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Intratumor childhood vaccine-specific CD4$^+$ T-cell recall coordinates antitumor CD8$^+$ T cells and eosinophils

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ABSTRACT

Background Antitumor mechanisms of CD4$^+$ T cells remain crudely defined, and means to effectively harness CD4$^+$ T-cell help for cancer immunotherapy are lacking. Pre-existing memory CD4$^+$ T cells hold potential to be leveraged for this purpose. Moreover, the role of pre-existing immunity in virotherapy, particularly recombinant poliovirus immunotherapy where childhood polio vaccine specific immunity is ubiquitous, remains unclear. Here we tested the hypothesis that childhood vaccine-specific memory T cells mediate antitumor immunotherapy and contribute to the antitumor efficacy of polio virotherapy.

Methods The impact of polio immunization on polio virotherapy, and the antitumor effects of polio and tetanus recall were tested in syngeneic murine melanoma and breast cancer models. CD8$^+$ T-cell and B-cell knockout, CD4$^+$ T-cell depletion, CD4$^+$ T-cell adoptive transfer, CD40L blockade, assessments of antitumor T-cell immunity, and eosinophil depletion defined antitumor mechanisms of recall antigens. Pan-cancer transcriptome data sets and polio virotherapy clinical trial correlates were used to assess the relevance of these findings in humans.

Results Prior vaccination against poliovirus substantially bolstered the antitumor efficacy of polio virotherapy in mice, and intratumor recall of poliovirus or tetanus immunity delayed tumor growth. Intratumor recall antigens augmented antitumor T-cell function, caused marked tumor infiltration of type 2 innate lymphoid cells and eosinophils, and decreased proportions of regulatory T cells (Tregs). Antitumor effects of recall antigens were mediated by CD4$^+$ T cells, limited by B cells, independent of CD40L, and dependent on eosinophils and CD8$^+$ T cells. An inverse relationship between eosinophil and Treg signatures was observed across The Cancer Genome Atlas (TCGA) cancer types, and eosinophil depletion prevented Treg reductions after polio recall. Pretreatment polio neutralizing antibody titers were higher in patients living longer, and eosinophil levels increased in the majority of patients, after polio virotherapy.

Conclusion Pre-existing anti-polio immunity contributes to the antitumor efficacy of polio virotherapy. This work defines cancer immunotherapy potential of childhood vaccines, reveals their utility to engage CD4$^+$ T-cell help for antitumor CD8$^+$ T cells, and implicates eosinophils as antitumor effectors of CD4$^+$ T cells.

WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ Intratumor reactivation, or recall, of memory T cells has been shown to mediate antitumor effects in preclinical models. Whether pre-existing immunity is an asset to intratumor virotherapy remains contentious, and the antitumor mechanisms of memory T-cell recall remain undefined.

WHAT THIS STUDY ADDS

⇒ Polio and tetanus-specific CD4$^+$ T cells mediate antitumor efficacy after intratumor recall by engaging antitumor functions of eosinophils and potentiating antitumor CD8$^+$ T-cell function; pre-existing, polio-specific CD4$^+$ T cells potentiate the antitumor efficacy a recombinant poliovirus (Lerapolturev) currently being tested in clinical trials.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

⇒ This study implies that intratumor delivery of childhood vaccine associated antigens can mediate cancer immunotherapy and defines eosinophils as key antitumor effectors of memory CD4$^+$ T cells.

INTRODUCTION

Although CD4$^+$ T cells are key mediators of adaptive immune functionality and memory,[1–3] routes to harness their potential for cancer immunotherapy are lacking. Adaptive immune memory enables robust immune responses to previously encountered pathogens. Accordingly, recall responses, that is, activation of adaptive memory cells by cognate antigen, orchestrate localized innate and adaptive inflammation.[4 5] Based upon recent work demonstrating cancer immunotherapy utility of intratumoral antiviral CD8$^+$ T-cell responses,[6 7] we hypothesized that tumor-localized, childhood vaccine-associated CD4$^+$ T-cell recall may engage antitumor functions of CD4$^+$ T cells.

PVSRIPO (now known as 'Lerapolturev'), the live-attenuated poliovirus type 1 (Sabin) vaccine modified with the internal ribosomal

entry site of human rhinovirus type 2,[8] has shown early evidence of efficacy in recurrent glioblastoma (rGBM)[9] and recurrent, non-resectable melanoma[10 11] after intratumor (i.t.) administration. Poliovirus (polio) vaccination is part of the standard pediatric immunization schedule worldwide, either with the live attenuated (Sabin), or the inactivated (IPOL) vaccines. The coding sequence of PVSRIPO is identical to the type 1 Sabin vaccine. Pre-existing serum anti-PVSRIPO/polio antibody reactivity was confirmed in all patients receiving PVSRIPO therapy.[9 10 12] Moreover, clinical use of PVSRIPO entails prior boost with trivalent IPOL at least 1 week before its i.t. administration, which caused serum PVSRIPO neutralizing antibody increases in all patients.[9 10] The antitumor effects of polio virotherapy encompass neoplastic cell damage and sublethal viral infection of myeloid cells driving sustained type I interferon (IFN) signaling.[12–15] Anti-polio immune memory likely impedes PVSRIPO replication within the tumor, but may provide an alternate antitumor mechanism of action through i.t. recall of polio vaccine specific T cells.

Using mouse tumor models of melanoma and breast cancer, we demonstrate that pre-existing immunity to polio potentiates the antitumor efficacy of polio virotherapy by engaging antitumor functions of $CD4^+$ T cells. I.t. polio or tetanus recall triggered marked $CD4^+$ T cell, type 2 innate lymphoid cell (ILC2), and eosinophil influx; mediating antitumor efficacy through $CD8^+$ T cells and eosinophils in a CD40L independent manner. In cohorts of patients with rGBM treated with Lerapolturev (PVSRIPO), higher levels of pretreatment polio neutralizing antibodies were associated with longer survival, and peripheral induction of eosinophils were observed in patients with melanoma after i.t. treatment with Lerapolturev. Thus, polio virotherapy and childhood vaccine associated antigens coordinate antitumor type I and II immunity via $CD4^+$ T-cell recall.

MATERIALS AND METHODS

Extended materials and methods are presented in online supplemental information.

Mice, cell lines, viruses, poly(I:C), and in vivo grade antibodies

hCD155-tg C57BL/6 mice were a gift of Satoshi Koike (Tokyo, Japan). Wildtype (wt) (#000664), CD8 knockout (k/o) (#002665), B-cell k/o (#002288), OT-I (#003831), and CD45.1 C57BL/6 mice (#002014) were from The Jackson Laboratory. OT-I and CD45.1 C57BL/6 mice were crossed to generate CD45.1+OT-I mice. B16.F10 (American Type Culture Collection), E0771 (G. Palmer, Duke University, USA), $E0771^{hCD155}$, $B16.F10^{hCD155}$, and $B16.F10.9^{hCD155}$-OVA cells were grown in high-glucose Dulbecco's Modified Eagle Medium (DMEM, Gibco) containing 10% fetal bovine serum (FBS) (Sigma-Aldrich). $B16.F10.9^{hCD155}$-OVA, $B16\text{-}F10^{hCD155}$, and $E0771^{hCD155}$ cells were previously derived.[12 13] All cell lines were confirmed to be mycoplasma negative. Laboratory grade PVSRIPO, mouse-adapted PVSRIPO (mRIPO), and UV-inactivated PVSRIPO (UVP) were generated in HeLa cells followed by size exclusion purification of the resultant supernatant as previously described.[12 13] PVSRIPO was used for vaccination; UVP was used to isolate effects of polio capsid (which contains polio vaccine-specific epitopes); mRIPO is a mouse adapted version of PVSRIPO that was used to test the full impact of polio virotherapy in mice. VacciGrade high molecular weight poly(I:C) (InvivoGen) was reconstituted per manufacturer instructions. In vivo grade antibodies to interleukin (IL)-5 (TRFK5) or control (HRPN), CD4 (GK1.5) or control (LTF-2), CD40L (MR-1) or control (#BP0091), and CD40 (FGK4.5) or control (2A3) were from Bio X Cell.

Vaccines, immunizations, and i.t. viral titers

Unless otherwise indicated, vaccines using PVSRIPO (1×10^7 plaque forming units (pfu)/mouse), tetanus toxoid (Tet) (MilliporeSigma; 0.5 µg/mouse), or hemocyanin-keyhole limpet (KLH, Sigma-Aldrich; 100 µg/mouse) were diluted in phosphate buffered saline (PBS) with Alhydrogel (1:1; InvivoGen); 50 µL of vaccine was administered bilaterally in the quadriceps muscles. Combined immunizations of IPOL and Tenivac (Sanofi Pasteur) were administered unilaterally for each vaccine. Vaccine boosts occurred 14 days later. For i.t. viral titers, tumors were harvested, weighed, and mechanically homogenized in 1 mL PBS. Homogenate was tested by plaque assay.[16]

Murine tumor model experiments

For B16 implantations, 2×10^5 cells were implanted subcutaneously into the flank of male and female mice; for E0771 implantations 5×10^5 cells were implanted into the fourth mammary fat pad of female mice. hCD155-tg C57BL/6 mice were used with mRIPO to recapitulate polio virotherapy (active viral replication ± pre-existing immunity); whereas wt C57BL/6 mice, which are non-permissive to polio, were used with UVP to isolate the effects of polio recall. Tumors were treated with either DMEM or PBS (vehicle), mRIPO (1×10^7 pfu), UVP (1×10^8 pre-inactivated pfu), Tet (0.5 µg), and/or poly(I:C) (30 µg) as indicated in figure legends. Treatment groups were randomized by tumor volume (caliper measurements, using the equation $L \times W \times W/2$) at the first day of treatment. Mice were euthanized when tumor volume exceeded 1000 mm^3, unless preceded by ulceration (which was infrequent and not associated with any particular treatment), in which case mice were excluded from the study. Group sizes were based on power assessments from pilot experiments, or prior experience.[12] Tumor measurements were performed blinded to the i.t. treatment group starting after the last dose of i.t. therapy/antigen. Mice with outlier starting tumor volume at the time of randomization (1 SD from the mean) were excluded.

Flow cytometry analysis of tumors

Tumors were harvested at time points denoted in figure legends and dissociated in RPMI-1640 media (Thermo Fisher) containing 100 µg/mL Liberase-TM (Sigma-Aldrich) and 10 µg/mL DNAse I (Roche) for 30 min at 37°C, followed by passage through a 70 µM (Olympus Plastics) cell strainer, centrifugation, and washing in PBS. For experiments using Zombie Aqua (BioLegend), cells were stained with Zombie Aqua in PBS (1:500) following manufacturer instructions. Cell suspensions were then incubated with 1:50 mouse TruStain FcX (BioLegend) followed by panel-specific staining in PBS containing 2% FBS. Staining of intracellular transcription factors, cytokines, and granzyme B was accomplished using the Foxp3/Transcription Factor Staining Buffer Set (Thermo Fisher) following manufacturer instructions. See online supplemental information for information about panels and antibodies used. Data were collected on a Fortessa X20 at the Duke Cancer Institute Flow Cytometry Core Facility; FCS files were analyzed using FlowJo V.10 (BD Biosciences). Gating strategies are presented in online supplemental information; isotype controls, fluorescence minus one controls, and comparison to established negative cell populations were used to define positivity.

Clinical trial associated analyses

PVSRIPO neutralization titer assays were performed per the clinical trial protocol,[9] and values for both rGBM clinical trials were used from clinical trial enrollment assays. Phase I rGBM (NCT01491893) polio titers were previously reported[9] and survival was updated as of April 29, 2020; phase II (NCT02986178) survival was updated as of April 27, 2021. Melanoma (NCT03712358)[10 11] clinical trial participant percentages of white blood cells were acquired from clinical complete blood count (CBC) tests.

Statistical analysis and clinical trial cohorts

Assay-specific statistical tests are indicated in the corresponding figure legends. GraphPad Prism V.8 was used to perform statistical analyses. Two-way analysis of variance (ANOVA) was used to assess the difference in tumor growth over time between groups, and ANOVA post hoc testing was used to account for multiple comparisons unless otherwise noted in the figure and/or figure legend. A statistical probability of ($p<0.05$) was used unless otherwise noted; all p values are two-tailed. All data points reflect individual mice or patients.

RESULTS

Polio immunization potentiates polio virotherapy

We first tested anti-polio antibody production (ie, seroconversion) in mice transgenic for the human poliovirus receptor CD155 (hCD155-tg) on immunization with IPOL or PVSRIPO (to mimic type 1 Sabin) with and without alum adjuvant (Alhydrogel; ALH), part of licensed vaccine formulations (eg, Pentacel, Pediarix, Kinrix).[17] A duration of 45 days between initial immunization and tumor implantation allowed establishment of immunological memory.[18] IPOL achieved limited seroconversion and PVSRIPO immunization elicited a stronger antibody response; ALH bolstered antibody responses to both (online supplemental figure S1A,B). To recapitulate high levels of anti-polio antibodies in patients with cancer,[9] we chose PVSRIPO+ALH (hereafter 'polio') vaccination to determine the role of pre-existing polio immunity in PVSRIPO immunotherapy. Murine tumor models and mice expressing hCD155 were previously developed to permit entry and replication of PVSRIPO, and PVSRIPO was adapted to murine cells to recapitulate viral replication in murine cancer cells (mRIPO).[13 19] In prior studies in polio vaccine naïve, syngeneic mouse tumor models, a single i.t. injection of mRIPO required programmed cell death protein-1 (PD1)/programmed death ligand-1 blockade or tumor expression of the immunogenic ovalbumin (OVA) protein to mediate durable antitumor effects.[12 13] However, i.t. mRIPO mediated durable antitumor efficacy in polio immunized mice in melanoma (B16) and breast (E0771) cancer models, relative to control (KLH) immunized counterparts (figure 1A,B, online supplemental figure S1C). Thus, prior polio immunization bolsters the antitumor efficacy of polio virotherapy.

Polio virotherapy is associated with T-cell inflammation within the tumor.[12 13] mRIPO replication within tumors was substantially reduced in polio immunized mice 2 days and 5 days post-treatment (figure 1C,D), consistent with high neutralizing antibody titers (online supplemental figure S1B). Yet, we observed increased total immune cell density ($CD45.2^+$) in polio immunized mice after mRIPO therapy, explained largely by an influx of conventional $CD4^+$ T cells and myeloid cells ($CD11b^+$, $Ly6G^{Neg}$, $F4/80^+$) (figure 1E, online supplemental figures S2A,S3). Subsequent analysis revealed that the increased $CD11b^+$ myeloid cells in polio immunized mice treated with mRIPO were eosinophils (figure 1F); levels of dendritic cells (DCs) were not significantly altered. Elevated tumor necrosis factor (TNF), granulocyte-macrophage colony-stimulating factor (GM-CSF), IL-1β and IL-17A levels in tumor homogenate and higher IL-4 and IL-5 in explanted tumor draining lymph node (TDLN) cultures from polio immunized mice treated with mRIPO indicated distinct inflammatory responses after mRIPO therapy (online supplemental figure S2B). GM-CSF and IL-5 are known inducers of eosinophil production and recruitment,[20] possibly explaining eosinophil influx in tumors (figure 1F). Conventional $CD4^+$ T cells and eosinophil infiltration in response to mRIPO treatment of polio immunized mice were also observed in a separate model, E0771 (online supplemental figure S2C,D). Thus, pre-existing immunity to polio accentuates inflammatory responses to mRIPO that are associated with enhanced antitumor efficacy.

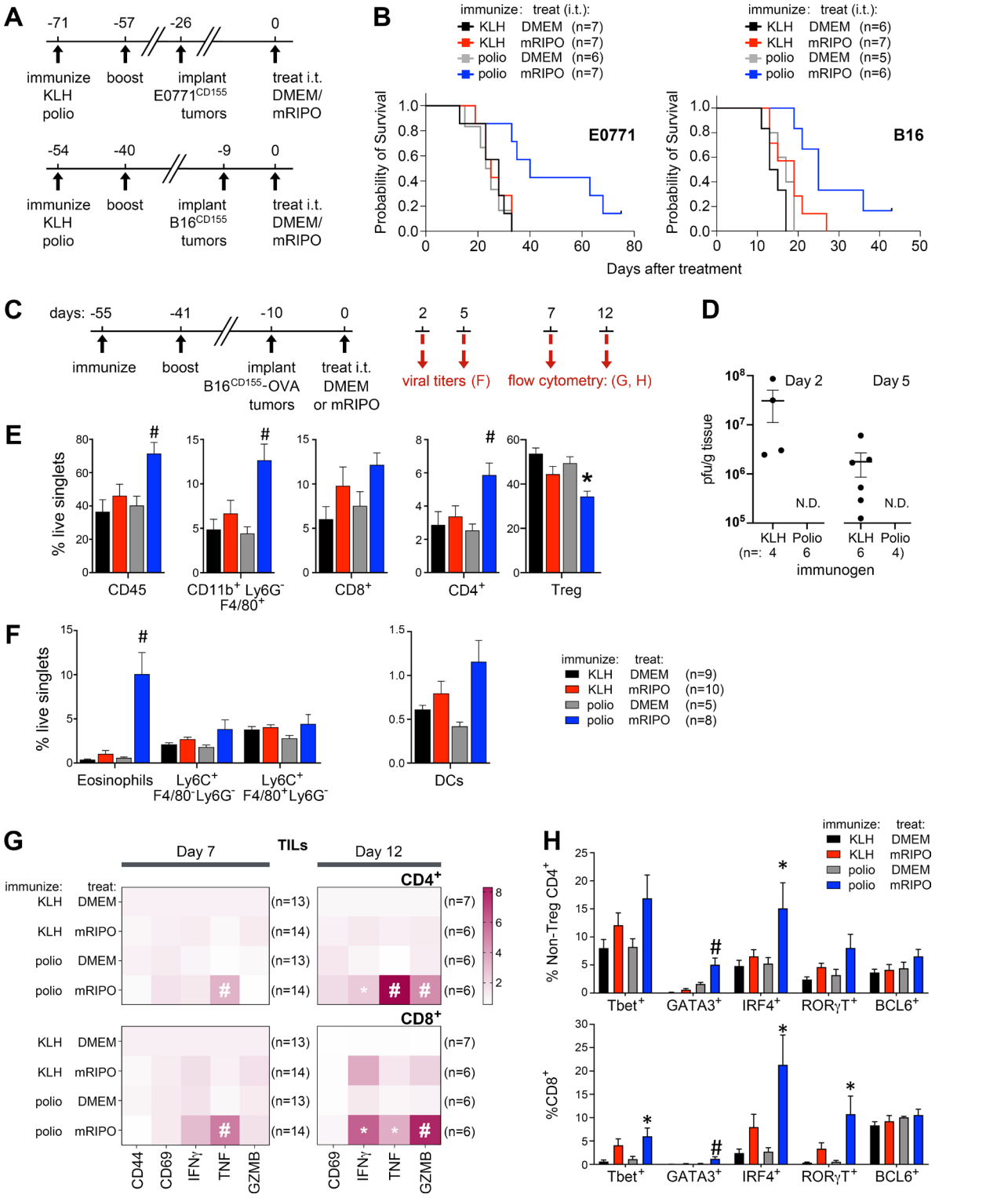

Figure 1 Polio immunization potentiates antitumor and inflammatory efficacy of polio virotherapy. (A) Polio or KLH immunized hCD155-tg mice bearing hCD155-tg B16 or E0771 tumors were treated with DMEM (control) or mRIPO. (B) Survival cut-off was tumor volume >1000 mm^3; see online supplemental figure S1C; representative from two experiments. (C) Schema for experiments in D–H. (D) Tumor homogenate viral titers post-mRIPO; N.D., not detected. (E) Flow cytometry analyses of tumors at day 7 (n=13/group mock; n=14/group mRIPO). (F) Flow cytometry analyses for myeloid cells and dendritic cells (DCs: $Ly6C^{Neg}$, $F480^{Neg}$, $CD11c^{+}$, IA/IE^{+}) at day 11. (G) Activation markers in TILs at days 7 and 12; pooled from two experiments; fold mean KLH-DMEM values are shown. (H) TIL transcription factor expression at day 12; same samples as day 12 of (A); representative data of three repeats are shown. See online supplemental figures S1–S4 for extended data. (E–H) Tukey's post hoc test $p<0.05$ versus mock controls (*) or all other groups (#). Data bars and brackets indicate mean+SEM. mRIPO, mouse adapted PVSRIPO; DMEM, Dulbecco's Modified Eagle Medium; IFN, interferon; i.t., intratumor; KLH, hemocyanin-keyhole limpet; OVA, ovalbumin; TIL, tumor-infiltrating lymphocyte; TNF, tumor necrosis factor.

Augmented T-cell functional phenotypes in polio immunized mice treated with mRIPO

$CD8^+$ and $CD4^+$ tumor-infiltrating lymphocytes (TILs) in polio vaccinated mice expressed higher levels of intracellular IFN-γ, TNF, and granzyme B post-mRIPO treatment, implying enhanced functional status (figure 1G, online supplemental figure S4). TILs from polio immunized mice treated with mRIPO also exhibited increased expression of the transcription factors Tbet, GATA3, and RORγt; as well as induction of IRF4, a promoter of T-cell activation and function (figure 1H).[21] Expression of the T-cell exhaustion markers PD1 and T cell immunoglobulin and mucin domain-containing protein 3 (TIM3) on $CD4^+$ T cells in the tumor and TDLNs of polio vaccinated mice treated with mRIPO were reduced (online supplemental figure S2E). Changes in T-cell activation/differentiation markers were consistent in the E0771 orthotopic breast cancer model (online supplemental figures S2F,G). Thus, recall responses to polio increase functional phenotypes in TILs after intratumoral polio virotherapy.

Polio and tetanus recall antigens mediate antitumor efficacy

Reovirus-specific memory $CD8^+$ T cells directly kill reovirus infected cancer cells during oncolytic reovirus therapy,[22] and tetanus-specific memory $CD4^+$ T cells kill cancer cells infected with *Listeria* expressing Tet.[23] Alternatively, inflammatory responses caused by memory T-cell recall in the tumor microenvironment (TME) may also bolster immune surveillance.[6 7] We hypothesized that recall-induced inflammation explained accentuated antitumor efficacy of polio virotherapy in polio vaccinated mice, since viral replication—required for production of viral antigen in tumor cells, oncolysis, and antiviral inflammation[12]—was sharply reduced in polio immunized mice (figure 1D). To probe antitumor effects of polio recall in the TME we used a model devoid of hCD155 (mice and tumors; wt C57BL/6 mice) and treated tumors with UV inactivated PVSRIPO (UVP) to preclude viral infection/replication. We included comparisons with another vaccine-associated recall antigen, Tet, which has been shown to mediate antitumor effects in other studies.[23 24]

I.t. therapy with UVP exerted antitumor efficacy exclusively in polio immunized mice; Tet treatment mediated transient antitumor effects in Tet immunized mice (figure 2A–C, online supplemental file 1). Natural recall responses occur in the presence of a localized innate immune response to pathogen replication. To mimic this, Tet or polio immunized mice were treated with poly(I:C) alone or in combination with UVP or Tet. Both UVP and Tet mediated pronounced antitumor effects in this context (figure 2D). These data reveal that i.t. recall of memory T cells in the TME—independent of the expression/major histocompatibility complex (MHC) presentation of their cognate antigen by malignant cells—mediates antitumor efficacy.

$CD4^+$ T cells mediate the antitumor efficacy of recall antigens

To determine which adaptive compartment(s) explain the antitumor efficacy of polio recall responses (independent of viral replication), we compared i.t. treatment with UVP in $CD8^+$ T-cell and B-cell k/o mice relative to wt mice (figure 3A). CD4 k/o mice were not tested due to the role of $CD4^+$ T cells in enabling both $CD8^+$ T-cell and B-cell responses to vaccination. As expected, polio immunization in wt and CD8 k/o mice, but not B-cell k/o mice, led to anti-PVSRIPO antibody production (figure 3B). B16 tumor growth was similar in each genetic context after mock treatment. Relative to wt mice, the antitumor efficacy of UVP in polio immunized mice was limited in CD8 k/o mice at later time points, but was enhanced in B-cell k/o mice (figure 3C; p=0.007 wt vs B-cell k/o UVP treated curves, two-way ANOVA). Moreover, B-cell k/o did not prevent the influx of eosinophils or $CD4^+$ TILs (online supplemental figure S6A) associated with polio recall (figure 1). Thus, the antitumor efficacy of polio recall is partially dependent on $CD8^+$ T cells, and is limited by B cells.

These observations, along with increased $CD4^+$ TILs after mRIPO therapy in polio immunized mice (figure 1E), imply that $CD4^+$ T cells dictate the antitumor efficacy of polio recall responses. Thus, we next tested the antitumor effect of polio recall with and without transient $CD4^+$ T-cell depletion (figure 3D, starting 1-day pretreatment). Despite robust depletion of $CD4^+$ T cells in the TDLN, $CD4^+$ T-cell depletion within the tumor was incomplete and preferentially reduced regulatory T cell (Treg) populations, possibly explaining modest antitumor effects and increased $CD8^+$ T-cell densities after $CD4^+$ T-cell depletion (figure 3D, online supplemental figure S6B). Nonetheless, $CD4^+$ T-cell depletion nearly ablated the antitumor efficacy of UVP in polio immunized mice and prevented recruitment of eosinophils in response to UVP (figure 3D); $CD4^+$ T-cell depletion also prevented the induction of granzyme B in $CD8^+$ T cells after UVP treatment (online supplemental figure S6B). Collectively these findings indicate that the antitumor and inflammatory effects of polio recall are $CD4^+$ T-cell dependent. Confirming that polio-specific $CD4^+$ T cells are sufficient to potentiate the antitumor efficacy of polio virotherapy (with active viral replication), adoptive transfer of $CD4^+$ T cells from spleens of polio immunized mice, but not that of Tet, bolstered the antitumor efficacy of mRIPO (figure 3E, online supplemental figure S6C).

I.t. recall antigen therapy potentiates antitumor $CD8^+$ T-cell function

Tumor-specific $CD4^+$ T cells were shown to directly kill tumor cells,[25–27] engage cytotoxic innate immune cells,[28 29] and provide help to effector $CD8^+$ T cells.[1–3 30] Consistent with the latter, antitumor effects of recall antigens were observed after delivery to the TME (figures 2 and 3), were partially dependent on $CD8^+$ T cells (figure 3C), and $CD8^+$ TILs had improved polyfunctional phenotypes after mRIPO therapy in polio vaccinated mice (figure 1G).

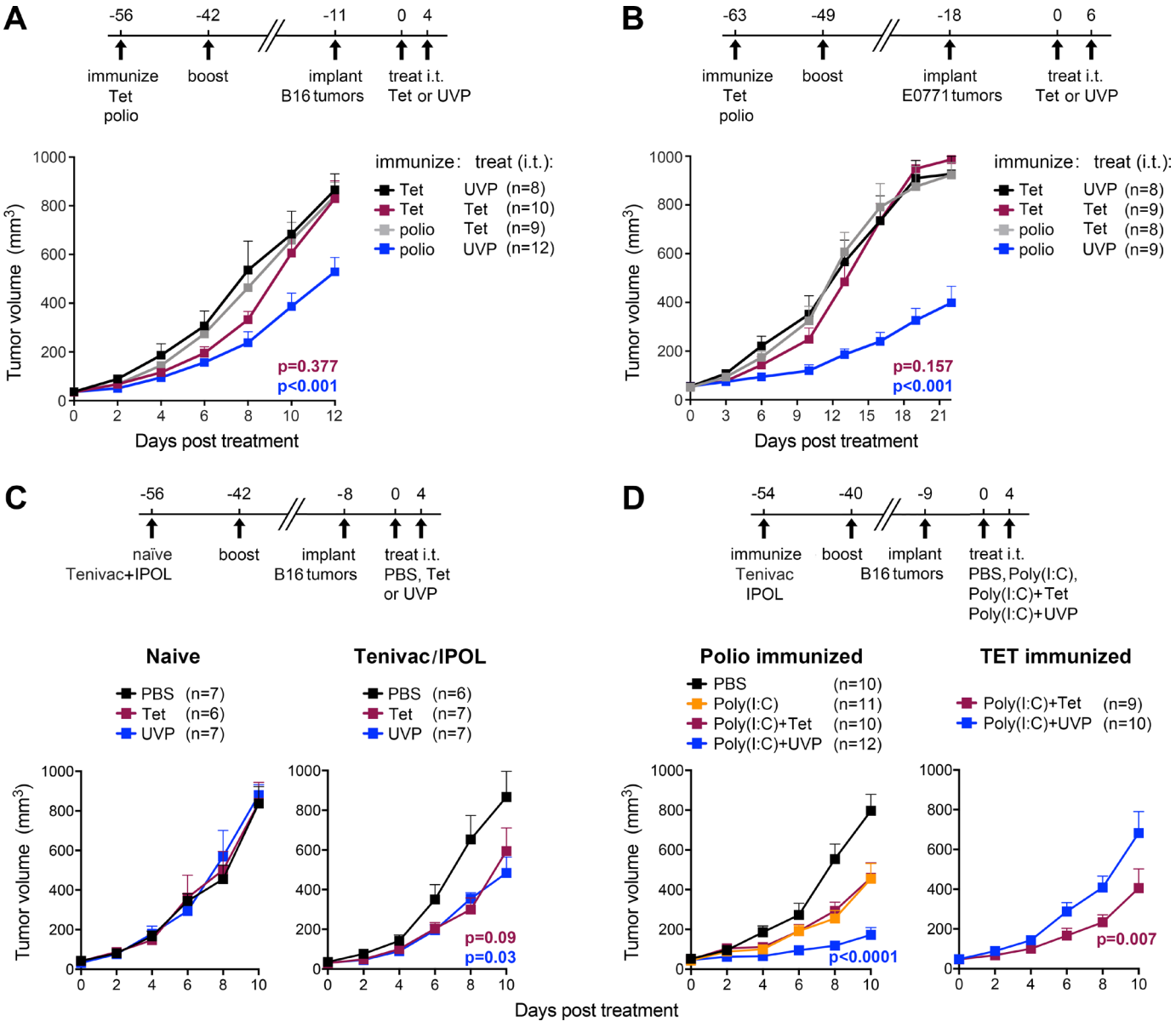

Figure 2 Polio and tetanus recall antigens mediate antitumor efficacy. B16 (A) or E0771 (B) tumor-bearing mice immunized with polio or Tet were treated intratumor with Tet or UV inactivated PVSRIPO (UVP). (C) Age-matched naïve or Tenivac and IPOL immunized mice were treated with PBS, Tet, or UVP. (D) Mice immunized as in (A, B) were treated intratumor with mock, poly(I:C) (30 µg), poly(I:C) + Tet, or poly(I:C) + UVP as shown. (A–D) Mean+SEM from a representative experiment of at least two repeats is shown; asterisks indicate Dunnett's test p<0.05 versus all other groups; online supplemental figure S5 presents extended data. IPOL, inactivated polio vaccine; i.t., intratumor; PBS, phosphate buffered saline; UVP, UV-inactivated PVSRIPO; Tet, tetanus toxoid.

Thus, we next sought to determine if polio (UVP) and Tet recall enhances the function of *antitumor* $CD8^+$ T cells. To this end, we adoptively transferred $CD45.1^+$ OT-I $CD8^+$ T cells (OVA-specific) to polio or Tet vaccinated mice and determined the impact of UVP and Tet-induced recall on B16-OVA OT-I TIL phenotypes (figure 4A). Induction of recall responses in the tumor after UVP or Tet was associated with delayed tumor growth and increased tumor infiltration of endogenous $CD45.2^+$ cells, eosinophils, and conventional $CD4^+$ T cells; notably, levels of antitumor OT-I T cells were non-significantly increased (figure 4B). However, analysis of tumor infiltrating OT-I $CD8^+$ T cells revealed enhanced granzyme B, TNF, and IFN-γ; and reduced expression of the terminal exhaustion marker TIM3 after polio or Tet recall (figure 4C). Varied Th-associated transcription factor expression in both OT-I and endogenous T cells was observed, including that of GATA3, RORγt, and BCL6 (figure 4C). Transcriptomic analysis of OT-I TILs after polio recall revealed increased expression of granzymes; genes linked with T-cell activation, function, or homeostasis (*Taok3, CD86, CCR5, Egr2, Adgre1, Vdr, IRF4,* and *BCL6*); genes associated with Th1 immunity (*Ptger4, Fgl2*); as well as genes associated with Th2 immunity (*Alox15, Ccl8,* and *GATA3*) (figure 4D,

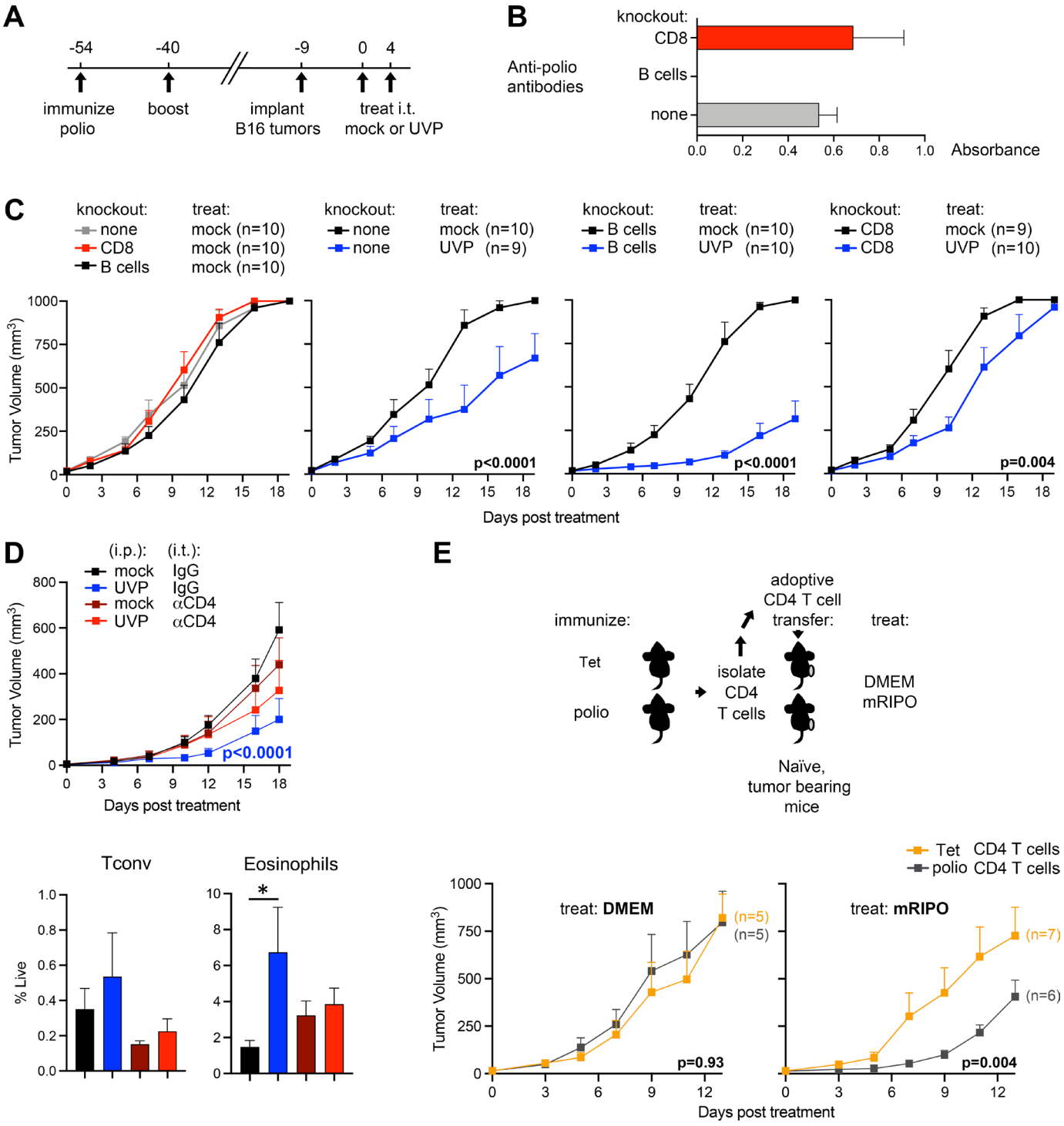

Figure 3 CD4+ T cells mediate antitumor efficacy of polio recall. (A) Design for experiments in (B, C). (B) ELISA for anti-polio antibodies in each genetic background at day 0 (n=4/group). (C) Mean tumor volume+SEM after mock treatment (left) or mock versus UVP treatment (right panels) for each genotype context; p values are from two-way analysis of variance (ANOVA) comparison of UVP to the control group. (D) Mice immunized with polio were treated with mock or UVP as in (A), with intraperitoneal (i.p.) injections of IgG (control) or CD4+ T-cell depleting antibody (250 µg delivered every 3 days starting at day −1); mean tumor volume+SEM and flow cytometry analysis of tumor infiltrating CD4 T cells and eosinophils are shown; n=9 per group; p value is from a two-way ANOVA comparing UVP IgG versus UVP αCD4; (*) indicates Tukey's post hoc p<0.05. (E) CD4+ T cells from spleens of mice immunized with Tet (control) or polio were adoptively transferred to naïve B16 tumor-bearing recipients 1 day-prior to intratumor treatment with DMEM or mRIPO. Mean tumor volume+SEM for mock or mRIPO treated mice for each CD4+ T-cell transfer condition; p values are from two-way ANOVA comparison of the two curves shown in each panel. See online supplemental figure S6 for extended data. DMEM, Dulbecco's Modified Eagle Medium; i.t., intratumor; UVP, UV-inactivated PVSRIPO; Tet, tetanus toxoid; mRIPO, mouse adapted PVSRIPO; Tconv, conventional CD4+ T cells.

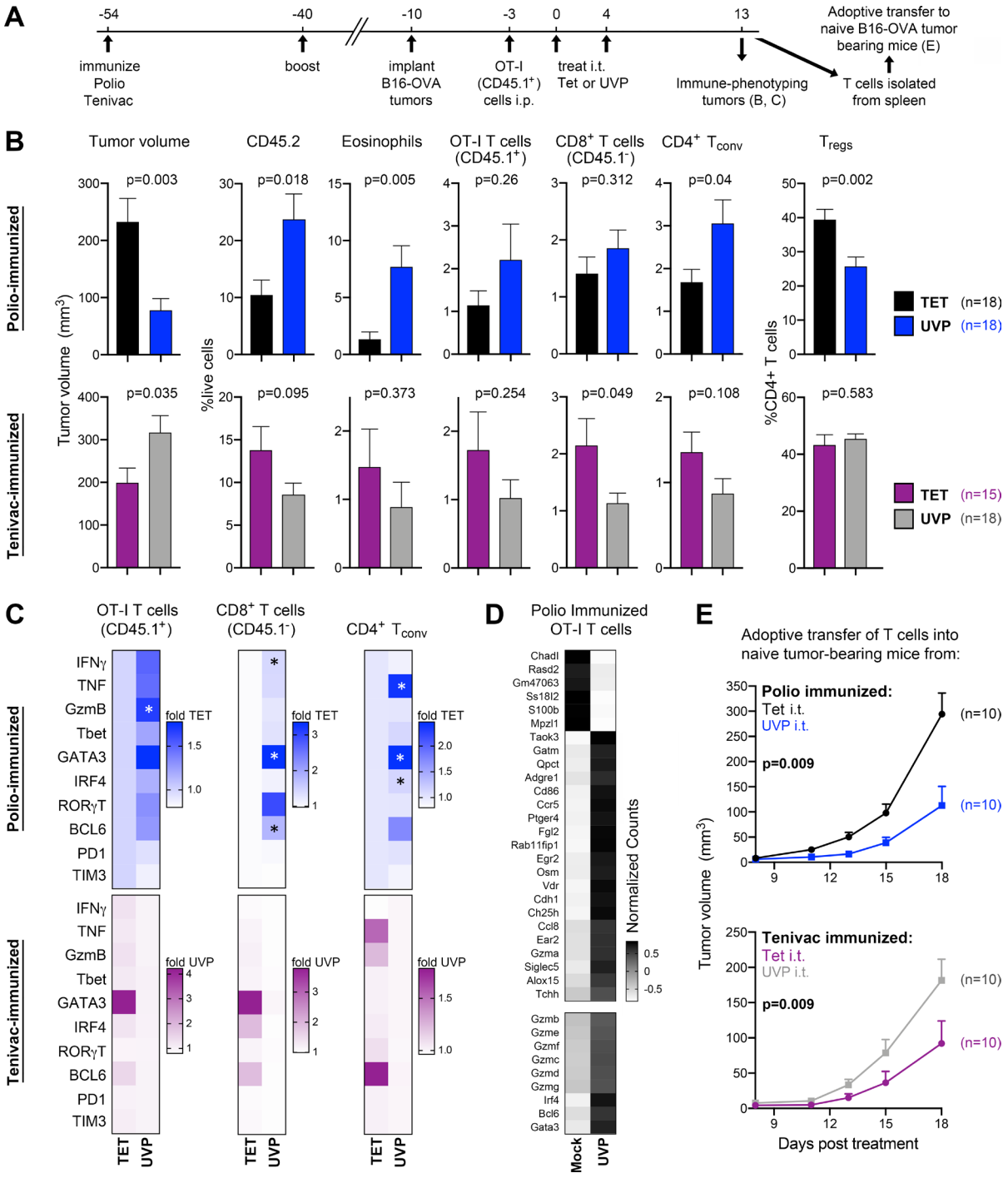

Figure 4 Intratumor recall antigen therapy potentiates antitumor CD8+ T-cell function. (A) Polio or Tet (Tenivac) immunized mice were implanted with B16-OVA tumors, followed by adoptive transfer of activated OT-I (CD45.1+) cells, and treatment with either Tet or UVP. (B) Tumor volume and flow cytometry analyses of immune cells (B) and TIL subsets (C); online supplemental figure S7 presents gating of OT-I TILs. (D) Transcriptomes of OT-I TILs isolated from polio immunized mice 12 days after treatment (DMEM or UVP) were analyzed. Center and scaled mean normalized counts are shown for transcripts that were significantly different after false discovery rate p value adjustment in two separate experiments (top panel, n=4/group and n=3/group, respectively) or for features relevant to (C) that approached significance in both data sets, including granzymes, IRF4, BCL6, and GATA3 (bottom panel); n=4 replicates/group are shown in heat map. Online supplemental figure S8B,C presents normalized counts for individual samples and extended data. (E) Tumor progression in naïve mice adoptively transferred with T cells from spleens of mice in A–C. Mean tumor volume+SEM is shown; p value is from two-way analysis of variance. All data bars represent mean+SEM; heatmaps in (C) were normalized by fold average of the mismatched antigen control; Tukey's post hoc test p<0.05 relative to all other groups (#) or respective DMEM control (*). (A–C) pooled results from two experiments; data in (E) were repeated twice. DMEM, Dulbecco's Modified Eagle Medium; TIM3, T cell immunoglobulin and mucin domain-containing protein 3; IFN, interferon; i.t., intratumor; OVA, ovalbumin; PD1, programmed cell death protein-1; UVP, UV-inactivated PVSRIPO; Tet, tetanus toxoid; TIL, tumor-infiltrating lymphocytes; TNF, tumor necrosis factor.

online supplemental figure 8C). We confirmed these observations by testing the impact of polio recall on endogenous TRP2-specific (an endogenous B16 antigen) CD8$^+$ TILs in B16 tumors without OVA expression. TRP2-specific CD8$^+$ TILs exhibited increased granzyme B and reduced TIM3 expression after polio recall in a CD4$^+$ T-cell dependent manner (online supplemental figure S9). Together, these data indicate improved cytolytic and functional phenotypes of antitumor T cells after polio recall.

Functionally demonstrating enhanced antitumor T-cell immunity after i.t. recall, T cells isolated from spleens of mice treated with i.t. recall antigen (UVP in polio vaccinated, or Tet in Tenivac vaccinated) delayed tumor growth after transfer to naive recipients (figure 4E). CD4$^+$ T-cell help promotes antitumor CD8$^+$ T-cell function in part through CD40L signaling to CD40 on antigen presenting cells,[1] but can also help antitumor CD8$^+$ T cells independent of CD40L.[31] CD40L blockade did not prevent UVP-induced eosinophil, conventional CD4$^+$ T cell, or antitumor OT-I T influx; it also did not antagonize antitumor effects (online supplemental figure S10). In addition, CD40 ligation did not recapitulate eosinophil influx observed after recall antigen therapy (online supplemental figure S10). Thus, i.t. CD4$^+$ T-cell recall potentiates the antitumor function of antitumor CD8$^+$ T cells in a CD40L independent manner.

Tumor infiltrating eosinophils inversely associate with Tregs in human tumors

Engagement of other antitumor immune effectors via cytokine secretion is a key CD40L-independent mechanism of CD4$^+$ T cells.[31 32] I.t polio capsid (UVP) and virotherapy (mRIPO) consistently caused robust i.t. infiltration of eosinophils (figures 1, 3 and 4), in a CD4$^+$ T-cell dependent manner (figure 3D). Tumor eosinophil influx associates with immunotherapy response,[33–35] and recent work demonstrated that CD4$^+$ T cells enlist antitumor functions of eosinophils after PD1 blockade via IL-5.[36] First, we asked if eosinophil density correlates with that of CD4$^+$ T cells or other features in the TME of human tumors by querying a pan-cancer data set from The Cancer Genome Atlas (TCGA).[37] Using CIBERSORT[38] prediction of cell infiltrates,[37] samples from each cancer type were stratified by presence or absence of detected eosinophil gene expression signatures (figure 5A, online supplemental figure S11,12); Testicular Germ Cell Tumors (TGCT) and Uveal Melanoma (UVM) were excluded due to limited cases with eosinophil enrichment (n<3). While limited association of eosinophil presence was observed with CD8$^+$ or CD4$^+$ T-cell enrichment, eosinophil presence was associated with significantly lower Treg signatures across all cancer types (figure 5A, online supplemental figure S11B,C). Eosinophil presence was associated with longer survival in Low Grade Glioma (LGG), where eosinophil density was also the highest (online supplemental figure S11A), but not in other tumor types (figure 5B, online supplemental figure S11D). Importantly, significant differences in Treg density on stratification by eosinophil enrichment were observed within several cancer types, with heterogenous relationships between CD4$^+$ and CD8$^+$ T-cell density (online supplemental figure S12). Notably, eosinophil influx was associated with reduced Treg proportions after polio recall in our studies (figures 1E and 4B, online supplemental figure S2). Together, these data may reflect a role for eosinophils in countering tumor infiltrating Tregs.

Antitumor type II immunity after mRIPO treatment of polio immunized mice

We next sought to determine the significance of eosinophil infiltration after polio recall in mice. Eosinophils are mediators of type II immune responses, which play roles in anti-helminth immunity and allergic inflammation.[39] Eosinophil recruitment can be mediated by other type II immune mediators, including ILC2s, which coordinate Th2 responses through direct interactions with CD4$^+$ T cells,[40] express the transcription factor GATA3 and eosinophil promoting cytokine IL-5, but lack T-cell receptor expression. Indeed, GATA3$^+$ CD3^{Neg} cells increased in tumors of mice after i.t. recall (figure 5C), possibly reflecting ILC2 influx. We next tested how eosinophils impact polio virotherapy (mRIPO) in polio immunized mice, and measured ILC2s directly. Eosinophil depletion (via IL-5 neutralization[41]) mitigated the antitumor effects of mRIPO in B16-OVA^{hCD155} bearing, polio immunized mice (figure 5D,E); did not reduce CD4$^+$ T-cell influx; but blocked reductions in Treg proportion (figure 5F). Moreover, i.t. polio virotherapy (mRIPO) led to ILC2 influx (figure 5F) and altered ILC2 phenotypes in polio immunized mice, with reduced IL-5 and induced PD1 and granzyme B expression (figure 5G). Aside from its role in controlling eosinophil levels, IL-5 is also critical for B-cell differentiation.[42] However, since the antitumor effects of polio recall are independent of—and possibly countered by—B cells (figure 3C), and because of the consistently robust eosinophil infiltration on polio recall, we conclude that IL-5 neutralization diminishes antitumor effects of polio recall via eosinophil depletion. Collectively, these data demonstrate antitumor roles for eosinophils and recruitment of ILC2s with altered phenotypes after polio recall and imply that eosinophils regulate i.t. Treg densities.

Pre-existing anti-polio antibodies associate with longer survival after Lerapolturev (PVSRIPO) therapy; peripheral eosinophils increase after Lerapolturev

In trials of Lerapolturev in rGBM[9] and melanoma,[10] all patients were confirmed seropositive for anti- PVSRIPO neutralizing antibodies (from polio vaccine cross-reacting antibodies) at the time of enrollment, a feature anticipated to correlate with pre-existing PVSRIPO-specific CD4$^+$ T-cell immunity. We queried both phase I (n=61)[9] and II (n=72 at the time of data cut-off) clinical rGBM cohorts for the relationship between pretreatment Lerapolturev neutralizing antibodies and survival. In both

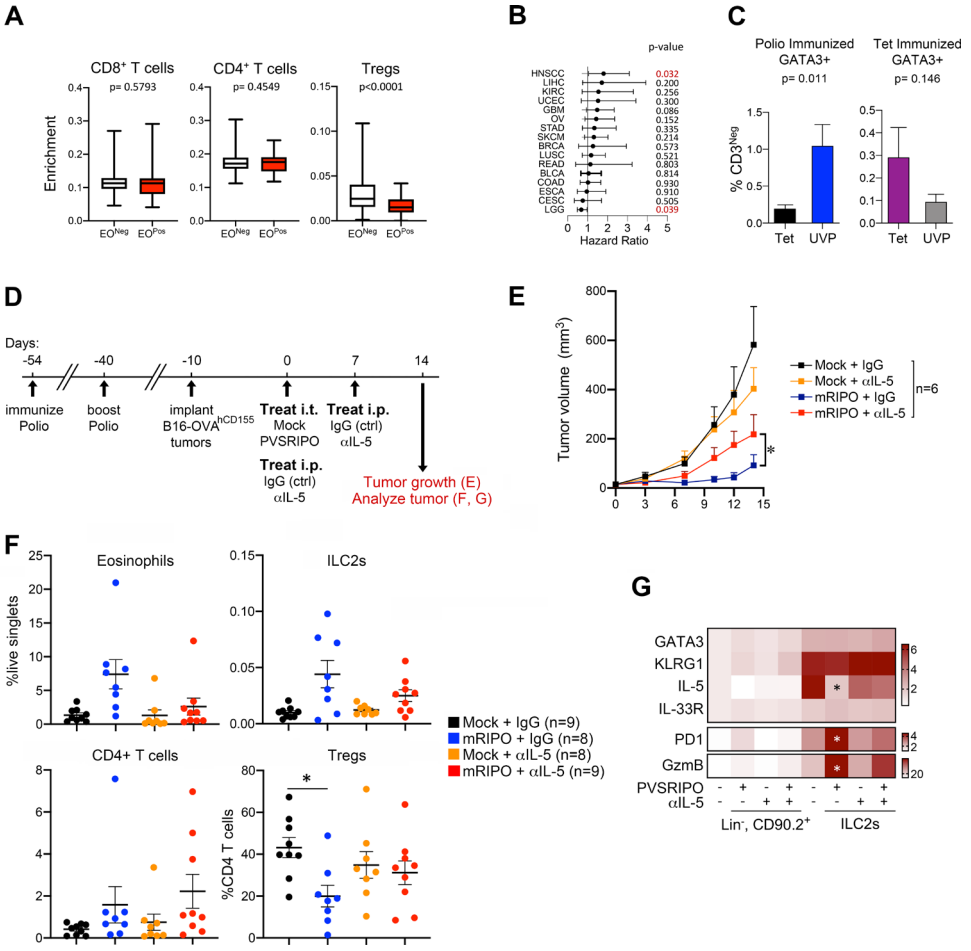

Figure 5 Eosinophils inversely associate with Tregs in human tumors; mRIPO induces antitumor type II immunity in polio immunized mice. (A) CIBERSORT deconvoluted T-cell subsets in The Cancer Genome Atlas cancer types (n=29) stratified by eosinophil status, p values from paired t-test. (B) HRs (survival) ± 95% CIs for eosinophil status by cancer type; cancer types with less than 20 deaths were excluded; p values are from Mantel-Cox log-rank test. (C) Percentage of GATA3+ $CD3^{Neg}$ cells from data in figure 4C, p values are from unpaired t-test. (D) Design for (E–H): polio immunized, B16-OVA^{hCD155} tumor bearing hCD155-transgenic mice were treated with mock or PVSRIPO ± eosinophil depletion (anti-IL-5 or control IgG, 1 mg weekly). Mean tumor volume+SEM (E), eosinophil and ILC2 density in tumors (F), and phenotype of tumor infiltrating ILC2s ($lineage^{Neg}CD90^{+}CD127^{+}CD25^{+}$) versus lineage negative $CD90^{+}$ cells for comparison are shown (G). (E) (*)Two-way analysis of variance p<0.05; (F–G) (*)Tukey's post hoc test p<0.05 versus mock+IgG control. Online supplemental figure S11–S13 presents extended data. IL, interleukin; ILC2, type 2 innate lymphoid cell; i.p., intraperitoneal; i.t., intratumor; OVA, ovalbumin; PD1, programmed cell death protein-1; UVP, UV-inactivated PVSRIPO; Tet, tetanus toxoid; Tregs, regulatory T cells; mRIPO, mouse adapted PVSRIPO.

cohorts, patients living >18 months post-Lerapolturev (typical median survival in rGBM is ~9 months)[9 43] had significantly higher pretreatment neutralizing antibody titers (figure 6A,B). To address whether or not type II immune responses may be engaged in patients treated with i.t. polio virotherapy, we analyzed longitudinal CBCs available from a small dose-escalation trial of Lerapolturev in recurrent, unresectable melanoma (phase I, n=12).[10 11] These data revealed increased blood eosinophil levels post-Lerapolturev in 8/12 patients (figure 6C), coinciding with a reduction in neutrophils (10/12 patients). These findings may indicate that pre-existing immunity contributes to the antitumor efficacy of polio virotherapy, and that polio virotherapy induces type II immune responses in patients with cancer.

DISCUSSION

This work reveals cancer immunotherapy potential of tumor-localized $CD4^{+}$ T-cell recall. $CD4^{+}$ T-cell help is key for generating fully functional antitumor $CD8^{+}$ T-cell immunity[1 44] and long-term memory.[2 45] Antitumor $CD8^{+}$ T cells exhibited greater polyfunctional phenotypes after recall, adoptive T-cell transfer from recall antigen treated mice delayed tumor growth in naïve recipients, and UVP antitumor effects were blunted in polio immunized mice lacking $CD8^{+}$ T cells. This indicates provision of $CD4^{+}$ T-cell help to antitumor $CD8^{+}$ T cells. Indeed, $CD4^{+}$ T-cell help is linked with exhaustion marker down-regulation, elevated TNF/IFN-γ/granzyme B expression, and Tbet/IRF4 induction in 'helped' effector T cells,[3] all of which occurred with polio virotherapy in immunized

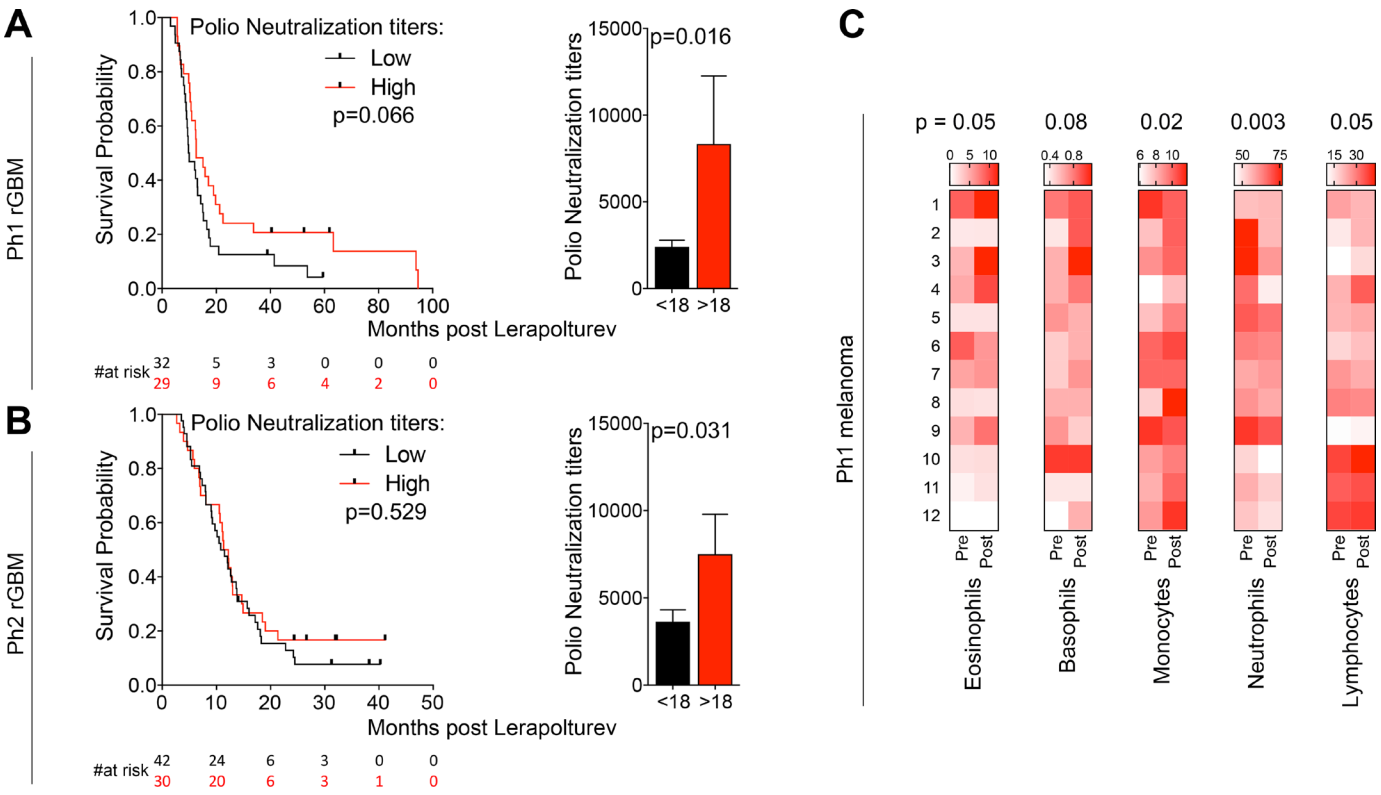

Figure 6 Pre-existing anti-polio antibodies associate with longer survival after Lerapolturev (PVSRIPO) therapy; peripheral eosinophils increase after Lerapolturev. (A, B) Pretreatment Lerapolturev serum neutralization titers were measured in recurrent glioblastoma (rGBM) phase I (A, Ph1, NCT01491893) and phase II (B, Ph2, NCT02986178) trials. Survival after separation by median neutralization titer (1:2000), alongside mean+SEM titer for patients surviving <vs> 18 months after Lerapolturev in the phase I (A) and phase II (B) trials. (A, B) Kaplan-Meier p values are from Mantel-Cox log-rank test; bar graph p values are from unpaired t-test. (C) Percentage of indicated cell types out of total white blood cells at the time of Lerapolturev administration ('pre') and follow-up ('post', 9–11 days after treatment) for all (n=12) patients treated in the phase I melanoma clinical trial (NCT03712358); p values above heatmap are from paired Wilcoxon test.

mice. Such T-cell help is likely multifaceted, including 'licensing' DCs; $CD4^+$ T-cell secretion of cytokines (eg, IL-21, IFN-γ); TME reprogramming; or through positive effects of recruited eosinophils on $CD8^+$ T-cell immune surveillance.[36 46] Possibly due to the activity of polio neutralizing antibodies limiting $CD4^+$ T-cell activation by reducing recall antigen availability or persistence, B cells were counterproductive to antitumor effects of polio recall.

Polio recall consistently mediated stronger antitumor and inflammatory responses than that of Tet in our studies. One possibility is that this is due to differences in the intensity and/or quality of pre-existing immunity induced after polio versus Tet vaccination in our model systems. In support of this possibility, antitumor effects of Tet and UVP were similar when mice were immunized with clinical grade IPOL vaccine (figure 2C), which induced weaker anti-polio immunity than the polio immunization strategy (PVSRIPO+ALH) used for other experiments in our study (online supplemental figure S1B). However, it is also well established that the nature of antigen (eg, epitope density, which is likely higher in the polio capsid) can influence adaptive immune responses.[47 48]

The antitumor efficacy of polio recall only partially depended on $CD8^+$ T cells. Recall antigen therapy caused eosinophil and ILC2 influx, and eosinophil depletion decreased antitumor effects in polio immunized mice treated with mRIPO. Highlighting the importance of context, both eosinophils and ILC2s were also shown to mediate protumorigenic effects.[49 50] In asthma, $CD4^+$ T cells recruit eosinophils[51] via ILC2s.[52] ILC2s express MHC class II and propagate Th2 responses in helminth infections.[40] In cancer, tumor eosinophil infiltration is linked with immunotherapy response,[33 34] eosinophils were shown to support $CD8^+$ T-cell immune surveillance,[46] and ILC2s contribute to anti-PD1 antitumor efficacy.[35 53] Our work indicates that antitumor functions of eosinophils, and possibly that of ILC2s, can be engaged by $CD4^+$ T cells. How $CD4^+$ T-cell recall recruits eosinophils to the tumor remains to be fully determined, however, recent work provides a potential explanation by showing that during PD1 blockade IL-5 secretion by $CD4^+$ T cells induces expansion of eosinophils and i.t. recruitment.[36] Moreover, cytokine analysis of tumor homogenates and TDLN explants after polio virotherapy also revealed increased eosinophil promoting GM-CSF and IL-5 in polio immunized mice (online supplemental figure S2B).

We also discovered an inverse relationship between tumor eosinophil influx and Treg density in human tumors, and recall antigen therapy led to decreased proportions of Tregs that covaried with eosinophil influx, implying a reciprocal role of eosinophils in controlling CD4+ T-cell biology. Determining precisely how eosinophils mediate antitumor effects after CD4+ T-cell recall, and whether ILC2s contribute to this process requires further study. Differences in eosinophil density alone does not appear to be prognostic in most cancer types, with potential exception of LGG, where eosinophil levels were also the highest among all other tumor types. Interestingly, a negative correlation between peripheral eosinophils and glioma grade has been reported[54]; and respiratory allergies and atopy associate with lower glioma incidence.[55]

We used Th2 polarizing vaccination strategies, consistent with the clinical use of polio and tetanus vaccines, to decipher antitumor potential of CD4+ T-cell recall. While—canonically—Th1/Tc1, Th2/Tc2, and Th17/Tc17 polarizations are mutually exclusive, recall antigen therapy in this context produced Th1 (CD8+ T-cell engagement, Tbet/IFN-γ expression in CD4+ T cells); Th2 (eosinophil/ILC2 recruitment, GATA3 expression in CD4+ T cells); and to a lesser extent, Th17 (RORγt expression in CD4+/CD8+ T cells) polarizing features. These data add to mounting evidence that diverse CD4+ T-cell polarizations, beyond that of Th1, can generate compatible and productive antitumor immune responses.[56–58]

Our work shows that, while pre-existing immunity limits viral replication within the tumor, it enhances the antitumor efficacy of polio virotherapy. A limitation of this work is that it was performed in murine systems that do not capture the heterogeneity of either polio/Tet memory CD4+ T-cell responses or the TME in humans. Moreover, due to the rapid growth of tumor models employed, tumors were relatively small at the time of treatment initiation. Nonetheless, suggesting applicability of our observations to humans, pretreatment polio neutralizing antibody titers were higher in patients with rGBM that lived longer after polio virotherapy, and blood eosinophil levels increased in the majority of patients with melanoma (8/12) after i.t. treatment with polio virotherapy (Lerapolturev). As a caveat, higher neutralizing polio antibody titers may reflect superior immune functional status. While we cannot exclude this possibility, these data at minimum reveal that pre-existing immunity does not preclude successful polio virotherapy in patients. Our observations also imply that multiple dosing of Lerapolturev may be warranted to accentuate antitumor effects of polio recall. Indeed, responses in patients with melanoma treated with Lerapolturev clustered in the cohort with the highest number of i.t. treatments.[10]

Given the eminent importance of CD4+ T cells in cancer immunotherapy,[1 32] growing efforts aim to leverage CD4+ T-cell help within tumors, for example, with CD40 agonistic antibodies,[59] or with peptide vaccines including MHC class II epitopes[44] that prime neoantigen specific CD4+ and CD8+ T cells.[60] Our work uncovers the potential of harnessing childhood vaccine-specific memory CD4+ T cells to engage multifaceted antitumor mechanisms of CD4+ T cells.

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Contributors MCB, GMB, AD, DMR, DMA, DDB, SKN, and MG contributed to study conception and design. MCB and MG administered the project and carried out statistical analyses. MCB, GMB, ZPM, and YY performed experiments and acquired data; MCB and MG wrote the manuscript and all authors participated in reviewing and editing. MCB accepts full responsibility for the finished work and/or the conduct of the study, had access to the data, and controlled the decision to publish.

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Competing interests MCB, AD, DMA, DDB, SKN, and MG own intellectual property related to Lerapolturev, which has been licensed to Istari Oncology. MCB, AD, MG, and DDB received consultancy fees from Istari Oncology; MG and DDB hold equity in Istari Oncology. All other authors declare they have no competing interests.

Patient consent for publication Consent obtained directly from patient(s).

Ethics approval This study involves human participants and was approved by Duke University Institutional Review Board, NCT01491893, NCT02986178, NCT03712358. Participants gave informed consent to participate in the study before taking part.

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