A number of antitumor vaccines have recently shown promise in upregulating immune responses against tumor antigens and improving patient survival. In this study, we examine the effectiveness of vaccination using interleukin (IL)-15-expressing tumor cells and also examine their ability to upregulate immune responses to tumor antigens. We demonstrated that the coexpression of IL-15 with its receptor, IL-15Rα, increased the cell-surface expression and secretion of IL-15. We show that a gene transfer approach using recombinant adeno virus to express IL-15 and IL-15Rα in murine TRAMP-C2 prostate or TS/A breast tumors induced antitumor immune responses. From this, we developed a vaccine platform, consisting of TRAMP-C2 prostate cancer cells or TS/A breast cancer cells coexpressing IL-15 and IL-15Rα that inhibited tumor formation when mice were challenged with tumor. Inhibition of tumor growth led to improved survival when compared with animals receiving cells expressing IL-15 alone or unmodified tumor cells. Animals vaccinated with tumor cells coexpressing IL-15 and IL-15Rα showed greater tumor infiltration with CD8+ T and natural killer (NK) cells, as well as increased antitumor CD8+ T-cell responses. Vaccination with IL-15/IL-15Rα-modified TS/A breast cancer cells provided a survival advantage to mice challenged with unrelated murine TUBO breast cancer cells, indicating the potential for allogeneic IL-15/IL-15Rα-expressing vaccines.

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INTRODUCTION

Tumor cell vaccines have shown preclinical promise and antitumor activity in patient trials in a number of malignancies, including breast, prostate and pancreatic cancer, and leukemia (reviewed in Keenan and Jaffee1). These vaccines have the advantage over single-antigen vaccines in that they can target multiple known and unknown tumor-associated antigens. The whole tumor cell vaccine platform that has been the furthest developed is GVAX (granulocyte-macrophage colony-stimulating factor (GM-CSF) gene-transfected tumor cell vaccine), which consists of irradiated autologous or allogeneic tumor cells genetically modified to secrete granulocyte-macrophage colony-stimulating factor. GVAX has been shown to induce the infiltration of dendritic cells to the vaccination site, and stimulate CD8+ T-cell responses, as well as antibody responses (reviewed in Eager and Nemunaitis2). The clinical outcomes of phase III trials of GVAX, however, have been disappointing. Two randomized, controlled phase III trials of GVAX in prostate cancer (VITAL-1 and VITAL-2) were terminated owing to a lack of efficacy compared with standard chemotherapy with docetaxel and prednisone.3,4 In the VITAL-2 study, an increase in patient deaths was also noted in the GVAX arm.

Interleukin-15 (IL-15) is a proinflammatory cytokine capable of stimulating the differentiation and proliferation of T, B and natural killer (NK) cells. It is essential for the differentiation and maintenance of memory CD8+ T cells and NK/T cells.5 It can also promote the development of dendritic cells.6 IL-15 function is mediated through its heterotrimetric receptor composed of IL-15Rs, IL-2R/IL-15Rβ (CD122), and the common cytokine receptor γ-chain (γc, CD123).7,8 IL-15 is tightly bound by IL-15Rα alone, anchoring it on the surface of antigen-presenting cells and limiting its secretion. Signaling is initiated by presentation of IL-15 by IL-15Rα in ‘trans’ to the IL-2R/IL-15Rβ and γc expressed on the effector cells.9 The coexpression of IL-15 and IL-15Rα on non-lymphoid cells such as antigen-presenting cells has been shown to be necessary to support its ‘trans’ presentation and activation of effector cells.9,10 In HIV infection, the coordinated dysregulation of IL-15 and IL-15Rα has been shown to occur in progressive disease underlying the importance of coexpressing IL-15 and IL-15Rα.11

We utilized IL-15 to develop a whole-tumor cell vaccine targeting murine breast and prostate cancer. We show that tumor cells transduced with IL-15 inhibited tumor growth in vivo and this was enhanced when IL-15Rα was also coexpressed by the tumor cells. Vaccination with modified tumor cells expressing IL-15 and IL-15Rα inhibited tumor formation and led to increased survival. Furthermore, we show that the immune responses induced by vaccination are mediated by CD8+ T cells and NK cells.

RESULTS

Tramp-C2 and TS/A cells express IL-15 following transduction with Ad.ml15 + Ad.ml15Rα

To examine if TRAMP-C2 and TS/A cells could be made to express IL-15, we transduced them with Ad.ml15, Ad.null or Ad.ml15 + Ad.ml15Rα and examined IL-15 secretion by enzyme-linked immunosorbent assay. We found that neither TRAMP-C2 nor TS/A cells natively secrete detectable levels of IL-15 and did not secrete IL-15 in response to transduction with a control vector, Ad.null. Both cell lines expressed IL-15 following transduction with Ad.ml15 alone or in combination with Ad.ml15Rα (Figures 1a and b).

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Significantly higher levels of IL-15 were detected in the supernatants of cells transduced with both Ad.mIL-15 and Ad.mIL-15R when compared with those infected with Ad.mIL-15 alone ($P < 0.01$). We confirmed the functional status of the secreted IL-15 by its ability to induce proliferation of CTLL-2 cells. Culture media from TRAMP-C2 or TS/A cells transduced with Ad.mIL-15 + Ad.mIL-15R induced the proliferation of CTLL-2 cells, whereas those transduced with Ad.null did not (Figure 1c). The media retained its ability to induce CTLL-2 proliferation to a dilution of 1:1000.

In order to determine the cellular localization of IL-15 following transduction with Ad.mIL-15, Ad.null or Ad.mIL-15 + Ad.mIL-15R, we examined transduced TS/A tumors by immunohistochemistry. TS/A tumors that had been infected with Ad.null did not exhibit any IL-15 staining, whereas those transduced with either Ad.mIL-15 alone or in combination with Ad.mIL-15R showed significant IL-15 staining (Figures 1d–f). TS/A cells transduced with Ad.mIL-15 alone expressed IL-15 throughout the cell, whereas those that had been transduced with both Ad.mIL-15 and Ad.mIL-15R exhibited IL-15 staining predominantly at the surface of the cell.

TRAMP-C2 and TS/A cells expressing IL-15 and IL-15R inhibited tumor growth

In order to examine the effects of IL-15 and IL-15R expression on tumor growth we transduced TS/A and TRAMP-C2 cells with Ad.mIL-15 with or without Ad.mIL-15R and subcutaneously injected them into syngeneic BALB/c or C57Bl/6 mice, respectively. We found that the expression of IL-15 alone or in combination with IL-15R inhibited the growth of TS/A (Figure 2a) and TRAMP-C2 tumors (Figure 2b) ($P < 0.05$). In both tumor lines, the added expression of IL-15R further inhibited tumor growth when compared with IL-15 alone. IL-15R alone also reduced tumor growth in TS/A ($P < 0.05$).

To further show that IL-15 expression by tumors could inhibit tumor growth, we injected Ad.mIL-15, Ad.mIL-15R, Ad.mIL-15 + Ad.mIL-15R or Ad.null into actively growing TS/A or TRAMP-C2 tumors in vivo. Ad.mIL-15 or Ad.mIL-15 + Ad.mIL-15R resulted in significant tumor growth reduction when compared with Ad.null ($P < 0.05$). The combination of IL-15 and IL-15R inhibited the growth of both TS/A (Figure 2c) and TRAMP-C2 (Figure 2d) tumors ($P < 0.05$). Ad.mIL-15R did not reduce the growth of either TS/A or TRAMP-C2 tumors compared with Ad.null and therefore this group was not continued.

Growth inhibition mediated by IL-15 and IL-15R is CD8$^+$ and NK dependent

To determine which cell population is important in the inhibition of tumor growth following transduction with IL-15 with or without IL-15R, we depleted CD8$^+$ T cells, CD4$^+$ T cells or NK cells and looked for an abrogation of tumor suppression. Depletion of CD4$^+$ cells did not affect tumor growth kinetics in animals treated with either IL-15 alone (Figure 3a) or in combination with IL-15R (Figure 3b). However, depletion of NK or CD8$^+$ cells inhibited IL-15-induced tumor growth suppression (NK, $P = 0.005$; CD8$^+$, $P = 0.001$) and IL-15 + IL-15R (NK, $P = 0.025$; CD8$^+$, $P < 0.001$), indicating a role of these cells in the antitumor effect. In animals with tumors transduced with IL-15 alone, the depletion of NK cells showed the greatest abrogation of tumor response, whereas when IL-15 was combined with IL-15R, CD8$^+$ depletion demonstrated greater attenuation of tumor responses.

A cellular vaccine expressing IL-15 and IL-15R increased survival

Given that the expression of IL-15 and IL-15R by tumor cells can induce an antitumor response that is CD8$^+$ mediated, we examined the ability of modified TRAMP-C2 and TS/A cells to act as a vaccine platform. To do this, we infected TRAMP-C2 and TS/A with Ad.mIL-15 with or without Ad.mIL-15R and treated them with mitomycin C (MMC) to inhibit their growth. We injected the cells into the flanks of BALB/c or C57Bl/6 mice and 2 weeks later challenged the mice with unmodified TS/A or TRAMP-C2 cells, respectively, on the opposite flank. Mice vaccinated with TS/A cells expressing IL-15 did not show significantly greater survival than those vaccinated with TS/A transduced with Ad.null (Figure 4a). However, mice vaccinated with TS/A expressing both IL-15 and IL-15R had significantly improved survival compared with those vaccinated with TS/A expressing IL-15 alone or Ad.null.
In order to assess the tumor-specific effects of the vaccine, we examined the ability of splenocytes from TRAMP-C2- and TS/A-vaccinated mice to lyse the respective unmodified tumor cells in either TRAMP-C2 or TS/A tumors. After stimulation with tumor antigens, splenic cells isolated from TRAMP-C2-Ad.null-treated mice demonstrated little lytic activity against TRAMP-C2 or MC38 cells (Figure 6a), whereas spleen cells isolated from TRAMP-C2-IL-15/IL-15Rα showed increased lytic activity against TRAMP-C2 cells, but not against murine MC38 colon cancer. Similarly, splenocytes isolated from TS/A-Ad.null-treated mice showed little lytic activity, whereas those isolated from TS/A-IL-15/IL-15Rα showed increased lytic activity against TS/A and TUBO cells, indicating a tumor-specific immune response (Figure 6b). Greater lytic activity was shown against TS/A cells as compared with TUBO cells. To confirm that the lytic activity seen was directed against the CD8+ T-cell infiltrates, we found no differences in the relative numbers of these cells in either TRAMP-C2 or TS/A tumors.

Vaccination with TRAMP-C2 or TS/A cells expressing IL-15 and IL-15Rα induces cell-mediated immune responses

In a proof-of-principle experiment designed to show the efficacy of the vaccine against an established tumor, we implanted highly aggressive and poorly immunogenic TS/A cells on one flank of the mouse, allowed the tumors to grow for 7 days, to a mean volume of 130 mm³, and then vaccinated the mice with Ad.null at 1 × 10⁹ plaque-forming unit. Arrows indicate the injection time point. Mice were evaluated daily for tumor growth. N = 10 per group. (A) TS/A or (B) TRAMP-C2 cells were transduced with Ad.null, Ad.IL-15, Ad.mIL-15Rα or Ad.IL-15 + IL-15Rα at an MOI of 100. After 24 h, 5 × 10⁵ cells were transplanted into mice. Mice were evaluated daily for tumor growth. N = 10 per group. Error bars = s.e.m.

(P = 0.001). Similarly, mice vaccinated with TRAMP-2 expressing both IL-15 and IL-15Rα showed significantly greater survival than those transduced with the Ad.null control vector; P = 0.004 (Figure 4b). Unlike TS/A cells, the transduction of TRAMP-C2 cells with IL-15 alone led to a significant survival advantage over that observed with Ad.null (P = 0.001); however, this was significantly less than that of IL-15 combined with IL-15Rα (P = 0.039). When we challenged mice vaccinated with TS/A expressing IL-15/IL-15Rα with the unrelated TUBO breast cancer cell line there were 2.5-fold more CD8+ and 2.7-fold more NK cells when compared with mice vaccinated with TS/A + Ad.null (Figure 5a). A similar effect was seen following vaccination with TRAMP-C2 cells expressing IL-15/IL-15Rα, with 2.5-fold more CD8+ cells and 2.8-fold more NK cells when compared with the tumors of unvaccinated animals (Figure 5b). When looking at CD4+ T-cell infiltrates, we found no differences in the relative numbers of these cells in either TRAMP-C2 or TS/A tumors.
IL-15 is a powerful proinflammatory cytokine that can enhance innate and adaptive immune responses. In preclinical studies, IL-15 has been shown to be active as a single agent or in combination with other immune-modulating agents, such as anti-CD40, anti-PD-L1 and anti-CTLA-4. Due to its immunostimulatory effects, IL-15 is also being studied as a vaccine adjuvant. We and others have demonstrated that the coexpression of IL-15 along with its receptor, IL-15Rα, as a vaccine, enhanced the biological activity of IL-15. Here we showed that murine TS/A and TRAMP-C2 tumor cells can be transduced with adenoviruses expressing IL-15 and IL-15Rα, and that the cells secrete functional IL-15.

In terms of function, we showed that coexpression of IL-15 and IL-15Rα induced a CD8+ T-cell-mediated anticancer immune response. 5 × 10^5 TS/A cells were transduced with (a) Ad.IL-15 alone or (b) Ad.IL-15 + Ad.IL-15Rα and transplanted into BALB/c mice. CD8+ CD8 and NK cells were depleted from the mice using injections of 200 μg of anti-CD4 (GK1.5) or anti-CD8 (2.43) or 50 μg of anti-NK (anti-asialo GM1) antibodies as described in Materials and methods. Mice were followed for tumor growth (10 mice per group). Error bars = s.e.m.

cells expressing IL-15/IL-15Rα secreted significantly more IFN-γ than those isolated from mice vaccinated with TRAMP-C2 or TS/A expressing Ad.null when exposed to SNC9-H8 or AH1, respectively (P < 0.01) (Figures 6c and d). No IFN-γ release was seen when exposed to OVA or p66, whereas IFN-γ was released from splenocytes of the IL-15/IL-15Rα or Ad.null-treated animals exposed to HEX486-494 or DP484, immunodominant epitopes of adenovirus. In addition to increased lytic activity or IFN-γ release, animals vaccinated with TRAMP-C2 expressing IL-15/IL-15Rα had more SPAS-1 tetramer-specific CD8+ cells (Figure 6e) compared with those vaccinated with TRAMP-C2 cells transduced with Ad.Null (Figure 6f), or untreated animals (data not shown).

DISCUSSION

IL-15 is a powerful proinflammatory cytokine that can enhance innate and adaptive immune responses (reviewed in Steel et al. 2014). Recombinant human IL-15 has entered clinical trials for treatment of patients with metastatic melanoma and renal cell carcinoma. In preclinical studies, IL-15 has been shown to be active as a single agent or in combination with other immune-modulating agents, such as anti-CD40, anti-PD-L1 and anti-CTLA-4. Due to its immunostimulatory effects, IL-15 is also being studied as a vaccine adjuvant. We and others have demonstrated that the coexpression of IL-15 along with its receptor, IL-15Rα, as a vaccine, enhanced the biological activity of IL-15. Here we showed that murine TS/A and TRAMP-C2 tumor cells can be transduced with adenoviruses expressing IL-15 and IL-15Rα, and that the cells secrete functional IL-15. Bergamaschi et al. reported that IL-15 and IL-15Rα bind intracellularly and that this interaction stabilizes the protein, allowing IL-15 to be secreted, whereas when IL-15 is expressed in the absence of IL-15Rα it is rapidly degraded, allowing only limited amounts to be secreted. In line with this, we showed that TS/A and TRAMP-C2 cells expressing both IL-15 and IL-15Rα secreted greater quantities of IL-15 than those cells that expressed IL-15 alone.

In humans, very little IL-15 is detected in the serum; rather IL-15 is predominantly found on the surface of cells bound to its receptor. Most IL-15 detected in the blood is thought to be bound to soluble IL-15Rα cleaved from the surface of cells. IL-15Rα has been shown to trans-present the cytokine to effector cells. The trans-presentation of IL-15 by IL-15Rα is the primary way in which IL-15 signals through the β- and γ-chains of the IL-15 receptor, and both IL-15 and IL-15Rα are required to be expressed by the same cell to allow trans-presentation to occur. This complex signaling mechanism may act to regulate the effects of IL-15 by allowing tightly controlled and directed delivery to the effector cells. From this, we posited that the coexpression of IL-15 and IL-15Rα on tumor cells would allow the surface trans-presentation by the tumor cells to effector cells to stimulate an antitumor response. We found that coexpression of IL-15 with IL-15Rα led to IL-15 expression on the surface of the tumor cells, whereas the expression of IL-15 alone showed diffuse cytoplasmic localization within the cells (Figure 1). The requirement for IL-15Rα for the cell surface expression of IL-15 is supported by Bergamaschi et al., who showed that little IL-15 is detected on the cell surface following the transfection with a plasmid expressing IL-15 alone, whereas when they combined IL-15 with IL-15Rα they were able to detect IL-15 expression on the surface of the cells.

In terms of function, we showed that coexpression of IL-15 and IL-15Rα in TS/A breast or TRAMP-C2 prostate cancer cells resulted in smaller tumors than when the cells expressed IL-15 alone. These effects were seen when the tumor cells were transduced with IL-15 and IL-15Rα prior to implantation or when IL-15 and IL-15Rα was delivered into pre-established tumors. A number of studies have shown that gene delivery of IL-15 into tumor cells can induce an antitumor response. In this study, we showed that tumor cells transduced with IL-15 alone did have an antitumor effect when compared with the Ad.null control; however, this was significantly less than that seen when IL-15 was combined with IL-15Rα.

IL-15 has been reported to induce tumor-cell killing through the stimulation of NK and CD8+ T cells (reviewed in Steel et al. 2014). We showed that the depletion of NK cells and CD8+ T cells (reviewed in Steel et al. 2014) limited the antitumor effect of treatment with IL-15 and its receptor, providing evidence that in our models NK and CD8+ T cells have an essential role. This is in line with the results shown by Dubois et al. using soluble IL-15/IL-15Rα-IgG1–Fc complexes. The ability of IL-15 and its receptor to induce a CD8+ T-cell response directed against the tumor following the transduction of the tumor cells with IL-15 and IL-15Rα pointed at this combination’s potential to be used as a vaccine. Genetically modified tumor cell vaccines such as GVAX, engineered to express granulocyte-macrophage colony-stimulating factor, that demonstrated activity in early clinical trials have failed to show efficacy in phase III trials. Further, in the GVAX phase III trial comparing GVAX and docetaxel to docetaxel and prednisone, there was greater risk of death associated with the vaccine...
Vaccinating mice with IL-15 and IL-15Rα significantly prolongs the survival of mice challenged with tumor. Mice were immunized with $1 \times 10^6$ MMC-treated TS/A or TRAMP-C2 cells transduced with Ad.null, Ad.IL-15 alone, Ad.IL-15Rα alone or Ad.IL-15 + Ad.IL-15Rα, respectively, into their left flanks. Two weeks later, the mice were challenged with $5 \times 10^5$ (a) TS/A, (b) TRAMP-C2, (c) TUBO or (d) MC38 cells in their right flanks, and evaluated for survival (10 mice per group). MMC-treated cells transduced with Ad.IL-15 + Ad.IL-15Rα improved survival in animals challenged with (a) TS/A ($P = 0.001$), (b) TRAMP-C2 ($P = 0.004$), (c) TUBO ($P = 0.043$) but not (d) MC38 cells ($P = 0.33$) compared with Ad.null. (e) Mice were implanted with $1 \times 10^5$ TS/A cells into their left flank. After 7, 10, 14 and 17 days, the animals were treated with $1 \times 10^6$ MMC-treated TS/A cells transduced with Ad.null or Ad.IL-15 + Ad.IL-15Rα into their right flanks. The mice were followed for survival ($N = 8$). MMC-treated cells transduced with Ad.IL-15 + Ad.IL-15Rα improved survival in animals with pre-established tumors ($P = 0.002$).

Figure 4. Vaccinating mice with IL-15 and IL-15Rα significantly prolongs the survival of mice challenged with tumor. Mice were immunized with $1 \times 10^6$ MMC-treated TS/A or TRAMP-C2 cells transduced with Ad.null, Ad.IL-15 alone, Ad.IL-15Rα alone or Ad.IL-15 + Ad.IL-15Rα, respectively, into their left flanks. Two weeks later, the mice were challenged with $5 \times 10^5$ (a) TS/A, (b) TRAMP-C2, (c) TUBO or (d) MC38 cells in their right flanks, and evaluated for survival (10 mice per group). MMC-treated cells transduced with Ad.IL-15 + Ad.IL-15Rα improved survival in animals challenged with (a) TS/A ($P = 0.001$), (b) TRAMP-C2 ($P = 0.004$), (c) TUBO ($P = 0.043$) but not (d) MC38 cells ($P = 0.33$) compared with Ad.null. (e) Mice were implanted with $1 \times 10^5$ TS/A cells into their left flank. After 7, 10, 14 and 17 days, the animals were treated with $1 \times 10^6$ MMC-treated TS/A cells transduced with Ad.null or Ad.IL-15 + Ad.IL-15Rα into their right flanks. The mice were followed for survival ($N = 8$). MMC-treated cells transduced with Ad.IL-15 + Ad.IL-15Rα improved survival in animals with pre-established tumors ($P = 0.002$).

Figure 4. Vaccinating mice with IL-15 and IL-15Rα significantly prolongs the survival of mice challenged with tumor. Mice were immunized with $1 \times 10^6$ MMC-treated TS/A or TRAMP-C2 cells transduced with Ad.null, Ad.IL-15 alone, Ad.IL-15Rα alone or Ad.IL-15 + Ad.IL-15Rα, respectively, into their left flanks. Two weeks later, the mice were challenged with $5 \times 10^5$ (a) TS/A, (b) TRAMP-C2, (c) TUBO or (d) MC38 cells in their right flanks, and evaluated for survival (10 mice per group). MMC-treated cells transduced with Ad.IL-15 + Ad.IL-15Rα improved survival in animals challenged with (a) TS/A ($P = 0.001$), (b) TRAMP-C2 ($P = 0.004$), (c) TUBO ($P = 0.043$) but not (d) MC38 cells ($P = 0.33$) compared with Ad.null. (e) Mice were implanted with $1 \times 10^5$ TS/A cells into their left flank. After 7, 10, 14 and 17 days, the animals were treated with $1 \times 10^6$ MMC-treated TS/A cells transduced with Ad.null or Ad.IL-15 + Ad.IL-15Rα into their right flanks. The mice were followed for survival ($N = 8$). MMC-treated cells transduced with Ad.IL-15 + Ad.IL-15Rα improved survival in animals with pre-established tumors ($P = 0.002$).

The strong proinflammatory characteristics of IL-15 and its requirement of trans-presentation for effector cell activation may allow IL-15/IL-15Rα-expressing tumor cells to induce a safer, more targeted immune response than previously studied tumor vaccines.

To examine the efficacy of an IL-15/IL-15Rα-expressing tumor cell vaccine, we transduced mouse TS/A breast cancer or TRAMP-C2 prostate cancer cells with Ad.IL-15 and Ad.IL-15Rα, and used these cells as a vaccine. We showed that this vaccine stimulated an antitumor immune response leading to a significant prolongation of survival when animals were challenged with TS/A or TRAMP-C2 cells. Tumors isolated from these animals had greater levels of survival when animals were challenged with TS/A or TRAMP-C2 cells expressing IL-15 and IL-15Rα, and secrete IFN-γ when stimulated with a CD8+ tumor cell vaccine was targeting an unknown antigen. Both TS/A and TUBO are murine breast cancer cell lines derived from BALB/c mice and may share a number of unidentified tumor-associated antigens. Tumor cells expressing IL-15 and IL-15Rα may have an advantage over other allogeneic tumor vaccines in that IL-15 can overcome immunodominance by enhancing immune responses to subdominant antigens. Tumor cells expressing IL-15 and IL-15Rα may have an advantage over other allogeneic tumor vaccines in that IL-15 can overcome immunodominance by enhancing immune responses to subdominant antigens. This may broaden the antitumor response allowing shared subdominant tumor antigens to be targeted, thereby increasing the potential for success when using IL-15 and IL-15Rα to augment allogeneic vaccinations. In addition, the use of whole tumor cells as the base of this vaccine allows the potential targeting of multiple uncharacterized tumor antigens, unlike other vaccine strategies such as genetically engineered dendritic cells or viral vaccines where specific antigens must be identified in the tumor and vectors designed around those antigens.

We also showed that IL-15/IL-15Rα-expressing tumor vaccines are effective in a clinically relevant therapeutic setting. Mice harboring poorly immunogenic and highly aggressive TS/A tumors could be effectively treated with IL-15/IL-15Rα-expressing tumor vaccines, leading to a significant prolongation of survival.
In summary, coexpression of IL-15 and IL-15Rα resulted in robust levels of IL-15 secretion by tumor cells to coexpress IL-15 and IL-15Rα can induce and enhance immune responses to subdominant antigens, increasing the potential to use allogeneic tumor cell vaccines. The findings in the current study provide the scientific rationale for the investigation of this vaccine platform in clinical trials on cancer to determine whether genetically modified tumor cells expressing IL-15 and IL-15Rα may induce anticancer responses.

**MATERIALS AND METHODS**

**Cell lines**

TS/A and TUBO mouse mammary carcinoma cell lines were derived from a BALB/c background and were gifts from Dr Patrizia Nanni (University of Bologna, Bologna, Italy) and were grown in Dulbecco’s modified Eagle medium (DMEM, BioSource, Gaithersburg, MD, USA) with 10% fetal bovine serum (FBS; Gemini, Calabassas, CA, USA) and 10 μg ml⁻¹ gentamicin sulfate (BioSource). Human embryonic kidney (HEK)-293 cells and murine TRAMP-C2 prostate cancer cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA) and were grown in DMEM with 10% FBS and 10 μg ml⁻¹ gentamicin sulfate (BioSource). MC38 murine colon cancer cells were grown in DMEM with 10% FBS and 10 μg ml⁻¹ gentamicin sulfate (BioSource). TRAMP-C2 and MC38 cells were derived on a C57Bl/6 background. CTL-2 cytotoxic T cells were purchased from the American Type Culture Collection (ATCC) and were cultured in RPMI 1640 supplemented with 10% FBS and 200 U ml⁻¹ IL-2.

**Adenoviruses**

The murine IL-15 and IL-15Rα cDNAs were provided by Dr Yutaka Tagaya (National Cancer Institute, Bethesda, MD, USA). Ad.mIL-15 and Ad.mIL-15Rα are E1, E3-deleted recombinant adenoviruses (rAd) expressing the murine IL-15 and IL-15Rα, respectively. Ad.null is an E1, E3-deleted rAd control vector expressing no transgene. All vectors were generated using the AdMax system (Microbix Biosystems, Mississauga, ON, Canada), double plaque-isolated, expanded on HEK-293 cells, purified on two-step and continuous CsCl gradients or an anion-exchange column (Sartorius Stedim, Bohemia, NY), titrated as plaque-forming units (pfu) ml⁻¹, and stored at −70 °C.

**Peptides**

Synthetic peptides SNC9H8 (STHVNHLC), a dominant TRAMP-C2 epitope, AHI (SPSVYWHQF), a dominant TS/A epitope, p66 (TVVPANSL), a dominant rat NEU epitope, HEX486–494 (KYSPSNVK), or FALSNAEIDL, dominant adenovirus epitopes, or OVA257–264 (SIINFEKL), an epitope from hen ovalbumin, were purchased from Genscript (Piscataway, NJ, USA).

**Animals**

Male 6–8-week-old C57Bl/6 mice and female 6–8-week-old BALB/c mice were obtained from the Division of Cancer Research and Treatment, National Cancer Institute (NCI; Frederick, MD, USA). All the animals were maintained in the NCI animal holding facility and their use adhered to the NIH Laboratory Animal Care Guidelines and was approved by the NCI Animal Care and Use Committee.

**Flow cytometry**

SPAS-1 tetramer was previously described. Cell surface expression of CD8, CD4, and CD94 was performed using antibodies from eBioscience (San Diego, CA, USA) and analyzed using FlowJo software (Tree Star, Inc., Ashland, OR, USA).

**IL-15 secretion and activity**

Culture supernatants were removed before and 48 h after infection of tumor cells with Ad.mIL-15 or Ad.mIL-15Rα or Ad.null, each at a multiplicity of infection (MOI) of 100. Supernatants were assayed for murine IL-15 by enzyme-linked immunosorbent assay (R&D Systems, Minneapolis, MN, USA) according to the manufacturer’s instructions. To assess the functionality of secreted IL-15, supernatants were diluted and incubated with CTL-2 cells. Proliferation of CTL-2 cells at 48 h was determined using the CellTiter 96 AQueous Non-Radioactive Cell Proliferation Assay (Promega, Madison, WI, USA) according to the manufacturer’s instructions.

**Immunohistochemistry**

Tumors from TS/A cells infected with Ad.mIL-15 or Ad.mIL-15Rα or Ad.null at each an MOI of 100 were resected, fixed in OCT (SAKURA-Finetek, Torrance, CA, USA) and sectioned by cryostat. Tissue sections were treated with 0.3% hydrogen peroxide, then treated with Avidin/Biotin blocking kit (Zymed, South San Francisco, CA, USA) according to the manufacturer’s instructions. The sections were stained with goat anti-mouse IL-15 (R&D Systems). The slides were washed and stained using the anti-goat HRP-DAB Cell and Tissue Staining Kit (R&D Systems) according to the manufacturer’s instructions and counterstained with hematoxylin.

**Treatment effect**

TS/A and TRAMP-C2 cells were seeded in 175 cm² tissue culture flasks at 1 × 10⁶ cells per flask and transduced with Ad.mIL-15, Ad.mIL-15Rα, Ad.mIL-15 + Ad.mIL-15Rα or Ad.null at an MOI of 100. Cells were harvested after 24 h and groups of 10 mice were subcutaneously injected with 5 × 10⁶ transduced cells. Mice were evaluated daily for tumor growth.

In a separate experiment, 5 × 10⁷ TS/A or TRAMP-C2 cells were injected subcutaneously into the flanks of BALB/c and C57Bl/6 mice, respectively (10 mice per treatment). When the tumors reached 75–125 mm³, they were intratumorally injected with Ad.mIL-15, Ad.mIL-15Rα, Ad.mIL-15 +
Immune cell depletion

Groups of BALB/c mice were depleted of specific immune cell populations.21 Briefly, CD4⁺ or CD8⁺ cells were depleted with anti-CD4 or anti-CD8 antibodies purified from the supernatants of hybridomas GK1.5 (ATCC) and 2.43 (ATCC), respectively. Five days before vaccination with TS/A transduced with IL-15, IL-15⁺IL-15Rα or Ad.null, mice were intraperitoneally injected with 200 µg of the appropriate antibody for three consecutive days and then continued every 3 days thereafter. Greater than 95% depletion of specific lymphocyte populations was confirmed by peripheral blood flow cytometry.

Vaccinations

TS/A and TRAMP-C2 cells were seeded at 1 × 10⁶ cells/flask into 175 cm² tissue culture flasks and transduced with Ad.mIL-15, Ad.mIL-15⁺Ad.mIL-15Rα or Ad.null at an MOI of 100. After 24 h, the cells were treated with MMC at 0.5 mg/ml⁻¹ for 30 min at 37°C to inhibit cell proliferation. Groups of BALB/c and C57Bl/6 mice were immunized with 1 × 10⁶ MMC-TS/A and MMC-TRAMP-C2 cells, respectively, into their left flanks. Two weeks later, the mice were challenged with 5 × 10⁵ TS/A, TUBO, MC38 or TRAMP-C2 cells in their right flanks and the mice were evaluated for survival.

In a separate experiment, 1 × 10⁵ TS/A cells were implanted into the left flank of BALB/c mice. Seven days later, when tumors were ~130 mm³, the mice were injected subcutaneously with 1 × 10⁶ MMC-TS/A expressing IL-15 + IL-15Rα or Ad.null (as above) in the right flank. Animals were similarly vaccinated on days 10, 14 and 17 and assessed for tumor growth (N = 8 per group).

Cytokine secretion and cytotoxicity assays

Spleen single-cell suspensions were prepared from mice killed on day 21 after MMC-TRAMP-C2 or MMC-TS/A vaccination and cultured with MMC-treated (0.5 mg/ml⁻¹) TRAMP-C2, MC38, TUBO or TS/A tumor cells at a ratio of 50:1, 10:1 and 1:1. Recombinant human IL-2 was added to a concentration of 10–15 U/ml⁻¹. After 4 days, cytotoxicity assays were performed: effector cells from each group were cultured with 10⁶ TRAMP-C2, MC38, TUBO or TS/A target cells per well in triplicate at varying E/T ratios and incubated at 37°C for 4 h. Cytotoxic activity was measured by LDH release using the CytoTox 96 Non-Radioactive Cytotoxicity Assay (Promega). The percentage of lysis was calculated as 100 × ((experimental release) – (effector spontaneous release)) – (target spontaneous release))/ (target maximum release) – (target spontaneous release)).
To detect a CD8\(^+\) response against the TRAMP-C2 or TS/A, splenocytes were assayed for IFN-\(\gamma\) secretion. Splenocytes from groups of animals (\(N = 3\)) vaccinated with MMC-TS/A or MMC-TRAMP transduced with Ad.mil-15, Ad.mil-15 + Ad.mil-15R or Ad.null were pooled and plated at \(2 \times 10^5\) cells per well in 24-well plates in triplicate. Splenocytes were cocultured with 10 \(\mu\)g/ml of the CD8\(^+\) dominant peptides: SNC9-H8, A1H, OVA257-264, p66, DBP43 or HEX486-494 for 72 h. Supernatants were collected and IFN-\(\gamma\) was measured by enzyme-linked immunosorbent assay (R&D Systems) according to the manufacturer’s instructions. All samples were tested in triplicate.

Statistical analysis

Statistical analysis was performed using JMP Statistical Software version 5.1 (SAS Institute, Cary, NC, USA). Kaplan–Meier nonparametric regression analyses were performed for tumor prevention experiments with significance determined by the log-rank test. The comparison of the effect of vaccination on antibody titers among different groups was analyzed by one-way analysis of variance followed by Tukey–Kramer honestly significant difference test or nonparametric Wilcoxon/Kruskal–Wallis test. A \(P\)-value of \(<0.05\) was considered significant.

CONFICT OF INTEREST

The authors declare no conflict of interest.

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