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Dissecting the heterogeneity of circulating and tissue-resident memory T cells

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6

Adenoviral vaccines promote protective tissue-resident memory T cell populations against cancer

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Abstract

Adenoviral vectors emerged as important platforms for cancer immunotherapy. Vaccination with adenoviral vectors is promising in this respect, however, their specific mechanisms of action are not fully understood. Here, we assessed the development and maintenance of vaccine-induced tumor-specific CD8⁺ T cells elicited upon immunization with adenoviral vectors. Adenoviral vaccine vectors encoding the full-length E7 protein from human papilloma virus (HPV) or the immunodominant epitope from E7 were generated, and mice were immunized intravenously with different quantities (10^7 , 10^8 or 10^9 infectious units). The magnitude, kinetics and tumor protection capacity of the induced vaccine-specific T cell responses were evaluated. The adenoviral vaccines elicited inflationary E7-specific memory CD8⁺ T cell responses in a dose-dependent manner. The magnitude of these vaccine-specific CD8⁺ T cells in the circulation related to the development of E7-specific CD8⁺ tissue-resident memory T (T_{RM}) cells, which were maintained for months in multiple tissues after vaccination. The vaccine-specific CD8⁺ T cell responses conferred long-term protection against HPV-induced carcinomas in the skin and liver, and this protection required the induction and accumulation of CD8⁺ T_{RM} cells. Moreover, the formation of CD8⁺ T_{RM} cells could be enhanced by temporal targeting CD80/CD86 costimulatory interactions via CTLA-4 blockade early after immunization. Together, these data show that adenoviral vector-induced CD8⁺ T cell inflation promotes protective T_{RM} cell populations, and this can be enhanced by targeting CTLA-4.

Introduction

After viral infection, naïve CD8⁺ T cells clonally expand and differentiate into effector cell populations, which turn into phenotypically diverse memory CD8⁺ T cell subsets that differ in their homing properties, cytokine polyfunctionality and cytolytic capacity (1). These memory T cell populations reside in the blood circulation, lymphoid organs but also in tissues, where they can become tissue-resident (2–5).

Defining the protective cellular subsets and the crucial molecular interactions that induce sufficient effective CD8⁺ T cell memory pools at the right location is decisive for the design of successful vaccines against malignant disease. Recombinant adenoviral vectors are widely studied to induce immunity against viral infections. Adenovirus-based vaccines have been developed against respiratory syncytial virus and hepatitis C virus and demonstrated effective immunogenicity (6,7). Adenoviral vectors are currently strong candidate vaccines against the recently emerged SARS-CoV-2 causing the pandemic COVID-19 (8–11). Adenoviruses are also frequently used as platforms for gene therapy, oncolytic therapy and vaccines in cancer treatment (12). Regarding the cancer vaccine platform, several adenoviral vectors are currently in clinical trials for (preventive) treatment against prostate cancer, pancreatic cancer and human papilloma virus (HPV)-associated cancer (12). The utilization of adenoviral vectors as vaccines relates to their genome that can be genetically engineered. Replication-deficient adenoviral vectors are generated by deletion of early proteins (E1/E3) that are essential for viral replication. However, these vectors still allow considerable transgene expression. The absence of replication and the lack of integration of the vector genome, is linked to the safety of these vaccine vectors (13, 14). The tropism of the adenoviral vectors is broad, nonetheless on systemic administration the liver is a major site of infection (15–17).

Moreover, adenoviral vaccine vectors are able to induce and sustain substantial memory CD8⁺ T cell populations. In experimental settings, recombinant replication-deficient HuAd5 vectors have been modified by a mini-gene strategy to express specific epitopes eliciting memory T cell inflation (18, 19) Memory inflation, a characteristic of cytomegalovirus (CMV) infection, is typified by the maintenance of large populations of virus-specific CD8⁺ T cells with an effector-memory-like phenotype (20, 21). The low-level intermittent persistence of antigen is critical for the induction of inflationary memory responses, and importantly, the

inflationary CD8⁺ T cells remain functional over time, which makes adenoviral vectors an ideal vaccine candidate (21). In contrast, chronic infections with high-level active replication elicit virus-specific exhausted CD8⁺ T cells (22). It became also apparent that both adenoviral vectors and CMV elicit circulating and tissue-resident memory T (T_{RM}) cells that are enriched in certain tissues (23–26). Whether memory T cell inflation is related to the formation and maintenance of T_{RM} cells on adenoviral-vector vaccination is not fully understood. Furthermore, which cellular subset is critical for protection against cancer progression is unknown.

Here, we aim to understand the connection between memory T cell inflation and the induction of tissue-residency of T cells in an adenoviral vaccine setting, and in addition assess the protective capacity of circulating and T_{RM} cell pools against solid tumors. We show that mice immunized with adenoviral vectors expressing the E7 oncogene of HPV or only the E7 immunodominant epitope induce high frequencies of circulating inflationary E7-specific CD8⁺ T cells, and the magnitude of these cells related to their development into T_{RM} cells. In particular, these vaccine-induced CD8⁺ T_{RM} cells provided long-term protection against HPV-positive tumors in the skin and liver, and enhancement of the T_{RM} cell formation by temporal CTLA-4 blockade, improved suboptimal antitumor vaccination. Thus, the induction of CD8⁺ T cell tissue-residency by adenoviral vectors is linked to memory inflation, and this induction is essential for protection against solid tumors.

Materials and methods

Mice

C57BL/6 mice were obtained from Charles River Laboratories (L'Arbresle, France) or Jackson Laboratory (Sacramento, California, USA). *Cd70*^{-/-} (27) and *Cd80/86*^{-/-} (28) and Ly5.1 (CD45.1) mice were bred in house. At the start of the experiments, mice were 6 to 8 week-old. Animals were housed in individually-ventilated cages under specific-pathogen free conditions at the animal facility at the Leiden University Medical Center (LUMC). All animal experiments were approved by the Animal Experiments Committee of LUMC and performed according to the recommendations and guidelines set by the LUMC and Dutch Experiments on Animals Act.

Adenoviral vaccine vectors

E1/E3-deleted replication-deficient adenoviral vectors (type 5) expressing full-length E7 (Ad-E7) or the E7 epitope (Ad-R9F) transgenes under control of the human CMV promoter were produced at the Jenner Institute Viral Vector Core Facility (University of Oxford), as previously described (19). Expression cassettes were flanked by an initiator methionine, stop codon and a SV40 polyA tail. Peptide sequence full-length E7: MHGDTPTLHE YMLDLQPETT DLYCYEQLND SSEEDEIDG PAGQAEPDRA HYNIVTFCK CDSTLRLCVQ STHVDIRTLE DLLMGTLGIV CPICSQKP. Peptide sequence E7 epitope (R9F): RAHYNIVTF.

HEK293A T-Rex cells were transfected using linearized plasmid and the presence of the antigen gene was confirmed by PCR. The integrity of the antigenic DNA sequence and absence of contaminating adenovirus was confirmed by Flank-Flank PCR. The virus was titred to obtain infectious units (IU) per mL, and assayed by spectrophotometry to quantify the number of virus particles per mL. The P:I ratios of virus particles to infectivity were 25 (Ad-E7) and 40 (Ad-R9F). The sterility of the virus was also confirmed by inoculation of TSB broth with 10 μ L of purified virus and incubation for 3 days at 35°C. Adenoviral vectors were stored at -80°C and injected intravenously via the retro-orbital route at a dose of 10⁷, 10⁸ or 10⁹ IU per mouse.

Isolation of T_{RM} cells

Mice were perfused with 20 mL PBS containing 2 mM EDTA to remove circulating blood cells from the organs of interest. The liver, lungs and tumors were cut into small pieces using

surgical knives. The liver was resuspended in 3.5 mL IMDM containing 250 U/mL collagenase type 1-A (C2674, Sigma) and 20 µg/mL DNase I (D5025, Sigma). Lung tissue was incubated with 1 mL IMDM and 250 U/mL collagenase and 20 µg/mL DNase. For both liver and lungs, collagenase and DNase were incubated for 25 min at 37°C. Tumors were incubated for 15 min at 37°C with IMDM-containing Liberase (#05401020001, Roche). Single cell suspensions were made using 70 µm cell strainers and subsequently lymphocytes were isolated using a Percoll gradient.

For intraepithelial lymphocyte (IEL) isolation, fat tissue and Peyer's patches were removed from the small intestine after which it was cut into 1 cm pieces. After washing, the intestine was incubated with 20 mL IMDM containing 10% FCS, 5 mM EDTA and 1 mM DTT (D0632, Sigma) for 30 min at 37°C. Supernatant containing the IEL fraction was isolated by filtering over a 70 µm cell strainer and lymphocyte fractions were isolated using a Percoll gradient. For skin T cell isolation, first remaining hair was removed using Veet hair removal cream after which 1 cm by 1.5 cm skin tissue (located at the subcutaneous tumor site) was dissected. Skin tissue was incubated in 1.5 mL of 2.5 mg/mL Dispase II (D4693, Sigma) at 37°C for 1.5 hours. The skin tissue was cut into small pieces using surgical knives after which it was incubated in 1.5 mL 3 mg/mL collagenase type IV (C5138 Sigma) and 5 µg/mL DNase I (D5025, Sigma) for 30 min at 37°C. Single cell suspensions were made using 70 µm cell strainers.

Tumor challenge models

The TC-1 tumor cell line (a kind gift from T.C. Wu, John Hopkins University, Baltimore, MD) was developed by retroviral transduction of C57BL/6 lung epithelial cells with the HPV16 E6/E7 and c-H-ras oncogenes (29). The tumor cell line C3 was generated by transfection of mouse embryonic cells with the HPV16 genome and an activated-ras oncogene (30). Mice were inoculated subcutaneously in the flank with 1×10^5 TC-1 tumor cells or 5×10^5 C3 tumor cells. Tumor size (mm^3) was measured two times a week using a caliper and calculated as $(L \times W \times H) \times 0.52$ (L: for length, W: width, H: height). Mice were euthanized when tumor size reached 500–1000 mm^3 in volume. *Mycoplasma* tests were frequently performed by PCR and were negative for all cell lines. Cell lines were authenticated with a microsatellite PCR.

Luciferase-expressing TC-1 tumor cells were generated by transducing the TC-1 cells with a lentiviral vector expressing IRIS-GFP and the luciferase gene luc2. Before performing

surgery, mice received 0.1 mg/kg buprenorphine (Temgesic) subcutaneously as analgesia and isoflurane for anesthesia. After opening the peritoneum, the tip of the spleen was lifted to inject 1×10^5 TC-1-luc2 tumor cells in the spleen. To visualize tumor outgrowth by bioluminescence imaging, mice were injected intraperitoneally (IP) with 100 mg/kg D-luciferin (Synchem, Germany) and imaged after 10 min with the IVIS Spectrum Imager. Bioluminescence signals were measured twice a week starting from day 2 after tumor challenge.

In vivo cytotoxicity assay

The cytotoxicity of CD8⁺ T cells was assessed by transferring target cells (splenocytes from Ly5.1 (CD45.1) mice) that were prior differentially labeled with CFSE and peptide. Target cells were either CFSE high labeled (5 μ M) and then loaded with specific peptide (RAHYNIVTF) or were CFSE low labeled (0.5 μ M), and then loaded with a-specific peptide. Subsequently, the two target cell populations were mixed in a 1:1 ratio and injected intravenously into recipient mice. Recipient mice (wild-type C57BL/6 mice, *Cd80/86*^{-/-} mice or mice containing CD70 blocking antibodies (clone FR70)) were either naïve or were previously vaccinated with 10^9 IU Ad-R9F for 50 days at the time of transfer. At 16 hours after transfer, the target cell killing in the spleens of recipient mice was determined by flow cytometry.

Adoptive T cell transfers

Spleens from Ly5.1 (CD45.1) mice were isolated and subsequently CD8⁺ effector-memory T (T_{EM} , CD44⁺CD62L⁻CD69⁺) and central-memory T (T_{CM} , CD44⁺CD62L⁺CD69⁺) cells were purified on a BD FACSAria cell sorter (BD Bioscience). T_{EM} and T_{CM} cells were adoptively transferred via retro-orbital injection into naïve Ly5.2 mice, TC-1 tumor-bearing mice or into mice previously vaccinated with 10^8 IU Ad-R9F.

In vivo antibody and FTY720 treatment

CD8⁺ T cell depleting monoclonal antibodies (clone 2.43) were administered twice per week, starting 4 days before tumor challenge, at 150 μ g per mouse by IP injection. For low-dose anti-CD8 treatment, CD8⁺ T cell depleting monoclonal antibodies (clone 2.43, Bio X Cell, West Lebanon, New Hampshire, USA) were administered twice per week, starting 4 days before tumor challenge, at 20 μ g per mouse by IP injection. To assess CD8⁺ T cell infiltration, the CD8⁺ T cells in the circulation were labeled by injecting mice retro-orbitally with 3 μ g CD8a

APC (clone 53.6-7, BioLegend). After 3 min mice were sacrificed and subsequently tumor, spleen and liver were analyzed.

CTLA-4 (clone 9H10) monoclonal antibodies (Bio X Cell) were administered IP twice per week for 2 weeks starting at the day of infection at 200 µg per mouse. CXCR3 (clone CXCR3-173, Bio X Cell) monoclonal antibodies were administered IP twice per week starting 4 days before tumor inoculation at 200 µg per mouse. CD80 (clone RM80) and CD86 (clone P03.1) blocking antibodies (31) were administered IP two times per week at 300 µg per mouse for 2 weeks starting the day of infection. CD70 (clone FR70) blocking antibody (32) was administered IP twice a week at 150 µg per mouse starting at the beginning of the experiment and continued for 3 weeks.

FTY720 (SML0700, SIGMA) was dissolved in 0,9% NaCl and administered IP twice per week at 50 µg starting 1 week before tumor challenge.

Flow cytometry

Fluorescently labeled monoclonal antibodies against the following mouse antigens were used: CD3 (clone 145–2 C11, BD Biosciences), CD4 (clone RM4-5, BioLegend), CD8a (clone 53–6.7, BioLegend), CD8b (clone YTS156.7.7, BioLegend), CD11a (clone M17/4, eBioscience), CD127 (clone A7R34, Thermo Fisher), KLRG-1 (clone 2F1, Thermo Fisher), CD44 (clone IM7, BioLegend), CD49a (clone Ha31/8, BD Biosciences), CD62L (clone MEL-14, BioLegend), CD69 (clone H1.2F3, BD Biosciences), CD38 (clone 90, Thermo Fisher) and CD103 (clone 2E7, Thermo Fisher). Cells were stained according to our previously published protocol (33). 7-AAD (A1310, Invitrogen) staining was used to exclude dead cells. E7-specific CD8⁺ T cells were quantified using MHC class I tetramers for the RAHYNIVTF epitope. Flow cytometric acquisition was performed on a BD Fortessa flow cytometer (BD Biosciences).

Statistics

Statistical analyzes were performed using GraphPad Prism (La Jolla, CA, Unites States). The One-way analysis of variance or Student's t-test was used for statistical analysis of unpaired data following a Gaussian (normal) distribution test. Difference in survival was tested with the log-rank (Mantel-Cox) survival test. All P values were two sided, and P<0.05 was considered statistically significant.

Results

Adenoviral vector vaccines induce CD8⁺ T cell memory inflation and associated quantities of T_{RM} cells

Mice were immunized with different dosages of adenoviral vectors encoding full-length HPV E7 protein (Ad-E7) or the immunodominant epitope RAHYNIVTF from E7 (Ad-R9F). Vaccination with 10⁸ and 10⁹ IU of Ad-E7 or Ad-R9F elicited high frequencies of circulating E7-specific CD8⁺ T cell populations in the blood, that is, 4%–12% of the total CD8⁺ T cell population, which were maintained for months (**Figure 1A**). In contrast, immunization with 10⁷ IU elicited E7-specific responses <2%. The higher vaccination dose correlated with an enhancement of circulating E7-specific CD8⁺ T cells with an effector-memory phenotype (ie, CD44⁺CD62L⁻CD127⁺KLRG1⁺) (**Figure 1B and S1A**).

Next, we interrogated the impact of adenoviral vaccination on the development of E7-specific CD8⁺ T cells in multiple organs. E7-specific CD8⁺ T cells with a T_{RM}-like phenotype (ie, CD69⁺ or CD69⁺CD103⁺) were present in liver, lungs, intestine and skin (**Figure 1C and S1B**). Especially, in the liver large quantities of E7-specific CD69⁺ CD8⁺ T cells were induced, which were stably maintained for more than 200 days after vaccination (**Figure 1D**). The E7-specific CD69⁺ CD8⁺ T cells in the liver also express other markers associated with CD8⁺ T cell tissue-residency such as CD38, CD11a and CD49a (**Figure 1E**) (34–36). The adenovirus-vectored vaccines also elicited circulating effector-memory cells (CD44⁺CD62L⁻KLRG1⁺CD69⁻) in these organs. Remarkably, T_{RM} cells increased particularly in the liver compared with the non-resident CD8⁺ T cells on high dose (10⁹ IU) vaccination. The increase in the total E7-specific CD8⁺ T cell population was mainly due to the rise in E7-specific CD8⁺ T_{RM} cell numbers (**Figure 1F**). In the lungs, mainly non-resident CD69⁻ E7-specific CD8⁺ T cells are increased in numbers after high dose vaccination (**Figure 1G**). Thus, high-dosed adenoviral vaccination elicits memory T cell inflation and specifically enhanced T_{RM} cell formation in liver.

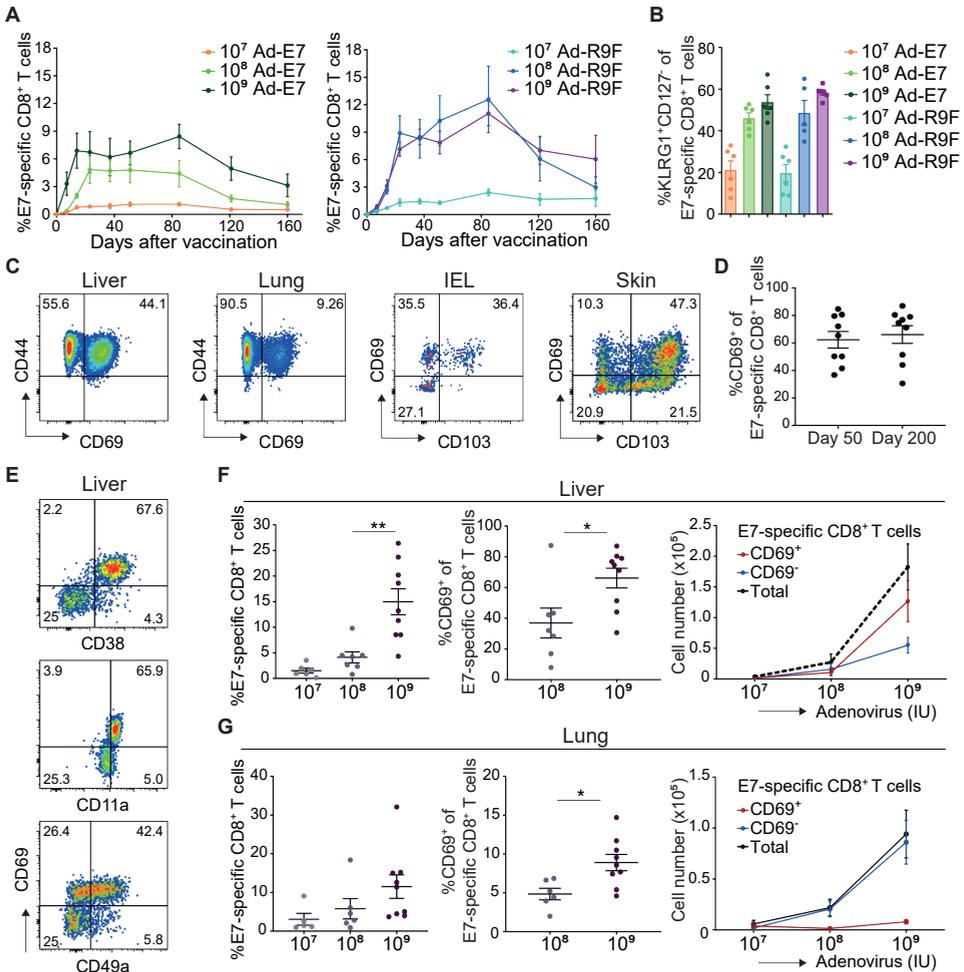


Figure 1. Adenoviral vaccine vectors induce memory CD8⁺ T cell inflation and T_{RM} cells. Mice were vaccinated IV with increasing dosages (10⁷, 10⁸ or 10⁹ IU) of Ad-E7 or Ad-R9F vaccine vectors. E7-specific CD8⁺ T cells were longitudinally monitored with MHC class I tetramers. (A) Kinetics of E7-specific CD8⁺ T cells in blood. (B) Phenotype of E7-specific CD8⁺ T cells in blood at day 50 after vaccination. (C) E7-specific CD8⁺ T_{RM} cells in liver, lung and IEL and CD8⁺ T_{RM} cells in skin 200 days after 10⁹ IU Ad-R9F vaccination. (D) Percentage of E7-specific CD8⁺ T_{RM} cells in liver at day 50 and day 200 after 10⁹ IU Ad-E7 or Ad-R9F vaccination. (E) Phenotype of E7-specific CD8⁺ T_{RM} cells in liver at day 50 after Ad-R9F vaccination. (F, G) Percentage and absolute numbers of E7-specific CD8⁺ T cells and T_{RM} cells after indicated doses of Ad-E7 or Ad-R9F in liver (F) and lung (G) 200 days after vaccination. Data shown are pooled from 2 to 3 independent experiments (n=3-5 per group). Data are expressed as mean±SEM. One-way ANOVA (three groups) or Student t-test (two groups) was used for statistical analysis. *P<0.05, **P<0.01. ANOVA, analysis of variance; IEL, intraepithelial lymphocyte; IU, infectious units; T_{RM}, tissue-resident memory T.

Rapid conversion of circulating vaccine-elicited CD8⁺ T cells into tissue-resident cells

To assess whether the inflationary CD8⁺ T cells induced by adenoviral vectors have the capacity to migrate to the liver and differentiate into T_{RM} cells, we performed adoptive transfers of the circulating CD8⁺ effector-memory (T_{EM}; CD44⁺CD62L⁻CD69⁻) or central-memory (T_{CM}; CD44⁺CD62L⁺CD69⁻) T cells (**Figure S2A**) from Ad-R9F vaccinated mice into naïve recipient mice or into recipient mice that were prior challenged with TC-1 tumor cells or prior vaccinated with Ad-R9F. When transferred into naïve mice, the T_{EM} and T_{CM} subsets largely retain their phenotype in the liver after 14 days of transfer (**Figure 2A**). However, in mice-bearing E7-expressing TC-1 tumors a substantial fraction of the transferred T_{EM} cells differentiated within 10 days into liver CD69⁺ T_{RM} cells (**Figure 2B and S2B**). This conversion was also evident in mice previously vaccinated with the same adenoviral vector (**Figure 2C and S2C**). A portion of the E7-specific CD8⁺ T_{CM} cells converted also converted into T_{RM} cells, although this conversion was to a lower extent as compared with their effector-memory counterparts. Thus, adenoviral vaccine-induced E7-specific CD8⁺ T cells that circulate in the body can convert rapidly into cells with a T_{RM} phenotype in an antigen-dependent manner.

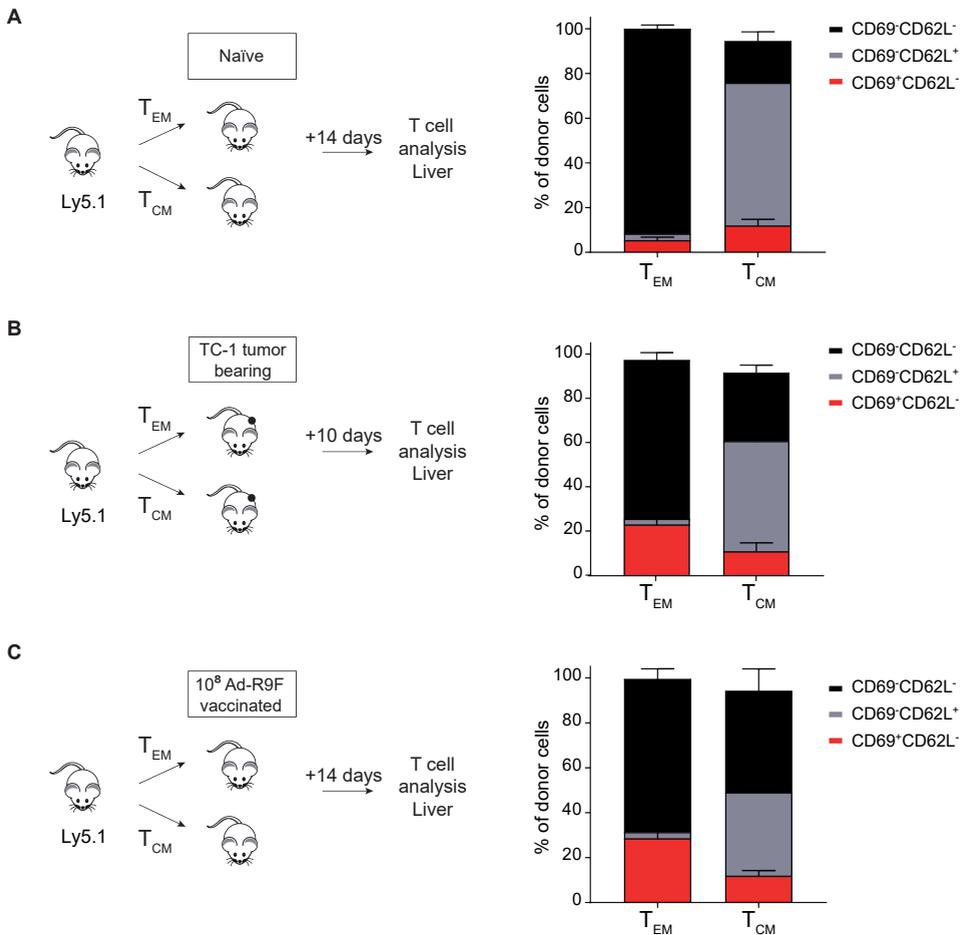


Figure 2. Circulating adenoviral vaccine-elicited CD8⁺ T cells convert rapidly into T_{RM} cells. (A) Effector-memory (T_{EM}^+ ; CD44⁺CD62L⁻CD69⁻) or central-memory (T_{CM}^+ ; CD44⁺CD62L⁺CD69⁺) CD8⁺ T cells were isolated from infected Ly5.1 (CD45.1⁺) mice and adoptively transferred into naïve recipient mice, the distribution and phenotype of donor cells was analyzed 14 days later. Bar graphs show the frequency of CD69⁺CD62L⁻, CD69⁺CD62L⁺ and CD69⁺CD62L⁻ T_{RM} cells within the donor CD45.1⁺ CD8⁺ T cell population in the liver. (B) Ly5.1 mice were vaccinated with 10^8 IU Ad-R9F. At day 50 day, the T_{EM}^+ or T_{CM}^+ CD8⁺ T cells were isolated and adoptively transferred into recipient mice, which were 10 days before transfer challenged with TC-1 tumor cells (subcutaneously in the flank). Bar graphs show the frequency of CD69⁺CD62L⁻, CD69⁺CD62L⁺ and CD69⁺CD62L⁻ cells within the donor CD45.1⁺ CD8⁺ T cell population in the liver. (C) Ly5.1 (CD45.1⁺) mice were vaccinated intravenously with 10^8 IU Ad-R9F. At day 50 day, the T_{EM}^+ or T_{CM}^+ CD8⁺ T cells were isolated and adoptively transferred into recipient mice, which were vaccinated intravenously with 10^8 IU Ad-R9F 23 days before transfer. Bar graphs show the frequency of CD69⁺CD62L⁻, CD69⁺CD62L⁺ and CD69⁺CD62L⁻ cells within the donor CD45.1⁺ CD8⁺ T cell population in the liver. Data shown are from two independent experiments (n=6 per group). Data are expressed as mean±SEM. IU, infectious units.

Adenoviral vector vaccination provides long-term CD8⁺ T cell dependent tumor protection

To investigate the potential of Ad-E7 and Ad-R9F vaccine vectors to provide tumor protection, mice received a subcutaneous TC-1 tumor challenge 50 days after vaccination. The strength of the vaccine-induced CD8⁺ T cell responses correlated with long-term protection against subcutaneously transplanted TC-1 tumors (**Figure 3A, B**). To confirm the importance of the vaccine-induced CD8⁺ T cells in tumor protection, these cells were specifically depleted by anti-CD8 antibodies. On CD8⁺ T cell depletion, none of the vaccinated mice were protected (**Figure 3A, B**). Subsequently, we validated in a second model the tumor protection efficacy of adenoviral vaccines. For this, we used the HPV16-transformed C3 tumor model. In this model, Ad-R9F vaccination resulted in substantial protection against C3 tumors in a dose-dependent manner, and this protection also required CD8⁺ T cells (**Figure 3C, D**). Thus, adenoviral vaccines elicit E7-specific CD8⁺ T cells that protect against subcutaneous tumors.

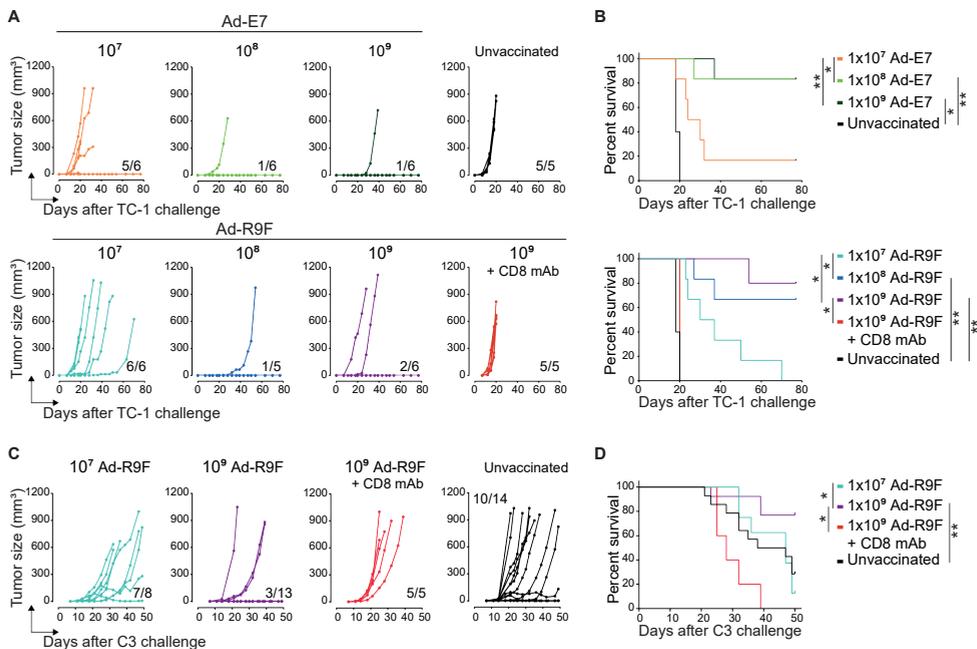


Figure 3. CD8⁺ T cells are essential for adenoviral vector-mediated protection against subcutaneous tumors.

Mice were vaccinated intravenously with increasing dosages (10⁷, 10⁸ or 10⁹ IU) of Ad-E7 or Ad-R9F vaccine vectors. Tumor outgrowth (A) and survival (B) of mice challenged with TC-1 tumor cells (subcutaneously in the flank) at day 160 after vaccination. CD8 depleting antibodies (150 µg IP) were administered twice per week starting 4 days before tumor challenge. (C, D) Tumor outgrowth (C) and survival (D) of mice challenged with C3 tumor cells (subcutaneously in the flank) at day 50 after vaccination with 10⁷ or 10⁹ IU Ad-R9F. CD8 depleting antibodies (150 µg IP) were injected 4 days before tumor challenge. Data shown are from two pooled experiments (n=3–8 per group). Log-rank (Mantel-Cox) survival test was performed. *P<0.05, **P<0.01. IU, infectious units.

The induction of T_{RM} cells by adenoviral vectors is important for tumor protection

To study the importance of circulating and tissue-resident $CD8^+$ T cells elicited by adenoviral vaccines for tumor protection, we trapped the circulating cells in the lymph nodes by the sphingosine 1-phosphate receptor modulator FTY720 (37) or depleted either the circulating $CD8^+$ T cells by low dose anti- $CD8$ antibody treatment or tissue-resident $CD8^+$ T cells by CXCR3 antibodies (**Figure 4A, B**). Compared with untreated vaccinated mice, FTY720 treatment resulted in a substantial reduction in tumor protection. Moreover, on low-dose anti- $CD8$ antibody treatment, none of the vaccinated mice were protected against tumor outgrowth (**Figure 4C, D**). Both FTY720 treatment and low dose anti- $CD8$ affected the total amount of $CD8^+$ T cells in the circulation (**Figure 4A**), and also those present in the tumor (**Figure S3**). Nevertheless, the few $CD8^+$ T cells that were present in the tumor still converted into T_{RM} cells, indicating that T_{RM} formation is not blocked but severely limited and is likely due to limited infiltration from the circulation (**Figure S3**). Thus, diminishing the number of circulating $CD8^+$ T cells prevent robust accumulation of T_{RM} cells in the tumor, indicating that the circulating $CD8^+$ T cells are an important source for sufficient formation of tumor-resident $CD8^+$ T cells.

To directly study the role of T_{RM} cells in tumor control, we tested whether CXCR3 antibodies, known to specifically deplete T_{RM} cells in the liver (38), also deplete T_{RM} cells in subcutaneous tumors. Treatment with CXCR3 antibodies resulted in a nearly complete depletion of liver T_{RM} cells and also of T_{RM} cells in the tumor (**Figure 4B**), whereas $CD8^+$ T cells in the circulation were not depleted (**Figure 4A**). CXCR3 treatment did however not affect skin T_{RM} (**Figure S4**), which corroborates the notion that circulation $CD8^+$ T cells are vital for the formation of T_{RM} cells in the tumor. To examine possible effects of CXCR3 antibody treatment on the migration of $CD8^+$ T cells, we labeled the circulating $CD8^+$ T cells by injecting APC-labeled $CD8$ antibodies 3 min before sacrifice. CXCR3 treatment did not alter the migration properties of $CD8^+$ T cells in the tumor, spleen or liver (**Figure S5**). Taken together, CXCR3 antibody treatment does not affect T cell migration into tissues or the number of skin T_{RM} cells but results in specific depletion of T_{RM} cells in the tumor. Importantly, on the specific T_{RM} cell depletion in the tumor by CXCR3 antibodies, Ad-R9F vaccination did not show any efficacy and all mice succumbed on tumor challenge (**Figure 4C, D**).

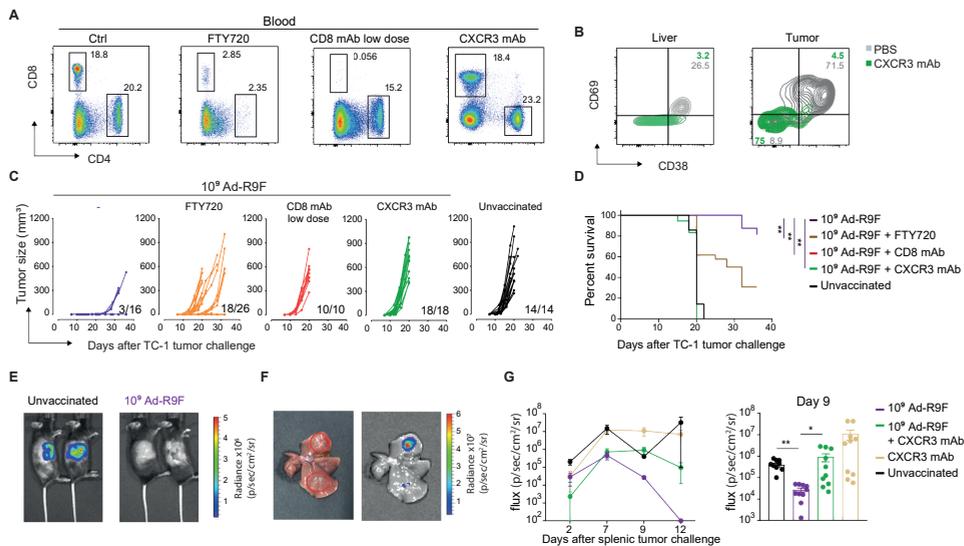


Figure 4. T_{RM} cells are important for adenovirus-mediated tumor protection. (A) Percentage of CD8⁺ T cells of total blood lymphocytes in untreated (Ctrl) mice or in mice treated for 2 weeks with FTY720, low-dose anti-CD8 mAb or CXCR3 antibodies. FTY720 (50 μ g IP), CD8 depleting antibody (20 μ g IP), and CXCR3 antibody (200 μ g IP) was administered twice per week. (B) Expression of CD69 and CD38 on E7-specific CD8⁺ T cells in liver and tumor of 10⁹ IU Ad-R9F intravenously vaccinated mice that were challenged with TC-1 tumor cells at day 50 post-vaccination and treated twice per week (starting 4 days before tumor challenge) with a depleting CXCR3 antibody (200 μ g IP). Analysis was performed at day 20 post-tumor challenge. (C, D) Tumor outgrowth (C) and survival (D) of mice challenged with TC-1 tumor cells (subcutaneously in the flank) at day 50 after intravenous vaccination with 10⁹ IU Ad-R9F. FTY720 (50 μ g IP), low-dose CD8 depleting antibody (20 μ g IP) or CXCR3 depleting antibody (200 μ g IP) was administered twice per week starting 4 days before tumor challenge. Data are pooled from 2 independent experiments (n=6–9 per group). (E) Mice were vaccinated intravenously with 10⁹ IU Ad-R9F and 44 days post-vaccination received luciferase-containing TC-1 (TC-1-luc2) tumor cells in the spleen, which metastasize to the liver. Shown are bioluminescence signals of unvaccinated and 10⁹ IU Ad-R9F vaccinated mice at day 7 after TC-1-luc2 splenic tumor challenge. (F) Image and bioluminescence signal of a representative liver from unvaccinated mouse at day 12 after TC-1-luc2 splenic tumor challenge. Bioluminescence signals were absent in all livers of vaccinated mice. (G) CXCR3 depleting antibody (200 μ g IP) was administered twice per week starting 4 days before splenic tumor challenge. Bioluminescence flux at liver side at indicated time points after tumor challenge is shown (n=10 per group). Data are expressed as mean \pm SEM. One-way ANOVA was used for statistical analysis. Log-rank (Mantel-Cox) survival test is performed for survival data. *P<0.05, **P<0.01. ANOVA, analysis of variance; IU, infectious units.

To corroborate the importance of T_{RM} cells for tumor protection, we setup a model to assess the efficacy of the E7-specific CD8⁺ T cell-eliciting adenoviral vectors against tumor development in the liver. Previous studies showed that on intrasplenic injection of tumor cells, the tumor cells are initially trapped in the liver vasculature followed by invasion of the surrounding tissue (39, 40). By performing this procedure with luciferase-expressing TC-1 tumor cells, we were able to track the metastatic tumor development in the liver by in vivo bioluminescence (Figure 4E, F). In contrast to unvaccinated mice, 10⁹ IU Ad-R9F vaccinated mice, with intact numbers of T_{RM} cells, were protected against liver tumor outgrowth. Strikingly, T_{RM} depletion

in vaccinated mice resulted in considerable outgrowth of the tumor cells (**Figure 4G**). Thus, T_{RM} cells mediate critical immunity against tumors growing in the skin and liver.

Costimulatory requirements for inflationary E7-specific CD8⁺ T cell responses and T_{RM} cell formation

Costimulatory molecules regulate T cell activation and differentiation, and can serve as therapeutic targets for modulating T cell responses. At day 3 after adenoviral vector vaccination, the costimulatory molecules CD80 and CD86 were upregulated on CD11b⁺CD11c⁺ cells in the liver (**Figure 5A**). To directly dissect the role of CD80/CD86-mediated costimulation in the induction of memory inflation and the formation of T_{RM} cells after adenovirus-vector vaccination, we evaluated mice deficient in these costimulatory molecules and in addition abrogated CD80/CD86 interactions at different time points post 10^9 IU Ad-R9F vaccination using blocking antibodies. *Cd80/86*^{-/-} mice were not able to generate inflationary E7-specific CD8⁺ T cell responses in the blood after Ad-R9F vaccination (**Figure 5B**). Consistently, in adenovirus-vector vaccinated wild-type mice, but not in vaccinated *Cd80/86*^{-/-} mice, a functional cytolytic E7-specific CD8⁺ T cell response was generated that killed E7⁺ target cells in vivo (**Figure 5C, D**). Blockade of CD80/CD86-mediated costimulation at different time points after adenovirus-vector vaccination indicated that predominantly the initial CD80/CD86 interactions are required for the initiation of inflationary E7-specific CD8⁺ T cell responses in the blood (**Figure 5B**). The deprived E7-specific CD8⁺ T cell response in mice with deficient CD80/CD86-mediated costimulation, related to a complete lack of E7-specific CD8⁺ T_{RM} cell formation in liver and lungs of these mice (**Figure 5E**). In contrast to CD80/CD86, CD70 was not upregulated on vaccination and consistently the abrogation of CD70 did not affect the generation or cytolytic function of E7-specific CD8⁺ T cells in the circulation or in the tissues (**Figure 5A–E**). Thus, in particular CD80/CD86-mediated costimulation is essential for the inflationary E7-specific CD8⁺ T cell response and development of T_{RM} cell formation on adenoviral vaccination.

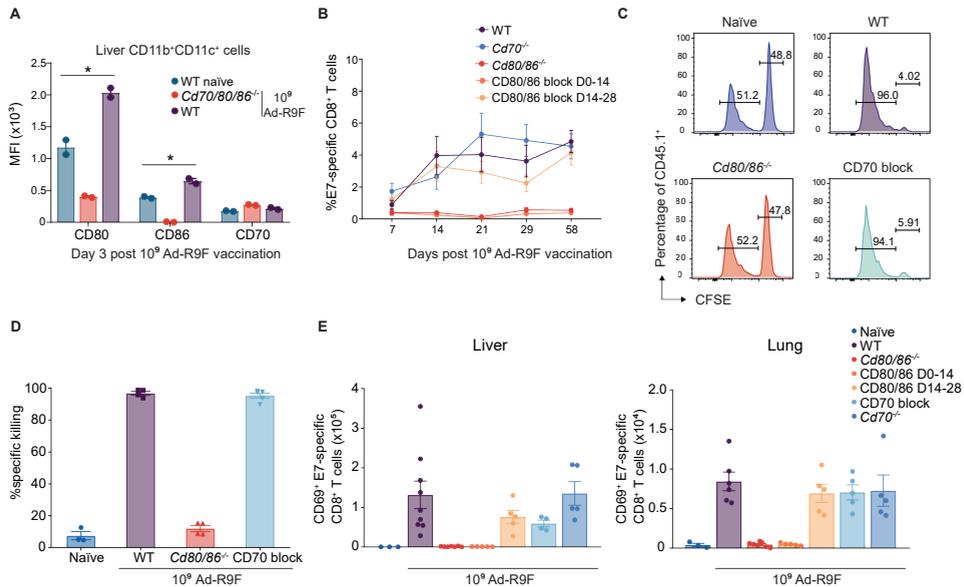


Figure 5. CD80/86-mediated costimulation is required for the development of inflationary E7-specific CD8⁺ T cell responses and T_{RM} cell formation. (A) Expression levels of CD80, CD86 and CD70 on CD11b⁺CD11c⁺ cells in the liver 3 days post-intravenous vaccination with 10⁹ IU Ad-R9F. One-way ANOVA was used for statistical analysis. *P<0.05. Experiments were performed twice with similar outcome. (B) Kinetics of E7-specific CD8⁺ T cells after 10⁹ IU Ad-R9F intravenous vaccination. CD80/86 or CD70 blocking antibodies were administered during indicated time periods. (C) In vivo cytotoxicity assay. Target ratio of specific (CFSE high) and non-specific (CFSE low) peptide-loaded CD45.1⁺ splenocytes 1 day after adoptive transfer in C57BL/6 mice 50 days after 10⁹ IU Ad-R9F vaccination. (D) In vivo cytotoxicity assay. Percentage of specific killing of target CD45.1⁺ cells. (E) E7-specific CD8⁺ T_{RM} cell numbers in the liver and lung at day 50 post 10⁹ IU Ad-R9F intravenous vaccination. Data are expressed as mean±SEM. ANOVA, analysis of variance; IU, infectious units.

Enhancement of T_{RM} cell formation by CTLA-4 blockade leads to delayed tumor outgrowth

Next, we evaluated whether enhancing CD80/86-mediated costimulation by blocking the inhibitory receptor CTLA-4, which competes with CD28 for CD80/86, could positively impact the formation of adenoviral vaccine-elicited CD8⁺ T_{RM} cells, and hence the protection against subcutaneous tumors. To create a therapeutic window, mice were vaccinated with 10⁷ IU Ad-R9F and subsequently treated with CTLA-4 blocking antibodies during the first 2 weeks after vaccination. Blockade of CTLA-4 delayed tumor outgrowth as compared with control treated vaccinated mice, and this delay of tumor outgrowth was dependent on T_{RM} cells (**Figure 6A, B**). Although CTLA-4 blockade resulted in similar frequencies of circulating E7-specific CD8⁺ T cells, a large portion of these cells displayed an effector-memory phenotype (**Figure 6C**).

Moreover, the formation of CD8⁺ T_{RM} cells in the liver was enhanced by CTLA-4 blockade (Figure 6D). Thus, temporal improving CD80/CD86-mediated costimulation increases CD8⁺ T_{RM} cell formation leading to delayed tumor outgrowth.

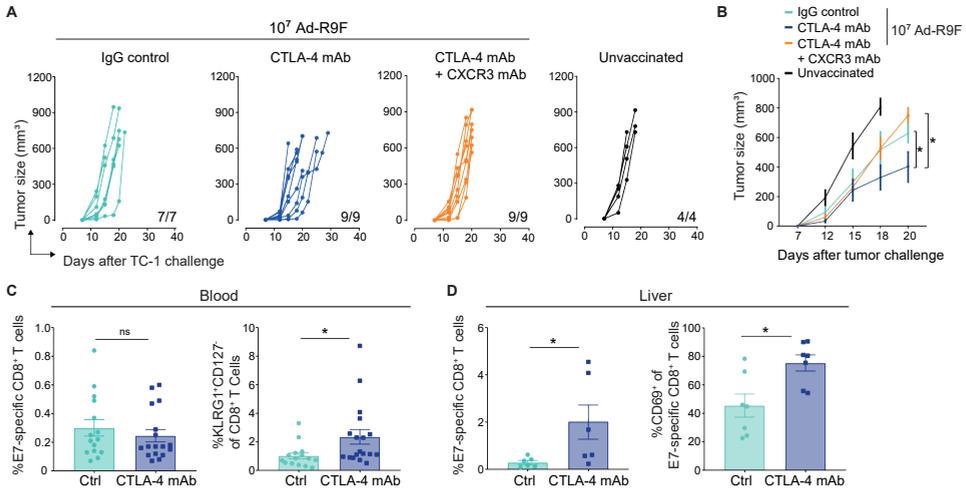


Figure 6. Enhancement of T_{RM} cell formation by CTLA-4 targeting leads to delayed tumor outgrowth. (A) Tumor outgrowth of mice challenged with TC-1 tumor cells (subcutaneously in the flank) at day 50 after 10⁷ IU Ad-R9F intravenous vaccination. CTLA-4 monoclonal antibody (200 µg IP) was administered twice per week for two weeks starting the day of vaccination. CXCR3 depleting antibody (200 µg IP) and control IgG antibody (200 µg IP) were administered twice per week starting 4 days before tumor challenge. (B) Mean tumor size of mice intravenous vaccinated with 10⁷ IU Ad-R9F, and treated with CTLA-4, CXCR3 or control IgG antibody IP. (C) Percentage and phenotype of CD8⁺ T cells in blood 21 days after intravenous vaccination with 10⁷ IU Ad-R9F and treatment with blocking CTLA-4 antibody (200 µg IP). (D) Percentage of E7-specific CD8⁺ T cells and E7-specific CD8⁺ T_{RM} cells in the liver at day 50 post vaccination and treatment with blocking CTLA-4 antibody (200 µg IP). CTLA-4 blockade was provided for two weeks post vaccination. Data shown are pooled from 2 independent experiments (n=3-6 per group). One-way ANOVA or Student's t-test was used for statistical analysis. Data are expressed as mean±SEM. *P<0.05, **P<0.01. ANOVA, analysis of variance; IP, intraperitoneally; IU, infectious units; ns, not significant.

Discussion

Here, we show that adenoviral vaccine vectors expressing E7 antigens from HPV induce E7-specific memory inflation and associated formation of T_{RM} cells. In particular, T_{RM} cell development was instrumental in providing protection against subcutaneous tumors and metastasized tumors in the liver. To this end, it was unknown which cellular subsets induced on adenoviral vaccination are implicated in protection. The induction of memory inflation and ensuing T_{RM} development depended on early CD80/CD86-mediated costimulatory interactions, and enhancing this costimulatory communication by blocking the interaction of CD80/CD86 with the inhibitory receptor CTLA-4, improved T_{RM} cell formation leading to increased protection.

We used replication-deficient adenoviral vaccine vectors (18, 19), modified to elicit CD8⁺ T cell responses against the E7 oncogene of HPV as a traceable model to study the development of tumor-specific memory T cell populations and their connection to protection. Large populations of circulating E7-specific memory CD8⁺ T cells with an effector-memory-like phenotype were maintained on high-dose vaccination. This resembles CMV infection, where the viral inoculum dose determines the degree of memory T cell inflation (21, 41). In contrast to CMV, adenoviral-vectors do not replicate, however, antigen likely persists at low levels. Bolinger *et al* showed that even without replication of the adenoviral vaccines, low-level antigen is expressed at late time points after vaccination. This persistence of antigen after vaccination was tested by adoptive transfer of CFSE-labeled TCR transgenic CD8⁺ T cells into previously vaccinated recipients (18). Even 100 days after vaccination, adoptively transferred T cells became activated and proliferated. Although the induction pathway of inflationary adenoviral induced T cells is distinct from inflationary CMV-specific T cells, the sustained memory inflation induced by adenoviral vectors and CMV is closely related, in both mouse and human (42, 43). Strikingly, we found that the induction and magnitude of inflationary CD8⁺ T cells is linked to an increase in T_{RM} cells.

For various infections, the route of infection is crucial for the magnitude and location of T_{RM} cell formation (reviewed in 44). Consistent with the liver being the site of infection on systemic administration of the adenoviral vaccine (15–17), we found that E7-specific CD8⁺ T_{RM} cells were stably maintained in the liver for months after adenoviral vaccination. The antigen persistence in the liver on adenoviral vector immunization may explain both the phenomenon of memory T cell inflation as well as the maintenance of liver T_{RM} cells, since T_{RM} cell formation is linked to memory inflation and the T_{RM} cells can be enhanced by both local antigen presentation and inflammation (45–49).

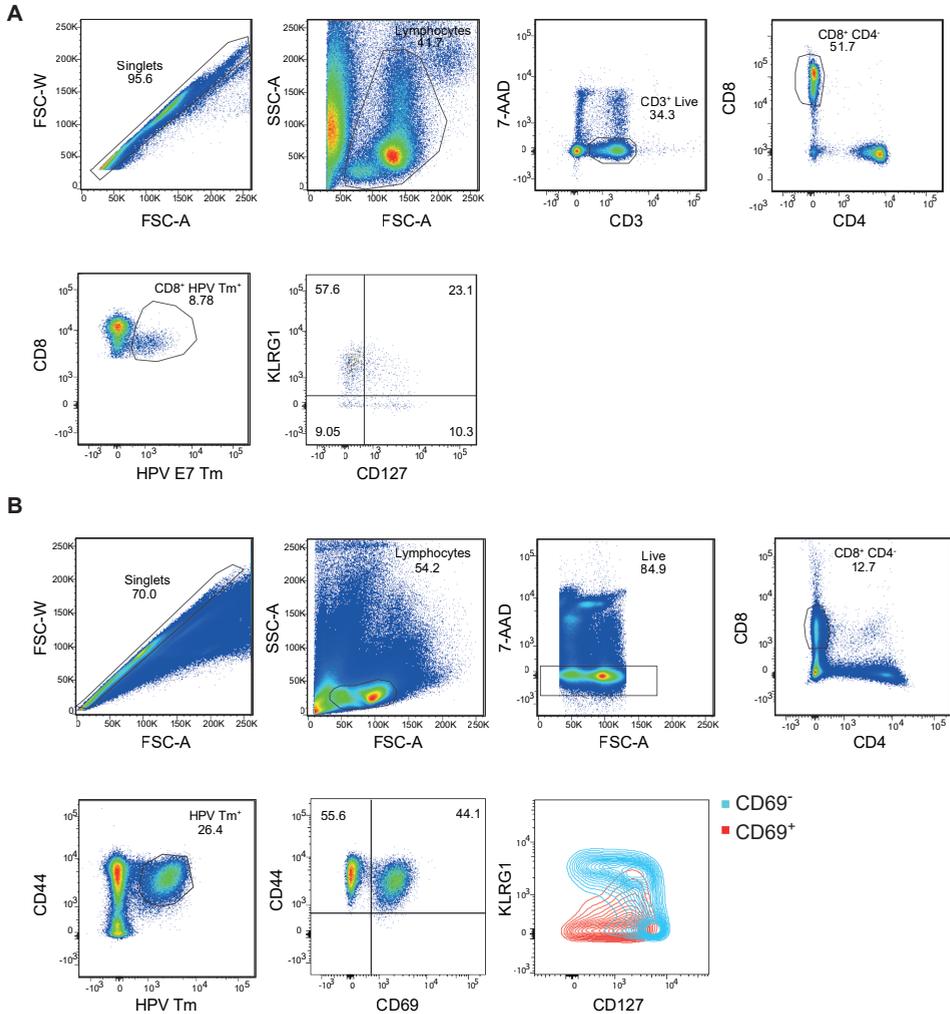
Previously, Ad35-based vectors eliciting E7-specific T cell responses showed protection against subcutaneous tumor protection but the mode of action remained unclear (50, 51). Here, we found for the first time a crucial role for T_{RM} cells for the adenovirus-mediated tumor protection in both liver and skin. CMV-based vaccines can also mediate tumor protection and this correlates to circulating inflationary CD8⁺ T cells (52), but whether T_{RM} cells are implicated for protection is unknown. Circulating T cells are also important, and our results indicate that these cells are a crucial source for the formation of tumor-resident CD8⁺ T cells. Recombinant

vaccinia viruses also mediate tumor protection that rely on both circulating and T_{RM} cells, and collaboration between these subsets leads to optimal antitumor immunity (53).

The characteristics of effector and memory antigen-specific $CD8^+$ T cell responses are shaped by pathogen-specific differential utilization of costimulatory pathways (54). Herein, we demonstrate that the initial expansion of adenovirus-vectored vaccine-specific $CD8^+$ T cells depends completely on CD80/CD86 costimulatory interactions, which is dissimilar to the induction of memory inflation on MCMV infection (33). In addition, CD70-mediated costimulation is redundant for adenoviral induced T cell responses whereas this type of costimulation is important for the development of mouse CMV-specific T cell immunity (55). CTLA-4 generates inhibitory signals that counteract the costimulation provided via CD28 by competing directly with CD28 for its ligands CD80/CD86 (56, 57), but CTLA-4 can also actively remove its ligands CD80/CD86 by internalizing them for degradation (58). Blocking CTLA-4 is thought to promote especially the priming of $CD8^+$ T cells (59, 60). In line with an early impact of CD80/CD86 costimulatory interactions, we here show that initial targeting of CTLA-4 after immunization increases $CD8^+$ T_{RM} cells and therefore also protective immunity. It is of interest to further explore whether directly targeting CD28 or targeting other costimulatory receptors could also lead to enhanced T_{RM} cell formation.

To conclude, here we show that $CD8^+$ T_{RM} cells are an important protective cellular subset induced by adenoviral vectors. Moreover, the formation of T_{RM} cells is linked to the magnitude of memory inflation in the circulation, and this process can be enhanced by targeting CD28-mediated costimulation. Thereby, this study provides new insights for future development of adenoviral vaccine strategies with an emphasis on the induction of T_{RM} cells.

Supplementary information



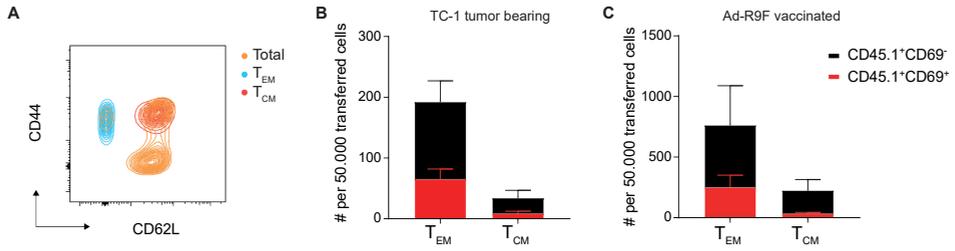


Figure S2. A) Phenotype of adoptively transferred CD8⁺ T cells. Expression of CD44 and CD62L on sorted effector and central-memory T cell subsets from Ly5.1 mice before adoptive transfer to recipient mice. B, C) Absolute number of CD45.1⁺CD8⁺CD69⁺ T cells and CD45.1⁺CD8⁺CD69⁻ T cells in liver per 50,000 transferred T_{EM} or T_{CM} cells. Transferred cells were isolated from Ad-R9F vaccinated mice and injected into TC-1 tumor bearing mice (B) or mice that were previously vaccinated with 10⁸ IU Ad-R9F (C).

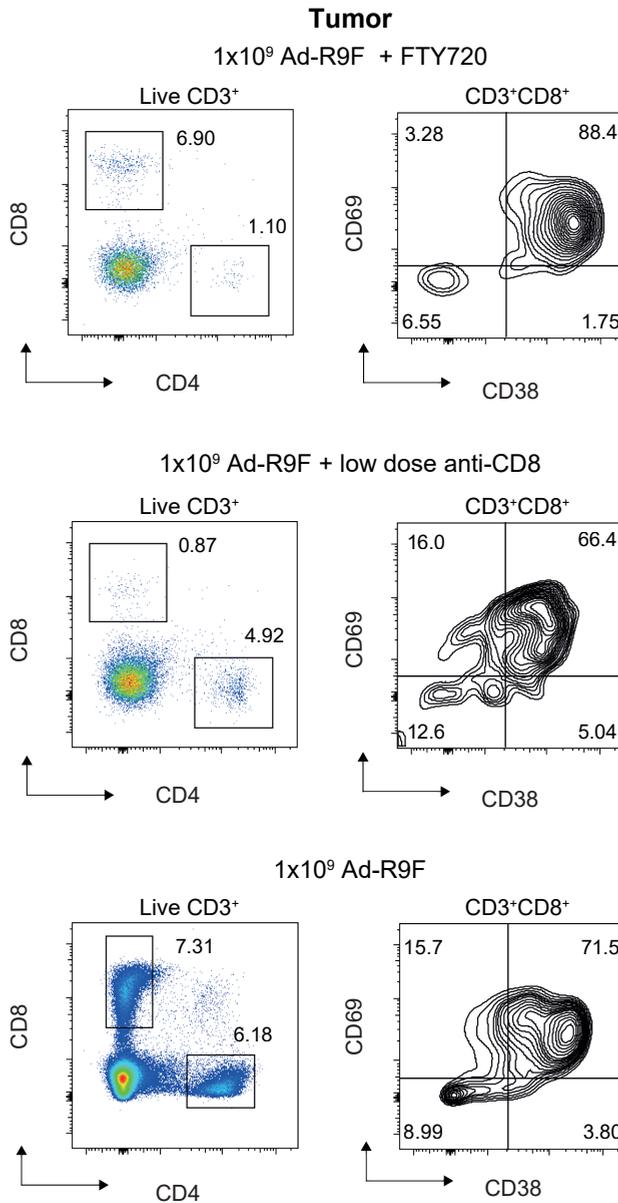


Figure S3. Phenotype of CD8⁺ T cells isolated from tumors at day 20 after TC-1 tumor challenge. Mice were challenged with TC-1 tumor cells (subcutaneously in the flank) at day 50 after IV vaccination with 10⁹ IU Ad-R9F. Low dose CD8 depleting antibody (20 μg IP) or FTY720 (50 μg IP) was administered twice per week starting 4 days before tumor challenge.

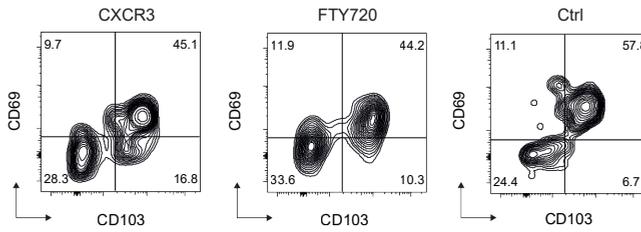


Figure S4. Phenotype of skin CD8⁺ T cells at the location of subcutaneous TC1 tumors at day 20 after tumor challenge. Mice were challenged with TC-1 tumor cells (subcutaneously in the flank) at day 50 after IV vaccination with 10⁹ IU Ad-R9F. CXCR3 depleting antibody (200 µg IP) or FTY720 (50 µg IP) was administered twice per week starting 4 days before tumor challenge.

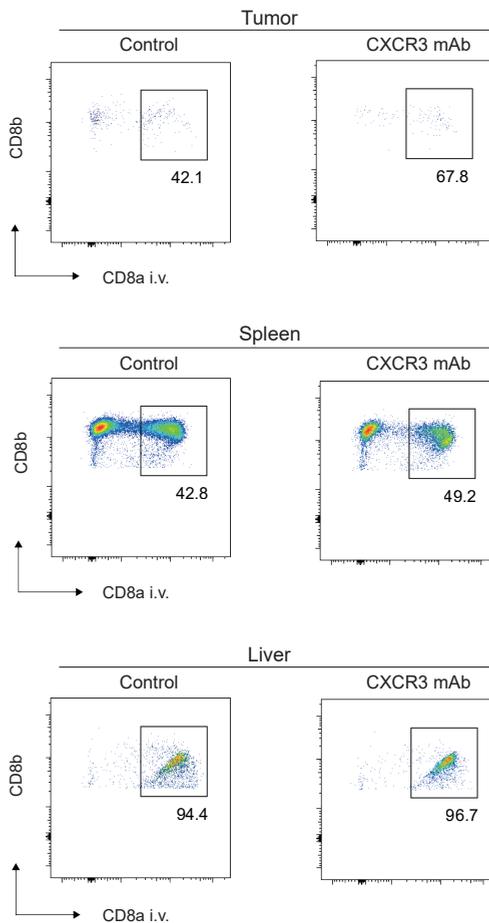


Figure S5. Infiltration of IV labeled CD8⁺ T cells in tumor, spleen and liver at day 20 after TC-1 tumor challenge. Mice were challenged with TC-1 tumor cells (subcutaneously in the flank) at day 50 after IV vaccination with 10⁹ IU Ad-R9F. CXCR3 depleting antibody (200 µg IP) was administered twice per week starting 4 days before tumor challenge. 3 minutes before sacrifice, mice were injected IV (retro-orbitally) with 3 µg CD8a APC (clone 53.6-7, BioLegend).

Ethics approval

All animal experiments were approved by the institutional Animal Experiments Committee and were executed according to the institutional animal experimentation guidelines and were in compliance with the guidelines of the Institutional, and European animal care and usage committees.

Author contributions

EG and RA contributed to the study conceptualization and study design. EG, MS, SD, AY and FB performed data acquisition. JC, LL, HY, KG, LH, FK, PK were involved in developing methodology and interpretation of data. The manuscript was written by EG and RA and edited by all authors. RA supervised the study.

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