Supplemental Information for

IL-15 enhanced antibody-dependent cellular cytotoxicity mediated by NK cells and macrophages

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Supplemental Materials & Methods

Reagents

Human IL-15 was provided by the Biopharmaceutical Department of the National Cancer Institute. Rituximab and Campath-1 were obtained from the NIH Pharmacy. Rat anti-asialo-GM1 was purchased from Wako Chemicals (Richmond, VA). The anti-NK1.1 antibody was obtained from BioXcell (West Lebanon, NH). Clodronate liposomes and control liposomes were purchased from FormuMax Scientific, Inc. (Sunnyvale, CA).

Cell lines

EL4-hCD20 cells were maintained in RPMI-1640 medium (Life Technologies) supplemented with 10% fetal bovine serum, 100 U/mL of penicillin, and 100 mg/mL of streptomycin. The EL4-hCD20 cell line was established by transfection of EL4 murine T-lymphoma cells with the Moloney-derived long terminal repeat (LTR)-CD20-LTR vector containing the human CD20 cDNA. The ATL cell line, MET-1, was established from the peripheral blood of a patient with acute ATL and the cells were maintained by serial transfer in SCID/NOD mice (Jackson Laboratories, Bar Harbor, ME). MET-1 cells have a distinct phenotype elucidated by fluorescein-activating cell sorting (FACS) analysis: $CD3^{dim}$, $CD4^{+/-}$, $CD7^{-}$, $CD20^{-}$, $CD25^{+}$ and $CD52^{+}$.

Therapeutic study

The therapeutic studies were performed in both C57BL/6 WT and FcγR-/- mice bearing EL4-hCD20 tumors. The therapeutic protocol is shown in Fig. 1A. Groups of EL4-hCD20 tumor-

bearing mice received hIL-15 i.p., 5μg per mouse every day, 5 days a week for 4 weeks starting at day 3 after injection of tumor cells or with the anti-CD20 antibody, rituximab, 100μg weekly for 4 weeks starting at day 5 or with a combination of hIL-15 with rituximab at the same dose and dosing schedules as those in the hIL-15 and rituximab groups. An additional group of EL4-hCD20 tumor-bearing mice that received PBS injections served as a control. The therapeutic studies in MET-1 xenograft model of ATL with both WT and FcγR-/- SCID/NOD mice were performed using the same therapeutic protocol as that in EL4-hCD20 model with the exception that the therapeutic trials were started on these mice when their serum soluble IL-2Rα (sIL-2Rα) levels were more than 1000 pg/mL, which occurs approximately 10 to 14 days after tumor inoculation (Fig.1B). Throughout the experiments, survival of the mice was recorded. The experiments were repeated.

Cytolytic activity assay

C57BL/6 WT and FcγR-/- mice were injected with EL4-hCD20 cells i.v. Three days later, groups of the tumor-bearing mice received PBS (control) or hIL-15 i.p at a dose of 5 μg/mouse for 5 continuous days. NK cells were purified from splenocytes of the mice at day 5 after therapy using negative isolation microbeads (Miltenyi Biotec Inc. Auburn, CA). EL4-hCD20 cells ($2x10^6$) were labeled with 0.1 mCi of chromium-51 (^{51}Cr) (Perkin-Elmer) for 30 minutes at 37°C in FBS. After washing with medium, the labeled target cells were coated with or without rituximab at a concentration of 10 μg/mL for 30 minutes at 4°C. Then the ^{51}Cr labeled rituximab-coated and uncoated target cells were incubated with isolated NK cells for 5 hours at various effector to target ratios. Radioactivity in the liquid phase was measured in a γ scintillation counter. Specific lysis was determined by using the formula: % lysis = 100x[(experiment cpm –

spontaneous cpm)/(maximum cpm – spontaneous cpm)]. The maximum release value was determined from target cells treated with 1% (v/v) Triton X-100 (Sigma). The data represent the mean ± SD of triplicates.

In vivo cell depletion experiments

The EL4-hCD20 tumor-bearing WT mice received combination therapy of hIL-15 with rituximab at the same dose and dosing schedules as above with the exception that the therapy lasted for 2 weeks instead of 4 weeks. The *in vivo* cell depletion experiment protocol is shown in Fig. 3A. NK cells were depleted *in vivo* by i.p. injections of anti-asialo-GM1 (50 μL) or anti-NK1.1 monoclonal antibody (100μg) and macrophages were depleted *in vivo* by i.v. injections of clodronate liposome (200 μL). One dose of anti-asialo-GM1, anti-NK1.1 antibody or clodronate liposome was administered one day before initiation of the combination therapy and subsequent doses were administered twice weekly for 2 weeks. An additional group of EL4-hCD20 tumor-bearing mice that received PBS injections served as a control and yet another control group of EL4-hCD20 tumor-bearing mice received the combination treatment and a control liposome. In the second *in vivo* cell depletion study, the EL4-hCD20 tumor-bearing WT mice received rituximab alone on days 1, 6, 11, and 15. Anti-asialo-GM1, anti-NK1.1 monoclonal antibody, clodronate liposomes and control liposomes were given on days -1, 1, 4, 7, 11 and 15. The experimental protocol is shown in Fig. s2A.

Conjugates of NK cells with EL4-hCD20 cells

NK cells were freshly isolated from WT mice treated with PBS or hIL-15, or with hIL-15 combined with clodronate liposomes as above, then labeled with CFSE (Life Technologies).

EL4-hCD20 cells were labeled with eFlour 670 dye (Life Technologies), then coated with or without rituximab at a concentration of 10ug/mL at 4°C for 30 minutes. The labeled NK cells and EL4-hCD20 cells were cocultured at a ratio of 1:2 at 37°C for 30 minutes. Then, the samples were gently vortexed and fixed with 1% paraformaldehye. Flow cytometry was performed to determine the numbers of double-positive events (NK cell–target cell conjugates). The percentages of total NK cells in the conjugates between NK cells from control mice and uncoated EL4-hCD20 target cells were presented as 1 and those of others were presented as fold changes.

Flow cytometry analysis

Surface and intracellular staining of splenocytes was performed using commercial FITC, PE, PerCP/Cy5.5, APC, pacific blue or BV510 conjugated antibodies (eBioscience, BioLegend). The samples were collected on a FACSCalibur or a FACSVerse flow cytometer (BD Biosciences, San Jose, CA) and analyzed using FlowJo cytometry analysis software (TreeStar, Inc., Ashland, OR).

Detection of serum concentration of the soluble IL-2Rα (sIL-2Ra)

SCID/NOD mice were injected with MET-1 cells and about 2 weeks later, the blood samples were taken from the mice. Measurement of the serum concentrations of the sIL-2Rα, a surrogate tumor marker of the IL-2Rα expressing ATL cells, was performed using an enzyme-linked immunosorbent assay (ELISA). The ELISA kit was purchased from R&D Systems (soluble Tac, catalogue no. DR2A00, Minneapolis, MN). The ELISAs were performed as indicated in the manufacturer's kit inserts.

Supplemental Results

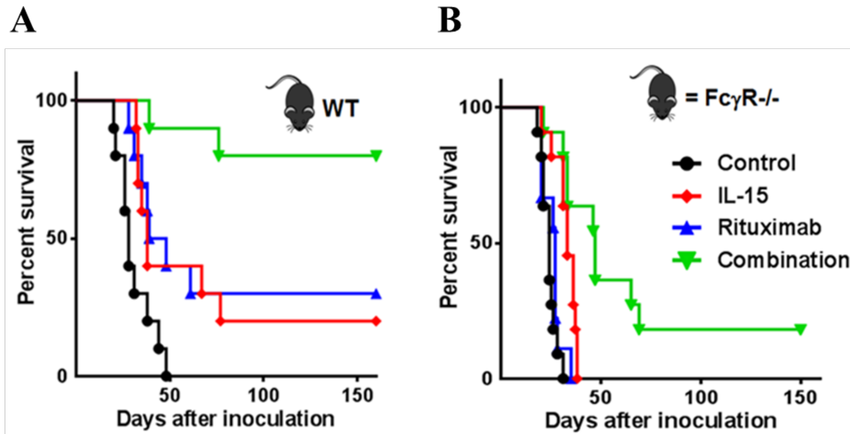
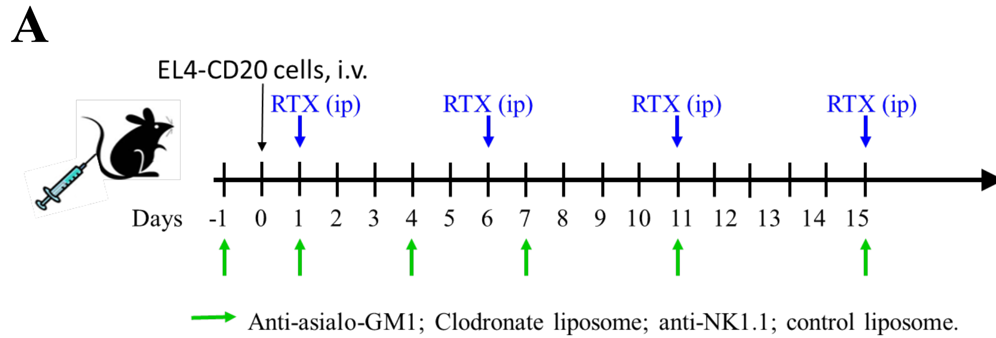


Figure s1. Related to Figure 1. The inclusion of hIL-15 in combination therapies increased the therapeutic efficacy of rituximab in WT C57BL/6 mice but not in FcγR-/- mice. In WT mice-bearing EL4-hCD20 tumors, treatments with either IL-15 or rituximab showed therapeutic efficacy when compared with the PBS control ($p<0.01$). The combination regimen with both IL-15 and rituximab showed a much greater therapeutic efficacy when compared with each single treatment alone, significantly augmenting tumor-free survival ($p<0.05$). In FcγR-/- mice, neither IL-15 or rituximab alone showed any significant therapeutic efficacy. Although the combination of IL-15 and rituximab showed a significant amount of activity when compared to all other groups ($p<0.01$). Still, this increase was considerably less than what we observed in WT mice. At 150 days, 20% of FcγR-/- mice remained, compared to 80% of the WT mice.

A



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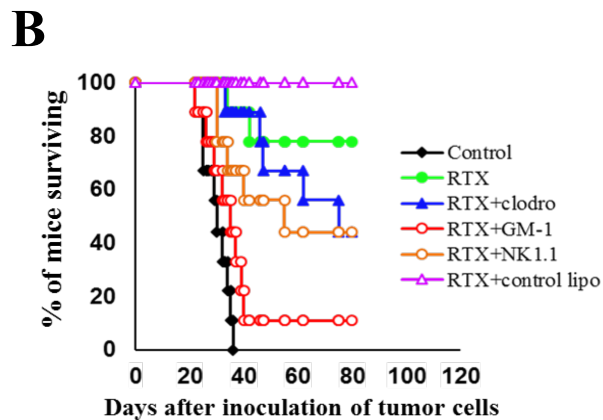

Figure s2. Related to Figure 3. Both NK cells and macrophages are necessary but not sufficient for the optimal therapeutic efficacy mediated by rituximab (n=9). The EL4-hCD20 tumor-bearing WT mice were treated with anti-NK1.1 antibody (100μg) or anti-asialo-GM1 (50 μL) or clodronate liposome (200 μL) to eliminate NK cells or macrophages, respectively, together with rituximab. (A) Experimental schema. (B) Kaplan-Meier survival plot of the mice in the therapeutic study. Treatment with rituximab or rituximab plus control liposome showed great therapeutic efficacy as seen by the prolonged survival of the EL4-hCD20 tumor-bearing mice when compared with the mice in the PBS control group ($p<0.001$). Compared with the group receiving rituximab alone, depletion of macrophages with clodronate or NK cells with the anti-NK1.1 antibody was associated with a reduction of the antitumor

efficacy although the differences were not statistically significant. Administration of anti-asialo-GM1 to eliminate NK cells was associated with an abrogation of the antitumor efficacy (p<0.01).

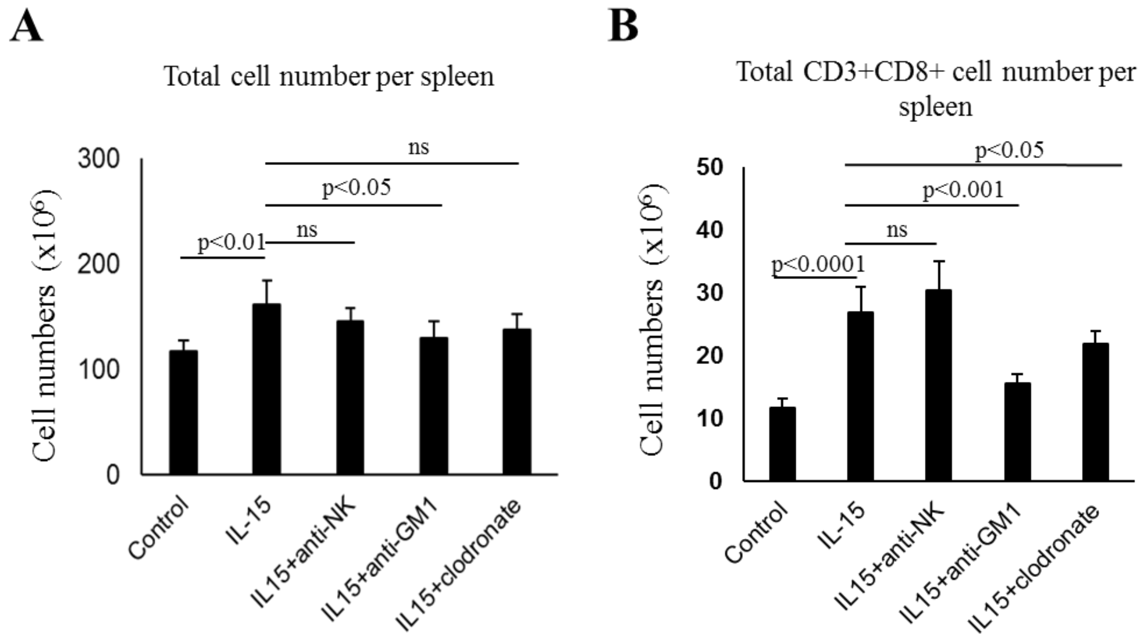

Figure s3. Related to Figure 4. Effects of cell depletion reagents on cell numbers induced by hIL-15 (n=3). WT C57BL/6 mice were treated with hIL-15 together with or without the cell depletion reagents for 6 days. At day 6 after initiation of the therapy, the mice were sacrificed, and spleens were taken. Splenocytes were separated and counted. Furthermore, the percentages of different cell types in spleens were analyzed by flow cytometry and the absolute cell numbers were calculated. Experimental schema was showed in Fig. 4A. (A) Total cell number per spleen and (B) Average absolute CD3+CD8+ cell number per spleen. Treatment with hIL-15 was associated with the increase in the total cell numbers (p<0.01) and CD3+CD8+ T-cell numbers in the spleens whereas clodronate and anti-asialo-GM1 reduced the numbers of CD3+CD8+ T-cells increased by IL-15.

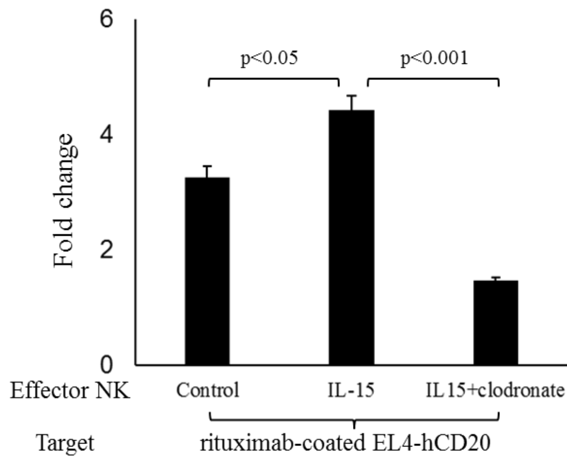

Figure s4. Related to Figure 4. Treatment with IL-15 enhanced the bindability of NK cells with target cells. NK cells were freshly isolated from WT C57BL/6 mice which were treated with PBS or hIL-15 or IL-15 plus clodronate for 6 days. The isolated NK cells and EL4-hCD20 cells were labeled with CSFE or eFlour 670 dye, respectively. Labeled EL4-hCD20 cells were coated with or without rituximab at a concentration of 10ug/mL at 4°C for 30 minutes. Then labeled NK and EL4-hCD20 cells were cocultured at a ratio of 1:2 at 37°C for 30 minutes. Then, the samples were gently vortexed and fixed with 1% PFA. Flow cytometry was performed to determine the number of double-positive events (NK cell–target cell conjugates). The conjugates between PBS-treated NK cells and un-coated EL4-hCD20 cells were presented as 1 and the conjugates in other groups were calculated as fold changed. NK cells isolated from IL-15 treated mice showed increased ability to form conjugates with rituximab-coated EL4-hCD20 cells. In contrast, treatment with clodronate liposomes abrogated the IL-15 increased bindability of NK cells to rituximab-coated EL4-hCD20 cells.