Augmented IL-15Rα Expression by CD40 Activation Is Critical in Synergistic CD8 T Cell-Mediated Antitumor Activity of Anti-CD40 Antibody with IL-15 in TRAMP-C2 Tumors in Mice

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Augmented IL-15Rα Expression by CD40 Activation Is Critical in Synergistic CD8 T Cell-Mediated Antitumor Activity of Anti-CD40 Antibody with IL-15 in TRAMP-C2 Tumors in Mice

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IL-15 has potential as an immunotherapeutic agent for cancer treatment because it is a critical factor for the proliferation and activation of NK and CD8+ T cells. However, monotherapy of patients with malignancy with IL-15 that has been initiated may not be optimal, because of the limited expression of the private receptor, IL-15Rα. We demonstrated greater CD8 T cell-mediated therapeutic efficacy using a combination regimen of murine IL-15 administered with an agonistic anti-CD40 Ab (FGK4.5) that led to increased IL-15Rα expression on dendritic cells (DCs), as well as other cell types, in a syngeneic established TRAMP-C2 tumor model. Seventy to one hundred percent of TRAMP-C2 tumor-bearing wild-type C57BL/6 mice in the combination group manifested sustained remissions, whereas only 0–30% in the anti-CD40–alone group and none in the murine IL-15–alone group became tumor free (p < 0.001). However, the combination regimen showed less efficacy in TRAMP-C2 tumor-bearing IL-15Rα−/− mice than in wild-type mice. The combination regimen significantly increased the numbers of TRAMP-C2 tumorspecific SPAS-I/SNC9-H8 tetramer+CD8+ T cells, which were associated with the protection from tumor development on rechallenge with TRAMP-C2 tumor cells. Using an in vitro cytolytic assay that involved NK cells primed by wild-type or IL-15Rα−/− bone marrow–derived DCs, we demonstrated that the expression of IL-15Rα by DCs appeared to be required for optimal IL-15–induced NK priming and killing. These findings support the view that anti-CD40–mediated augmented IL-15Rα expression was critical in IL-15–associated sustained remissions observed in TRAMP-C2 tumor-bearing mice receiving combination therapy. The Journal of Immunology, 2012, 188: 000–000.

Interleukin-2 has been approved by the U.S. Food and Drug Administration (FDA) for use in the treatment of patients with metastatic malignancy (1, 2). However, IL-2 is not optimal as an agent to inhibit tumor growth because it is associated with capillary leak syndrome and because it induces a series of checkpoints that inhibit immune responses that include its role in activation-induced cell death (AICD) and maintenance of IL-2–dependent regulatory T cells. In contrast, IL-15, with its ability to activate CD8 T cells and NK cells, its inhibition of AICD, and its role in the persistence of CD8 memory T cells, might be a better choice. Attempts to prevent tumor growth and to treat various mouse tumor models with IL-15 proved effective (3–8). In contrast to IL-2, IL-15 is a membrane-associated molecule that induces signaling at an immunological synapse between APCs and target NK or CD8 T cells (9). IL-15Rα on surfaces of monocytes and dendritic cells (DCs) presents IL-15 in trans to cells that express the other two IL-15 receptors, IL-2/IL-15Rβγ (CD122) and common γ-chain (γc) alone (9). Mice with genetic deletions of IL-15 or its private receptor, IL-15Rα, are characterized by decreased numbers of NK, NKT, CD8+/CD44high T, TCRγ/δ+ T, and intestinal intraepithelial CD8α+/β− T cells, suggesting that physiologically relevant IL-15 signals require IL-15Rα expression (10, 11). The FDA and National Cancer Institute (NCI) have approved a phase I dose-escalation trial of recombinant human IL-15 in patients with metastatic melanoma and renal cell cancer, and this trial has been initiated.

Although IL-15 administration may ultimately show efficacy in the treatment of metastatic malignancy, it may not be optimal when used in monotherapy. As noted for IL-15 long-term persistence and its optimal transpresentation to NK and CD8 T cells, it must be bound to IL-15Rα on the surface of DCs and monocytes (9–13). However, there is only modest expression of IL-15Rα on unactivated DCs; therefore, the level of IL-15Rα expression would be limiting in therapeutic trials that use IL-15 alone. In the current study, we examined the use of an agonistic anti-CD40 Ab to in...
duce IL-15Rα expression that could be given to circumvent this problem.

CD40 is a member of the TNFR superfamily that plays a critical role in both cellular and humoral immune responses (14). CD40 ligation triggers a series of cellular functions, including activation of APCs (15, 16). Agonistic anti-CD40 Abs were shown to promote T cell-mediated immunity and effective treatment of neoplastic diseases in animal models (17, 18). It was reported that a stimulatory CD40 Ab indirectly activated NK cells by inducing IL-12 secretion by DCs, which resulted in significant antitumor effects (19). In another potential contribution to effective therapy of tumors, we demonstrated that activation of CD40 was associated with an increased expression of IL-15Rα by DCs and an enhanced antitumor efficacy in murine models (6). This induction of IL-15Rα expression by CD40 activation provided the scientific basis for this study in which we demonstrated therapeutic efficacy mediated by the combination regimen of murine IL-15 (mIL-15) and an agonistic anti-CD40 Ab in the TRAMP-C2 model of prostatic cancer. Furthermore, we used IL-15Rα−/− mice and DCs from such mice to demonstrate that induction of IL-15Rα expression by an agonistic anti-CD40 Ab contributes to the synergistic antitumor effect observed with the combination therapy. The findings from this study suggest that the combination regimen is very promising for the treatment of patients with cancer.

Materials and Methods

Reagents

mIL-15 was purchased from PeproTech (Rocky Hill, NJ). Rat anti-mouse CD40 (FGK4.5) and rat anti-mouse CD8 (clone 2.43) mAbs were obtained from Bio X Cell (West Lebanon, NH). Rat anti-asialo-GM1 was purchased from Wako Chemicals (Richmond, VA).

Tumor cell line and mouse model

The TRAMP-C2 cell line was derived from a prostate tumor of a male TRAMP mouse. MC38 is a metastatic murine colon cancer cell line syngeneic to C57BL/6 mice. IL-15Rα−/− mice (Jackson Laboratory, Bar Harbor, ME) were backcrossed to the C57BL/6 strain for 10 additional generations. The tumor model was established by s.c. injection of 5 × 10^3 TRAMP-C2 cells into the right flank of male C57BL/6 (NCI-Frederick) or IL-15Rα−/− mice. All animal experiments were approved by the NCI Animal Care and Use Committee and were performed in accordance with NCI Animal Care and Use Committee guidelines.

Therapeutic study

The therapeutic studies were performed in both C57BL/6 wild-type and IL-15Rα−/− mice bearing TRAMP-C2 tumors. Therapy started when the tumor volumes reached 40–110 mm³. Groups of TRAMP-C2 tumor-bearing mice received mIL-15 i.p., 2.5 μg/mouse every day, 7 d a week for 2 wk; the anti-CD40 Ab, 200 μg on day 0 and then 100 μg on days 3, 7, and 10; or a combination of mIL-15 with anti-CD40 Ab at the same doses and dosing schedules as those in the mIL-15 and the anti-CD40 Ab groups. An additional group of TRAMP-C2 tumor-bearing mice that received PBS injections served as a control. Throughout the experiments, survival of mice was recorded, and TRAMP-C2 tumor growth was monitored by measuring tumor size in two orthogonal dimensions. The tumor volume was calculated using the equation: 1/2(long dimension)(short dimension)^2.

Luciferase–GFP–lentivirus infection and bioluminescence imaging

TRAMP-C2 cells were seeded into a 24-well plate at the concentration of 5 × 10^4/ml/well and cultured overnight. Then, luciferase-GFP fusion protein expressing lentivirus (pol2-fluc-eGFP; Advanced Technology Program, NCI-Frederick) was added to the culture for 2 d. The GFP+ cells were collected by sorting. Male C57BL/6 wild-type mice were injected with luciferase-GFP–transduced TRAMP-C2 (TRAMP-C2/luc-GFP) cells s.c., and the therapeutic study was performed using the same therapeutic protocol as above. The bioluminescence images were obtained 1 d before and 16 and 40 d after initiation of therapy with an in vivo-imaging system, and the images were analyzed using Living Imaging software (both from Xenogen, Alameda, CA).

In vivo cell-depletion experiment

The TRAMP-C2 tumor-bearing wild-type mice received combination therapy of mIL-15 with the anti-CD40 Ab at the same doses and dosing schedule as above. NK and/or CD8+ T cells were depleted in vivo by i.p. injections of anti-asialo-GM1 (50 μl) or purified rat anti-mouse CD8 Ab (200 μg). One dose of anti-asialo-GM1 and anti-mouse CD8 Ab was administered 1 d before initiation of combination therapy; subsequent doses were administered three times weekly for 2 wk. Depletion of NK and CD8+ cells in PBMCs was evaluated by flow cytometry during the experiment. Survival of mice was recorded, and TRAMP-C2 tumor growth was monitored throughout the experiments.

Immune parameters following treatments in TRAMP-C2 tumor-bearing mice

TRAMP-C2 tumor-bearing wild-type mice were treated using the same therapeutic protocol as above. At day 12 after initiation of the treatment, all of the mice (four mice/group) were sacrificed, and the spleens were obtained. The proportion and absolute numbers of NK1.1+, CD8+, CD44^high CD8+, and TRAMP-C2 tumor-specific SPAS-1/SNC9-H2 (20) tetramer+ CD8+ cells in the spleens were analyzed by flow cytometry.

Rechallenge of the surviving tumor-free mice from the combination group

Surviving tumor-free mice from the group receiving the combination regimen of mIL-15 and anti-CD40 Ab were rechallenged with 5 × 10^3 TRAMP-C2 tumor cells or with 1 × 10^6 MC38 tumor cells, an irrelevant tumor cell line, at 40 d or 3.5 mo after initiation of therapy to assess the effect on tumor engraftment. One group of the surviving tumor-free mice received i.p. injections of purified rat anti-mouse CD8 Ab (200 μg/injection) three times weekly for 2 wk, and one dose of anti-CD8 Ab was administered 1 d before the rechallenge. Another group of mice received combination therapy initially but did not receive the TRAMP-C2 tumor cells before the rechallenge. The mice in the control groups did not receive either the initial TRAMP-C2 challenge or any therapeutic reagents prior to “rechallenge.”

Flow cytometry analysis

Surface staining to quantitate the number of NK1.1, CD8, CD44, and SPAS-1/SNC9-H2 tetramer-expressing cells in PBMCs or splenocytes was performed using commercial FITC-, PE-, or allophycocyanin-conjugated Abs (eBioscience), with the exception of the PE-conjugated SPAS-1/SNC9-H2 tetramer, which was provided by Dr. J.P. Allison. IL-15Rα expression on cell surfaces was detected using biotinylated anti-mIL-15Rα Ab or bio- tinylated normal goat IgG as an isotype control (R&D Systems), followed by PE-labeled streptavidin (eBioscience). CD40 expression on TRAMP-C2 cell surfaces was analyzed using the primary Ab FGK4.5 or normal rat IgG as an isotype control, followed by PE-labeled mouse anti-rat Ig (BD Biosciences, San Jose, CA). The samples were collected on a FACSscan flow cytometer (BD Biosciences) and analyzed using FlowJo cytometry analysis software (TreeStar, Ashland, OR).

Activation of NK cells primed by bone marrow-derived DCs

NK cells were purified from RAG1−/− mice using negative-isolation MicroBeads (Miltenyi Biotec). Bone marrow-derived DCs (BMDCs) from wild-type or IL-15Rα−/− mice were generated using 10 ng/ml IL-4 and 20 ng/ml GM-CSF (both from PeproTech). The BMDCs were pretreated with LPS (1 μg/ml; Sigma, St. Louis, MO), anti-CD40 Ab (10 μg/ml), or medium alone for 20 h. Then BMDCs were washed and cocultured with NK cells at a 2:1 ratio for an additional 20 h in the presence or absence of LPS (1 μg/ml), anti-CD40 Ab (10 μg/ml), mIL-15 (2.5 ng/ml), or anti-CD40 Ab plus mIL-15. 51Cr-labeled TRAMP-C2 cells were incubated with NK cells and BMDCs for 5 h at various E:T ratios. Radioactivity in the liquid phase was measured in a gamma counter (PerkinElmer). The percentage of specific lysis was determined using the following formula: percentage lysis = 100 × [(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).

Statistical analysis

Comparison of cell numbers in spleens and luminescent signals of images among different treatment groups was analyzed using the Student t test. The
statistical significance of differences in the survival of mice in different groups was determined using the log-rank test and the GraphPad Prism program (GraphPad, San Diego, CA).

Results

Combination therapy of mIL-15 with an agonistic anti-CD40 Ab led to regression of established TRAMP-C2 tumors in wild-type mice

We investigated the therapeutic efficacy of the combination regimen of mIL-15 with an agonistic anti-CD40 Ab in an established TRAMP-C2 tumor model. The rationale for this approach is that expression of IL-15R\(\alpha\) was induced by CD40 activation (6, 21). Therapy started when TRAMP-C2 tumors were well established (average tumor volume = 80 mm\(^3\)). Treatment with mIL-15 alone at a dose of 2.5 \(\mu\)g/mouse, 5 d a week for 2 wk, provided a modest inhibition of tumor growth (Fig. 1A) and prolonged survival of the TRAMP-C2 tumor-bearing mice compared with mice in the PBS control group (Fig. 1B, \(p < 0.05\)). Treatment with anti-CD40 Ab at a dose of 200 \(\mu\)g/mouse on day 0, followed by 100 \(\mu\)g/mouse on days 3, 7, and 10, significantly inhibited tumor growth (Fig. 1A) and prolonged survival of TRAMP-C2 tumor-bearing mice compared with mice in the PBS control group or the mIL-15–alone group (Fig. 1B, \(p < 0.001\)). Critically, combination therapy with mIL-15 and the anti-CD40 Ab provided greater therapeutic efficacy than did monotherapy with either mIL-15 or anti-CD40 Ab (Fig. 1A, 1B, \(p < 0.001\)). The therapeutic study was repeated, and comparable results were obtained. All mice in the PBS control and mIL-15–alone groups died of tumor progression by day 40, and 70–100% of mice in the anti-CD40 Ab-alone group died of tumor progression within 2 mo (Fig. 1B). In contrast, combination treatment resulted in a highly significant prolongation of survival, with 70–100% of mice in the combination group becoming and remaining tumor free (Fig. 1B). Flow cytometry analysis showed that TRAMP-C2 cells do not express CD40 on their cell surfaces (data not shown).

Bioluminescence imaging confirmed efficacy of the combination treatment

Groups of six wild-type mice bearing TRAMP-C2/luc-GFP tumors were treated using the same therapeutic protocol as above. As

![FIGURE 1](http://www.jimmunol.org/)

**Combination therapy of mIL-15 and agonistic anti-CD40 Ab led to regression of established TRAMP-C2 tumors in wild-type C57BL/6 mice.** Therapy was initiated after tumors were well established, with an average volume of 80 mm\(^3\). Changes in tumor volume during the therapeutic course (A) and Kaplan–Meier survival plot of mice (B) in one of the two therapeutic studies (\(n = 9–10\)). Treatment with mIL-15 alone at a dose of 2.5 \(\mu\)g/mouse, 5 d a week for 2 wk, inhibited tumor growth slightly and prolonged survival of TRAMP-C2 tumor-bearing mice compared with mice in the PBS control group (\(p < 0.05\)), whereas treatment with anti-CD40 Ab (200 \(\mu\)g/mouse on day 0 and then 100 \(\mu\)g/mouse on days 3, 7, and 10) provided greater inhibition of tumor growth and prolonged survival of the TRAMP-C2 tumor-bearing mice compared with mice in the PBS control group or mIL-15–alone group (\(p < 0.001\)). Furthermore, combination therapy with mIL-15 and anti-CD40 Ab provided a greater therapeutic efficacy, as demonstrated by the fact that all of the mice in the combination group were alive at day 60, with 80% becoming tumor free, whereas only 20% of the mice in the anti-CD40 Ab-alone group and none of the mice in the PBS control or mIL-15–alone group were alive at that time. (C) Bioluminescence imaging of TRAMP-C2/luc-GFP tumor-bearing mice confirmed efficacy of combination treatment. Groups of six mice bearing TRAMP-C2/luc-GFP tumors were treated using the same therapeutic protocol. The bioluminescence images were taken at different time points. Treatment with anti-CD40 Ab significantly delayed tumor growth. However, at day 40 after initiation of therapy, only one of six mice in the anti-CD40 group was tumor free. In contrast, the combination treatment led to regression of tumors, with all six mice in the group becoming and remaining tumor free.
shown in Fig. 1C, the average tumor volumes among groups were comparable at the beginning of therapy. Treatment with anti-CD40 Ab significantly inhibited tumor growth, with average total luminescent signals of $1.0 \times 10^9$ photons/s on day 16, which were much lower than $1.5 \times 10^9$ and $7.7 \times 10^8$ photons/s in the PBS control and mIL-15 groups, respectively, on the same day ($p < 0.01$). However, only one of six mice in the anti-CD40 group was tumor free. In contrast, combination treatment led to regression of tumors, with all six mice becoming and remaining tumor free.

**Compared with wild-type mice, IL-15Rα−/− mice showed markedly reduced therapeutic efficacy when treated with the combination regimen of mIL-15 and anti-CD40 Ab**

IL-15 and its private receptor, IL-15Rx, are both essential for the support of NK and CD8+ T cell homeostasis. Expression of IL-15Rx specifically on DCs is critical for the trans-presentation of IL-15 and activation of NK cells. A series of studies was performed to determine whether increased expression of IL-15Rx, mediated by administration of agonistic anti-CD40 Ab, played a role in the augmented IL-15-mediated therapeutic efficacy observed in the combination-treatment group. Previously, we reported that treatment with anti-CD40 Ab augmented expression of IL-15Rx on the CD11c+ population of splenocytes and increased serum concentrations of IL-15Rx (6). In the current study, we performed a therapeutic trial in the TRAMP-C2 tumor model using IL-15Rα−/− mice. We used the same doses and dosing schedules for mIL-15 and anti-CD40 Ab as in the therapeutic studies in TRAMP-C2 tumor-bearing wild-type mice (Fig. 1).

Treatment with mIL-15 showed very little therapeutic efficacy in IL-15Rα−/− mice, as reflected by the very modest inhibition of tumor growth (Fig. 2A) and the prolongation of survival of the TRAMP-C2 tumor-bearing mice compared with those in the control group (Fig. 2B, $p < 0.001$). Treatment with anti-CD40 Ab inhibited tumor growth and prolonged the survival of TRAMP-C2 tumor-bearing IL-15Rα−/− mice compared with mice in either the PBS control or mIL-15–alone group (Fig. 2, $p < 0.001$). However, treatment with anti-CD40 Ab at the same dose and dosing schedule showed much less therapeutic efficacy in IL-15Rα−/− mice (Fig. 2) compared with that observed in wild-type mice (Fig. 1). Although the combination regimen in IL-15Rα−/− mice provided greater therapeutic efficacy compared with monotherapy consisting of either mIL-15 or anti-CD40 Ab (Fig. 2, $p < 0.001$), only 10–20% of the mice in the combination group became tumor free, in contrast with 70–100% of the wild-type mice receiving combination therapy (Fig. 1). The therapeutic study in IL-15Rα−/− TRAMP-C2 tumor-bearing mice was repeated, and comparable results were obtained. IL-15Rα−/− mice had decreased numbers of NK and CD44highCD8+ T cells (10, 11); therefore, the reduced therapeutic efficacy of combination therapy in IL-15Rα−/− mice might be due to the lower numbers of cytolytic cells.

To determine whether the killing activity of cytolytic cells from IL-15Rα−/− mice was lower than that of cells from wild-type mice, the lysis activity of NK cells toward TRAMP-C2 cells was examined ex vivo (Supplemental Fig. 1). Compared with NK cells isolated from TRAMP-C2 tumor-bearing wild-type mice that received PBS injections as a control, NK cells isolated from both TRAMP-C2 tumor-bearing wild-type and IL-15Rα−/− mice that received the combination therapy for 5 d showed increased lysis activity toward TRAMP-C2 tumor cells (Supplemental Fig. 1). However, NK cells isolated from wild-type mice that received the combination regimen showed greater lysis activity against tumor cells than did NK cells from IL-15Rα−/− mice (Supplemental Fig. 1). This suggested that both decreased numbers of NK and CD44highCD8+ T cells and lower lysis activity per cytolytic cell contributed to the reduced antitumor activity obtained using the combination regimen in IL-15Rα−/− mice bearing TRAMP-C2 tumor (Fig. 2) compared with that in wild-type mice (Fig. 1).

**CD8+ T cells played a major role in the combination regimen-mediated antitumor efficacy**

To investigate possible involvement of NK or CD8+ T cells as effectors in the combination regimen-mediated antitumor activity, we treated TRAMP-C2 tumor-bearing mice with anti–asialo-GM1 or purified rat anti-mouse CD8 Ab to eliminate NK or CD8+ T cells, together with the combination regimen. One dose of the anti–asialo-GM1 and anti-CD8 Ab was administered 1 d before the initiation of combination therapy, and subsequent doses were administered three times weekly for 2 wk. FACS analysis showed that >90% of NK cells (Supplemental Fig. 2A) and CD8+ cells (Fig. 3A) from PBMCs were depleted by administration of anti–asialo-GM1 and anti-CD8 Ab, respectively. Compared with the group receiving the combination therapy alone, depletion of CD8+ cells significantly, but not completely, eliminated antitumor efficacy (Fig. 3B, $p < 0.001$). Administration of anti–asialo-GM1 decreased antitumor efficacy (Fig. 3B, $p < 0.001$). Simultaneous depletion of CD8+ and NK cells abrogated antitumor efficacy mediated by the combination therapy (Supplemental Fig. 2B). It should be noted that administration of anti–asialo-GM1 depleted NK

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**FIGURE 2.** TRAMP-C2 tumor-bearing IL-15Rα−/− mice showed reduced efficacy with the combination regimen compared with wild-type mice. Changes in tumor volume during the therapeutic course (A) and Kaplan–Meier survival plot of the mice (B) in one of the two therapeutic studies ($n = 10$). The protocol for this study was the same as that in Fig. 1. Treatment with mIL-15 alone showed very little therapeutic efficacy in TRAMP-C2 tumor-bearing IL-15Rα−/− mice. Treatment with anti-CD40 Ab significantly delayed tumor growth and prolonged survival of TRAMP-C2 tumor-bearing mice compared with mice in the PBS control or mIL-15–alone group ($p < 0.001$). Furthermore, combination therapy with mIL-15 and anti-CD40 Ab provided greater therapeutic efficacy, as seen by the prolonged survival, with 20% of mice becoming tumor free, whereas none of the mice in other groups were tumor free ($p < 0.001$). The results are from one of two experiments.
cells (Supplemental Fig. 2A), as well as a subset of CD8+ cells, especially CD44brightCD8+ and TRAMP-C2 tumor Ag-specific SPAS-1/ SNC9-H8 tetramer+CD8+ cells (Fig. 3A). TRAMP-C2 cells isolated from in vivo tumors expressed high levels of MHC class I (MHCI) (Supplemental Fig. 2C). TRAMP-C2 cells cultured in vitro with IFN-γ increased total cell numbers and absolute numbers of CD8+ cells isolated from in vivo tumors expressed high levels of MHC class I (MHCI) (Supplemental Fig. 2C). TRAMP-C2 cells cultured in vitro with IFN-γ increased total cell numbers and absolute numbers of CD8+ cells (Supplemental Fig. 2D). These studies support the view that CD8+ T cells played a major role in combination regimen-mediated antitumor efficacy, whereas NK cells might also contribute to the therapeutic efficacy observed.

FIGURE 3. CD8+ T cells played a major role in the combination regimen-mediated antitumor activity in the TRAMP-C2 model. TRAMP-C2 tumor-bearing mice were treated with anti–asialo-GM1 (50 μL) or purified rat anti-mouse CD8 Ab (200 μg/injection), together with the combination regimen of mIL-15 plus anti-CD40 Ab. (A) Representative FACS analysis of PBMCs from the mice at day 19 after initiation of the therapy. The results showed that the addition of an anti-CD8 Ab was associated with the depletion >90% of CD8+ cells. Administration of anti–asialo-GM1 reduced the number of CD8+ cells, especially CD44brightCD8+ and TRAMP-C2 tumor-specific SPAS-1/SNC9-H8 tetramer+CD8+ cells. (B) Kaplan–Meier survival plot of the mice. Compared with the group receiving combination therapy alone, depletion of CD8+ T cells with anti-CD8 Ab plus combination treatment nearly abrogated antitumor efficacy (p < 0.001), and administration of anti–asialo-GM1 reduced antitumor activity (p < 0.001). The experiment was repeated, and the results of the two experiments were pooled together.

To further elucidate the effectors underlying the synergistic efficacy of mIL-15 plus anti-CD40 Ab, we defined the impact of combination therapy on numbers of splenic tumor Ag-specific CD8+ T cells using the TRAMP-C2 tumor-specific SPAS-1/SNC9-H8 tetramer. TRAMP-C2 tumor-bearing mice were treated with the same therapeutic protocol used in the therapeutic studies shown in Fig. 1. At day 12 after initiation of therapy, all mice (four mice/group) were sacrificed. The percentages of CD8+, CD44brightCD8+, and TRAMP-C2 tumor Ag-specific SPAS-1/SNC9-H8 tetramer+CD8+ cells in spleens were analyzed by flow cytometry, and absolute cell numbers were calculated. Treatment with mIL-15, anti-CD40 Ab, or their combination increased total cell numbers and absolute numbers of CD8+ and CD44brightCD8+ cells in the spleens compared with those in the control group (Fig. 4A–C, p < 0.05). Most critically, the combination regimen dramatically increased the absolute number of TRAMP-C2 tumor Ag-specific SPAS-1/SNC9-H8 tetramer+CD8+ cells in the spleens compared with those of all other groups (Fig. 4D, p < 0.05).

Protection from tumor development on rechallenge with TRAMP-C2 tumor cells was associated with increased numbers of TRAMP-C2 tumor Ag-specific SPAS-1/SNC9-H8 tetramer+CD8+ cells

Surviving tumor-free mice from the combination-therapy group in studies shown in Fig. 1 were rechallenged at day 40 (Fig. 5A) or 3.5 mo (Supplemental Fig. 3) after initiation of therapy with 5 × 105 TRAMP-C2 cells s.c. Another group of these surviving tumor-free mice was rechallenged with 1 × 106 MC38 cells, an irrelevant tumor cell line (Fig. 5B). Mice that initially received the TRAMP-C2 tumor and that became and remained tumor free after receiving the combination regimen demonstrated resistance to TRAMP-C2 tumor development compared with mice in the control group that had not received either TRAMP-C2 tumor cells or any previous treatment (Fig. 5A, Supplemental Fig. 3A, p < 0.001). However, such tumor-free mice did not show any resistance to MC38 tumor development (Fig. 5B). FACs analysis showed that the percentages of CD44bright CD8+ and TRAMP-C2 tumor Ag-specific SPAS-1/SNC9-H8 tetramer+CD8+ cells in PBMCs of mice surviving the initial tumor challenge were higher than those of mice in the control groups (Fig. 5C, Supplemental Fig. 3B). The protection from TRAMP-C2 tumor development mediated by the combination therapy was nearly abrogated by depletion of CD8+ cells (Fig. 5A, p < 0.01). To further verify that the protection from tumor development is tumor specific, we treated normal C57BL/6 mice with the combination regimen and then challenged the mice with TRAMP-C2 cells at day 40. The percentages of CD8+ and CD44brightCD8+ cells in the PBMCs of these mice increased, whereas the percentage of TRAMP-C2 tumor Ag-specific SPAS-1/SNC9-H8 tetramer+CD8+ cells did not (Supplemental Fig. 4A); these mice did not show resistance to TRAMP-C2 tumor development on rechallenge (Supplemental Fig. 4B). These findings support the view that protection from tumor development on rechallenge correlated with development of TRAMP-C2 tumor Ag-specific SPAS-1/SNC9-H8 tetramer+CD8+ T cells. Surviving tumor-free mice from the combination-therapy group shown in Fig. 1C were rechallenged at day 40 after initiation of therapy with 5 × 105 TRAMP-C2/luc-GFP cells s.c. on the left flank, and photographs were taken at day 23 after rechallenge. These surviving tumor-free mice demonstrated resistance to TRAMP-C2/luc-GFP tumor development compared with mice in the control group that had not received either TRAMP-C2/luc-GFP tumor cells or any previous treatment (Fig. 5D).

Administration of an agonistic anti-CD40 Ab induced the expression of IL-15Ra

We reported that administration of the anti-CD40 Ab increased the expression of IL-15Ra on the CD11c+ population of splenocytes (6). In the current study, we further examined IL-15Ra expression on other cell types by flow cytometry analysis. We demonstrated that
treatment with anti-CD40 Ab increased the expression of IL-15Rα on CD11c+ cells, as well as on other cell types, such as B cells, the CD11b+ population, and CD8+ cells in spleen, compared with PBS-treated mice (Fig. 6A).

Anti-CD40–induced IL-15Rα expression on BMDCs appears critical for optimal NK cell lytic activity

It was shown that murine DCs require IL-15Rα to prime NK cells (22). To further investigate the mechanism of synergetic antitumor effect of the combination regimen, we used an approach similar to that used by Koka et al. (22) to examine the abilities of BMDCs derived from wild-type or IL-15Rα−/− mice to support NK cytolytic activity. BMDCs were pretreated with LPS, anti-CD40 Ab, or their combination increased the total cell numbers and the absolute numbers of CD8+ cells and CD44highCD8+ cells in the spleen compared with the control group (p < 0.05). Critically, only the combination regimen significantly increased the numbers of SPAS-1/SNC9-H8 tetramer+CD8+ cells compared with all other groups (p < 0.05).

FIGURE 5. Enhanced TRAMP-C2 tumor-specific SPAS-1/SNC9-H8 tetramer+CD8+ T cells were associated with protection from a rechallenge with TRAMP-C2 tumor cells. (A) Kaplan–Meier survival plot of mice that received rechallenge of TRAMP-C2 cells at day 40 after initiation of original therapy. Compared with control mice that did not receive TRAMP-C2 cells or any previous treatment, mice surviving the first challenge with TRAMP-C2 cells (○) demonstrated resistance to TRAMP-C2 tumor development (p < 0.001). Depletion of CD8 cells (▲) nearly abrogated protection mediated by combination therapy (p < 0.01). (B) Kaplan–Meier survival plot of mice that received rechallenge of MC38 cells at day 40 after original therapy. Mice surviving the challenge with TRAMP-C2 cells did not show resistance to development of an irrelevant tumor (MC38) (p > 0.5). (C) Representative FACS analysis of PBMCs from mice just before rechallenge. Mice that initially received the TRAMP-C2 tumor and became and remained tumor free after receiving the combination regimen manifested an increase in the percentages of CD8+, CD44highCD8+ T cells, especially SPAS-1/SNC9-H8 tetramer+CD8+ T cells, compared with mice in the control group that had not received either TRAMP-C2 tumor cells or any previous treatment. (D) Bioluminescence images. Surviving tumor-free mice in the combination group from Fig. 1C were rechallenged with TRAMP-C2/luc-GFP cells at day 40 after initiation of the therapy. The mice surviving the first challenge with TRAMP-C2/luc-GFP cells demonstrated resistance to tumor development compared with mice in the control group.
The presence of mIL-15 alone, suggesting that expression of IL-15Rα by BMDCs is critical for the synergistic effect of the combination regimen on the activation of NK cells.

Cytolytic activities of NK cells primed by wild-type or IL-15Rα−/− BMDCs stimulated by LPS, anti-CD40, or medium alone. Compared with BMDCs cultured with medium, wild-type BMDCs stimulated with LPS or anti-CD40 Ab enhanced cytolytic activity of NK cells, whereas IL-15Rα−/−-comparably stimulated BMDCs did not. Cytolytic activities of NK cells cocultured with wild-type BMDCs in the presence or absence of anti-CD40 Ab, mIL-15, or their combination. With anti-CD40 Ab or mIL-15, NK cells showed enhanced cytolytic activity toward target cells compared with NK cells cocultured with BMDCs in medium alone. Furthermore, with both anti-CD40 Ab and mIL-15, NK cells showed the greatest cytolytic activity toward target cells compared with NK cells in all other cocultures. Cytolytic activities of NK cells cocultured with IL-15Rα−/− BMDCs in the presence or absence of anti-CD40 Ab, mIL-15, or their combination. In contrast to wild-type BMDCs, NK cells cocultured with IL-15Rα−/− BMDCs in the presence of anti-CD40 Ab did not show increased lysis activity toward target cells compared with NK cells cocultured with IL-15Rα−/− BMDCs in medium alone. With both anti-CD40 Ab and mIL-15, NK cells only showed a comparably increased lysis activity toward target cells as did NK cells cocultured with IL-15Rα−/− BMDCs in the presence of mIL-15 alone, suggesting that expression of IL-15Rα by BMDCs is critical for the synergistic effect of the combination regimen on the activation of NK cells.

TRAMP-C2 cells. NK cells cocultured with wild-type BMDCs in the presence of LPS or anti-CD40 Ab showed stronger cytolytic activity compared with those in medium alone (Fig. 6B). In contrast, lytic activity of NK cells primed by IL-15Rα−/− BMDCs that were stimulated with LPS or anti-CD40 Ab did not increase (Fig. 6B). When mIL-15 was added to the coculture with anti-CD40 Ab, wild-type BMDC-primed NK cells demonstrated stronger cytolytic activity to the target cells than did the wild-type BMDC-primed NK cells in the presence of either mIL-15 or anti-CD40 Ab alone (Fig. 6C). In contrast, IL-15Rα−/−-BMDC-primed NK cells in the presence of both mIL-15 and anti-CD40 Ab showed cytolytic activity that was similar to that of IL-15Rα−/−-BMDC-primed NK cells in the presence of mIL-15 alone (Fig. 6D). These findings suggest that anti-CD40 Ab-induced expression of IL-15Rα by BMDCs is critical for the synergistic effect of the combination regimen on NK cell activation.

**Discussion**

Receptors for IL-2 and IL-15 are heterotrimeric and both contain the γc (23) and IL-2/IL-15Rβ (24–27). In addition, the high-affinity forms of IL-2R and IL-15R contain a unique cytokine-specific α subunit (28). In light of the common receptor components, the two cytokines share several functions (13, 25, 26, 29, 30). In addition, there are distinct differences between the actions of IL-2 and IL-15 (30–33). In contrast to IL-2, IL-15 has no marked effect on regulatory T cells and is an antiapoptotic factor that inhibits IL-2–induced AICD in select systems (32). A critical factor in the functional differences between IL-2 and IL-15 involves distinct modes of action of these two cytokines. IL-2 is a secreted molecule that binds to preformed high-affinity heterotrimeric receptors (34, 35). In contrast, IL-15 is predominantly membrane bound and induces signaling in the context of an immunological synapse (9). Furthermore, IL-15Rα on activated DCs presents IL-15 in trans to cells that express IL-2/IL-15Rβ and γc but not IL-15Rα (9).

IL-2 has been approved by the FDA for the treatment of patients with metastatic malignancy (1, 2). However, IL-15 might be superior to IL-2 for cancer therapy (13, 25). IL-15 administration has shown therapeutic efficacy in animal models (4, 6, 36–38); however, it is not optimal as a single agent. IL-15Rα expression on monocytes and DCs is critical for transpresentation of IL-15 (9–11, 13). However, there is only a low level of expression of IL-15Rα on nonactivated DCs; therefore, IL-15Rα would be very limiting in therapeutic trials involving IL-15 alone.

In the current study, combination therapy with both mIL-15 and anti-CD40 Ab showed enhanced efficiency compared with monotherapy with either mIL-15 or anti-CD40 Ab (Fig. 1). With the combination treatment there was a highly significant prolongation of survival of TRAMP-C2 tumor-bearing mice, with 70–100% of the mice with established tumors becoming tumor free. Furthermore, surviving tumor-free mice in the combination group demonstrated resistance to TRAMP-C2 tumor development when they were rechallenged (Fig. 5, Supp. Fig. 3). A number of studies was performed to define the mechanisms underlying this synergy between IL-15 and anti-CD40 Ab. In contrast to our previous study (6), in which we demonstrated that NK cells appear to be the primary effector cells mediating the killing of CT26 and MC38 tumors after combination treatment, in the TRAMP-C2
model, CD8+ T cells were shown to play a major role in the combination regimen-mediated antitumor efficacy (Fig. 3). Depletion of CD8+ T cells significantly reduced the antitumor efficacy mediated by the combination therapy (Fig. 3). Furthermore, the combination regimen dramatically increased the absolute number of TRAMP-C2 tumor-specific SPAS-1/SNC9-H4 tetramer+CD8+ T cells compared with that of all other groups (Fig. 4D), and the protection from tumor development on rechallenge with TRAMP-C2 tumor cells was associated with an increased number of TRAMP-C2 tumor-specific tetramer+CD8+ T cells (Fig. 5). Therefore, the combination therapy of IL-15 plus anti-CD40 Ab has the potential to enhance the antitumor efficacy of both MHCI- and non-MHCI–restricted cytotoxic cells, which may broaden its application in human cancer therapy.

Nevertheless, there was also evidence to suggest that NK cells might also contribute to the therapeutic efficacy in the TRAMP-C2 model. It is known that, in addition to supporting NK cell survival, IL-15 supports NK cell activation. In the current study, the administration of anti–asialo-GM1 decreased antitumor efficacy mediated by the combination regimen (Fig. 3). Although anti–asialo-GM1 largely eliminated the NK cell population (Supplemental Fig. 2A), it also reduced the proportion of CD8+ T cells, especially CD44^highCD8+ and TRAMP-C2 tumor-specific tetramer+CD8+ T cells (Fig. 3A). Previously, it was demonstrated that non-NK cell expression of IL-15Rα is essential to support the generation, proliferation, and survival of NK cells (39). We demonstrated that administration of anti-CD40 Ab increased the expression of IL-15Rα on DCs (6), as well as other cell types, such as B cells, the CD11b+ population, and CD8+ cells. Furthermore, we showed that IL-15Rα on activated human monocytes and DCs presents IL-15 in trans to NK cells not expressing IL-15Rα (9). Thus, expression of IL-15Rα on DCs, as well as other cell types, appears to be critical for the activation of NK cells and for cytotoxicity against tumor cells.

Our present studies were directed toward defining whether anti-CD40–induced IL-15Rα expression is an important feature of antitumor efficacy of the combination therapy of IL-15 plus agonistic anti-CD40 Ab. Although the combination treatment was quite effective in the therapy of TRAMP-C2 tumor-bearing wild-type mice (Fig. 1), treatment with the combination regimen showed less therapeutic efficacy on tumor growth in IL-15Rα−/− mice (Fig. 2). Furthermore, NK cells isolated from wild-type mice that received the combination regimen manifested greater lysis activity toward TRAMP-C2 tumor cells than did NK cells from IL-15Rα−/− mice, suggesting that both decreased numbers of NK cells and lower lysis activity per living cell contributed to reduced antitumor activity. Although these studies in IL-15Rα−/− mice support the view that anti-CD40 Ab-induced IL-15Rα expression contributes to efficacy of the combination regimen, the results are difficult to interpret because IL-15Rα−/− mice are deficient in NK and CD44^highCD8+ T cells. In an attempt to circumvent this problem, we used an approach similar to that initiated by Koka et al. (22). These scientists found that mIL-15Rα−/− DCs failed to support NK cell elaboration of IFN-γ and cytolytic activity. In parallel with their studies, we analyzed the ability of BMDCs derived from wild-type or IL-15Rα−/− mice to prime NK cell cytolytic activity. We used anti-CD40 Ab to preactivate BMDCs and then cocultured these cells with freshly isolated NK cells in the presence of mIL-15, anti-CD40 Ab, or the combination of mIL-15 plus anti-CD40 Ab. Subsequently, these NK cells were assayed for their ability to kill TRAMP-C2 cells. NK cells cocultured with wild-type BMDCs in the presence of LPS or anti-CD40 Ab showed increased cytolytic activity. In contrast, the lytic activity of NK cells primed by IL-15Rα−/− BMDCs stimulated with LPS or anti-CD40 Ab did not increase over control levels. Koka et al. (22) demonstrated that wild-type and IL-15Rα−/− BMDCs expressed comparable levels of the activation/maturity markers CD40 and CD86 both before and after activation and that elaboration of IL-12 by DCs after stimulation was comparable between wild-type and IL-15Rα−/−. However, in studies of Koka et al. (22) and ourselves IL-15Rα−/− BMDCs were unable to fully activate NK cells to produce IFN-γ secretion or prime them to become cytolytic effectors. Taken together, these studies support the view that CD40 activation induces IL-15Rα expression on the surface of BMDCs, and this cytokine receptor presents IL-15 in trans to NK cells, leading to NK cell activation and increased cytolytic activity. This augmented IL-15Rα expression mediated by anti-CD40 Ab appears to be a critical element for an optimal response in IL-15–associated sustained remissions observed in TRAMP-C2 tumor-bearing mice receiving combination therapy.

In summary, given the multiple mechanisms of action of agonistic anti-CD40 Abs, we cannot conclude that the sole mode of action in the current study was the observed augmentation of IL-15Rα expression on DCs, as well as other cell types. Nevertheless, in addition to its other antitumor actions, the administration of agonistic anti-CD40 Ab and its associated increased expression of IL-15Rα facilitate the transactivation action of IL-15 on target effector CD8+ and NK cells. These findings in the current study provide the scientific basis for human clinical trials of patients with cancer to determine whether a similar synergistic antitumor activity is observed after administration of agonistic Abs to CD40 in combination with IL-15.

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Disclosures

The authors have no financial conflicts of interest.

References


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Figure s1
Figure s2
Figure s3

(A) Graph showing the percentage of mice surviving over time after re-challenge.

(B) Flow cytometry plots comparing CD8+ cells between control and re-challenge conditions.

Legend:
- CD8+ cells
- Tetramer
- CD8
Figure s4

A

CD8+ cells

Control

No tumor treated

NK1.1

CD8

CD44

Tetramer

B

Days after tumor cell injection

No. of mice surviving

Control

No tumor treated
Supplementary figure legends

Figure s1. Lysis activity of NK cells mediated by the combination regimen was examined ex vivo. TRAMP-C2 tumor bearing wild type or IL-15Rα/- mice were treated with the combination regimen or with PBS as a control. At day 5 after initiation of the therapy, the mice were killed and the NK cells were isolated from the spleens. $^{51}$Cr-labeled TRAMP-C2 cells were coincubated with freshly isolated NK cells at various effector to target ratios for 5 hours. Compared with the NK cells isolated from the control wild type mouse, the NK cells isolated from both wild type (WT) and IL-15Rα/- mice (KO) that received the combination regimen showed increased lysis activity toward the TRAMP-C2 cells. However, the NK cells isolated from the wild type mice that received the combination regimen showed greater lysis activity to the tumor cells than did the NK cells isolated from IL-15Rα/- mice. The data represent the mean of triplicates.

Figure s2. CD8+ T-cells played a major role in the combination regimen mediated antitumor efficacy. (A) Depletion of NK1.1+ cells from the PBMCs by anti-asialo-GM1. Blood samples were taken at day 5 after initiation of the therapy from the mice shown in Figure 3. FACS analysis of the PBMC showed that more than 90% of NK1.1+ cells were depleted by administration of anti-asialo-GM1 when compared with combination alone. (B) Kaplan-Meier survival plot. TRAMP-C2 tumor bearing mice were treated using the same protocol as that used in the experiments shown in Fig. 3. Depletion of both CD8+ and NK cells abrogated the therapeutic efficacy mediated by the combination therapy. (C) MHC-I expression on TRAMP-C2 tumor cells analyzed by FACS. TRAMP-C2 cells isolated from in vivo tumors expressed high levels of MHC-I (solid line). TRAMP-C2 cells cultured in vitro also expressed MHC-I
(solid line) and culture of the TRAMP-C2 cells with IFN-γ increased the expression of MHC-I on their cell surfaces (dotted line). Grey shades represent isotype control. (D) Cytolytic activity assay of CD8+ cells toward TRAMP-C2 cells ex vivo. Spleen cells were harvested from TRAMP-C2 tumor bearing mice and cultured for 7 days with IFN-γ pre-treated and irradiated TRAMP-C2 cells and 10u/mL of hIL-2. Then CD8+ cells were isolated from the cultured spleen cells for assay of cytolytic activity toward 51Cr-TRAMP-C2 cells pre-cultured with or without IFN-γ.

Figure s3. Rechallenge of the tumor free mice from the combination group in the therapeutic studies shown in Figure 1 at 3½ months after initiation of the original therapy. (A) Kaplan-Meier survival plot of mice that received rechallenge of TRAMP-C2 cells and (B) Representative FACS analysis of PBMC from the mice just before rechallenge. When compared with control mice which did not receive TRAMP-C2 cells and any treatment previously, mice surviving the first challenge with TRAMP-C2 cells demonstrated resistance to TRAMP-C2 tumor development on rechallenge (p<0.001) and an increased percentage of SPAS-1/SNC9-H8 tetramer+CD8+ T-cells.

Figure 4. s4. Protection from TRAMP-C2 tumor development was associated with the enhanced TRAMP-C2 tumor specific SPAS-1/SNC9-H8 tetramer+CD8+ T-cells. (A) Representative FACS analysis of PBMC from mice just before challenge with TRAMP-C2 cells and (B) Kaplan-Meier survival plot. When compared with control mice which did not receive any treatment previously, mice received the combination regimen, but not TRAMP-C2 cells, manifested an increased percentage of CD8+ and CD44hiCD8 T-cells. However, these
mice did not show any increase in SPAS-1/SNC9-H8 tetramer+CD8+ T-cells and did not show resistance to TRAMP-C2 tumor challenge when compared with the mice in the control group.