Augmented interleukin (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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Abstract

Interleukin-15 (IL-15) has potential as an immunotherapeutic agent for cancer treatment because it is a critical factor for the proliferation and activation of natural killer (NK) and $CD8^+$ T cells. However, monotherapy of patients with malignancy with IL-15 that has been initiated may not be optimal due to limited expression of the private receptor, IL-15Rα. We demonstrated greater CD8 T-cell mediated therapeutic efficacy of a combination regimen of murine IL-15 (mIL-15) administered with an agonistic anti-CD40 antibody (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-100% 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 mIL-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 that in wild type mice. The combination regimen significantly increased the numbers of TRAMP-C2 tumor specific SPAS-1/SNC9-H_8 $tetramer^+CD8^+$ 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 (BMDCs), 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.

Keywords: interleukin-15, interleukin-15Rα, anti-CD40, CD8 T-cell, mouse model

Introduction

Interleukin-2 (IL-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 IL-2 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 (Treg) cells. By contrast, interleukin-15 (IL-15) with its ability to activate CD8 T-cells and natural killer (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 have proven effective (3–8). In contrast to IL-2, IL-15 is a membrane-associated molecule that induces signaling at an

immunological synapse between antigen-presenting cells (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-15Rβ (CD122) and γc alone (9). Mice with genetic deletions of IL-15 or its private receptor, IL-15Rα , are characterized by decreased numbers of NK, NK-T, $CD8^{+}/CD44^{high}$ T, $TCR\gamma^{+}/\delta^{+}$ T and intestinal intraepithelial $CD8\alpha^{+}/\beta^{-}$ T cells, suggesting that physiologically relevant IL-15 signals require IL-15Rα expression (10, 11). The FDA and NCI have approved a phase I dose escalation trial of rhIL-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, IL-15 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 and therefore the level of IL-15Rα expression would be limiting in therapeutic trials that employ IL-15 alone. In the present study we examined the use of an agonistic anti-CD40 antibody to induce IL-15Rα expression that could be given to circumvent this problem.

CD40 is a member of the tumor necrosis factor receptor 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 antibodies have been shown to promote T-cell mediated immunity and effective treatment of neoplastic diseases in animal models (17, 18). It has been reported that a stimulatory CD40 antibody 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 mIL-15 and an agonistic anti-CD40 antibody in the TRAMP-C2 model of prostatic cancer. Furthermore, we used $IL\text{-}15R\alpha^{-/-}$ mice and DCs from such mice to demonstrate that induction of IL-15Rα expresion by an agonistic anti-CD40 antibody 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

Murine IL-15 was purchased from PeproTech (Rocky Hill, NJ). Rat anti-mouse CD40 (FGK4.5) and rat anti-mouse CD8 (clone 2.43) monoclonal antibodies 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. The 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 subcutaneous (s.c.) injection of 5×10^5 TRAMP-C2 cells into the right flank of male C57BL/6 (NCI-Frederick, Frederick, MD) or IL-15Rα−/− mice. All animal experiments were approved by the National Cancer Institute Animal Care and Use Committee (NCI ACUC) and were performed in accordance with NCI ACUC 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 mm^3. Groups of TRAMP-C2 tumor-bearing mice received mIL-15 i.p., 2.5μg per mouse everyday, 5 days a week for 2 weeks or the anti-CD40 antibody, 200μg on day 0, then 100μg on days 3, 7 and 10 or a combination of mIL-15 with anti-CD40 antibody at the same doses and dosing schedules as those in the mIL-15 and the anti-CD40 antibody 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 by using the formula $\frac{1}{2}$(long dimension)(short dimension)^2.

Luciferase-GFP-lentivirus infection and bioluminescence imaging

TRAMP-C2 cells were seeded into 24-well plate at the concentration of 5×10^4/mL/well and cultured overnight. Then, luciferase-GFP fusion protein expressing lentivirus (pol2-ffluc-eGFP) (Advanced Technology Program, NCI-Frederick, Frederick, MD) was added to the culture for 2 days. The GFP positive 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 one day before and 16 as well as 40 days after initiation of therapy with an in vivo imaging system (Xenogen Corporation, Alameda, CA) and the images were analyzed using Living Imaging software (Xenogen Corporation).

In vivo cell depletion experiment

The TRAMP-C2 tumor-bearing wild type mice received combination therapy of mIL-15 with the anti-CD40 antibody 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 (50μL) or purified rat anti-mouse CD8 antibody (200μg). One dose of anti-asialo-GM1 and anti-mouse CD8 antibody was administered one day before initiation of combination therapy and subsequent doses were administered three times weekly for 2 weeks. Depletion of NK and $CD8^+$ cells in PBMC 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

The 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 (4 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-H_8 (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 antibody were rechallenged with 5×10^5 TRAMP-C2 tumor cells or with 1×10^5 MC38 tumor cells, an irrelevant tumor cell line, at 40 days or at 3½ months 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 antibody (200μg/injection) 3 times weekly for 2 weeks and one dose of anti-CD8 antibody was administered one day 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, SPAS-1/SNC9-H_8 tetramer expressing cells in PBMCs or splenocytes was performed using commercial FITC- or PE- or APC-conjugated antibodies (eBioscience) except for the PE-conjugated SPAS-1/SNC9-H_8 tetramer which was provided by Dr. J. P. Allison. IL-15Rα expression on cell surfaces was detected by using biotinylated anti-mIL-15Rα antibody or biotinylated 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 by using the primary antibody, FGK4.5 or normal rat IgG as an isotype control, followed by PE-labeled mouse anti-rat Ig (BD Biosciences). The samples were collected on a FACScan flow cytometer (BD Biosciences, San Jose, CA) and analyzed using FlowJo cytometry analysis software (TreeStar, Inc., Ashland, OR).

Activation of NK cells primed by BMDCs

NK cells were purified from RAG1−/− mice using negative isolation microbeads (Miltenyi Biotec). BMDCs from wild type or IL-15Rα−/− mice were generated using 10ng/mL of IL-4 (PeproTech) and 20ng/mL of GM-CSF (PeproTech). The BMDCs were pretreated with LPS (1μg/mL; Sigma, St. Louis, MO) or anti-CD40 antibody (10μg/mL) or medium alone for about 20 hours. Then BMDCs were washed and cocultured with NK cells at a ratio of 2:1 for an additional 20 hours in presence or absence of LPS (1μg/mL), anti-CD40 antibody (10μg/mL), mIL-15 (2.5ng/mL) or anti-CD40 antibody plus mIL-15. Chromium-51 (^{51}Cr) labeled TRAMP-C2 cells were incubated with the NK cells together with BMDCs for 5 hours at various effector to target ratios. Radioactivity in the liquid phase was measured

in a γ counter (PerkinElmer). The percentage of specific lysis was determined by using the formula: % 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, St. Louis, MO).

Statistical analysis

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

Results

Combination therapy of mIL-15 with an agonistic anti-CD40 antibody 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 antibody in an established TRAMP-C2 tumor model. The rationale for this approach is that the expression of IL-15Rα was induced by CD40 activation (6, 21). Therapy started when TRAMP-C2 tumors were well established (the average tumor volume was 80 mm^3). Treatment with mIL-15 alone at a dose of 2.5μg/mouse, 5 days a week for 2 weeks provided a modest inhibition of tumor growth (Fig. 1A) and prolonged survival of the TRAMP-C2 tumor-bearing mice when compared with mice in the PBS control group (Fig. 1B, $p<0.05$). Treatment with anti-CD40 antibody at a dose of 200μg/mouse on day 0, then 100μg/mouse on days 3, 7 and 10, significantly inhibited tumor growth (Fig. 1A) and prolonged survival of the TRAMP-C2 tumor-bearing mice when compared with the mice in either PBS control or mIL-15 alone group (Fig. 1B, $p<0.001$). Critically, combination therapy with both mIL-15 and the anti-CD40 antibody provided greater therapeutic efficacy than that observed with monotherapy with either mIL-15 or anti-CD40 antibody (Fig. 1A & B, $p<0.001$). The therapeutic study was repeated and comparable results were obtained. All mice in groups of PBS control and mIL-15 alone died from tumor progression by day 40 and 70–100% of the mice in the anti-CD40 antibody alone group died from tumor progression within 2 months (Fig. 1B). In contrast, combination treatment resulted in a highly significant prolongation of survival, with 70–100% of the 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).

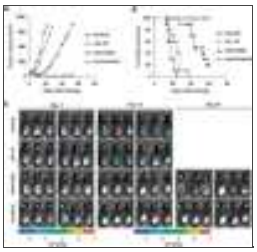

Figure 1

The combination therapy that involved mIL-15 and an agonistic anti-CD40 antibody led to the regression of established TRAMP-C2 tumors in wild type C57BL/6 mice

Bioluminescence imaging confirmed efficacy of the combination treatment

Groups of 6 wild type mice bearing TRAMP-C2/luc-GFP tumors were treated using the same therapeutic protocol as above. As shown in Figure 1C, the average tumor volumes among groups were comparable at the beginning of therapy. Treatment with anti-CD40 antibody significantly inhibited the tumor growth with average total luminescent signals of 1.0×10^9 photons/second (p/s) at day 16 , that were much lower than 1.5×10^{10} p/s and 7.7×10^9 p/s in PBS control and mIL-15 groups, respectively at the same day ($p<0.01$). However, only one out of 6 mice in the anti-CD40 group was tumor free. In contrast, combination treatment led to regression of tumors with all 6 mice becoming and remaining tumor free.

IL-15Rα–/– mice when compared to wild type mice showed markedly reduced therapeutic efficacy when treated with the combination regimen of mIL-15 and anti-CD40 antibody

IL-15 and its private receptor, IL-15Rα, are both essential for the support of NK and $CD8^+$ T-cell homeostasis. Expression of IL-15Rα 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 if increased expression of IL-15Rα mediated by administration of agonistic anti-CD40 antibody played a role in the augmented IL-15 mediated therapeutic efficacy observed in the combination treatment group. Previously, we reported that treatment with the anti-CD40 antibody augmented expression of IL-15Rα on the $CD11c^+$ population of splenocytes and increased serum concentrations of IL-15Rα.(6) In the present study, we performed a therapeutic trial in the TRAMP-C2 tumor model using IL-15Rα–/– mice. The same doses and dosing schedules of mIL-15 and the anti-CD40 antibody were used as were employed in therapeutic studies in TRAMP-C2 tumor-bearing wild type mice shown in Figure 1. Treatment with mIL-15 showed very little therapeutic efficacy in IL-15Rα–/– mice as seen by the very modest inhibition of the tumor growth (Fig. 2A) and the prolongation of survival of the TRAMP-C2 tumor-bearing mice when compared with those in the control group (Fig. 2B, $p<0.001$). Treatment with anti-CD40 antibody inhibited the tumor growth and prolonged the survival of the TRAMP-C2 tumor-bearing IL-15Rα–/– mice when compared with the mice in either PBS control or mIL-15 alone group (Fig. 2A & B, $p<0.001$). However, the treatment with the anti-CD40 antibody at the same dose and dosing schedule showed much less therapeutic efficacy in IL-15Rα–/– mice (Fig. 2) when compared to that observed in wild type mice (Fig. 1). Although the combination regimen in IL-15Rα–/– mice provided greater therapeutic efficacy when compared with monotherapy with either mIL-15 or the anti-CD40 antibody (Fig. 2A & B, $p<0.001$), only 10–20% of the mice in the combination group

became tumor free, in contrast with the 70–100% of the mice that became tumor free in wild type mice receiving the combination therapy as shown in Figure 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 $CD44^{high}CD8^{+}$ T-cells (10, 11), therefore, the reduced therapeutic efficacy of combination therapy in IL-15Rα−/− mice might be due to the lower numbers of the cytolytic cells.

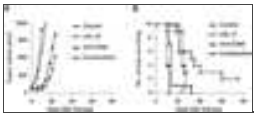

Figure 2

TRAMP-C2 tumor-bearing IL-15Rα−/− mice showed reduced efficacy with the combination regime when compared to wild type mice

To determine whether the killing activity of the cytolytic cells from IL-15Rα−/− mice was lower than that of the cells from wild type mice, the lysis activity of NK cells toward TRAMP-C2 cells was examined ex vivo (Fig. s1). Compared with NK cells isolated from the 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 days showed increased lysis activity toward TRAMP-C2 tumor cells (Fig. s1). 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 (Fig. s1), suggesting that both decreased numbers of NK and $CD44^{high}CD8^{+}$ T cells and lower lysis activity per cytolytic cell contributed to the reduced antitumor activity obtained in the combination regimen in IL-15Rα−/− mice bearing the TRAMP-C2 tumor (Fig. 2) when compared to that in the 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 antibody to eliminate NK or $CD8^{+}$ T-cells, together with the combination regimen. One dose of the anti-asialo-GM1 and anti-CD8 antibody was administered one day before the start of the combination therapy and subsequent doses were administered three times weekly for 2 weeks. FACS analysis showed that more than 90% of NK cells (Fig. s2A) and $CD8^{+}$ cells (Fig. 3A) from PBMC were depleted by administrations of anti-asialo-GM1 and anti-CD8 antibody, respectively. Compared with the group receiving the combination therapy alone, depletion of $CD8^{+}$ cells significantly but not completely eliminated the antitumor efficacy (Fig. 3B, $p<0.001$). Administration of anti-asialo-GM1 decreased the antitumor efficacy (Fig. 3B, $p<0.001$). Simultaneous depletion of both $CD8^{+}$ and NK cells abrogated antitumor efficacy mediated by the combination therapy (Fig. s2B). It should be noted that administration of anti-asialo-GM1 depleted not only NK cells (Fig. s2A) but also a subset of $CD8^{+}$ cells, especially $CD44^{high}CD8^{+}$ and TRAMP-C2 tumor antigen specific SPAS-1/SNC9-H_8 $tetramer^{+}CD8^{+}$ cells (Fig. 3A). TRAMP-C2 cells isolated from in vivo tumors expressed high levels of MHC-I (Fig. s2C). TRAMP-C2 cells cultured in vitro with IFN-γ increased the expression of MHC-I on their cell surfaces ((Fig. s2C), that was associated with the

increased cytolytic activity of $CD8^+$ T-cells toward the TRAMP-C2 cells ex vivo (Fig. s2D). 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.

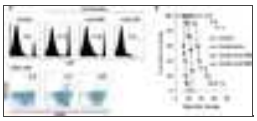

Figure 3

$CD8^+$ T-cells played a major role in the combination regimen mediated antitumor activity in the TRAMP-C2 model

TRAMP-C2 tumor antigen specific SPAS-1/SNC9-H_8 tetramer$^+$$CD8^+$ T-cells were increased in TRAMP-C2 tumor-bearing mice by the combination regimen

To further elucidate the effectors underlying the synergistic efficacy of mIL-15 plus anti-CD40 antibody, we defined the impact of combination therapy on numbers of splenic tumor antigen specific $CD8^+$ T-cells using the TRAMP-C2 tumor specific SPAS-1/SNC9-H_8 tetramer. TRAMP-C2 tumor-bearing mice were treated using the same therapeutic protocol as used in the therapeutic studies shown in Figure 1. At day 12 after initiation of the therapy, all mice (4 mice/group) were sacrificed. The percentages of $CD8^+$, $CD44^{high}CD8^+$, and TRAMP-C2 tumor antigen specific SPAS-1/SNC9-H_8 tetramer$^+$$CD8^+$ cells in spleens were analyzed by flow cytometry and the absolute cell numbers were calculated. Treatment with mIL-15 or anti-CD40 antibody or their combination increased total cell numbers and absolute numbers of $CD8^+$ and $CD44^{high}CD8^+$ cells in the spleens when compared with those in the control group (Fig. 4A–C, $p<0.05$). Most critically, the combination regimen dramatically increased the absolute number of TRAMP-C2 tumor antigen specific SPAS-1/SNC9-H_8 tetramer$^+$$CD8^+$ cells in the spleens when compared with those of all other groups (Fig. 4D, $p<0.05$).

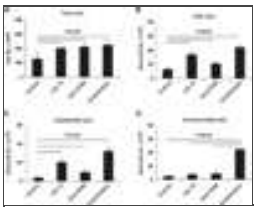

Figure 4

The combination of mIL-15 with the anti-CD40 antibody led to a significant increase in the absolute numbers of TRAMP-C2 tumor specific SPAS-1/SNC9-H_8 tetramer$^+$$CD8^+$ cells in the spleens of TRAMP-C2 tumor-bearing wild type mice

Protection from tumor development on rechallenge with TRAMP-C2 tumor cells was associated with increased numbers of TRAMP-C2 tumor antigen specific SPAS-1/SNC9-H_8 tetramer$^+$$CD8^+$ cells

Surviving tumor free mice from the combination group in therapeutic studies shown above in Figure 1 were rechallenged at day 40 (Fig. 5A) or 3½ months (Fig. s3) after initiation of therapy with 5×10^5 TRAMP-C2 cells s.c.. Another group of these surviving tumor free mice was rechallenged with 1×10^5 MC38 cells, an irrelevant tumor cell line (Fig. 5B). Mice that initially received the TRAMP-C2 tumor that became and remained tumor free after receiving the combination regimen demonstrated resistance to the TRAMP-C2 tumor development when compared with mice in the control group that

had not received either the TRAMP-C2 tumor cells or any treatment previously (Fig. 5A & Fig. s3A, $p<0.001$). However, such tumor free mice did not show any resistance to MC38 tumor development (Fig. 5B). FACS analysis showed that the percentages of $CD44^{high}CD8^{+}$ and TRAMP-C2 tumor antigen specific SPAS-1/SNC9-H_8 $tetramer^{+}CD8^{+}$ cells in PBMC of the mice surviving the initial tumor challenge were higher than those of the mice in the control groups (Fig. 5C & Fig. s3B). The protection from the 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 $CD44^{high}CD8^{+}$ cells in the PBMC of these mice increased whereas the percentage of TRAMP-C2 tumor antigen specific SPAS-1/SNC9-H_8 $tetramer^{+}CD8^{+}$ cells did not increase (Fig. s4A) and these mice did not show resistance to TRAMP-C2 tumor development on challenge (Fig. s4B). These findings support the view that protection from tumor development on rechallenge correlated with development of TRAMP-C2 tumor antigen specific SPAS-1/SNC9-H_8 $tetramer^{+}CD8^{+}$ T-cells. Surviving tumor free mice from the combination group shown above in Figure 1C were rechallenged at day 40 after initiation of therapy with 5×10^5 TRAMP-C2/luc-GFP cells s.c. on the left flank and the images were taken at day 23 after rechallenge. These surviving tumor free mice demonstrated resistance to TRAMP-C2/luc-GFP tumor development when compared with mice in the control group that had not received either the TRAMP-C2/luc-GFP tumor cells or any treatment previously (Fig. 5D).

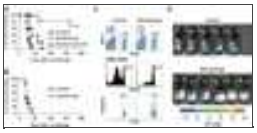

Figure 5

Enhanced TRAMP-C2 tumor specific SPAS-1/SNC9-H_8 $tetramer^{+}CD8^{+}$ T-cells were associated with protection from a rechallenge with TRAMP-C2 tumor cells

Administration of an agonistic anti-CD40 antibody induced the expression of IL-15Rα

We have reported that administration of the anti-CD40 antibody increased the expression of IL-15Rα on the $CD11c^{+}$ population of splenocytes (6). In the present study, we further examined the IL-15Rα expression on other cell types by flow cytometry analysis. We demonstrated that treatment with the anti-CD40 antibody increased the expression of IL-15Rα not only on the $CD11c^{+}$ cells but also on other cell types such as B cells, the $CD11b^{+}$ population and $CD8^{+}$ cells in the spleens when compared with the PBS treated mice (Fig. 6A).

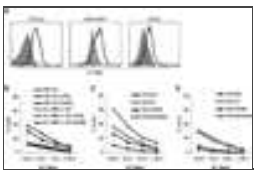

Figure 6

CD40 activation induced IL-15Rα expression and increased levels of IL-15Rα on the BMDCs appear critical for NK cell lytic activity

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 or anti-CD40 antibody or medium alone for 20 hours and then cocultured with freshly isolated NK cells for a further 20 hours in the presence of LPS or anti-CD40 antibody or mIL-15 or the combination of mIL-15 plus the anti-CD40 antibody. Subsequently, these NK cells were assayed for their ability to kill ^{51}Cr-labeled TRAMP-C2 cells. NK cells cocultured with wild type BMDCs in the presence of LPS or anti-CD40 antibody showed stronger cytolytic activity when 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 antibody did not increase (Fig. 6B). When mIL-15 was added to the coculture together with the anti-CD40 antibody, wild type BMDC-primed NK cells demonstrated stronger cytolytic activity to the target cells than did the wild type BMDC-primed NK cells in presence of either mIL-15 or anti-CD40 antibody alone (Fig. 6C). In contrast, IL-15Rα−/− BMDC-primed NK cells in presence of both mIL-15 and anti-CD40 antibody showed cytolytic activity that was similar to that of IL-15Rα−/− BMDC-primed NK cells in presence of mIL-15 alone (Fig. 6D). These findings suggest that the anti-CD40 antibody induced expression of IL-15Rα by the 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 Treg cells and is an anti-apoptotic 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 pre-formed 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 non-activated DCs and therefore, IL-15Rα would be very limiting in therapeutic trials involving IL-15 alone.

In the present study, combination therapy with both mIL-15 and anti-CD40 antibody showed enhanced efficiency when compared with monotherapy with either mIL-15 or anti-CD40 antibody (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 & Fig. s3). A number of studies were performed to define the mechanisms underlying this synergy between IL-15 and anti-CD40 antibody. In contrast to our previous study (6) where we demonstrated that NK cells appear to be the primary effector cells mediating the killing of CT26 and MC38 tumors after the 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-H_8 $tetramer^+CD8^+$ T-cells when compared to that of all other groups (Fig. 4D) and the protection from tumor development on rechallenge with TRAMP-C2 tumor cells was associated with the increased number of TRAMP-C2 tumor specific $tetramer^+CD8^+$ T-cells (Fig. 5). Therefore, the combination therapy of IL-15 plus anti-CD40 antibody has the potential to enhance the antitumor efficacy of both MHC-I- and non-MHC-I-restricted cytotoxic cells which may broaden its application in human cancer therapy.

Nevertheless, there was also evidence that suggests 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 present 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 (Fig. s2A), it also reduced the proportion of $CD8^+$ T-cells especially $CD44^{high}CD8^+$ and TRAMP-C2 tumor specific $tetramer^+CD8^+$ T-cells (Fig. 3A). Previously it had been 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 antibody 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 IL-15Rα non-expressing NK-cells (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 therapy with the combination regimen of IL-15 and an agonistic anti-CD40 antibody. Whereas 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 then 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 antibody induced IL-15Rα expression contributes to the efficacy in the combination regimen, the results are difficult to interpret since IL-15Rα−/− mice are deficient in NK and $CD44^{high}CD8^{+}$ T-cells. In an attempt to circumvent this problem we used an approach similar to that initiated by Koka and coworkers (22). These scientists found that murine IL-15Rα−/− DCs failed to support NK cell elaboration of IFN-γ and cytolytic activity. In parallel with the studies of Koka and coworkers, we analyzed the ability of BMDCs derived from wild type or IL-15Rα−/− mice to prime NK cell cytolytic activity. We used anti-CD40 antibody to preactivate BMDCs and then cocultured these cells with freshly isolated NK cells in the presence of mIL-15, anti-CD40 antibody or the combination of mIL-15 with anti-CD40 antibody. Subsequently these NK cells were assayed for their ability to kill TRAMP-C2 cells. NK cells cocultured with wild type BMDCs in presence of LPS or anti-CD40 antibody showed increased cytolytic activity. In contrast, the lytic activity of NK cells primed by IL-15Rα−/− BMDCs stimulated with LPS or anti-CD40 antibody did not increase over control levels. Koka et al.(22) demonstrated that wild type and IL-15Rα−/− BMDCs expressed comparable levels of the activation/maturation 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 both 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 antibody 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 antibodies, we cannot conclude that the sole mode of action in the present 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 an agonistic anti-CD40 antibody 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 present study provide the scientific basis for human clinical trials to determine if there is a similar synergistic antitumor activity observed by the administration of agonistic antibodies to CD40 in combination with IL-15 for the treatment of patients with cancers.

Supplementary Material

1

Click here to view.(181K, pdf)

2

Click here to view.(41K, doc)

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