Molecules and Cells

Cell-Based IL-15:IL-15Rα Secreting Vaccine as an Effective Therapy for CT26 Colon Cancer in Mice

Van Anh Do Thi[1], Hyung Min Jeon[1], Sang Min Park[1], Hayyoung Lee[2,*], and Young Sang Kim[1,*]

[1]Department of Biochemistry, College of Natural Sciences, Chungnam National University, Daejeon 34134, Korea, [2]Institute of Biotechnology, Chungnam National University, Daejeon 34134, Korea
*Correspondence: young@cnu.ac.kr (YSK); hlee@cnu.ac.kr (HL)
https://doi.org/10.14348/molcells.2019.0188
www.molcells.org

Interleukin (IL)-15 is an essential immune-modulator with high potential for use in cancer treatment. Natural IL-15 has a low biological potency because of its short half-life and difficulties in mass-production. IL-15Rα, a member of the IL-15 receptor complex, is famous for its high affinity to IL-15 and its ability to lengthen the half-life of IL-15. We have double-transfected IL-15 and its truncated receptor IL-15Rα into CT26 colon cancer cells to target them for intracellular assembly. The secreted IL-15:IL-15Rα complexes were confirmed in ELISA and Co-IP experiments. IL-15:IL-15Rα secreting clones showed a higher anti-tumor effect than IL-15 secreting clones. Furthermore, we also evaluated the vaccine and therapeutic efficacy of the whole cancer-cell vaccine using mitomycin C (MMC)-treated IL-15:IL-15Rα secreting CT26 clones. Three sets of experiments were evaluated; (1) therapeutics, (2) vaccination, and (3) long-term protection. Wild-type CT26-bearing mice treated with a single dose of MMC-inactivated secreted IL-15:IL-15Rα clones prolonged survival compared to the control group. Survival of MMC-inactivated IL-15:IL-15Rα clone-vaccinated mice (without any further adjuvant) exceeded up to 100%. This protection effect even lasted for at least three months after the immunization. Secreted IL-15:IL-15Rα clones challenging trigger anti-tumor response via $CD4^+$ T, $CD8^+$ T, and natural killer (NK) cell-dependent cytotoxicity. Our result suggested that cell-based vaccine secreting IL-15:IL-15Rα, may offer the new tools for immunotherapy to treat cancer.

Keywords: cell-based vaccine, CT26 colon carcinoma, IL-15:IL-15Rα complex, immunotherapy, interleukin-15, interleukin 15 receptor alpha

INTRODUCTION

The disadvantages of current cancer therapies such as chemotherapy or radiation therapy are the lack of specificity, the drug resistance after tumor recurrence, and the impairment of hematopoietic potential. Recently, immunotherapy, e.g., immune checkpoint inhibitors and CAR-T cell therapy are rapidly emerging as a promising alternative for cancer treatment by exploiting a patient's natural anti-tumor immunity. The key to success in cancer immunotherapy is employing tumor-associated antigens (TAAs) to make an effective cancer vaccine. Furthermore, to make cancer therapy more tumor-specific, a wide range of engineered tumor cell vaccines have been in clinical trials (de Gruijl et al., 2008; Kim, 2009; Kozlowska et al., 2013; Mackiewicz and Mackiewicz, 2010; Srivatsan et al., 2014). Although inactivated whole cancer cell vaccines can introduce a wide range of TAAs, they are not enough to overcome the local immunosuppression within tumor microenvironment. Genetically modified tumor cells to express exogenous immune stimulating cytokine is one way to break local immunosuppressive effects and evoke anti-tumor responses (Chiang et al., 2015; Srivatsan et al., 2014). Cytokine-based immune therapy focusing on stimulating the an-

Received 22 August, 2019; revised 3 November, 2019; accepted 6 November, 2019; published online 25 November, 2019

eISSN: 0219-1032
©The Korean Society for Molecular and Cellular Biology. All rights reserved.
This is an open-access article distributed under the terms of the Creative Commons Attribution-NonCommercial-ShareAlike 3.0 Unported License. To view a copy of this license, visit http://creativecommons.org/licenses/by-nc-sa/3.0/.

ti-tumor response via cytokine gene transfer has been reported (Do Thi et al., 2018; Kim, 2009). Although a few cytokines have been listed as pro-tumor agents such as TGF-β, IL-17, or VEGF (Alahari et al., 2015; Do Thi et al., 2016), numerous anti-tumor cytokines including IFN-γ, GM-CSF, TNF-α, IL-4, IL-2, IL-12, or IL-9, have been intensely studied, and some of them have been tested in phase I/II clinical trials (Lee and Margolin, 2011; Podhajcer et al., 2007). Furthermore, exogenous cytokine-expressing tumor cell vaccine has employed cancer cell lines to induce robust and specific anti-tumor response through tumor antigen presentation to overcome tumor. Plenty of cancer cell vaccines expressing ectopic cytokines have been tested in clinical trial phase I, II or III such as AGI-101H and GVAX for IL-6 and GM-CSF, respectively, and melanoma cellular vaccines engineered to express other cytokines (Kozlowska et al., 2013; Mackiewicz and Mackiewicz, 2010).

Colorectal cancer (CRC) is known as the third most common and fourth leading cancer-associated death worldwide (Sarvizadeh et al., 2019). Up to now, surgery and adjuvant chemotherapy is the standard for colon cancer treatment. However, the fact that 40% of colon cancer re-appears within five years after diagnosis is raising the demand to introduce new therapies to treat colon cancer. Developing cancer vaccines is one of the possible approaches. Unlike metastatic melanoma and renal cell carcinoma, CRC was considered a poor candidate for immunotherapy due to its low immunogenicity (Chaurasiya and Warner, 2017). As a result, the development of CRC cancer vaccine is facing a plenty of difficulties. A developing CRC vaccine that combined autologous cancer cells with BCG vaccine showed a little clinical effect of improved tumor-free survival (Uyl-de Groot et al., 2005). Modified autologous colon cancer cell vaccine with a pathogenic strain of Newcastle disease virus also reduced the recurrent rate from 81% to 61% in stage II level (Sarvizadeh et al., 2019).

IL-15 belongs to the cytokine members that share the common gamma (γC) chain receptor, including IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21. IL-15 plays critical roles in the development, expansion, and maintenance of effector NK and memory T cells. Although IL-15 and IL-2 share a common heterodimeric receptor called IL-2/IL-15Rβγ, IL-15 specially binds to its private α receptor chain, IL-15Rα. IL-15 acts through the trans-presentation model in which IL-15 assembles with membrane-embedded IL-15Rα to form the IL-15:IL-15Rα complex. In turn, IL-15:IL-15Rα complex engages the IL-2/IL-15Rβγ on the neighboring cells to trigger signaling (Dubois et al., 2002; Fehniger and Caligiuri, 2001). Recently, IL-15 became a promising cytokine for the treatment of cancer. Several products including ALT-803 (Kim et al., 2016; Wrangle et al., 2018), P22339 (Hu et al., 2018), the chimeric IL-15 Apo A-I (Ochoa et al., 2013) or combinations of IL-15-Fc and IL-15Rα-Fc (Dubois et al., 2008; Epardaud et al., 2008) illustrated the promising effect of IL-15 in cancer treatment.

In this study, we focused on engineering a whole-cell CT26 colon cancer vaccine, which secretes a complex of IL-15 and IL-15Rα (IL-15:IL-15Rα) through co-transfection. Although the secreted IL-15:IL-15Rα complex showed similar effect on spleen cell proliferation compared to secreted IL-15 *in vitro*, the complex did better in eliminating CT26 colon cancer cells both *in vitro* and *in vivo*. IL-15:IL-15Rα complex expressing tumor clones failed to form tumor in mice. Furthermore, immunization with IL-15:IL-15Rα complex expressing clones evoked long-term anti-CT26 colon cancer protection in mice. Splenocytes subset analysis revealed that the IL-15:IL-15Rα complex did enhance both the proliferation of splenocytes and their cytotoxicity, effector, and memory function both *in vitro* and *in vivo*. Furthermore, the promising capability to use the IL-15:IL-15Rα expressing CT26 cells as an anti-tumor drug was proven in therapeutic assay. Treatment with IL-15:IL-15Rα expressing CT26 cells significantly delayed wild-type CT26 tumor growth *in vivo* and eventually extended those mice's survival.

MATERIALS AND METHODS

Animal and tumor cell lines

BALB/c mice (female, 6- to 8-week old) were purchased from the Korea Research Institute of Chemical Technology (Korea). All animal procedures were approved and guided by the Institutional Animal Care and Use Committee (IACUC) of Chungnam National University (CNU-01056). The murine colon cancer CT26 and the YAC-1 lymphoma cell lines were cultured in RPMI-1640 (Gibco-BRL, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS; GIBCO-BRL), 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin (Sigma, USA) in humidified 5% CO_2 at 37°C. G-418 (0.5 mg/ml; Santa Cruz, USA) and hygromycin B (0.3 mg/ml; Merck, Germany) were used as a selective agent for transfections.

Plasmid construction and transfection

Mouse splenocyte cDNA was used as a template to amplify IL-15 and IL-15Rα cDNAs. To ensure the assembly of IL-15Rα and its ligand IL-15 as well as to enhance the expression level of them (Bamford et al., 1998), the IL-15 or IL-15Rα signal sequence was exchanged by that from IL-2 using the 3-steps polymerase chain reaction (PCR) strategy. To construct pcDNA3.1(neo)/IL-15Rα, pcDNA3.1(neo)/IL-15, and pcDNA3.1(hygro)/IL-15, the primers specific for each mRNA in the Supplementary Table S1 were used to amplify the respective cDNA fragments. In short, the PCR fragments encoding for amino acid sequences of IL-15 from 30 to 162 and IL-15Rα from 34 to 205 (extracellular domains) were generated by using specific primers. PCR fragments, pcDNA 3.1(+)/neo and pcDNA3.1(−)/hygro were digested with *BamHI*/*XhoI*. Each PCR fragment was then subcloned into the proper pcDNA3.1 expression vector. The constructs were confirmed by DNA sequencing (Macrogen, Korea). CT26 colon cancer cells were transfected with these constructs or empty vectors using Lipofectamine 2000 Transfection Reagent (Invitrogen, USA). After 24 h, the cells were plated in G418 (0.5 mg/ml) and/or hygromycin B (0.3 mg/ml) containing medium. Drug-resistant colonies were usually visible 2 to 3 weeks after transfection. To screen drug-resistant colonies, total RNA was extracted using a Hybrid-R kit (Geneall, Korea). Of total RNA, 1 μg was reverse transcribed using oligo (dT) primers and AccuPower RT premix (Bioneer, Korea) at 42°C for 1 h.

Semi-quantitative PCR was performed using HSTag premix (Geneall) on a DNA thermal Cycler (Bio-Rad, USA) using the proper detection primers (Supplementary Table S1).

ELISA

For IL-15, the ELISA assay used to measure the concentration of bioactive IL-15 and IL-15:IL-15Rα in culture supernatants. In shorts, 1 × 10^6 cells were incubated in 1 ml of culture medium in 24-well culture plates at 37°C for 24 h. IL-15 or IL-15:IL-15Rα complexes in the culture supernatants was measured with Mouse IL-15 DuoSet ELISA kit (DY447-05; R&D System, USA) or mouse IL-15/IL15R Platinum ELISA (BMS6023; Affymetrix, USA) respectively. For interferon γ (IFN-γ) assay, control and surviving mice from vaccination experiment were injected with 1 × 10^6 MMC inactivated wild-type CT26 cells and splenocytes were collected after 1 week. Each suspension of splenocytes (2 × 10^6 cells/well) was seeded with 600 μl complete culture media (RPMI; 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, 50 μM/ml of 2-ME) and treated with cancer cell lysate (2 × 10^5 boiled CT26 cells/well). After 72 h, 100 μl of culture supernatant from those stimulated splenocytes were subjected to IFN-γ ELISA (IFN-γ ELISA Kit; BioLegend, USA).

Antibodies and reagents

The following antibodies were used for flow cytometry, Western blot, and cytotoxicity assay. Anti-mouse IL-15Rα antibody (sc-374023; Santa Cruz), anti-mouse IL-15 antibody (Ab7213; Abcam, UK), FITC-rat anti-mouse CD4 (553729; BD, USA), PE-rat anti-mouse CD4 (553730; BD), FITC-rat anti-mouse CD8 (553031; BD), APC-rat anti-mouse CD62L (104411; BioLegend), PE-rat anti-mouse CD44 (553134; BD), purified hamster anti-mouse CD3 (557306; BD), FITC-goat anti-hamster (Armenian) IgG (405502; BioLegend), APC-rat anti-mouse CD49b (108909; BioLegend), purified anti-mouse CD8 (100701; BioLegend), purified anti-mouse (100401; BioLegend), FITC-rat anti mouse CD11b (557396; BD), PE-rat anti-mouse MHC class II I-A/I-E M5/114 (562010; BD), anti-L^d MHC class I (24-14-8S), FITC-secondary goat anti-mouse IgG (sc-2005; Santa Cruz).

Flow cytometric analysis

FACSCanto System was used for multicolor flow cytometric analysis. Cells were incubated with proper antibody diluted in staining buffer (1× phosphate-buffered saline [PBS] containing 0.02% sodium azide and 2% FBS) for 1 h at 4°C, in dark. After washing off the unbound antibody with the staining buffer, stained cells were analyzed with the flow cytometer (FACSCanto; Becton Dickinson, USA).

MTT assay

To examine the *in vitro* cell proliferation of transfected tumor clones, 1 × 10^4 cells were plated on a 96-well plate. The cells were cultured for 48 h and their proliferation was determined by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay (DyneBio, Korea).

To confirm the biological property of IL-15 and IL-15:IL-15Rα complex, the spleen cell proliferation assay was performed. Cells (1 × 10^6) from each tumor clones were cultured in 1 ml culture media in a 24-well plate, and the culture supernatants were collected after 24 h. The spleen cells from normal BALB/c mice were collected, then red blood cells were removed. The splenocytes were treated with the mixture between each culture supernatants and fresh culture media with the ratio 1:1. 2-ME was added to culture media to maintain the final concentration (50 μM/ml). MTT assay was used to determine the proliferation of 72 h after the treatment.

Tumor challenge

For primary tumor challenge, syngeneic BALB/c mice (n = 5) were injected subcutaneously into their right lower back quadrants with 1 × 10^6 wild-type, mock or transfected CT26 clones in 100 μl PBS. Tumor size was measured with calipers and tumor volume was calculated according to the following formula: $0.52 \times S^2 \times L$, where L is length and S is the width of the tumor. Bodyweight was also monitored daily. The survival of mice in each group was calculated using survival function of Origin Pro 8.1 (OriginLab Corporation, USA).

For secondary and tertiary tumor challenge, one month after the first challenge with IL-15:IL-15Rα transfected clones, all the tumor-free mice were subcutaneously injected with 1 × 10^6 wild-type CT26 cells into their left flank. As control, a group of BALB/c mice (n = 5) were injected with the same volume of CT26 cells. Tumor growth and survival of the mice were analyzed. Two months after the second challenge, all tumor-free mice were subcutaneously re-challenged with 1 × 10^6 wild-type CT26 cells into their right flank. As control, a group of BALB/c mice (n = 5) was injected with the same volume of CT26 cells. Tumor growth and survival of the mice were analyzed. At day 18 after the injection, the animals were sacrificed in a CO_2-containing chamber, the spleens and tumor masses were collected for further analysis.

Co-culture experiments of MMC-treated tumor cells and splenocytes

To inhibit tumor cell proliferation, the pre-seeded 1 × 10^5 cells/well were treated with 100 μg/ml mitomycin C (MMC; Roche, USA) in 24-well plate for 1 h. Before the experiment, the cells were extensively washed to remove all MMC (3 times in PBS). The mice were pre-injected with MMC treated wild-type CT26 cells. Two weeks after the injection, a mouse was sacrificed to collect the spleen. The spleen was ground and gently mashed through a 70 μm cell strainer (Corning, USA) in sterile conditions. After red blood cell removal by Red Blood Cell lysing buffer (Sigma), the spleen cells were stored in sterile culture medium. For co-culture of splenocytes with tumor cells, 5 × 10^6 splenocytes and 1 × 10^6 of MMC treated wild-type or transfected tumor clones were plated per well in RPMI media. After 48 h, the co-culture pictures were photographed under the microscope.

Cytotoxicity assay against CFSE-labelled target cells

To prepare effector cells, the spleens from pre-immunized mice with MMC treated wild-type CT26 were collected. After red blood cell lysis, 5 × 10^6 spleen cells were seeded together with 0.5 × 10^6 MMC treated wild-type CT26 in 24-well plate. The equal amount of culture supernatants from control, IL-15

or IL-15:IL-15Rα transfected clones were changed every day. After 48 h, the stimulated splenocytes were harvested as effector cells. Carboxyfluorescein succinimidyl ester (CFSE) is a fluorescent cell staining dye. To prepare CFSE labeling target cells, 1×10^7 wild-type CT26 cells were rinsed in 2.5 μM CFSE containing PBS for 20 min, in dark. After incubation, the remaining CFSE was eliminated by washing twice with culture medium (containing 10% FBS). For cytotoxic assay, effector and CFSE labeling target cells were mixed at different ratios (50:1 and 20:1). After 6 h, all the cells were collected by trypsin-EDTA and the CFSE dead cells were analyzed with propidium iodide (PI) staining using BD FACSCanto machine. The percentage of $CFSE^+PI^+$ cells were compared between groups.

For the CD4 or CD8 blocking assay, 10 μg/ml anti-mouse CD8a (100401; BioLegend) or anti-mouse CD4 (100701; BioLegend) was added to CT26 stimulated effector cells, incubated at room temperature (RT) for 30 min. Then the CFSE labeling CT26 cells were added at the effectors:target cells ratio 50:1. After incubation for 6 h, the cells were harvested and cell dead phenotype was determined by PI staining. The percentage of $CFSE^+PI^+$ cells were analyzed. To deplete both $CD4^+$ and $CD8^+$ T cells in the cytotoxic assay, 10 μg/ml each of anti-CD8a and anti-CD4 was added to CT26 stimulated splenocytes (effector) and incubated at RT for 30 min. Then the CFSE labeling CT26 (target) cells were added at the ratio of 30:1 (effectors:target). After incubation for 6 h, the cells were harvested, and dead cells were determined by PI staining. The percentage of $CFSE^+PI^+$ cells were analyzed.

NK cytotoxicity against Yac-1 lymphoma cells

The above effector cells stimulated by wild-type CT26 cells and culture supernatants from transfected clones were used as the effectors in this experiment. To prepare CFSE labeling target cells, 1×10^7 YAC-1 cells were rinsed in 2.5 μM CFSE containing PBS for 20 min, in dark. After extensively washing off the abundant CFSE, CFSE labeling YAC-1 cells were used as the target cells. For NK cytotoxic assay, CT26 stimulated effector cells and CFSE labeling YAC-1 were mixed at the ratio 50:1. After 6 h, the mixed cells were collected and stained with PI. The percentage of $CFSE^+PI^+$ cells were analyzed.

Therapeutics experiment

To examine therapeutic effect of IL-15:IL-15Rα against CT26 cancer, 0.1×10^6 wild-type CT26 cells were subcutaneously implanted into the right lower back quadrants of BALB/c mice (n = 15). Three days later, 1×10^6 MMC treated wild-type CT26 or MMC treated transfected clones (n = 5 for each group) were subcutaneously injected into a near position. Tumor growth and survival of those mice were analyzed.

Vaccination experiment

To estimate the ability of IL-15:IL-15Rα transfectants against CT26 cancer, BALB/c mice (n = 5) were subcutaneously vaccinated with 0.5×10^6 MMC treated clones (wild-type CT26 or S/IL-15 transfectants). Fourteen days later, 0.5×10^6 live wild-type CT26 cells were subcutaneously implanted in those mice. Tumor growth and survival of those mice were analyzed. At day 18 after the tumor implantation, the animals were sacrificed in a CO_2-containing chamber, the spleens and tumor masses were collected for further analysis.

Immunoprecipitation and Western blot

Culture supernatant from 2×10^7 cells was harvested at confluency. The culture supernatant was concentrated using 10MWCO centricon filter by centrifuging at 3,000 rpm for 15 min. For immunoprecipitation, IL-15Rα antibody (sc-374023; Santa Cruz) or IL-15 antibody (ab7213; Abcam) was added to the concentrated cell culture supernatants, incubated 1 h, 4°C. After the incubation, 20 μl Santa Cruz IP bead (protein A/G) was added and then incubated 30 min, 4°C. The beads were pelleted by centrifugation at 1,000*g*, 5 min, 4°C and washed with 200 μl TBST for 3 times. After 20 μl of 2× SDS PAGE loading dye was added, the pelleted beads were boiled 10 min. After chilling, 10 μl of the supernatant solution was analyzed by western blot. Recombinant IL-15 (0.2 μg) were loaded as a positive control.

For electrophoresis, gels with 4% stacking and 15% separating acrylamide gels were used. For western blot, protein from gels was transferred to PVDF membrane. After blocking, the membrane was overnight incubated with primary antibodies IL-15 (rabbit IgG; Abcam) or IL15Rα (mouse IgG; Santa Cruz) at, 4°C. After deep washing with TBST (3 times), the membrane was incubated with the proper secondary antibodies for 1.5 h at room temperature. After 3 times washing with TBST, the ECL Western Blotting Substrate solution (Thermo Fisher Scientific, USA) were applied on the membrane and the blot were recorded using Ez Capture II machine (ATTO Corporation, Japan).

Statistical analysis

All data was presented as mean ± SEM (error bars). GraphPad Prism 7 (GraphPad Software, USA) was used to analyze one-way or two-way ANOVA to point out significant differences between groups, as indicated in the figure legends. Survival data was analyzed using "Kaplan–Meier survival estimates" feature of Origin Pro 8.1 (OriginLab Corporation, USA).

RESULTS

Generation of tumor clones expressing IL-15 or IL-15:IL-15Rα complex

To ectopically express IL-15 or secretory form of IL-15:IL-15Rα complex, pcDNA3.1(+)/neo-IL-15 vector and pcDNA3.1(–)/hygro-IL-15Rα expression vector were constructed. While the IL-15 cDNA insert contains DNA sequence for the whole protein chain of IL-15 (aa 88-486), the IL-15Rα cDNA contains sequence for the extracellular region of IL-15Rα (aa 100-615) (Fig. 1A). The signal sequence of IL-15 or IL-15Rα were replaced with the sequence of IL-2 to enhance expression level. The CT26 colon carcinoma cells were transfected with either IL-15 expression vector only or both IL-15 and IL-15Rα expression vectors. As a mock vector control, the CT26 parent cells were co-transfected with both pcDNA3.1(+)/neo and pcDNA3.1(–)/hygro vectors. The expression of IL-15 or IL-15:IL-15Rα complex from the IL-15 and IL-15:IL-15Rα complex transfected clones were quantitated by ELISA (Fig. 1B). An IL-15 transfected clone expressing the highest

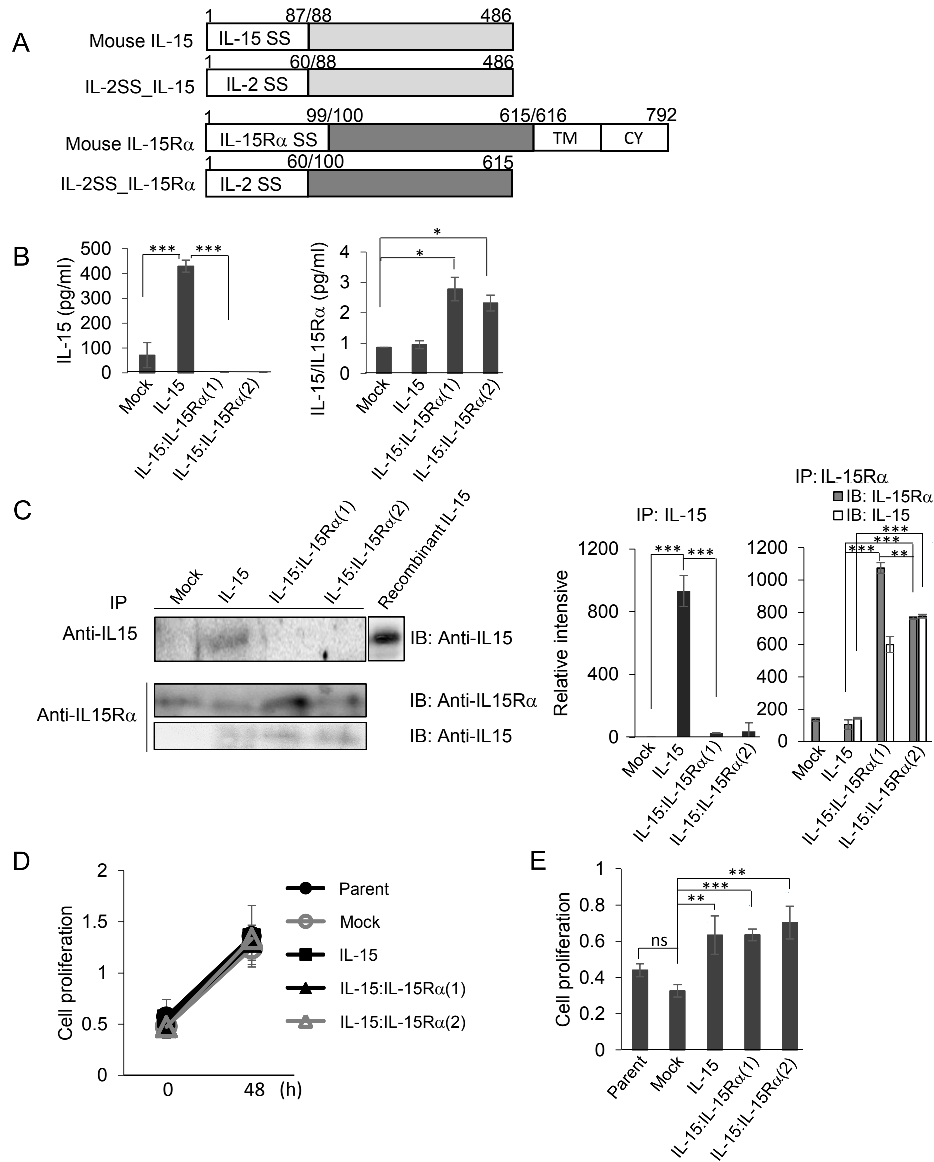

Fig. 1. Expression of exogenous IL-15 and/or IL-15Rα in CT26 colon carcinoma cells. (A) Construction of expression vectors encoding IL-15 or IL-15Rα. In the expression vectors, the natural signal peptide sequence (SS) of IL-15 or IL-15Rα were replaced with that of IL-2. TM, transmembrane domain; CY, cytoplasmic domain. (B) Quantitation of IL-15 or IL-15:IL-15Rα complex, secreted from 1×10^6 cells of transfected clones for 24 h incubation by ELISA. The monoclonal antibody used to detect IL-15 (DuoSet ELISA kit; R&D System) did not bind to the IL-15:IL-15Rα complex. The monoclonal antibody to the IL-15:IL-15Rα complex (Platinum ELISA; Affymetrix) did not bind to IL-15 alone. (C) Immunoprecipitation (IP) of IL-15 and IL-15Rα from the culture supernatants of the indicated transfectants. The amount of immunoprecipitated proteins were analyzed by immunoblotting (IB) with the indicated antibodies. Then, the relative intensive was shown in the bar graph. (D) Transfected cell proliferation analysis *in vitro* by MTT assay. The cell proliferation was correlated with the absorbance at optical density 570 nm. (E) Secreted exogenous IL-15 and IL-15:IL-15Rα complexes stimulated splenocyte proliferation *in vitro*. Culture supernatants from 1×10^6 cells of each transfected tumor clone were collected after 24 h. The splenocytes from normal BALB/c mice were re-suspended in the medium containing the indicate culture supernatants for 72 h. Then, the proliferation of splenocytes was assayed by MTT assay. Data are mean ± SEM of three independent experiments. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; ns, not significant.

level of IL-15 was chosen and named as IL-15. In the case of double transfectants of IL-15 and IL-15Rα, two tumor clones secreting high level of IL-15:IL-15Rα complex to the supernatant were chosen and named as IL-15:IL-15Rα(1) and IL-15:IL-15Rα(2). The monoclonal antibody used to detect IL-15 (DY447-05; R&D System) cannot bind to the IL-15:IL-15Rα complex, and the monoclonal antibody to the IL-15/IL-15Rα complex (BMS6023; Affymetrix) cannot bind to IL-15 alone. The immunoblot results showed that CT26 cells expressed no endogenous IL-15 protein and a little amount of endogenous

IL-15Rα (Fig. 1C). Co-precipitation of IL-15 using the transfectant culture supernatants with IL-15 antibodies showed that the relative intensity of IL-15 expression in IL-15 transfectant was 24.8-fold stronger than the other groups. Pull-down of IL-15Rα from transfectant culture supernatants with IL-15Rα antibodies showed that 7.4- to 10.3-fold enhanced the expression of IL-15Rα in IL-15:IL-15Rα(1) and IL-15:IL-15Rα(2). The relative level of the co-precipitation of IL-15 with IL-15Rα from IL-15:IL-15Rα groups are 4.2- to 5.4-fold higher than those in the IL-15 group. Together with ELISA results, these data indicated that IL-15:IL-15Rα complex was successfully formed and secreted in these clonal tumor cells.

The IL-15 and IL-15:IL-15Rα expression in tumor cells did not exert the autocrine effect on the proliferation of tumor cells (Fig. 1D). However, IL-15 is known to drive the proliferation of lymphocytes (Drake et al., 2016; Rhode et al., 2016). To analyze the biological activity of IL-15 and IL-15:IL-15Rα complex secreted by tumor clones, spleen cells from normal BALB/c mice were incubated with culture supernatants from these tumor clones for 48 h. Then the proliferation of treated spleen cells was analyzed by the MTT assay. The supernatants from tumor clones expressing IL-15 or IL-15:IL-15Rα complex stimulated spleen cell proliferation better than that from wild-type CT26 cells or mock-transfected clone. This result supports the notion that IL-15 drives spleen cell proliferation (Fig. 1E).

Expression of IL-15 or IL-15:IL-15Rα complex inhibited tumor growth *in vivo* and induced systemic anti-tumor immunity

To evaluate the influence of IL-15 or IL-15:IL-15Rα complex in tumor growth *in vivo*, tumor clones were injected subcutaneously into BALB/c mice at the right flank. Then the tumor growth and survival were monitored daily. Tumor growth in mice implanted with the IL-15 secreting tumor clone was delayed, and their survival was extended compared to the survival of control groups (Figs. 2A and 2B). Surprisingly, tumor clones expressing IL-15:IL-15Rα complexes, IL-15:IL-15Rα(1) and (2), completely inhibit the transfectants to form tumors, suggesting a strong anti-tumor stimulatory effect of IL-15:IL-15Rα complex. Remarkably, the tumors in IL-15:IL-15Rα(1) group that was formed in the early stage, regressed spontaneously. Therefore, all of mice implanted with wild-type tumor, mock-transfected clone, and tumor clone expressing IL-15 died of tumor after 45 days, and all of mice injected with IL-15 or IL-15:IL-15Rα survived. Notably, there was no significant body weight reduction in the mice injected with the tumor clones expressing IL-15 or IL-15:IL-15Rα complex, suggesting the absence of severe cytokine toxicity (Fig. 2C).

Anti-tumor effect of spleen cells activated *in vitro* with IL-15:IL-15Rα

Next, we tried to study the immune-stimulatory function of IL-15 or IL-15:IL-15Rα in tumor cell clones *in vitro*. The immunized BALB/C mice with MMC-inactivated wild-type CT26 cells were sacrificed. The splenocytes were isolated and mix cultured with MMC-inactivated tumor clones expressing IL-15 or IL-15:IL-15Rα. MMC permanently crosslinks DNA through *N*-alkylation, and, therefore, the treated cells cannot divide and grow (Cockerill et al., 2016; Volpe et al., 2010). In this study, MMC was used at the proper concentration to kill cancer cells completely four days after the treatment. As shown in Figure 3A, the addition of spleen cells severely

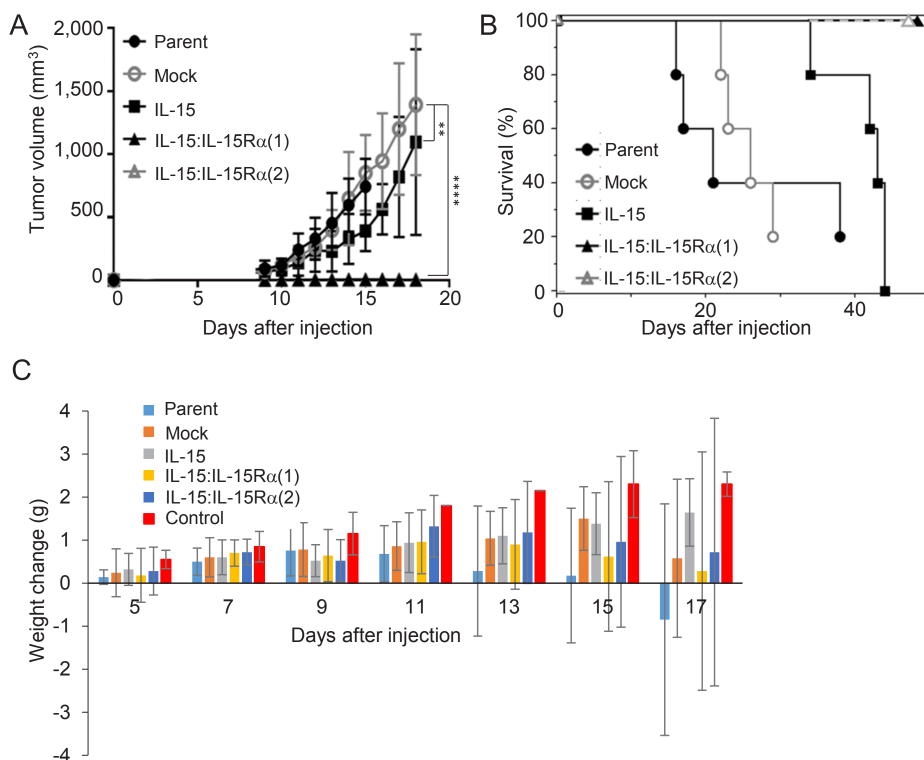

Fig. 2. Tumorigenicity of CT26 tumor clones expressing IL-15 or IL-15:IL-15Rα complexes. After implantation of tumor clones (1 × 10^6 cells/mouse) expressing IL-15 or IL-15:IL-15Rα into BALB/c mice (n = 5) subcutaneously at the right flank, tumor growth and survival of mice were monitored daily basis. (A) Tumor size was measured with a Vernier calipers and the tumor volume was calculated. $^{**}P < 0.01$; $^{***}P < 0.001$. (B) Long term survival analysis. Plot of the percentage of survival rate of the groups. (C) The changes in body weight after the tumor cell implantation was shown. Body weight of same age mice (n = 2) without tumor implantation was observed as a control.

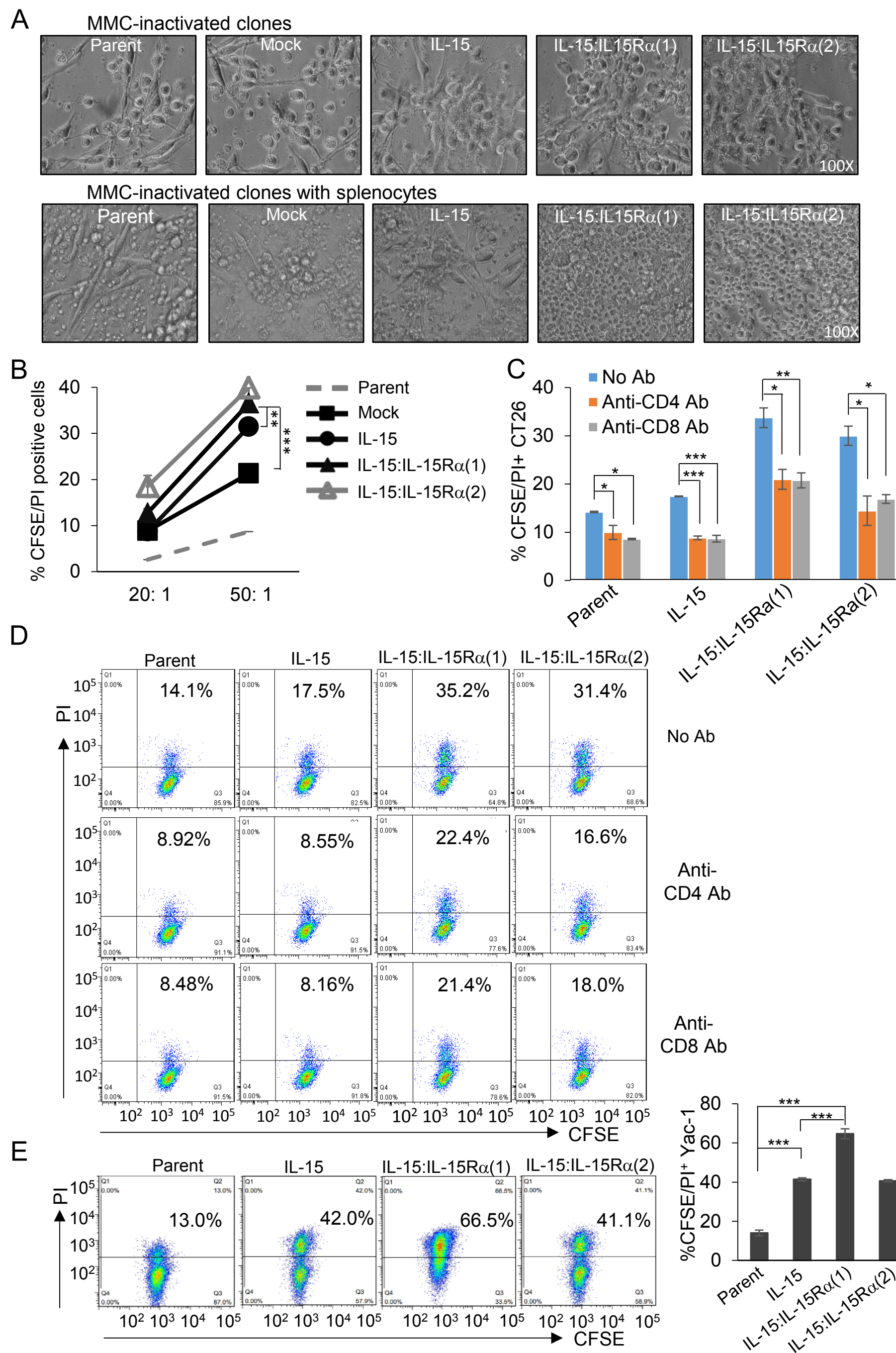

Fig. 3. IL-15:IL-15Rα complexes stimulate anti-tumor cytotoxic effect through CD4+, CD8+, and NK cells *in vitro*. (A) Splenocytes, obtained from BALB/c pre-immunized with CT26, were mixed and cultured with MMC-inactivated tumor clones expressing IL-15 or IL-15:IL-15Rα. The ratio of splenocytes to tumor clones is 50:1. After 48 h, the morphology of MMC-inactivate tumor clones in the mixed-cell cultures was recorded under a microscope. A representative result was shown from 3 independent experiments. (B) Pre-immunized splenocytes were stimulated *in vitro* with MMC-inactivated wild-type cells (antigen sources) and culture supernatants of tumor clones expressing IL-15:IL-15Rα complexes (cytokine sources) for 48 h. The culture supernatant from a parent or mock-transfected clones was used as control. The stimulated splenocytes and CFSE-labeled wild-type CT26 target cells were mixed for at ratio of 20:1 or 50:1. After 6 h incubation, the cell mixtures were collected and stained with PI. The percentage of CFSE+PI+ cells (dead cells) were counted by FACS. (C and D) The activated splenocytes, as described in (B) were pre-incubated with 10 μg/ml anti-CD4 or anti-CD8 antibodies for 30 min before mixing with CSFE-labeled CT26 target cells. After 6 h incubation, the percentage of CFSE+PI+ cells were analyzed by FACS. (E) The activated splenocytes, as described in (B) were mixed with CFSE-labeled YAC-1 target cells (ratio 50:1). After 6 h incubation, the cells were harvested and stained with PI. CFSE+PI+ cells were counted by FACS. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

destroyed the morphology of MMC-inactivated tumor clone cells expressing IL-15:IL-15Rα(1) and (2) compared to the other MMC-inactivated stimulator groups. Cellular morphology of MMC-inactivated wild-type, mock-transfected, and IL-15 expressing tumor clones were well maintained.

We next examine whether the cytotoxic stimulatory effect came from the culture supernatants of IL-15:IL-15Rα expressing tumor clones. The splenocytes from the immunized BALB/C mice with MMC-inactivated wild-type CT26 cells were activated by MMC-inactivated wild-type CT26 cells and the culture supernatants of the designated tumor clones for 48 h. The cytotoxicity of the activated splenocytes was assayed against CFSE-labeled wild-type CT26 cells 6 h after the co-culture. As shown in Figure 3B, splenocytes activated with culture supernatant from tumor clones expressing IL-15:IL-15Rα complex exerted higher cytotoxicity activity compared to those in the other groups, especially in the ratio of 50:1 (splenocytes vs CFSE-CT26 cells). IL-15:IL-15Rα(1) was 4-fold higher than wild-type CT26 group and 1.3-fold than IL-15 clone. This data confirmed the role of IL-15:IL-15Rα complex in culture supernatants as a stimulator for cytotoxic function of splenocytes.

To identify the effect of IL-15:IL-15Rα complex in culture supernatants from IL-15:IL-15Rα(1) and IL-15:IL-15Rα(2) clones on the functional subset of lymphocytes, the blocking effect of anti-CD4 or anti-CD8 antibody was examined. The splenocytes were activated by the culture supernatants from IL-15, IL-15:IL-15Rα(1) and IL-15:IL-15Rα(2) clones as mentioned above. The effector splenocytes were incubated with the blocking anti-CD4 or anti-CD8 antibodies before being mixed with the CSFE-labeled target cells. The incubation with anti-CD4 or anti-CD8 antibodies blocked the cytotoxic effect of the splenocytes (Figs. 3C and 3D). The abolition by anti-CD4 and anti-CD8 antibodies indicated that both $CD4^+$ and $CD8^+$ T cells could directly recognize target cancer cells by major histocompatibility complex (MHC) molecules. Hence, the surface expression of both MHC class II and MHC class I on cancer cell surface was examined. CT26 cancer cells expressed at the detectable level of both MHC class II and I (Supplementary Fig. S1), which is consistent with a previous report (Kreiter et al., 2015). To examine the participation of innate immunity in this anti-tumor model, the cytotoxicity of activated splenocytes after depleting $CD4^+$ and $CD8^+$ T cells by co-treatment with anti-CD4 and anti-CD8 antibody was estimated. Co-treatment with anti-CD4 and anti-CD8 antibodies significantly reduced the cytotoxicity of splenocytes activated with IL-15:IL-15Rα complexes against CFSE-CT26 target cells (Supplementary Fig. S2). Moreover, no difference in cytotoxic activities were found after co-treating with anti-CD4 and anti-CD8 antibodies between all experimental groups. NK cells, an innate lymphocyte, has been proven to be an important anti-tumor effector cell. NK cells exert the direct killing effect via the "missing-self" (MHC class I negative) recognition mechanism (Bern et al., 2019; Mocikat et al., 2003). Since CT26 cancer cells expressed a high level of surface MHC class I (Supplementary Fig. S2), CT26 cancer cell cannot be a proper target for NK mediated cytotoxicity *in vitro*. To further clarify the impact of IL-15 or IL-15:IL-15Rα produced by tumor clones on NK cell activation, splenocytes were stimulated as same as above. Then, the cytotoxicity of the stimulated effector cells to CFSE-labeled YAC-1 cells, an MHC class I negative target cells, was analyzed. Treatment effector splenocytes against CT26 cells with culture supernatants from the tumor clones expressing IL-15 or IL-15:IL-15Rα enhanced NK cytotoxic function up to 5-fold compared to treatment with culture supernatant from the parent (Fig. 3E). Altogether, these results indicate that IL-15:IL-15R produced by tumor clones plays a role in the activation of T cell- and NK cell-mediated cytotoxic effects.

Tumor growth of CT26 cells was delayed by post-treatment of the MMC-inactivated tumor clones expressing IL-15:IL-15Rα complex

To investigate the therapeutic effect of tumor clones expressing IL-15:IL-15Rα complex against CT26 cells, 5 mice per group were first subcutaneously implanted with live wild-type CT26 cells. Three days later, the tumor-bearing mice were treated with a single dose of MMC-inactivated tumor clones expressing IL-15:IL-15Rα clone (1) or (2) by subcutaneous injection in nearby sites. A group of mice was injected with MMC-inactivated mock CT26 as a control (Fig. 4A). Tumor growth and survival were monitored on a daily basis. Treatment with MMC-inactivated tumor clones expressing IL-15:IL-15Rα exerted a marked delaying effect on tumor growth (Fig. 4B). On day 17 post-tumor inoculation, the volume of tumor in control group was double that of the groups treated with MMC-inactivated IL-15:IL-15Rα(1) and (2) clones. The survival was also extended significantly in groups injected with MMC-inactivated tumor clones expressing IL-15:IL-15Rα complex compared to that in the control group (Fig. 4C). For 50% survival, MMC-inactivated IL-15:IL-15Rα group lasted until 60 days after injection, while the control group took less than 40 days. These results indicate that the tumor clones expressing IL-15:IL-15Rα complex exerts significant therapeutic effect against CT26 tumor growth.

The tumor clones expressing IL-15:IL-15Rα complex exerted a prophylactic effect against CT26 tumor cells through induction NK and memory T cells

We next assessed whether MMC-inactivated tumor clones expressing IL-15:IL-15Rα could be used as a tumor cell vaccine. Five mice per group were immunized with a single dose of MMC-inactivated tumor clones expressing mock or IL-15:IL-15Rα clone (1) or (2). Two weeks later, all immunized mice were challenged with 0.5 million live wild-type CT26 cells (Fig. 4D). Within three weeks after the wild-type CT26 challenging, all of the mice immunized with MMC-inactivated mock CT26 cells died of tumor. Surprisingly, almost all of the immunized mice with MMC-inactivated IL-15:IL-15Rα tumor clones (80% for clone 1, 100% for clone 2) rejected wild-type CT26 cells (Figs. 4E and 4F). At day 16 after the live tumor challenging, the tumor volume in mock control group was bigger 12-fold and 16-fold than that in IL-15:IL-15Rα(1) group and in IL-15:IL-15Rα(2) group, respectively (Fig. 4E). Furthermore, the initially formed tumor in mice vaccinated with tumor clones expressing IL-15:IL-15Rα group (1) or (2) regressed later. These results indicate that the tumor clones expressing IL-15:IL-15Rα exerts prophylactic effect against

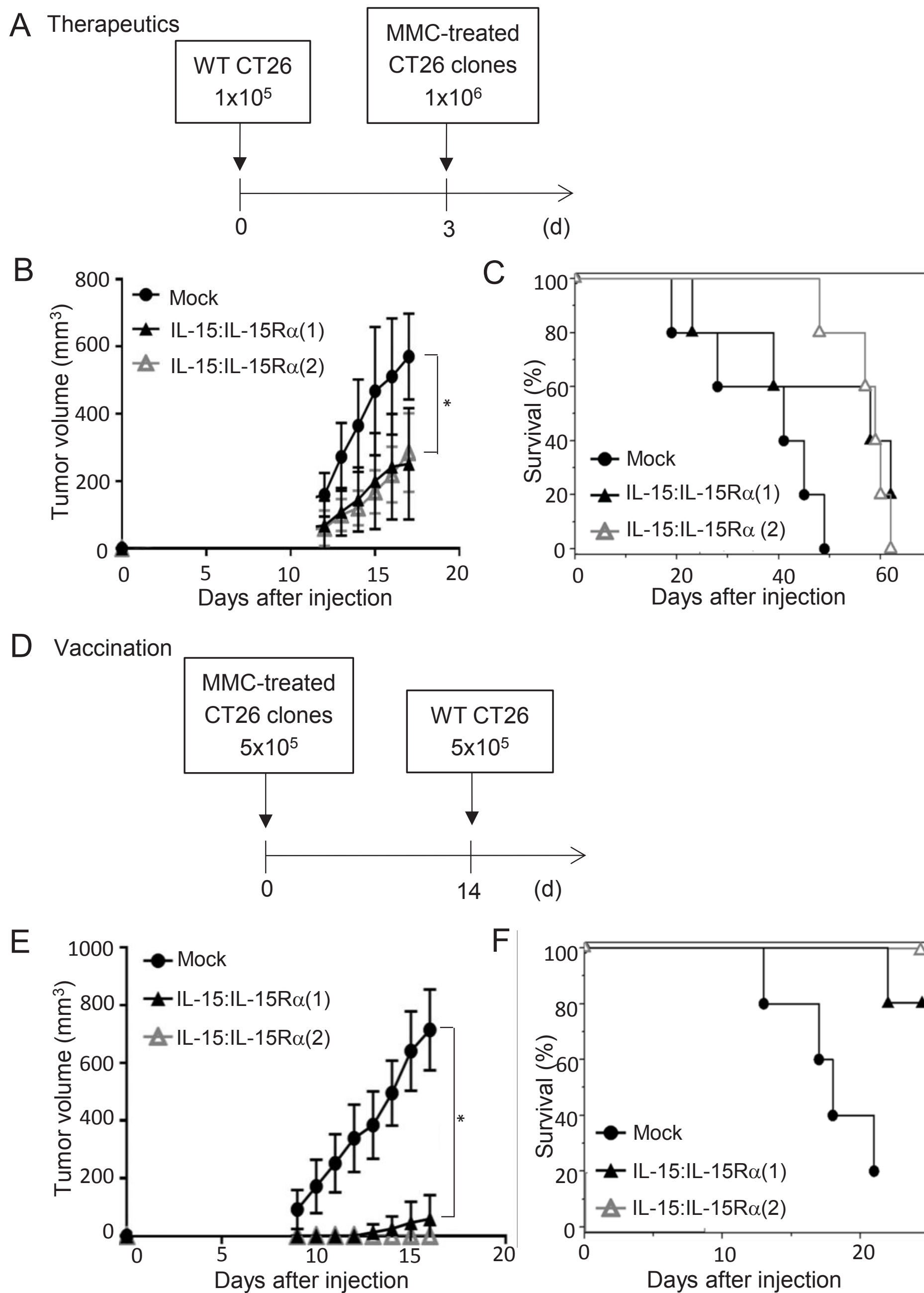

Fig. 4. Therapeutic and vaccination effects of tumor clones expressing IL-15:IL-15Rα complexes against CT26 cells. (A) The schedule of tumor cell injection for therapeutic effect. Normal mice were implanted with wild-type (WT) CT26 cells (1×10^5 cells/mouse) subcutaneously at the right flank, and injected with MMC-inactivated mock or IL-15:IL-15Rα tumor clones (1×10^6 cells/mouse) at near sites after 3 days (n = 5 for each group). (B and C) Tumor growth (B) and survival (C) of mice were monitored. (D) The schedule of tumor cell injection for vaccination. Mice were subcutaneously vaccinated with MMC-inactivated mock or tumor clones expressing IL-15:IL-15Rα (5×10^5 cells/mouse) at right flank. After two weeks, WT CT26 cells (5×10^5 cells/mouse) were subcutaneously implanted at the left flank. (E and F) Tumor growth (E) and survival (F) of mice were monitored. $^*P < 0.05$.

CT26 tumor cells.

We next analyzed which lymphocyte subsets in the mice displayed significant levels of anti-tumor immunity. After mice were primed and challenged as shown in Figure 4D, the primary anti-tumor response in the tumor-bearing mice was analyzed at day 18 of wild-type CT26 challenging. A tumor-bearing mouse from each vaccinated group was sacrificed. The total number of lymphocytes was increased 7-fold in the spleens from mice vaccinated with IL-15:IL-15Rα expressing tumor cells than those from mice vaccinated with mock CT26 cells (Fig. 5A), implying the increase in the proliferation of splenocytes from IL-15:IL-15Rα(1 or 2) tumor clone-immunized mice. Both the central memory ($CD44^+$, $CD62L^+$) and effector memory ($CD44^+$, $CD62L^-$) T cell population of $CD4^+$ or $CD8^+$ phenotype increased in those mice. (Figs. 5B and 5C). The number of central memory cells increased as $CD8^+CD44^+CD62L^+$ population increased up to 6.1-fold, and $CD4^+CD44^+CD62L^+$ population increased up to 5.0-fold. The more significant increase in effector phenotypes was observed as the number of effector memory $CD8^+$ and $CD4^+$ T cells increased up to 10.9-fold and 14.5-fold, respectively. NK cell population ($CD49b^+$) enhancement was even more obvious. Effector NK ($CD49b^+CD11b^+$) and NKT ($CD49b^+CD3^+$) cells of IL-15:IL-15Rα(1 or 2) tumor clone-im-

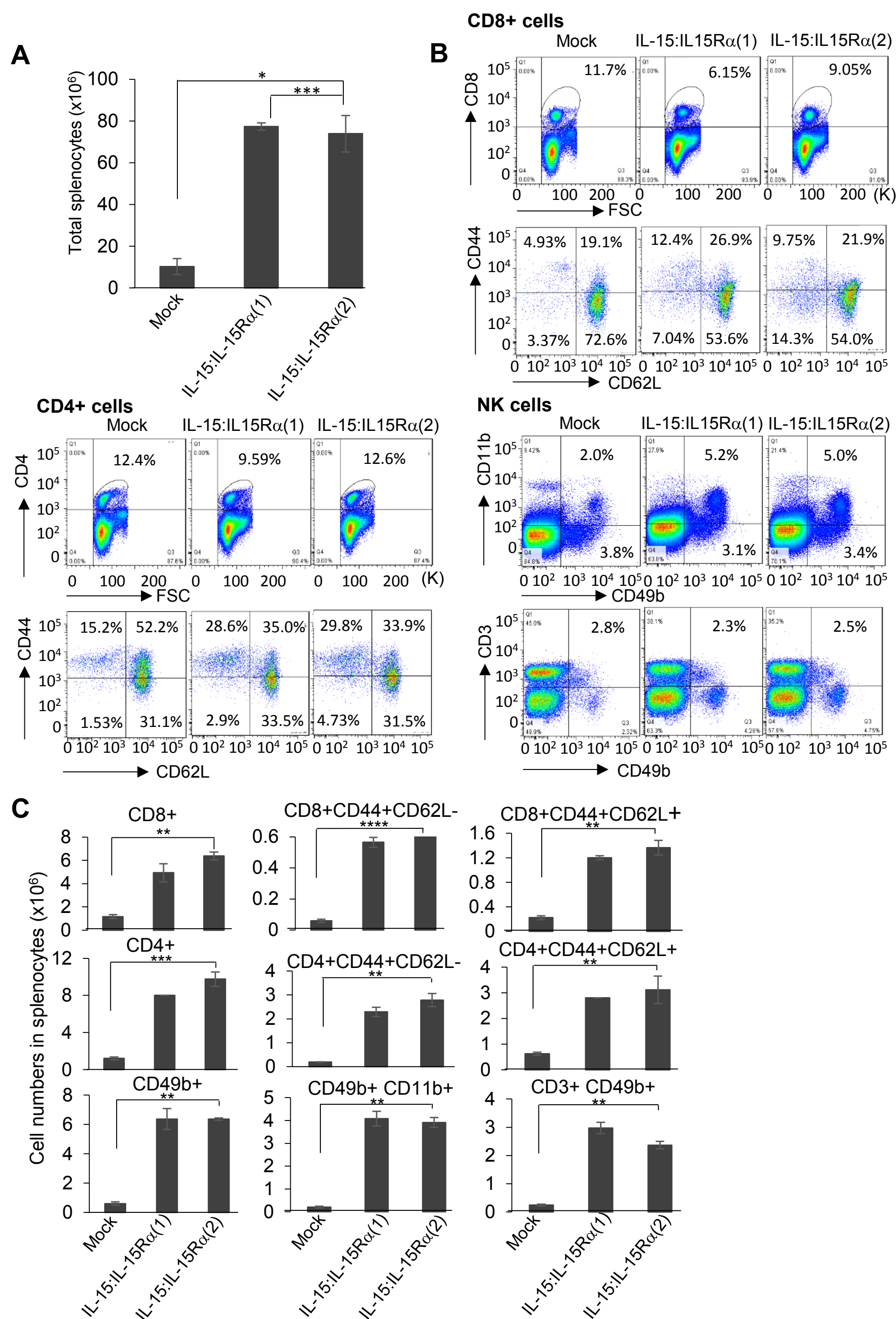

Fig. 5. Vaccination with MMC-inactivated tumor clones expressing IL-15:IL-15Rα complexes, stimulates memory phenotypes in splenic cells of mice. (A) The splenocytes from sacrificed vaccinated mice described in Figures 4D-4F were counted. (B) They were stained to analyze for percentages of effector memory ($CD44^+$ $CD62L^-$) and central memory ($CD44^+$ $CD62L^+$) of $CD4^+$ or $CD8^+$ T cells and NK subsets (total NK cells; $CD49b^+$, effector NK cells; $CD49b^+$ $CD11b^+$, NKT cells; $CD3^+$ $CD49b^+$). (C) The indicated numbers of $CD4^+$, $CD8^+$, and NK cells in splenocytes were graphed. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$.

munized mice increased up to 20.9-fold and 11.9-fold, respectively, compared to those in the control group.

Mice that rejected live IL-15:IL-15Rα expressing clones can also survive after being challenged with wild-type CT26 cells

To analyze the acquisition of systemic anti-tumor immunity, the surviving mice from the initial tumor clone implantation (described in Fig. 2) were challenged with live wild-type CT26 cells subcutaneously (2nd round) at the opposite side of flank from the initial tumor clone implantation (Fig. 6A). At the first round of wild-type CT26 challenging, all of the age-matched normal mice (n = 8) died of tumor within 38 days. However, the surviving mice from IL-15:IL-15Rα(1) expressing tumor clones were protected against the challenging with wild-type CT26 as around 50% of challenged mice survived and those from IL-15:IL-15Rα(2) rejected cancer cells and thus all of them survived (Figs. 6B and 6C). These results indicate that the mice that once rejected tumor clones expressing IL-15:IL-15Rα might acquire certain level of systemic anti-tumor

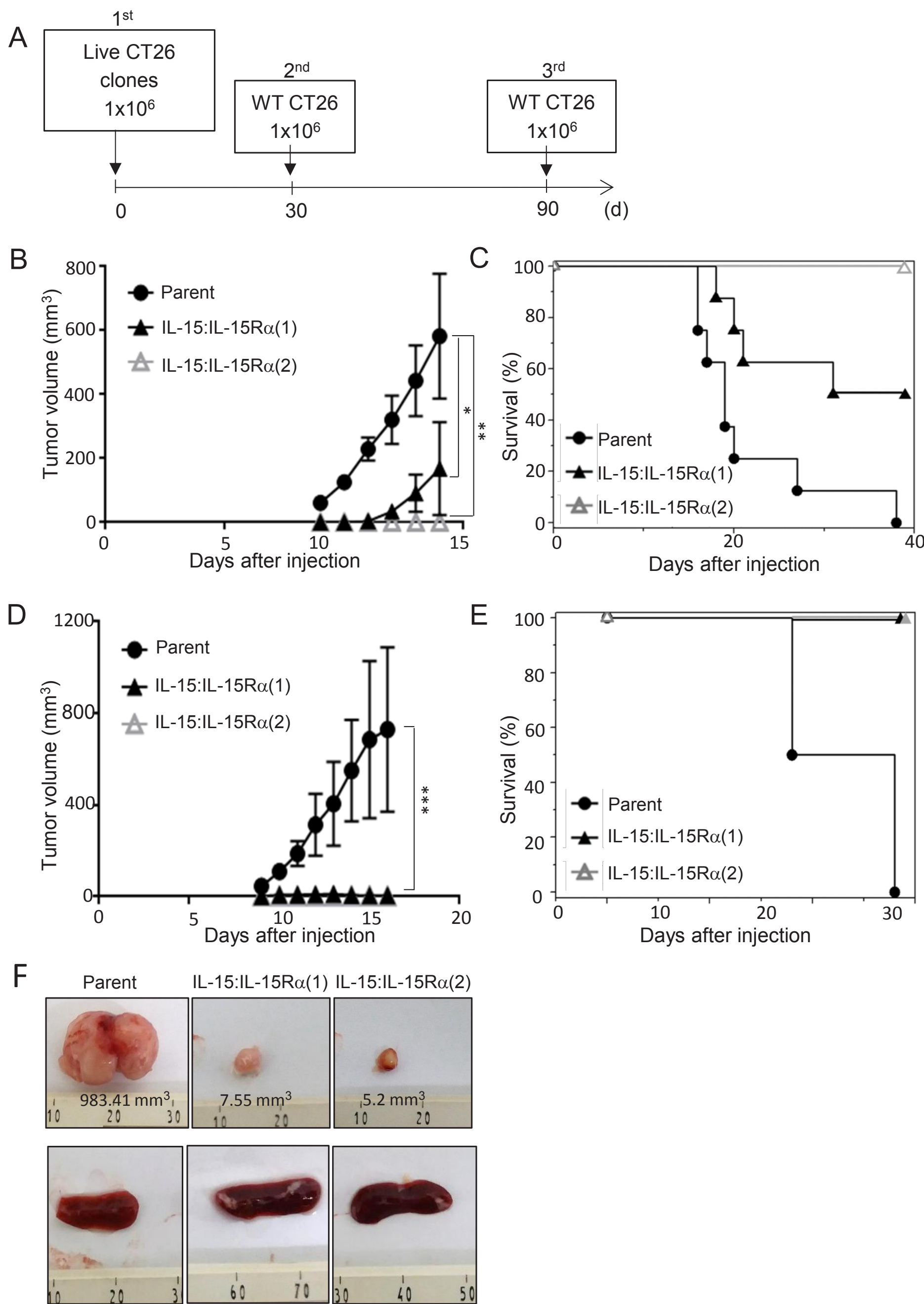

Fig. 6. Long-term anti-tumor memory immune response is acquired by vaccination with MMC-inactivated tumor clones expressing IL-15:IL-15Rα complexes. (A) The schedule of tumor implantation. (1st) Mice were subcutaneously primed tumor clones expressing IL-15:IL-15Rα (1×10^6 cells/ mouse) at day 0 on the left flank. (2nd) One month after the priming, tumor-free mice were subcutaneously challenged with 1×10^6 cells of live wild-type (WT) CT26 cells on the right flank. (3rd) Two months after the boosting step, the tumor-free mice were re-challenged subcutaneously with 1×10^6 cells of live WT CT26 cells on the left flank. (B and C) After the boosting step, the average tumor growth (B) and the survival of mice (C) were monitored daily basis. (D-F) After the re-challenging step with WT CT26, the tumor growth (D) and survival of mice (E) were recorded. On day 18 after the re-challenging, mice with tumor growth were sacrificed, and spleens and tumor masses (F) were photographed. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

immunity. To investigate the maintenance of the anti-tumor immunity, two months after the 2nd round of live tumor challenging, the surviving mice, 3 mice from IL-15:IL-15Rα(1) group and 5 mice from IL-15:IL-15Rα(2) group, were re-challenged with wild-type CT26 cells (3rd round); then tumor growth and survival were monitored (Figs. 6D and 6E). All of the mice experienced with IL-15:IL-15Rα expressing tumor clones survived while normal control mice implanted with wild-type CT26 cells died of the tumor (n = 2). Therefore, challenging with IL-15:IL-15Rα expressing tumor clones provided long-term protection against wild-type CT26 in 100% mice in IL-15:IL-15Rα groups (1) or (2). A representative tumor mass and spleen sample on day 18 after live wild-type CT26 challenging from each group is shown in Figure 6F. Tumor sizes of IL-15:IL-15Rα experienced groups were reduced 150-fold compared to the control group. These results suggest that the immunity once raised by the tumor clones expressing IL-15:IL-15Rα maintained for a long time, at least three months.

To analyze memory immune response in the long-term protection model, the tumor-bearing mice from experiments of Figures 6D-6E were sacrificed. The lymphocyte subsets from the spleens of those mice were analyzed (Fig. 7). The total spleen cells from IL-15:IL-15Rα experienced groups

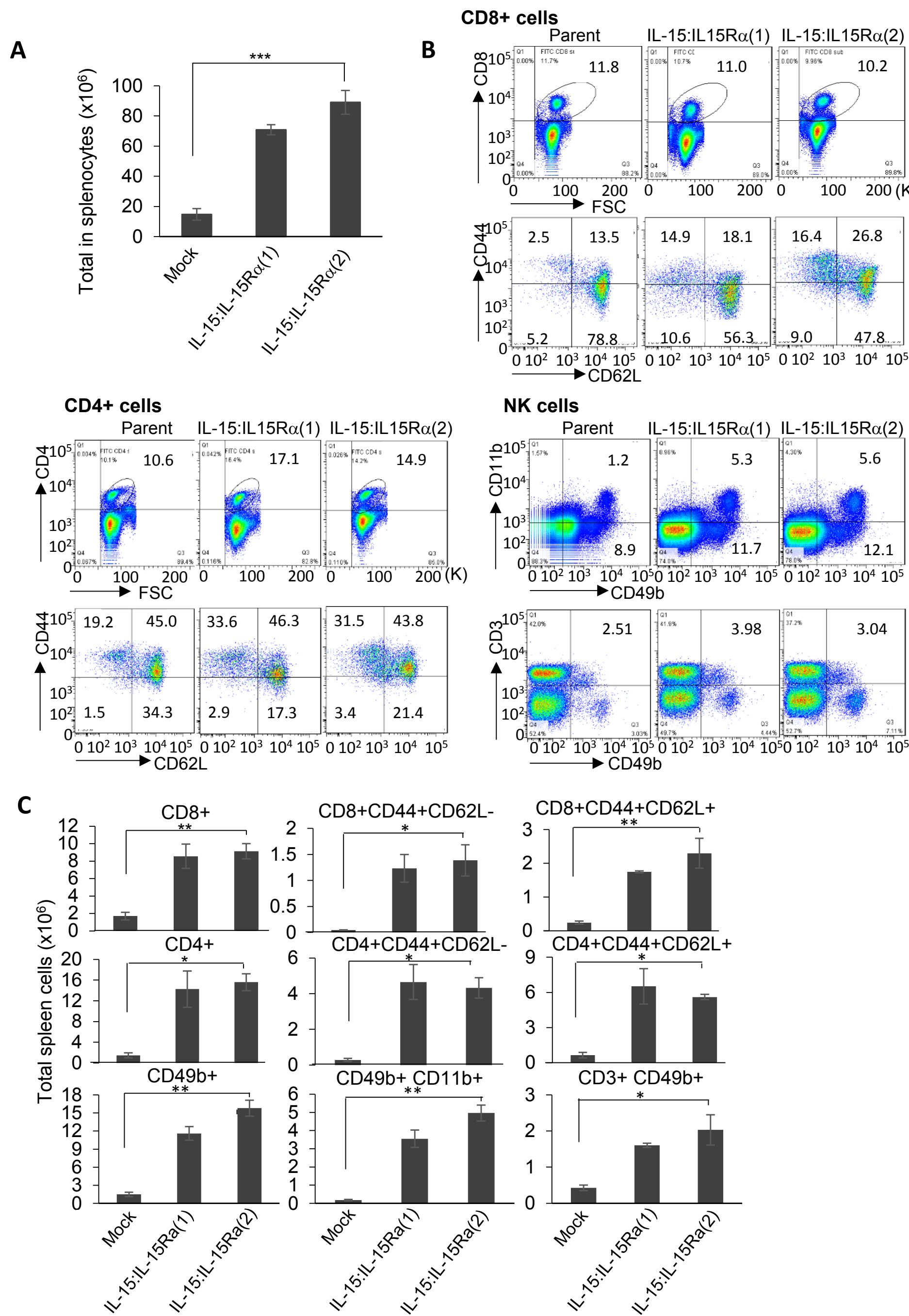

Fig. 7. Challenging with live tumor clones expressing IL-15:IL-15Rα stimulated long-term memory phenotypes in splenic cells. (A) Tumor-bearing mice same as Figure 6F were sacrificed, and the total number of splenocytes were counted. (B and C) The splenocytes obtained from the indicated mice were stained with antibodies to analyze the indicated $CD4^+$, $CD8^+$, and NK cell subsets. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

were 5- to 6-fold higher than those from the control group. The number of central memory cells increased as the $CD8^+CD44^+CD62L^+$ population increased up to 9.5-fold and the $CD4^+CD44^+CD62L^+$ cells enhanced up to 10-fold. The number of effector memory $CD8^+$ and $CD4^+$ T cells increased up to 31.4-fold and 16-fold, respectively. Furthermore, that of effector NK ($CD49b^+CD11b^+$) and NKT ($CD49b^+CD3^+$) cells increased up to 27-fold and up to 4.8-fold respectively, compared to those in the control group. Re-exposure with tumor antigens in the 2nd challenging round worked as a booster for the anti-tumor response. The increase in the number of effector memory $CD8^+$ and $CD4^+$ cells in the spleens of mice in Figure 7 were around 3-fold and 1.3-fold than those in Figure 5. The significant increase was also observed in the number of central memory $CD8^+$ and $CD4^+$ cells such as 1.5-fold and 2.0-fold, respectively. These results support the notion that the strong and effective anti-tumor cytotoxic effect induced by tumor clone expressing IL-15:IL-5Rα involves both the memory T cells and NK cells.

The antigen specificity of anti-CT26 cancer memory re-

sponse was analyzed by detection of IFN-γ secretion through CT26 antigen stimulation. Survivor mice from vaccination experiment (Fig. 4D) or control mice (vaccinated with wild-type CT26 cells) were sacrificed to collect the splenocytes. After *in vitro* stimulation with CT26 cell lysate for 72 h, the culture supernatants from the stimulated splenocytes were subjected to IFN-γ ELISA. Splenocytes from mice vaccinated with IL-15:IL-15Rα expressing CT26 produced 1.6-fold higher amount of IFN-γ after re-exposure to CT26 cell lysate compared to those from control group (Supplementary Fig. S3).

DISCUSSION

Cytotoxic immune effector cells play a crucial role to protect humans from pathogenic diseases. Although the cancer cells express strange antigens called tumor antigens, these cells exploit multiple mechanisms to escape the immune surveillance and to support their rapid growth (Galluzzi et al., 2015; Schreiber et al., 2011; Tennant et al., 2010). A challenge for immunologists is finding a way to boost the tumor-specific immune response to prohibit cancer growth. Some of them have been tried to identify various TAAs as candidates for tumor vaccine or therapy (Coulie et al., 2014). Most of them did not get effective anti-tumor results. Only a few TAAs such as WT-1, CD19, or HER1 (Bergado Baez et al., 2018; Phuphanich et al., 2013) were further developed as promising cancer therapies. Unlike TAAs-based vaccines, whole tumor cell-based vaccines are available for all cancers and target multiple epitopes. Therefore, they can induce a stronger immune response and avoid drug resistance phenomena (Chiang et al., 2015). Our cytokine-expressing whole tumor cell-based therapy has some further advantages from other systems. First, the TAAs were well presented to immune cells. Second, the expression of IL-15:IL-15Rα complex stimulated strong tumor-specific immune response against the TAAs presented on the tumor cell surface without any sign of systemic toxicity.

In this report, we exploited the potential of IL-15 in a complex with its specific receptor IL-15Rα to direct the immune response against CT26 colon cancer TAAs by stable co-expression IL-15 and a truncated IL-15Rα in the same CT26 cells. Both of them were targeted to the same area in endoplasmic reticulum, which facilitated their assembly to make a secreted IL-15:IL-15Rα complex by using IL-2 signal peptide (Fig. 1A). The assembly of IL-15 and IL-15Rα to the soluble cytokine complexes in culture supernatants was confirmed in ELISA and co-immunoprecipitation data (Figs. 1B and 1C). The IL-15 signal peptide has been reported to impede the translation and the secretion of IL-15, and therefore endogenous IL-15 cannot be detected in the extracellular area (Bamford et al., 1998). The replacement IL-15 or IL-15Rα signal peptide by signal peptide from IL-2 also enhanced the secretion of IL-15:IL-15Rα complex.

Although the ectopic expression of IL-15:IL-15Rα complex on CT26 cells did not change the proliferation rate of tumor cell *in vitro* (Fig. 1D), IL-15:IL-15Rα expression successfully inhibited CT26 cell growth in mice (Figs. 2, 4, and 5). Moreover, the immunization with IL-15:IL-15Rα expressing CT26 cell produced long-term protection against wild-type CT26 cancer up to three months in this study (Fig. 6). IL-15:IL-15Rα overexpressing clones demonstrated their outstanding anti-tumor effect over the cells overexpressing IL-15 alone. The overexpression of IL-15 delayed the tumor growth in mice for a little while. On the other hand, the overexpression of IL-15:IL-15Rα promoted strong and effective anti-tumor response to eliminate tumor in mice and finally completely cured those tumor-inoculated mice (Fig. 2). Even both IL-15 and IL-15:IL-15Rα in the culture supernatant triggered anti-cancer responses through CD4+, CD8+, or NK dependent manner (Figs. 3D and 3E), the anti-tumor response stimulated by IL-15:IL-15Rα complex were much more effective for eliminating cancer cells *in vitro* and *in vivo* than that stimulated by IL-15 (Figs. 2, 3A, and 3B). Furthermore, the robust anti-CT26 tumor immune response was acquired by immunization with IL-15:IL-15Rα expressing CT26 cells. As a result, up to 100% survival mice resisted against the later challenging of wild-type CT26 tumor cells (Figs. 4 and 6). Our data demonstrated that the ectopic IL-15:IL-15Rα complexes expressing tumor cells were the better candidate than IL-15 expressing clones in terms of tumor drug and vaccine development.

Recombinant IL-15-Fc and IL-15Rα-Fc were reported as a promising cancer immunotherapy (Epardaud et al., 2008; Kim et al., 2016; Wrangle et al., 2018). However, their anti-cancer effect against solid tumor was not shown to be strong enough in animal models. One of the reasons may be lacking a tumor-specific targeting system. The tumor-specific targeting system must be added at the same time with cytokine signals for effective cancer therapy. With a broad spectrum of TAAs on the surface, cancer cells became the best TAA presenting cell to direct the immune response against solid tumor. We reported here that a single dose treatment of MMC-inactivated IL-15:IL-15Rα complexes expressing clones prolonged the survival of tumor-bearing mice for a month (Figs. 4A-4C). Interestingly, 80~100% mice vaccinated ahead with MMC-inactivated IL-15:IL-15Rα complexes expressing clones were protected from the challenges with live CT26 wild-type tumor cells (Figs. 4D-4F). No sign of toxicity was observed when IL-15:IL-15Rα complexes expressing clones were inoculated in mice (Fig. 2C). Pre-immunizing mice with IL-15:IL-15Rα clones evoked long-lasting systemic anti-tumor responses. Those immunized mice were protected against wild-type CT26 challenging on the distal position from the immunization site, for at least 3 months (Fig. 6). These data strongly supported our hypothesis that combining IL-15:IL-15Rα complex with TAAs on the tumor cell surface improved the specificity of the immune response toward seeking and destroying tumor cells.

IL-15:IL-15Rα has been described as a potential stimulator for effector and memory function of NK and CD8 T cells (Epardaud et al., 2008; Kim et al., 2016; Rubinstein et al., 2006). Recently, evidence has emerged that tumor-reactive cytotoxic CD4+ T cell (ThCTL cells) plays a role in anti-tumor immunity (Kitano et al., 2013; Marshall et al., 2017; Nelles et al., 2014). Our results showed that the anti-tumor cytotoxicity of IL-15:IL-15Rα complexes stimulated spleen cells which depended not only upon NK and CD8+ T cells but also the cytotoxicity function of CD4+ T cells (Fig. 3). Splenic NK cells,

especially in effector NK (CD49b⁺CD11b⁺) population, were significantly enlarged when the mice were vaccinated with IL-15:IL-15Rα expressing clones. Notably, immunized mice with IL-15:IL-15Rα expressing clones triggered the significant augment of splenic effector memory (T_{EM}) and splenic central memory (T_{CM}) in both $CD8^+$ and $CD4^+$ T cells (Figs. 5 and 7). T_{CM} and T_{EM} have been proven as professional anti-tumor immune cells in terms of the strength and length of cell-mediated effector function as well as IFN-γ production (Yang et al., 2011). Moreover, the T_{CM} and T_{EM} subsets can directly induce the phenotypic, functional, and metabolic differentiation of naïve T lymphocytes (Klebanoff et al., 2016). Besides initial antigen signal strength and co-stimulation signal condition, the level of particular cytokines including IL-2, IL-7, and IL-15 can adjust the relative ratio of T_{CM}, T_{EM}, and naïve T cells in response to antigen (Willinger et al., 2005). Unlike IL-2, which keeps the key role in the differentiation and homeostasis of T cells, or IL-7, an important cytokine to maintain the survival of memory precursor T cells (Bordon, 2015), IL-15 is vital to the differentiation and proliferation of memory T cells (Pangrazzi et al., 2017). Recently, the essential role of IL-15 in the presence of tissue resident effector memory T cells was reported (Holz et al., 2018; Mackay et al., 2015). Taken together, the above evidence and our suggested that the success of IL-15:IL-15Rα complex expressing tumor cell vaccine might come from the ability of the vaccine to i) induce the formation of anti-tumor memory T cell subsets, and ii) improve the capability of seeking and killing tumor cells by the effector lymphocytes. Furthermore, our results are the first evidence to prove the potential role of IL-15:IL-15Rα complex in stimulation $CD4^+$ dependent cytotoxicity.

Our study firmly proved the potential roles of combining IL-15:IL-15Rα and TAAs to stimulate a strong and specific anti-tumor response by reviving tumor specific memory and effector CD8 and CD4 T cells. Although combinations with other immunotherapy such as immune checkpoint blockages are necessary to enhance the anti-tumor immunity, this approach is promising for providing an affordable and precise immunotherapy to cancer patients.

Note: Supplementary information is available on the Molecules and Cells website (www.molcells.org).

Disclosure

The authors have no potential conflicts of interest to disclose.

ACKNOWLEDGMENTS

This research was supported by a grant from Chungnam National University.

ORCID

Van Anh Do Thi https://orcid.org/0000-0003-4670-6386
Hyung Min Jeon https://orcid.org/0000-0003-2070-2969
Sang Min Park https://orcid.org/0000-0001-7144-8230
Hayyoung Lee https://orcid.org/0000-0002-8163-2880
Young Sang Kim https://orcid.org/0000-0002-2360-2595

REFERENCES

Alahari, S.V., Dong, S., and Alahari, S.K. (2015). Are macrophages in tumors good targets for novel therapeutic approaches? Mol. Cells *38*, 95-104.

Bamford, R.N., DeFilippis, A.P., Azimi, N., Kurys, G., and Waldmann, T.A. (1998). The 5′ untranslated region, signal peptide, and the coding sequence of the carboxyl terminus of IL-15 participate in its multifaceted translational control. J. Immunol. *160*, 4418-4426.

Bergado Baez, G., Hernandez Fernandez, D.R., Mazorra Herrera, Z., and Sanchez Ramirez, B. (2018). HER1-based vaccine: simultaneous activation of humoral and cellular immune response. Semin. Oncol. *45*, 75-83.

Bern, M.D., Parikh, B.A., Yang, L., Beckman, D.L., Poursine-Laurent, J., and Yokoyama, W.M. (2019). Inducible down-regulation of MHC class I results in natural killer cell tolerance. J. Exp. Med. *216*, 99-116.

Bordon, Y. (2015). T cell memory: new insight on old-timers. Nat. Rev. Immunol. *15*, 331.

Chaurasiya, S. and Warner, S. (2017). Viroimmunotherapy for colorectal cancer: clinical studies. Biomedicines *5*, E11.

Chiang, C.L., Coukos, G., and Kandalaft, L.E. (2015). Whole tumor antigen vaccines: where are we? Vaccines (Basel) *3*, 344-372.

Cockerill, P.A., Knoedler, J.J., Frank, I., Tarrell, R., and Karnes, R.J. (2016). Intravesical gemcitabine in combination with mitomycin C as salvage treatment in recurrent non-muscle-invasive bladder cancer. BJU Int. *117*, 456-462.

Coulie, P.G., Van den Eynde, B.J., van der Bruggen, P., and Boon, T. (2014). Tumour antigens recognized by T lymphocytes: at the core of cancer immunotherapy. Nat. Rev. Cancer *14*, 135-146.

de Gruijl, T.D., van den Eertwegh, A.J., Pinedo, H.M., and Scheper, R.J. (2008). Whole-cell cancer vaccination: from autologous to allogeneic tumor- and dendritic cell-based vaccines. Cancer Immunol. Immunother. *57*, 1569-1577.

Do Thi, V.A., Park, S.M., Lee, H., and Kim, Y.S. (2016). The membrane-bound form of IL-17A promotes the growth and tumorigenicity of colon cancer cells. Mol. Cells *39*, 536-542.

Do Thi, V.A., Park, S.M., Lee, H., and Kim, Y.S. (2018). Ectopically expressed membrane-bound form of IL-9 exerts immune-stimulatory effect on CT26 colon carcinoma cells. Immune Netw. *18*, e12.

Drake, A., Kaur, M., Iliopoulou, B.P., Phennicie, R., Hanson, A., and Chen, J. (2016). Interleukins 7 and 15 maintain human T cell proliferative capacity through STAT5 signaling. PLoS One *11*, e0166280.

Dubois, S., Mariner, J., Waldmann, T.A., and Tagaya, Y. (2002). IL-15Ralpha recycles and presents IL-15 In trans to neighboring cells. Immunity *17*, 537-547.

Dubois, S., Patel, H.J., Zhang, M., Waldmann, T.A., and Muller, J.R. (2008). Preassociation of IL-15 with IL-15R alpha-IgG1-Fc enhances its activity on proliferation of NK and CD8+/CD44high T cells and its antitumor action. J. Immunol. *180*, 2099-2106.

Epardaud, M., Elpek, K.G., Rubinstein, M.P., Yonekura, A.R., Bellemare-Pelletier, A., Bronson, R., Hamerman, J.A., Goldrath, A.W., and Turley, S.J. (2008). Interleukin-15/interleukin-15R alpha complexes promote destruction of established tumors by reviving tumor-resident CD8+ T cells. Cancer Res. *68*, 2972-2983.

Fehniger, T.A. and Caligiuri, M.A. (2001). Interleukin 15: biology and relevance to human disease. Blood *97*, 14-32.

Galluzzi, L., Pietrocola, F., Bravo-San Pedro, J.M., Amaravadi, R.K., Baehrecke, E.H., Cecconi, F., Codogno, P., Debnath, J., Gewirtz, D.A., Karantza, V., et al. (2015). Autophagy in malignant transformation and cancer progression. EMBO J. *34*, 856-880.

Holz, L.E., Prier, J.E., Freestone, D., Steiner, T.M., English, K., Johnson, D.N., Mollard, V., Cozijnsen, A., Davey, G.M., Godfrey, D.I., et al. (2018). CD8(+) T cell activation leads to constitutive formation of liver tissue-resident

memory T cells that seed a large and flexible niche in the liver. Cell Rep. *25*, 68-79.e4.

Hu, Q., Ye, X., Qu, X., Cui, D., Zhang, L., Xu, Z., Wan, H., Zhang, L., and Tao, W. (2018). Discovery of a novel IL-15 based protein with improved developability and efficacy for cancer immunotherapy. Sci. Rep. *8*, 7675.

Kim, P.S., Kwilas, A.R., Xu, W., Alter, S., Jeng, E.K., Wong, H.C., Schlom, J., and Hodge, J.W. (2016). IL-15 superagonist/IL-15RalphaSushi-Fc fusion complex (IL-15SA/IL-15RalphaSu-Fc; ALT-803) markedly enhances specific subpopulations of NK and memory CD8+ T cells, and mediates potent anti-tumor activity against murine breast and colon carcinomas. Oncotarget *7*, 16130-16145.

Kim, Y.S. (2009). Tumor therapy applying membrane-bound form of cytokines. Immune Netw. *9*, 158-168.

Kitano, S., Tsuji, T., Liu, C., Hirschhorn-Cymerman, D., Kyi, C., Mu, Z., Allison, J.P., Gnjatic, S., Yuan, J.D., and Wolchok, J.D. (2013). Enhancement of tumor-reactive cytotoxic CD4+ T cell responses after ipilimumab treatment in four advanced melanoma patients. Cancer Immunol. Res. *1*, 235-244.

Klebanoff, C.A., Scott, C.D., Leonardi, A.J., Yamamoto, T.N., Cruz, A.C., Ouyang, C., Ramaswamy, M., Roychoudhuri, R., Ji, Y., Eil, R.L., et al. (2016). Memory T cell-driven differentiation of naive cells impairs adoptive immunotherapy. J. Clin. Invest. *126*, 318-334.

Kozlowska, A., Mackiewicz, J., and Mackiewicz, A. (2013). Therapeutic gene modified cell based cancer vaccines. Gene *525*, 200-207.

Kreiter, S., Vormehr, M., van de Roemer, N., Diken, M., Lower, M., Diekmann, J., Boegel, S., Schrors, B., Vascotto, F., Castle, J.C., et al. (2015). Mutant MHC class II epitopes drive therapeutic immune responses to cancer. Nature *520*, 692-696.

Lee, S. and Margolin, K. (2011). Cytokines in cancer immunotherapy. Cancers (Basel) *3*, 3856-3893.

Mackay, L.K., Wynne-Jones, E., Freestone, D., Pellicci, D.G., Mielke, L.A., Newman, D.M., Braun, A., Masson, F., Kallies, A., Belz, G.T., et al. (2015). T-box transcription factors combine with the cytokines TGF-β and IL-15 to control tissue-resident memory T cell fate. Immunity *43*, 1101-1111.

Mackiewicz, J. and Mackiewicz, A. (2010). Gene-modified cellular vaccines: technologic aspects and clinical problems. Transpl. Proc. *42*, 3287-3292.

Marshall, N.B., Vong, A.M., Devarajan, P., Brauner, M.D., Kuang, Y., Nayar, R., Schutten, E.A., Castonguay, C.H., Berg, L.J., Nutt, S.L., et al. (2017). NKG2C/E marks the unique cytotoxic CD4 T cell subset, ThCTL, generated by influenza infection. J. Immunol. *198*, 1142-1155.

Mocikat, R., Braumuller, H., Gumy, A., Egeter, O., Ziegler, H., Reusch, U., Bubeck, A., Louis, J., Mailhammer, R., Riethmuller, G., et al. (2003). Natural killer cells activated by MHC class I(low) targets prime dendritic cells to induce protective CD8 T cell responses. Immunity *19*, 561-569.

Nelles, M.E., Moreau, J.M., Furlonger, C.L., Berger, A., Medin, J.A., and Paige, C.J. (2014). Murine splenic CD4(+) T cells, induced by innate immune cell interactions and secreted factors, develop antileukemia cytotoxicity. Cancer Immunol. Res. *2*, 1113-1124.

Ochoa, M.C., Fioravanti, J., Rodriguez, I., Hervas-Stubbs, S., Azpilikueta, A., Mazzolini, G., Gurpide, A., Prieto, J., Pardo, J., Berraondo, P., et al. (2013). Antitumor immunotherapeutic and toxic properties of an HDL-conjugated chimeric IL-15 fusion protein. Cancer Res. *73*, 139-149.

Pangrazzi, L., Naismith, E., Meryk, A., Keller, M., Jenewein, B., Trieb, K., and Grubeck-Loebenstein, B. (2017). Increased IL-15 production and accumulation of highly differentiated CD8(+) effector/memory T cells in the bone marrow of persons with cytomegalovirus. Front Immunol. *8*, 715.

Phuphanich, S., Wheeler, C.J., Rudnick, J.D., Mazer, M., Wang, H., Nuno, M.A., Richardson, J.E., Fan, X., Ji, J., Chu, R.M., et al. (2013). Phase I trial of a multi-epitope-pulsed dendritic cell vaccine for patients with newly diagnosed glioblastoma. Cancer Immunol. Immunother. *62*, 125-135.

Podhajcer, O.L., Lopez, M.V., and Mazzolini, G. (2007). Cytokine gene transfer for cancer therapy. Cytokine Growth Factor Rev. *18*, 183-194.

Rhode, P.R., Egan, J.O., Xu, W., Hong, H., Webb, G.M., Chen, X., Liu, B., Zhu, X., Wen, J., You, L., et al. (2016). Comparison of the superagonist complex, ALT-803, to IL15 as cancer immunotherapeutics in animal models. Cancer Immunol. Res. *4*, 49-60.

Rubinstein, M.P., Kovar, M., Purton, J.F., Cho, J.H., Boyman, O., Surh, C.D., and Sprent, J. (2006). Converting IL-15 to a superagonist by binding to soluble IL-15R{alpha}. Proc. Natl. Acad. Sci. U. S. A. *103*, 9166-9171.

Sarvizadeh, M., Ghasemi, F., Tavakoli, F., Sadat Khatami, S., Razi, E., Sharifi, H., Biouki, N.M., and Taghizadeh, M. (2019). Vaccines for colorectal cancer: an update. J. Cell Biochem. *120*, 8815-8828.

Schreiber, R.D., Old, L.J., and Smyth, M.J. (2011). Cancer immunoediting: integrating immunity's roles in cancer suppression and promotion. Science *331*, 1565-1570.

Srivatsan, S., Patel, J.M., Bozeman, E.N., Imasuen, I.E., He, S., Daniels, D., and Selvaraj, P. (2014). Allogeneic tumor cell vaccines: the promise and limitations in clinical trials. Hum. Vaccin. Immunother. *10*, 52-63.

Tennant, D.A., Duran, R.V., and Gottlieb, E. (2010). Targeting metabolic transformation for cancer therapy. Nat. Rev. Cancer *10*, 267-277.

Uyl-de Groot, C.A., Vermorken, J.B., Hanna, M.G., Jr., Verboom, P., Groot, M.T., Bonsel, G.J., Meijer, C.J., and Pinedo, H.M. (2005). Immunotherapy with autologous tumor cell-BCG vaccine in patients with colon cancer: a prospective study of medical and economic benefits. Vaccine *23*, 2379-2387.

Volpe, A., Racioppi, M., D'Agostino, D., Cappa, E., Filianoti, A., and Bassi, P.F. (2010). Mitomycin C for the treatment of bladder cancer. Minerva Urol. Nefrol. *62*, 133-144.

Willinger, T., Freeman, T., Hasegawa, H., McMichael, A.J., and Callan, M.F. (2005). Molecular signatures distinguish human central memory from effector memory CD8 T cell subsets. J. Immunol. *175*, 5895-5903.

Wrangle, J.M., Velcheti, V., Patel, M.R., Garrett-Mayer, E., Hill, E.G., Ravenel, J.G., Miller, J.S., Farhad, M., Anderton, K., Lindsey, K., et al. (2018). ALT-803, an IL-15 superagonist, in combination with nivolumab in patients with metastatic non-small cell lung cancer: a non-randomised, open-label, phase 1b trial. Lancet Oncol. *19*, 694-704.

Yang, S., Gattinoni, L., Liu, F., Ji, Y., Yu, Z., Restifo, N.P., Rosenberg, S.A., and Morgan, R.A. (2011). In vitro generated anti-tumor T lymphocytes exhibit distinct subsets mimicking in vivo antigen-experienced cells. Cancer Immunol. Immunother. *60*, 739-749.