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Combined delivery of IL12 and an IL18 mutant without IL18BP-binding activity by an adenoviral vector enhances tumor specific immunity

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Cytokines play pivotal roles in anticancer immune response. We previously reported that adenovirus armed with an IL18 variant (DR18) that overcomes IL18BP neutralizing effect displayed powerful therapeutic effects in local and distant tumors when delivered intratumorally. Here, we tested a combined delivery of IL12 and DR18 in tumor models since IL12 and IL18 are known to act synergistically in potentiating IFNγ production and antitumor immunity. To minimize adverse effects associated with systemic delivery, we constructed oncolytic adenoviruses (oAd) harboring DR18 and IL12 (oAd.DR18/IL12). IL12 was expressed as a single chain IL12 (scIL12) peptide composed of the IL12/p40 and IL12/p35 subunits. Intratumoral administration of oAd.DR18/IL12, oAd-expressing DR18 (oAd.DR18), or oAd-expressing IL12 (oAd.IL12) showed antitumor effect in syngeneic colorectal tumor models. Compared to oAd.DR18 or oAd.IL12, administration of oAd.DR18/IL12 improved the antitumor effects as well as increased survival rate in these models. We detected enhanced tumor infiltrating T lymphocytes and NK cells in oAd.DR18/IL12-treated mice than those from mock-treated or individually treated groups. Moreover, mice received oAd.DR18/IL12 had more robust tumor-specific cytotoxicity. Importantly, mice that had tumor regression after oAd.DR18/IL12 treatment established anti-tumor specific immune memory. These results show that adenovirus armed with engineered cytokines boosts tumor specific immunity and antitumor effect.

Cytokines are key components of the immune system and have been diligently/intently tested in cancer immunotherapy[1–3]. Both IL12 and IL18 are important cytokines that exert a synergistic effect in the production of IFNγ by natural killer cells and cytotoxic T lymphocytes[4]. IL18 was originally identified as an IFNγ-inducing factor and has demonstrated activity to induce natural killer cell and cytotoxic T lymphocyte activation[5]. Early studies using recombinant IL18 have demonstrated minimal efficacies in antitumor studies[6]. Recent studies showed that this could be derived from the limited availability of IL18 in tumor microenvironment[7]. Specifically, tumor cells produce an IL18-binding protein (IL18BP), a natural antagonist of IL18, which functions as a decoy receptor since it binds to IL18 with an exceptionally high affinity, thus counteracts IL18 activities[8]. This drawback can be overcome with the newly discovered IL18 variants (decoy-resistant IL18, DR18) since these variants retain full biological functions of IL18 while avoiding the neutralizing effect of IL18BP[7]. We showed recently that a mouse DR18 delivered using an adenoviral vector elicited potent and durable antitumor response[9].

The antitumor effect of IL18 can be enhanced by IL12 since IL12 up-regulates the expression of the IL18 receptor on T cells, Th1 cells, and B cells, resulting in a synergistic effect through enhanced IFNγ production[10]. A previous study demonstrated that IL12 and IL18 together stimulated Th1 immunity, resulting in the generation of highly effective tumor-reactive T cells[11]. This combination also enhanced the cytolytic effect and IFNγ production, and T cell proliferation[4,12]. Moreover, previous studies have indicated that IL12 in combination with IL18 has the capacity to inhibit tumor angiogenesis, which may ultimately result in tumor regression[13]. IL12 by itself is a pro-inflammatory cytokine that regulates T-cell and natural killer-cell responses, induces IFNγ

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production in both innate and adaptive immune-cell populations. IL12 reprograms CAR-expressing natural killer T cells to long-lived Th1-polarized cells with potent antitumor activity[14]. It was recently reported that combined expression of IL12 and DR18, a decoy-resistant variant of IL18, using mRNA techniques enhanced the antitumor immunity of engineered T cells[15], promoting us to use adenovirus-based vector as a delivery vehicle for combined delivery of these cytokines.

Adenovirus vectors are the most commonly employed vector for gene therapy and for cancer therapy. The virus can be engineered as replication-competent (oncolytic) vectors to replicate preferentially in cancer cells and to display oncolytic properties through the natural process of lytic virus replication[16,17]. Lysis of tumor cells releases tumor-specific antigens that trigger both the innate and adaptive immune systems. Oncolytic adenoviruses (oAds) armed with proinflammatory cytokines augment the cytotoxic and immunostimulatory activities to optimize immune-mediated tumor eradication. In addition, the virus has a highly favorable safety profiles for tumor immunotherapy[18,19]. Thus, adenovirus-based delivery system is among the most commonly used vectors for antitumor clinical trials.

In this study, we constructed adenoviruses for the expression of IL12 and DR18 and investigated for their antitumor activity using the CT26 or MC38 tumor models. Combined delivery of DR18 and IL12 (oAd.DR18/IL12) by intratumoral administration improved antitumor effects over that of oAd.DR18 or oAd.IL12 alone. Importantly, oAd co-expressing DR18 and IL12 promoted strong IFNγ production and antitumor specific immunity. Here we report the construction and antitumor effect of oAd armed with T cell-activating cytokines IL18 (DR18) and IL12.

Materials and methods

Ethics statement

All animal work was performed in accordance with an approved protocol (D2202080) for the use and care of laboratory animals that was approved by the Institutional Animal Care and Use Committee (IACUC) of Nanjing University. We confirm that all methods were performed in accordance with the relevant guidelines and regulations. All methods are reported in accordance with ARRIVE guidelines[20].

Cells

The murine colorectal carcinoma CT26 and MC38 cell lines, human lung carcinoma A549 cell line and HEK293 cell line were purchased from CellBank of China (Shanghai, China). The cell lines were cultured in Dulbecco's modified Eagle medium (Hyclone, Utah, USA) supplemented with 10% fetal bovine serum and antibiotics (Life Technologies, CA, USA). The cell lines were routinely checked for *Mycoplasma* using a Mycoplasma PCR Detection Kit (Beyotime, Shanghai, China) and were found free of *Mycoplasma* contamination.

Construction of cytokine-armed adenoviruses

The adenoviruses were constructed by using a simplified system for generating oncolytic adenovirus vectors as previously reported[21,22]. The conditional replication of the adenoviruses is controlled by a 24-base pair deletion in the E1A region (E1Δ24). The transgenes were inserted in the E1 region under the control of a CMV promoter. oAd.null is a control oncolytic adenovirus without any insertion of foreign transgene in the region.

The construction of oAd.DR18 was recently reported using a reported sequence[7,9]. Since IL12 is a heterodimeric protein, we used a design of a single chain protein as reported for bioactive IL12 expression[23,24]. In brief, the construct for IL12 expression was consisted of a cDNA encoding mouse IL12p40 subunit without a stop codon followed by linker sequence for (Gly4Ser)3, followed by a coding sequence for mouse mature IL12p35 subunit (without the signal peptide, thus p35ΔL) and a stop codon (IL12, p40-linker-p35ΔL). For the generation of DR18/IL12, a cDNA encoding for peptide P2A and then IL12 (p40-linker-p35ΔL) was inserted after the DR18 coding sequence (in frame, prior to the stop codon of DR18). The P2A can induce ribosomal skipping during translation, thus, was introduced artificially to generate DR18 and IL12 polyproteins from a single open-reading frame[25]. The backbone and shuttle vector plasmids were transferred to *E. coli*BJ5183 for homologous recombination. After confirming the resulting plasmid, linearized DNA was transfected to HEK293 cells to rescue the viruses. The viruses were amplified in HEK293 cells and purified by ultracentrifugation on a continuous cesium chloride gradient[26]. Virus concentration was determined by measuring the absorbance at OD260 with Nanodrop 2000 (ThermoFisher) and a conversion factor of 1 OD260 = 1.1×10^{12}VP was used[27].

Cytokine production assays

For cytokine production, A549 cells (12-well plate, 2×10^5 cells/well) were infected with 100 VPs/cell for 72 h. The culture medium was collected for measurement of IL12 (Sangon Biotech) and IL18 (SinoBiological) secretion by enzyme-linked immunosorbent assays using commercial kits.

Mouse tumor models

Female BALB/c and C57BL/6 mice of 6–8 weeks old (from SiPeiFu Biotechnology Co, Ltd., Beijing, China) were quarantined for one week before tumor implantation. They were housed in a specific pathogen-free condition within a controlled environment (temperature at 22–24 °C on a 12-h light-dark cycle).

To establish syngeneic tumor models, 5×10^5cells CT26 or MC38 cells were subcutaneously implanted into the right rear flank of BALB/c or C57BL/6 mice, respectively. When the tumor sizes were about 100 mm^3, the mice were randomly grouped and used for tumor treatment studies. The groups included: PBS control group, oAd.null (oAd without an insert), oAd.DR18, oAd.IL12, and oAd.DR18/IL12. The oAds were administered by intratumor injection (1×10^9 VPs in 50 µL PBS per injection, or PBS 50 µL as a control) every other day for 4 times and tumor growth was monitored. The mice were monitored for signs of sickness and for tumor

growth. Tumor sizes were measured using a digital caliper and calculated using the following formula: tumor volume = (width^2× length)/2. For survival studies, a mouse was considered died when the tumor size reached approximately 2000 mm^3. Mice were euthanized using CO_2 followed by cervical dislocation.

To test for antitumor memory, mice were re-challenged with CT26 cells 2 months after the final treatment. Tumor volumes were measured and recorded.

Tumor microenvironment analysis

We measured tumor infiltrating lymphocytes and gene expression to determine oAd induced changes in tumor microenvironment in CT26 tumor model. Mice were inoculated with CT26 cells and were treated by intratumoral injection on day 0 and day 2 with PBS or oAds when the tumors were approximately 300 mm^3. Tumor-bearing mice were sacrificed 2 days after the last injection and tumor tissues and spleens were removed and used for the analyses. For tumor infiltrating lymphocytes, the tumors were processed into single-cell suspension and stained with fluorescently conjugated antibodies to determine the frequencies of tumor-infiltrating leukocytes by flow cytometry. Fluorescently conjugated antibodies and dyes were purchased from eBioscience (San Diego, CA): CD45-PE-Cyanine7 (#25045182), CD3-FITC (#11003282), CD8-PE (#12008182), CD69-APC (#17069182), IFNγ-PerCP-Cyanine5.5 (#45731180), granzyme B-FITC (#11889882), and Fixable Viability Dye eFluor 780 (#65086514).

To measure transgene expression and gene induction, tumor samples were processed with Trizol reagent to extract total RNA. The RNA was reversed transcribed using HiScript III RT SuperMix (R323-01, Vazyme) according to the manufacturer's instructions. Gene expression was quantified by real-time reverse transcription PCR (RT-qPCR) using ChamQ SYBR Color qPCR Master reagent mix (Q411, Vazyme) on an ABI ViiA 7 PCR system (ThermoFisher). GAPDH expression was used as an internal control for normalization. The primers are listed in Table 1.

Cytotoxicity assay

A lactate dehydrogenase (LDH) release assay was adopted to determine cytotoxicity of spleen cells[28,29]. The splenocytes in suspension were mixed with CT26 tumor cells in a ratio of 100:1 with the spleen cell acted as the effector cell and CT26 as the target cell. We used 4T1 cells as a negative naïve control. After 4 h incubation, cytotoxicity was measured using an LDH release assay kit by following the manufacturer's instruction (Beyotime, C0016). The cytotoxicity of spleen cells on CT26 killing was calculated using the following formula: cytotoxicity (%) = (U value of LDH in the measurement tube-natural release tube)/(U value of LDH in the maximum release tube-natural release tube) × 100%.

Measurement of antitumor-specific immunity by ELISpot

To assess the specific antitumor immune response, mouse spleens from tumor microenvironment analysis were aseptically harvested and a suspension of unicellular splenocytes (5×10^5 cells/well) were co-cultured with CT26 cell lysate (corresponding to 5×10^4 cells/well) or 4T1 cell lysate as a control for ELISpot assay using a mouse interferon-γ ELISpot kit by following the manufacturer's instructions (DAKEWE, DKW22-2000-096). The cell lysates were prepared by repeated freezing and thawing of a 5×10^6 cells/ml suspension in serum-free DMEM and was stored at − 80 °C. The spots were captured with IRIS™ FluoroSpot/ELISpot reader (Mabtech, Sweden).

Statistical analysis

Statistics described were generated using unpaired Student's t-tests or one-way analysis of variance (ANOVA) with Least Significant Difference (LSD) test. Survival significance between groups was determined by Log-rank (Mantel-Cox) test. Data are presented as mean ± SD. Calculated p values are displayed as * $p<0.05$; ** $p<0.01$.

Mouse gene	Sequence	Accession #
Il12	F: 5'-AGACATCACACGGGACCAAAC R: 5'-CCAGGCAACTCTCGTTCTTGT	NM_001159424
Gapdh	F: 5'-TTGTCATGGGAGTGAACGAGA R: 5'-CAGGCAGTTGGTGGTACAGG	NM_008085
Tnfa	F: 5'-CCCTCACACTCAGATCATCTTCT R: 5'-GCTACGACGTGGGCTACAG	NM_013693
Ifng	F: 5'-ATGAACGCTACACACTGCATC R: 5'-CCATCCTTTTGCCAGTTCCTC	NM_008337
Prf1	F: 5'-AGCACAAGTTCGTGCCAGG R: 5'-GCGTCTCTCATTAGGGAGTTTTT	NM_011073
Gzmb	F: 5'-CCACTCTCGACCCTACATGG R: 5'-GGCCCCCAAAGTGACATTTATT	NM_013542

Table 1. Primers for gene expression analysis. The primers were selected from a primer bank of validated primers (https://pga.mgh.harvard.edu/primerbank/) and the primers for DR18 transgene detection were designed based on Y09278.1 (GenBank accession number) and the sequences were as the following: 5'-GCAC CACCGCGGTGATCAG (forward) and 5'-GGTCTGCGGTTCGCTCGCG (reverse).

Results

Construction of oncolytic adenoviruses harboring cytokines

We generated oAds using the E1A del24 backbone for this study since this vector can selectively infect and replicate in retinoblastoma 1 protein (Rb)-deficient human tumor cells but not normal cell with intact Rb1 pathways[30,31]. The viruses included those for expression of scIL12 (oAd.IL12), of DR18 (oAd.DR18), and DR18/scIL12 (oAd.DR18/IL12), and a control oAd without any insert in the corresponding region, oAd.null (Fig. 1A). Since IL12 is composed of a p35 and p40 subunit, we constructed IL12 as a fusion protein by connecting the p35 and p40 subunits with a (G4S)3 peptide linker. For DR18 secretion, we used a human albumin signal peptide to replace the native leader sequence since IL18 maturation requires caspase activation.

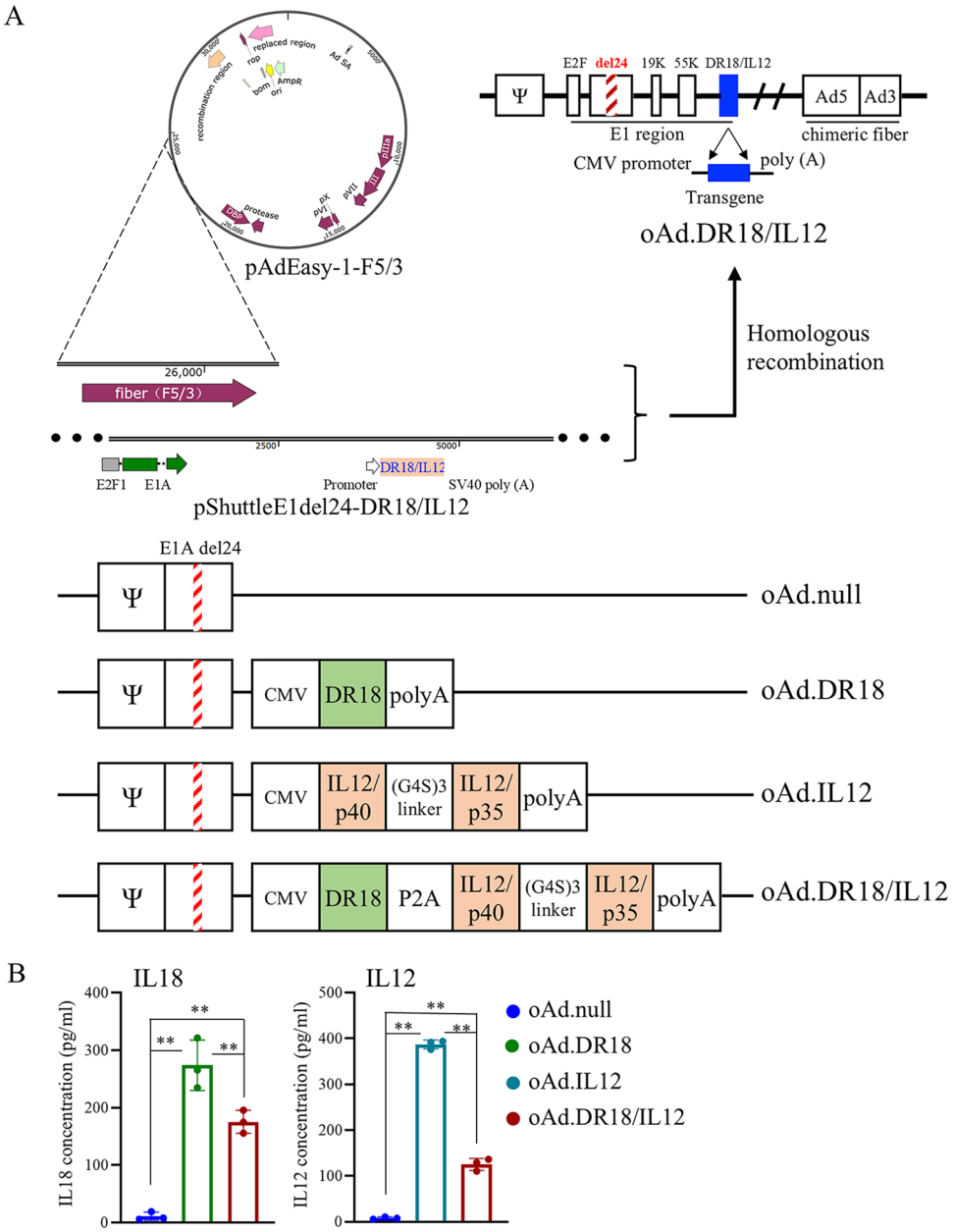

Fig. 1. Construction of cytokine-armed adenoviruses (**A**) Schematic illustration of adenovirus construction and region of cytokine insertion in the E1 region. Shadow indicates a 24-bp deletion at Rb binding site of E1A region. The decoy-resistant IL18 (DR18) contains a signal peptide of human albumin following by a cDNA encoding a reported IL18 variant devoid of IL18BP-binding activity. IL12 was constructed as a single chain peptide, consisting of a signal peptide followed by the IL12/p40 subunit, then a peptide linker of (G4S)3, and a mature form IL12A/p35 subunit. For DR18/IL12 construction, a P2A peptide was used for expression of DR18 and single chain IL12. (**B**) Confirmation of cytokine production by oncolytic adenovirus delivery. A549 cells were infected with oAds (100 VP/cell) for 72 h. Cytokine secretion to the cultures were detected by quantitative ELISA. Data are mean ± SD of triplicate samples, * $p < 0.05$, ** $p < 0.01$.

The delivery of transgenes by oAds was confirmed in A549 cells, a susceptible cell line to adenovirus infection. Cytokine secretion to culture medium was measured by quantitative ELISA (Fig. 1B). At 72 h post infection, significant production of the corresponding cytokines was detected from oAd.IL12 and oAd.DR18-infected cells compared to those of control of oAd.null-infected samples. We detected secretion of both IL12 and IL18 from oAd.DR18/IL12-infected samples although the production of IL12 or IL18 seemed to be at a reduced level compared to that by oAd.IL12 or oAd.DR18 delivery. These results demonstrated that oAd.DR18/IL12 was able to mediate both IL12 and DR18 production.

Antitumor effect of cytokine-armed oncolytic adenoviruses

Next, we tested the antitumor effect of the viruses in syngeneic mouse models. We injected 5×10^5CT26 cells subcutaneously into BALB/c mice. When the tumor volume averaged 100 mm^3, mice were injected intratumorally with PBS, oAd.null, oAd.IL12, oAd.DR18, or oAd.DR18/IL12 once every other day for a total of four injections (Fig. 2A). Tumor growth was monitored and mouse survival was recorded. As presented in Fig. 2B, control tumors treated with PBS showed aggressive growth, leading to a tumor volume of 614 ± 198 mm^3 on day 7 after the first treatment, while tumors treated with oAd.null reached 525 ± 91 mm^3. For comparison, tumors in oAd.IL12, oAd.DR18, and oAd.DR18/IL12-treated groups were measured on day 7 at 425 ± 53, 351 ± 83 and

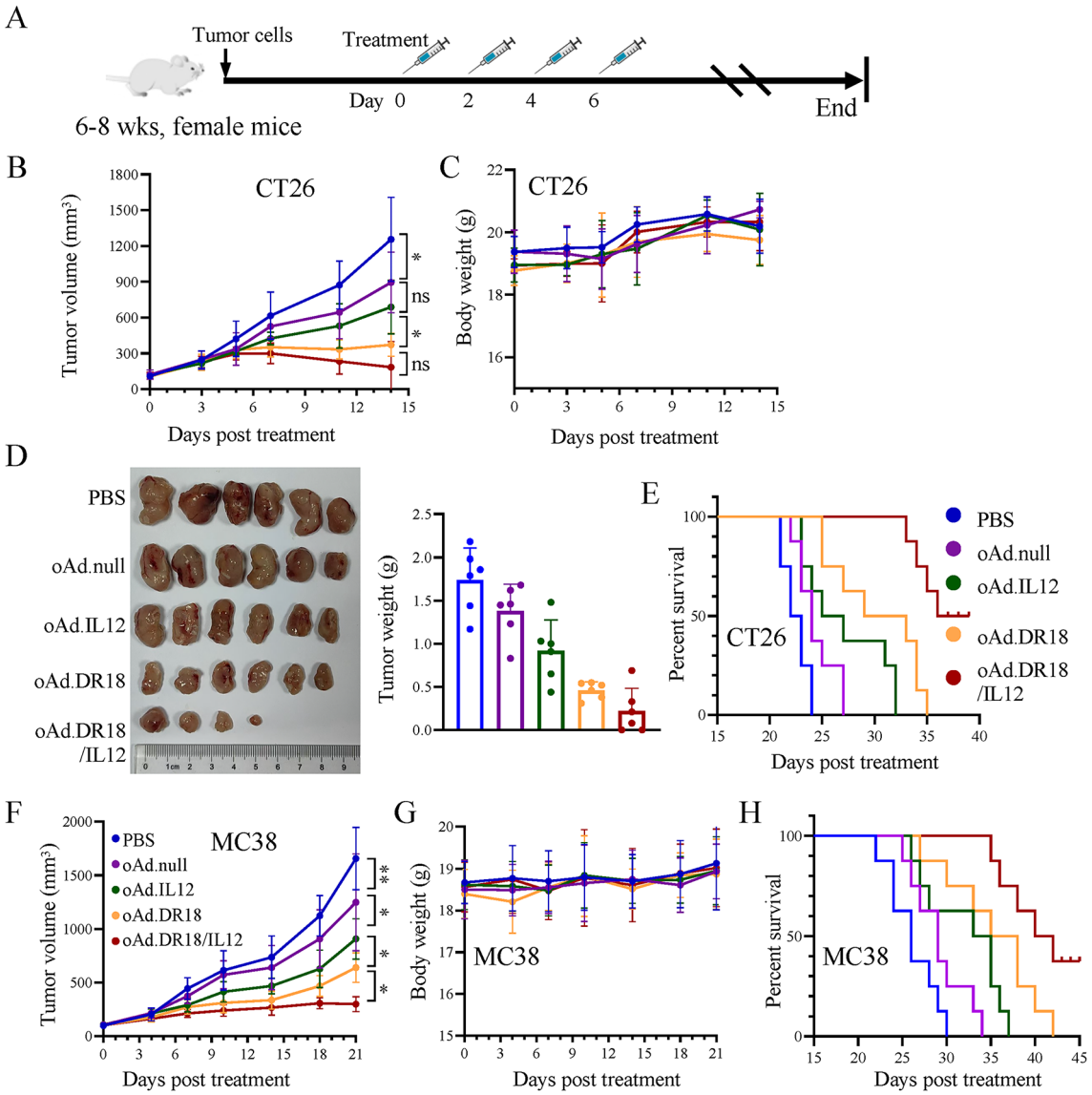

Fig. 2. Antitumor effect of cytokine-armed adenoviruses (**A**) Illustration of the experimental design. Murine CT26 and MC38 cells were implanted in the rear flank of syngeneic mice. When tumor size reached approximately 100 mm^3, oAds were administered by intratumor injection (n=8). The tumor growth (**B**), body weight (**C**) and tumor weight and photograph (**D**), survival rate in CT26 (n=8) (**E**) and in MC38 (**F**-**H**) were recorded. Data are presented as mean ± SD. For survival studies, a mouse was considered died when a tumor reached 2000 mm^3. Survival significance between groups was determined by Log-rank (Mantel-Cox) test.

300 ± 86 mm^3, respectively. By day 14 following the first treatment, tumor growth in oAd.DR18/IL12 treated group maintained strong inhibition (184 ± 214 mm^3, $P < 0.01$), whereas those in oAd.IL12 and oAd.DR18-treated groups reached 688 ± 225 and 371 ± 96 mm^3, respectively. The treatment had no obvious effect on mouse body weight changes (Fig. 2C). We measured tumor weight which confirmed that combined delivery of IL12 and DR18 had more potent anti-tumor effect (Fig. 2D). In parallel experiment, we found that mice in oAd.DR18/IL12-treated group had a higher survival rate compared to those in oAd.IL12 and oAd.DR18-treated groups ($P < 0.01$) (Fig. 2E). Importantly, we noticed half of the mice (4/8) in oAd.DR18/IL12-treated group had tumor regression on day 40.

The antitumor effect was also observed in MC38 tumor model. We observed similar inhibitory effect on tumor growth and prolonged survival in oAd.DR18/IL12-treated group (Fig. 2F-H). Importantly, mice in oAd.DR18/IL12-treated group had a better survival rate (3/8) than those in oAd.DR18 or oAd.IL12-treated groups (Fig. 2H). These results together demonstrated that combined expression of IL12 and DR18 with oAd.DR18/IL12 significantly enhanced the antitumor effect compared to single cytokine treatment.

oAd treatment alters tumor microenvironment for antitumor activity

We further monitored tumor infiltrating leukocytes by flow cytometry analysis. Notably, the numbers of total T and $CD8^+$ T cells were further elevated in oAd.DR18/IL12-treated group (Fig. 3A, Fig. B). Upon further analysis we found that these $CD8^+$ T cells from oAd.DR18/IL12-treated group were more positively stained for CD69, an early-activation marker expressed by cells, compared to other groups (Fig. 3C). Similarly, IFNγ and Granzyme B production was significantly elevated in tumor-infiltrated $CD8^+$ T cells in oAd.DR18/IL12-treated group (Fig. 3D, Fig. E). NK cells and those corresponding to IFNγ production were also elevated significantly in oAd.DR18/IL12 group (Fig. 3F, Fig. G). These together demonstrated that oAd.DR18/IL12 treatment promoted lymphocyte infiltration and activation.

We performed quantitative RT-PCR to analyze transgene expression and gene induction in the CT26 tumor tissues. As shown in Fig. 3H, we detected significant expression of IL12 and DR18 transgene at mRNA levels in oAd-treated tumor samples. Importantly, co-expression of IL12 and DR18 significantly increased IFNγ and TNFα gene expression, markers of IL18-stimulation and proinflammation, respectively. Moreover, oAd.DR18/IL12 also increased granzyme B and perforin levels, indicators of granule-mediated cytotoxicity. Taken together, these results implied that combination of DR18 with IL12 presented potential antitumor effect by modifying tumor microenvironment for antitumor activity.

Combined delivery of IL12 and DR18 promotes tumor-specific immune response

Results from flow cytometry and gene expression assays indicated that oAd.DR18/IL12 might have potentiated a tumor microenvironment favorable to activation of tumor-specific immune cells. We thus performed ELISpot assay to evaluate tumor specific immunity by measuring IFNγ production. In this aspect, the spleens were obtained aseptically from oAd-treated mice and single-cell suspension of splenocytes were co-cultured with CT26 tumor cells for 24 h. The number of IFNγ-producing cells was markedly increased in mice treated with oAd.DR18/IL12 compared with those in other groups (Fig. 4A, Fig. B). The effect was also elaborated with results from a cytotoxicity assay. As shown in Fig. 4C, splenocytes from oAd.DR18/IL12-treated mice had significant higher cytotoxic effect against CT26 cells than those oAd.DR18 or oAd.IL12 individually treated or in control groups, demonstrating that co-expression of IL12 and DR18 elicited potent tumor-specific effect.

Evaluation of long-term antitumor memory

We observed complete regression of subcutaneous CT26 tumors among the oAd.DR18/IL12-treated group in the survival study. We addressed whether intratumoral administration of oAd.DR18/IL12 had elicited immune memory against tumor implants (Fig. 5A, Fig. B). Two months following the final treatment, mice that had been cured of CT26 tumors from the oAd.DR18/IL12-treated group were re-challenged with CT26 tumor cells. The cohort of mice previously cured off CT26 tumors did not display any occurrence of the secondary inoculated tumors within 22 days after re-challenge (Fig. 5A). In contrast, all mice in the age-matched naïve control group developed tumors that grew in accordance with the expected kinetics in treatment-naïve mice implanted with the CT26 cells (Fig. B). This result thus strongly suggested that oAd.DR18/IL12 treatment enabled the establishment of long-term antitumor memory.

Discussion

The widespread clinical use of cytokines has been limited by severe side effects caused by low specificity and unfavorable biodistribution[2,32]. The local administration of recombinant protein into tumors demonstrated the mitigation of systemic toxicity; however, the rapid diffusion from the tumor microenvironment also limited the effective time window for treatment. In contrast, local delivery of cytokines to the tumor site using a carrier is a safer and more feasible approach. Cytokines were the first modern strategy for cancer immunotherapy[33]. Nevertheless, the systemic administration of cytokines at a therapeutic dose can result in significant toxicity in patients with cancer, as well as in animal models of cancer[34,35]. The success of GM-CSF delivered with a genetically modified herpes simplex virus has reinvigorated combined use of cytokines with oncolytic virotherapy in tumor immunotherapy[16].

In this study, we studied combined delivery of IL12 and an IL12 variant using oncolytic adenovirus. IL18 synergizes with IL12 for IFNγ production and activates proinflammatory signaling pathway for tumor immunotherapy[36–38]. We constructed cytokine-armed oncolytic adenoviruses and tested their effects in mouse CT26 and MC38 colorectal tumor models. We compared the effect of individual cytokines and combined delivery of DR18 and IL12 and found that combined expression of DR18 and IL12 had tumor-specific immunity.

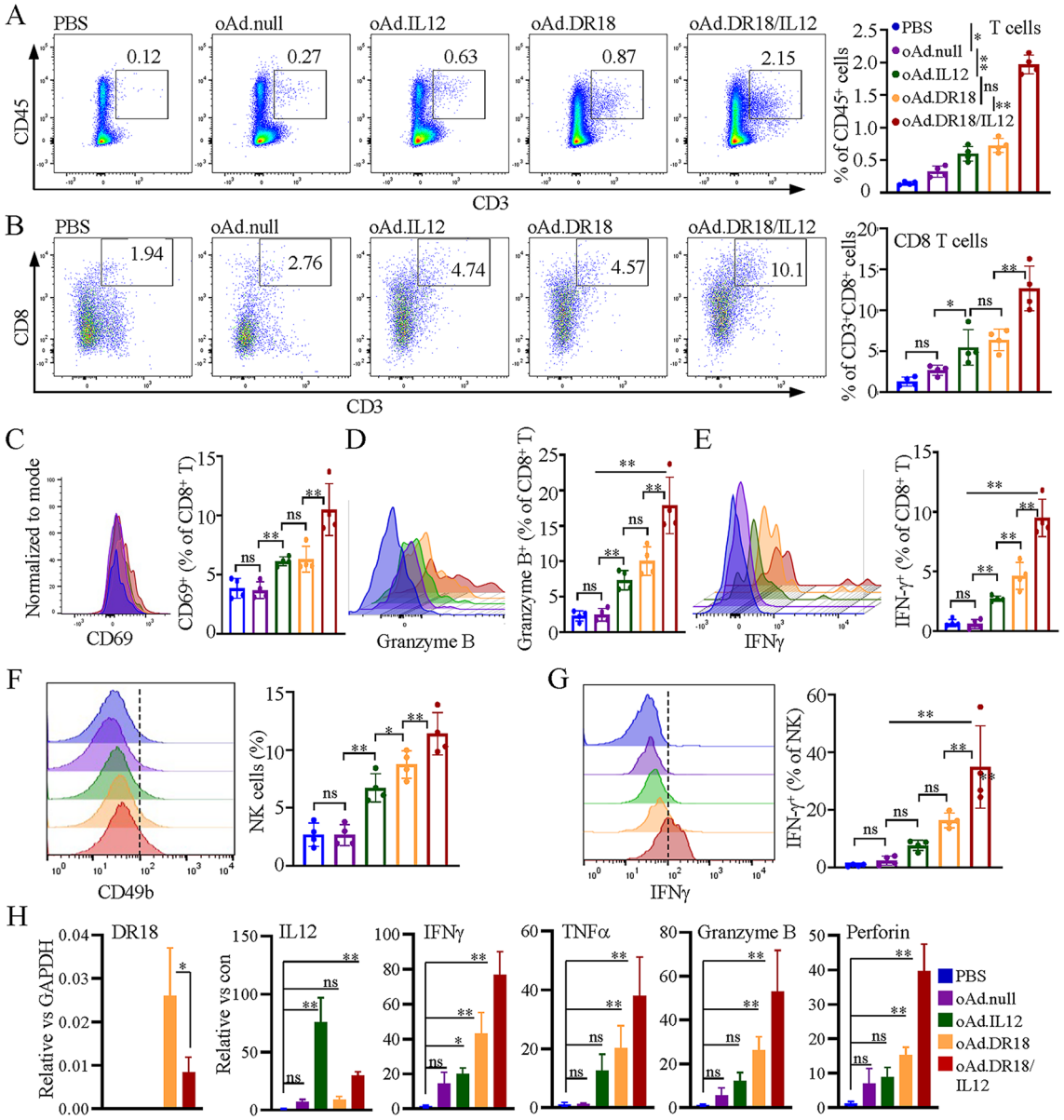

Fig. 3. Treatment with oAds alters tumor microenvironment for antitumor response. CT26 tumor-bearing mice were administered intratumorally with PBS or an oAd on day 0 and day 2. Mice were killed 2 days after the second injection. Tumor infiltrating cells were detected by flow cytometry and gene expression by RT-qPCR. (**A**) Representative flow plots and quantification of percentage of $CD45^+$ $CD3^+$ T cells in tumor in CT26 tumor model, (n = 4). (**B**) Representative flow plots and quantification of percentage of $CD8^+$ T cells by flow cytometry, (n = 4). The percentage of CD69-positive (**C**), granzyme B-positive (**D**) and IFNγ-positive (**E**) cells in the $CD8^+$ T cell population, (n = 4). (**F**) Representative flow plots and quantification of NK cells. (**G**) The percentage of IFNγ-positive NK cells. Data are mean ± SD (n = 4). (**H**) Expression of IL12 and DR18 transgenes and gene induction in tumor tissues was determined by RT-qPCR. GAPDH expression was used for normalization. Data are presented as mean ± SD (n = 3), * $p < 0.05$, ** $p < 0.01$.

Thus, intratumoral administration of oAd co-expressing IL12 and DR18 suppresses colorectal tumor growth by altering tumor microenvironment for effective anti-tumor immunity.

Mouse IL12 is composed of the p40 and p35 subunits. We constructed IL12 as a single chain cytokine since previous studies showed fusion of the p40 and p35 subunits did not affect IL12 activity. IL18 is an IL1 family cytokine whose secretion is strictly regulated by caspase-mediated events. For effective release, we used a signal peptide of human albumin for IL18 secretion, independent of inflammasome activity. In oAd.DR18/IL12, we used porcine teschovirus-1 derived 2A peptide (P2A) for expression of the two proteins since it gives the highest cleavage efficiency in human cell lines[39]. The advantage of the 2A peptide is its short length and close to stoichiometric expression of multiple proteins flanking the 2A peptide[39,40]. Indeed, we detected efficient cytokine production and release from oAd.DR18/IL12 transduced cells. Natural killer (NK) cell activity is

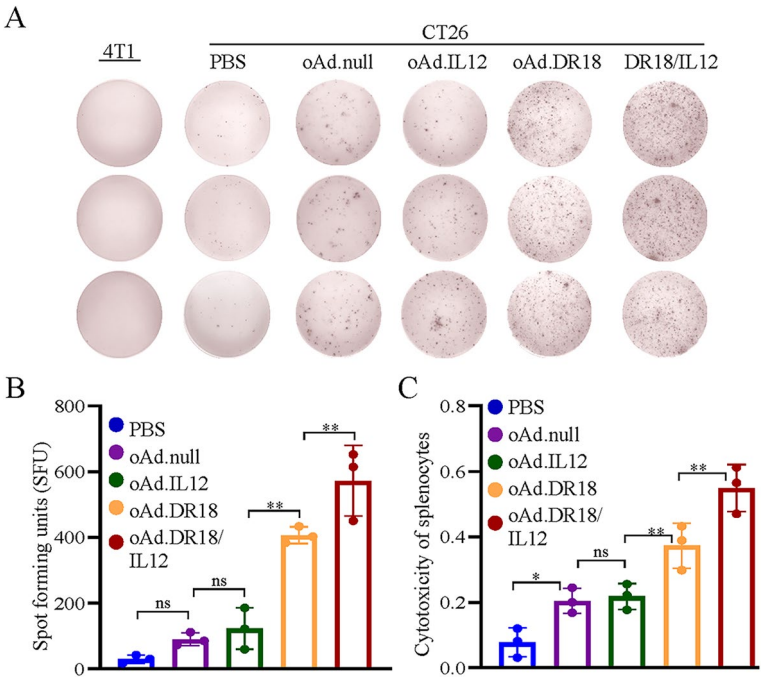

Fig. 4. Induction of tumor specific immunity detected by ELISpot. (**A**) ELISpot assay for IFNγ of splenocytes. CT26 tumor-bearing mice were administered intratumorally with PBS or an oAd on day 0 and day 2. The spleens were removed 2 days after the second injection and splenocytes were used for IFNγ secretion assay by ELISpot. 4T-1 cells were included as a control for the assay. The representative membranes were photographed and the spots were counted and presented as average ± SD triplicate samples, *n*=3. (**B**) The quantification of spot forming units (SFU) in ELISpot assay. (**C**) Cytotoxicity measurement of splenocytes. Splenocytes from mice treated with PBS or oAd-treated mice were incubated with CT26 cells or 4T-1 cells at a ratio of 100 effector-to-target (E: T). Cytotoxicity was determined by measuring LDH release. Results are expressed as mean ± SD, *n*=3.

essential for initiating antitumor responses and may be linked to immunotherapy success[41]. IL18-deficient mice have defective NK cell activity and Th1 response[42–44]. Accordingly, we detected increases in tumor infiltrating of T lymphocytes and NK cells.

IL12 is a potent, pro-inflammatory cytokine that has been studied as a potential immunotherapy for cancer. Unfortunately, early clinical trials in the mid-1990's showed that systemic delivery of IL12 incurred dose-limiting toxicities, prompting the development of gene therapy vectors able to express this cytokine locally in tumors[45,46]. IL18 induces the sequential activation of natural killer cells and cytotoxic T lymphocytes to display antitumor effect in immunocompetent mice[5]. Recombinant IL18 however has shown limited antitumor efficacy in preclinical models and clinical trials since the availability of IL18 in tumor tissues is normally significantly reduced due to the presence of IL18BP[7,47,48]. Thus, recombinant DR18, but not wild type IL18, has enhanced therapeutic potentials since the variant has the ability to escape IL18BP neutralization[7,49].

Recombinant adenoviruses (rAds) have become important players of new therapeutic strategy for cancer treatment. Adenoviral vectors can be engineered in different ways so as to change the tumor microenvironment from cold tumor to hot tumor. Thus, in addition to minimizing systemic exposure of directly delivered recombinant proteins, intratumoral expression of cytokines by virotherapy may also potentiate the antitumor effect associated with innate immune activation by viral vectors. Ads have been modified to express cytokines, tumor specific antigens, and immune-modulatory molecules such as TNFα, IL2, GM-CSF, and IFNα that have shown promising outcome in treatment of cancer. The approval of nadofaragene firadenovec, a replication-deficient rAd that delivers human interferon alfa-2b cDNA for genitourinary malignancies, has provided a promising alternative for BCG-unresponsive non-muscle-invasive bladder cancer[50,51]. Oncorine and ONYX-015 are oncolytic adenoviral vectors that have demonstrated efficacy in the treatment of certain types of cancer[52]. We used a design to deliver both IL12 and DR18 using an oncolytic adenoviral vector with 24 bp deletion in the E1 region since combined delivery of engineered cytokines with oAds. This vector system has the ability to boost long-term antitumor immunity[30,31]. Thus, this vector combined with IFNγ-inducing cytokines like DR18/IL12 used here could have potent antitumor effect against human tumors.

Cancer immunotherapy has rapidly become an indispensable mode of treatment for a multitude of solid tumor cancers[53]. We tested the antitumor effect using colorectal tumor models. Colorectal cancer (CRC) is the third most common cancer and one of the most lethal malignancies in the general population[54]. Surgical resection, supplemented by systemic chemotherapy and local pelvic radiotherapy is currently the main treatment for CRC[55]. Despite recent advances in systemic treatment of CRC, prognostic outcomes have remained to be poor, particularly for metastatic CRCs[56]. Immunotherapy is a new and exciting modality of

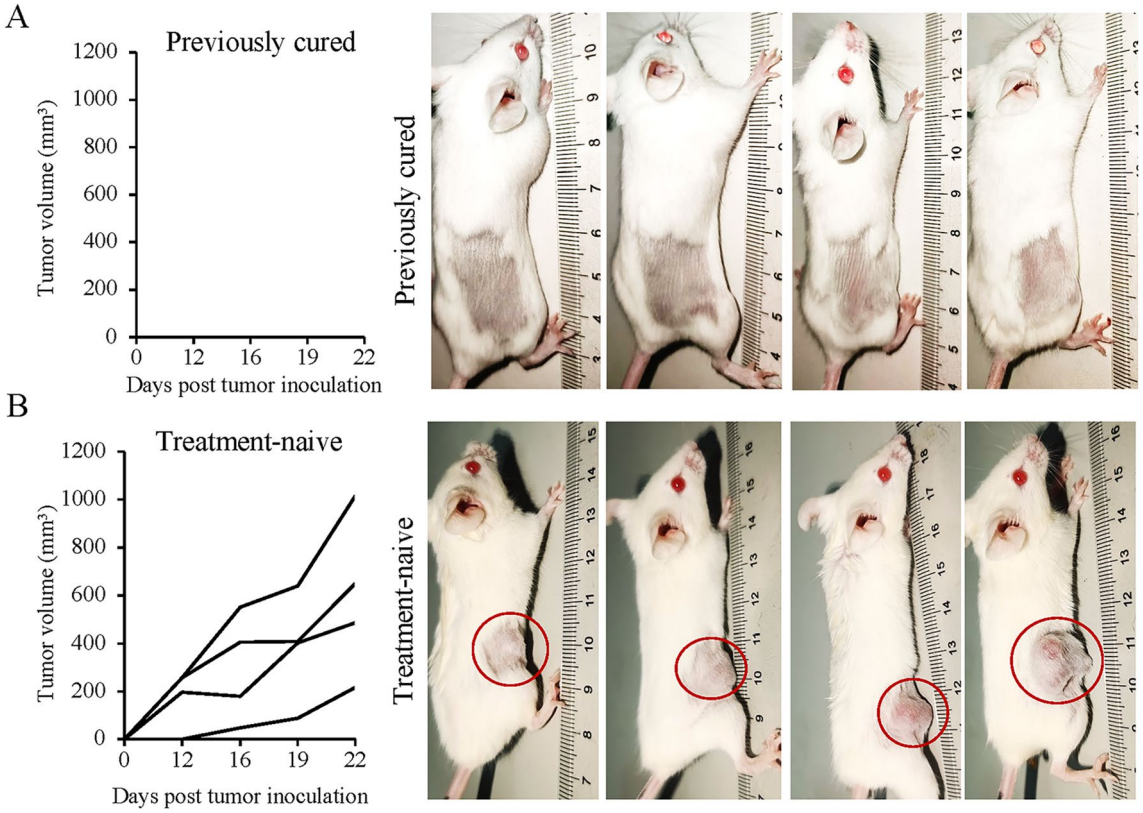

Fig. 5. Intratumoral administration of oAd.DR18/IL12 establishes long-term antitumor memory Tumor growth of CT26 cells on previously cured from CT26 tumors (**A**) or with age-matched treatment-naïve mice (**B**). For previously cured mice, CT26 cells were implanted approximately 2 months after the final treatment. The tumor growth was monitored and the mice were photographed 22 days after tumor inoculation.

cancer treatments[57]. Immunotherapy in cancer therapy is based on the concept by reversing regulatory T-cell-mediated immunosuppression since it is one of the main immune evasion techniques used by cancer cells. Tumors can manipulate cytokines that promote T regulatory cells and myeloid derived suppressor cells to escape cytotoxic T cell function. Thus, virotherapy combined with the use of anti-tumor immunity boosting cytokines may elicit tumor specific immunity against colorectal cancers.

Data availability

All data supporting the findings of this study are available within the paper.

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Author contributions

Yan Cheng, conceptualization, curation, investigation, data analysis, writing of original draft, reviewing and editing. Renjie Luo, investigation, reviewing. Erguang Li, conceptualization, funding acquisition, supervision, writing and editing.

Declarations

Competing interests

The authors declare no competing interests.

Ethic statement

The use and care of laboratory animals was approved by the Institutional Animal Care and Use Committee (IACUC) of Nanjing University. We confirm that all methods were performed in accordance with the relevant guidelines and regulations. All methods are reported in accordance with ARRIVE guidelines.

Additional information

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