and FlowJo software.

Immunoflurescence Microscopy and Analysis. A total of 1.5×10^5 wt. ILR or uc.ILR HEK-293 stably transfected cells were seeded overnight on glass coverslips pretreated with poly-D-lysine (Sigma Aldrich). Kit225 cells were starved overnight in a cytokine-deprived medium. For IL-15 *trans*-presentation experiments, 7.5×10^4 Kit225 cells were added to each type of HEK-293 for 1 h at 37 °C. Subsequently, cells were stained with Alexa-647–conjugated anti-CD4 antibody, fixed with 1% paraformaldehyde for 15 min at room temperature and permeabilized with PBS containing 0.05% Triton ×100 (Sigma Aldrich) for 10 min at room temperature. Coverslips were then blocked with PBS for 30 min at 4 °C and incubated with FITC-conjugated anti–IL-15 antibody or FITC-conjugated mouse IgG1 isotype control for 30 min at room temperature. The slides were mounted by using Prolong Gold Antifade reagent with DAPI (Life Technologies) and images were recorded using a Nikon A1 RS confocal microscope (60× NA 1.40 oil). Images processing and orthogonal view representations were done with FIJI software. Three-dimensional visualizations were done with Amira 5.5.0 software (FEI).

Statistical Analysis. Unpaired *t* tests were used for comparisons between two normally distributed groups. Differences were considered significant at $P < 0.05$. All statistical analyses were performed by using Prism 4 (GraphPad). Significance levels of statistical tests are: not significant (ns); $^{*}P < 0.05$; $^{**}P < 0.01$; or $^{***}P < 0.001$.

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2. Mortier E, Bernard J, Plet A, Jacques Y (2004) Natural, proteolytic release of a soluble form of human IL-15 receptor alpha-chain that behaves as a specific, high affinity IL-15 antagonist. *J Immunol* 173(3):1681–1688.
3. Mortier E, et al. (2006) Soluble interleukin-15 receptor alpha (IL-15R alpha)-sushi as a selective and potent agonist of IL-15 action through IL-15R beta/gamma. Hyperagonist IL-15 x IL-15R alpha fusion proteins. *J Biol Chem* 281(3):1612–1619.

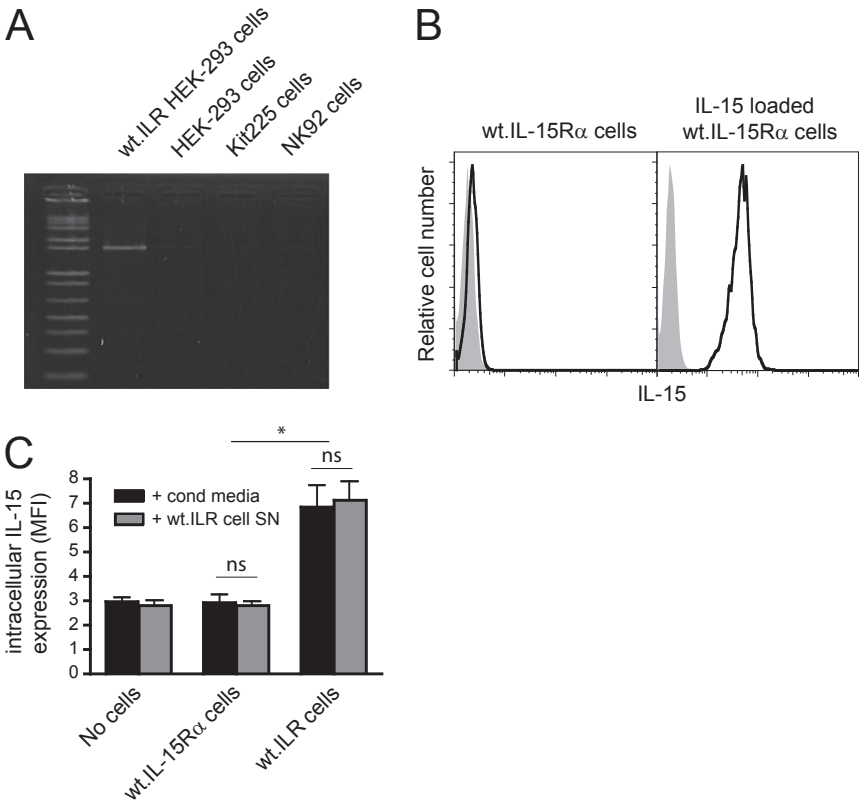

Fig. S1. (*A*) RT-PCR analysis of IL-15 expression in ILR transfected HEK-293 cells, HEK-293, Kit225, and NK92 cell lines. (*B*) Representative flow cytometric analysis of IL-15 at the surface of overnight IL-15 loaded IL-15Rα transfected HEK-293 cells. (*C*) Flow cytometric analysis of intracellular IL-15 expression (MFI) within Kit225 cells cocultured with wt.ILR or wt.IL-15Rα HEK-293 transfected expressing cells in the presence (gray bar) or in the absence (black bar) of soluble ILR released from wt.ILR HEK-293 cells. Wt.ILR cells were cultured 24 h and cell supernatant were harvested, after which Kit225 cells were cocultured with no cells, wt.IL-15Rα cells, or wt.ILR cells supplemented with wt.ILR-conditioned supernatant. All data are representative of at least three separate experiments. ns, not significant; $*P < 0.05$.

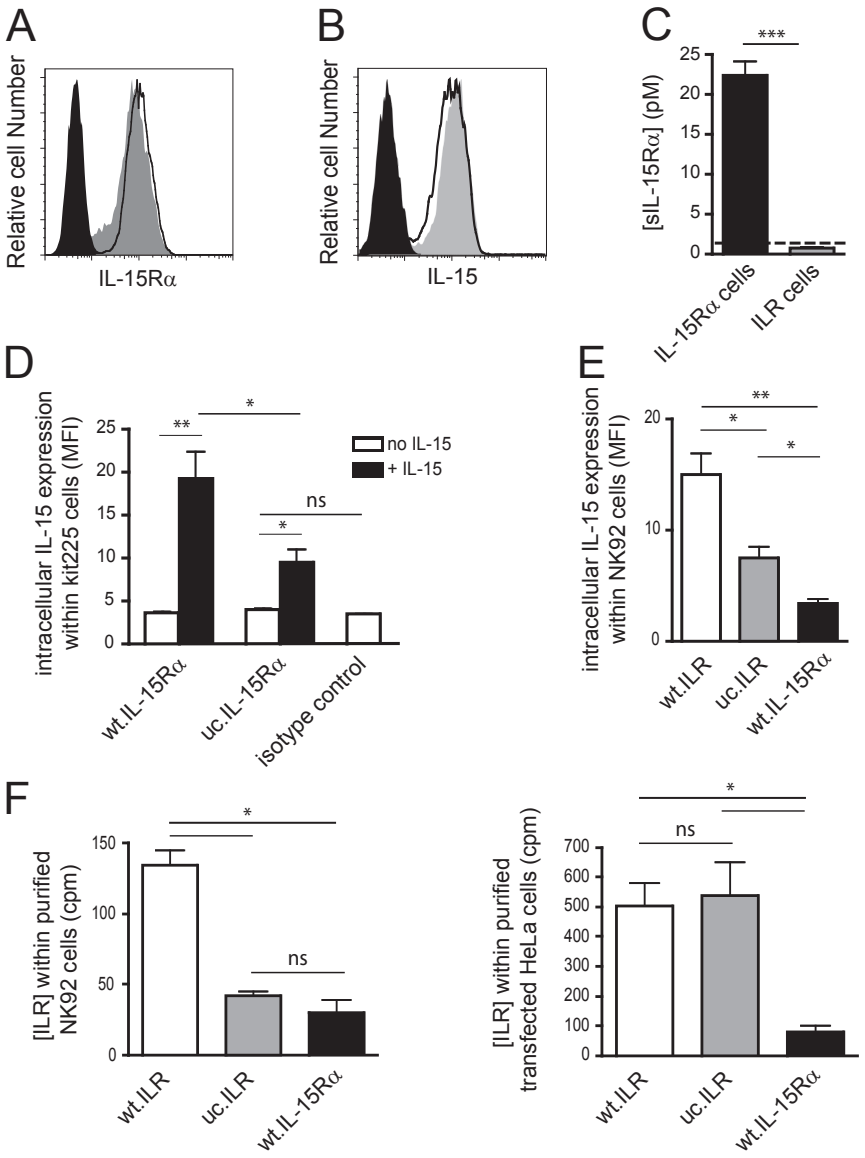

Fig. S2. (*A*) Flow cytometric analysis of IL-15Rα expression at the surface of wt.IL-15Rα (black histogram) and uc.IL-15Rα stably transfected HEK-293 cells (gray filled histogram). Isotype control staining is shown as black filled histogram. (*B*) Flow cytometric analysis of expression of IL-15 at the surface of wt.ILR (black histogram), uc.ILR cells (gray filled histogram), and wt.IL-15Rα transfected HEK-293 cells (black filled histogram). (*C*) ELISA detection of sIL-15Rα released in the culture supernatant from wt.IL-15Rα and wt.ILR transfected HEK-293 cells after 24 h of culture. Dashed line indicates the ELISA detection threshold (1 pM). (*D*) Flow cytometric analysis of IL-15 expression within Kit225 responding cells cocultured for 24 h with wt.IL-15Rα or uc.IL-15Rα HeLa cells, previously loaded overnight with 500 pM IL-15 or not. (*E*) Flow cytometric analysis of intracellular IL-15 expression (MFI) within NK92 responding cells following 24 h coculture with annoted cells. (*F Left*) ELISA measurement of IL-15.IL-15Rα complexes in lysates from purified NK92 cells (*Left*) previously cocultured for 24 h with wt.ILR, uc.ILR, or wt.IL-15Rα HeLa transfected cells. (*F Right*) ELISA measurement of IL-15.IL-15Rα complexes in lysates from wt.ILR, uc.ILR, or wt.IL-15Rα HeLa cells. Results are shown in cpm. All data are representative of at least two separate experiments. ns, not significant; $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

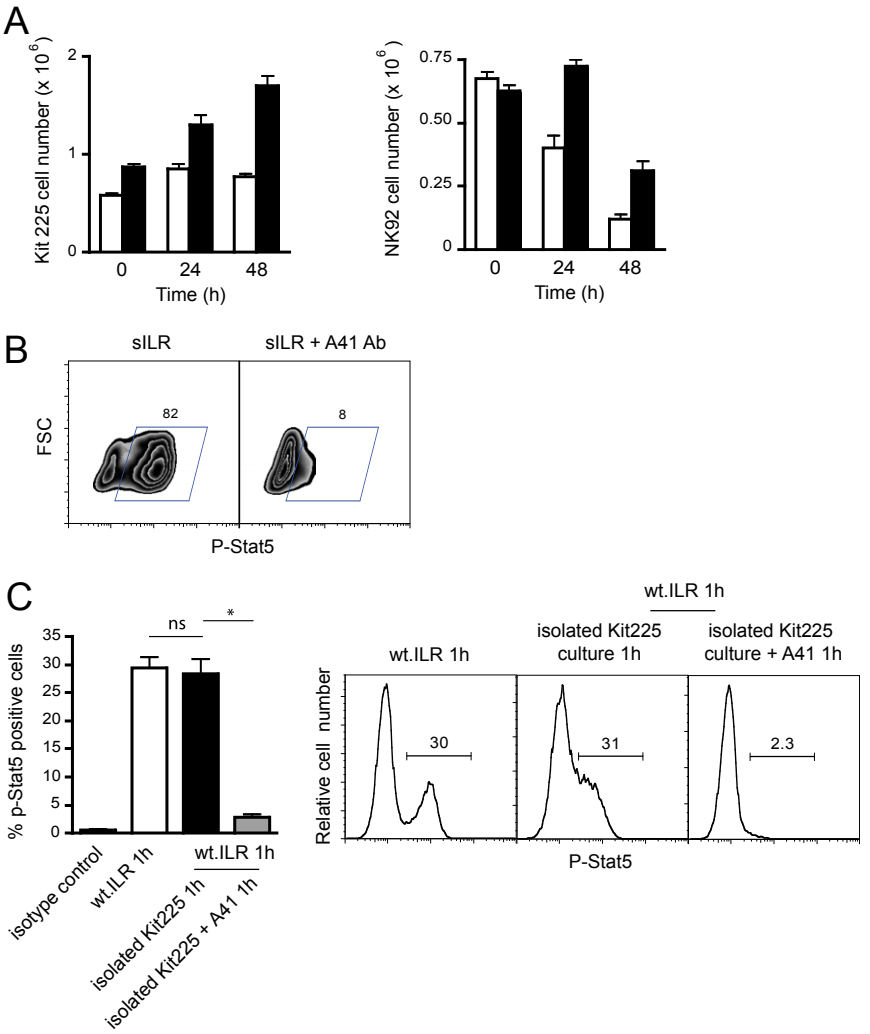

Fig. S3. (*A*) Kit225 (*Left*) or NK92 (*Right*) cells were cultured with 500 pM of soluble ILR overnight to allow IL-15 to enter within cells. Then, cells were washed and cultured in IL-15–free medium and counted at different time points. (*B*) Flow cytometric analysis of Stat5 phosphorylation within Kit225 responding cells treated with 50 pM of soluble ILR for 1 h in the presence or in the absence of A41 anti-CD122 antibody. (*C*) Flow cytometric analysis of Stat5 phosphorylation within Kit225 cells following 1 h coculture with wt.ILR transiently transfected HeLa cells, or 1 h coculture with wt.ILR transiently transfected HeLa cells and separated from presenting cells for one other hour, in the presence or in the absence of A41 anti-CD122 antibody. All data are representative of at least two separate experiments. ns, not significant; $*P < 0.05$.