

Overcoming T cell dysfunction in acidic pH to enhance adoptive T cell transfer immunotherapy

Flor Navarro ^a, Noelia Casares ^a, Celia Martín-Otal ^a, Aritz Lasarte-Cía ^a, Marta Gorraiz^a, Patricia Sarrión ^a, Diana Llopiz^a, David Reparaz ^a, Nerea Varo ^b, Juan Roberto Rodríguez-Madoz ^c, Felipe Prosper ^{c,d}, Sandra Hervás-Stubbs ^a, Teresa Lozano ^{a*}, and Juan José Lasarte ^{a*}

^aImmunology and Immunotherapy Program, University of Navarra, IdiSNA, Pamplona, Spain; ^bDepartment of Clinical Biochemistry, Clínica Universidad de Navarra, University of Navarra, IdiSNA, CIBERONC, Pamplona, Spain; ^cProgram, Center for Applied Medical Research (CIMA), University of Navarra, IdiSNA Hemato-Oncology, Pamplona, Spain; ^dDepartment of Hematology, Clínica Universidad de Navarra, University of Navarra, IdiSNA, CIBERONC, Pamplona, Spain

ABSTRACT

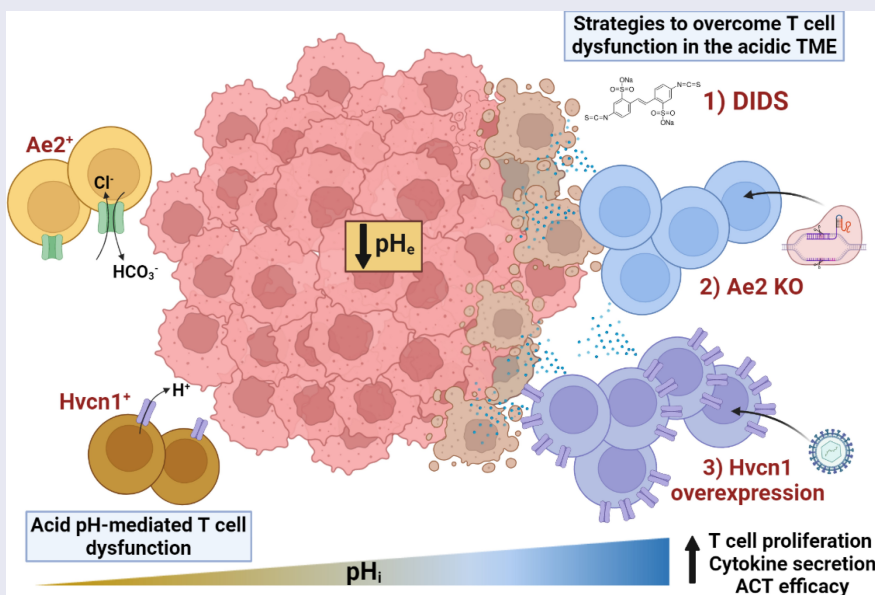
The high metabolic activity and insufficient perfusion of tumors leads to the acidification of the tumor microenvironment (TME) that may inhibit the antitumor T cell activity. We found that pharmacological inhibition of the acid loader chloride/bicarbonate anion exchanger 2 (Ae2), with 4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid (DIDS) enhanced CD4⁺ and CD8⁺ T cell function upon TCR activation *in vitro*, especially under low pH conditions. *In vivo*, DIDS administration delayed B16OVA tumor growth in immunocompetent mice as monotherapy or when combined with adoptive T cell transfer of OVA-specific T cells. Notably, genetic Ae2 silencing in OVA-specific T cells improved CD4⁺/CD8⁺ T cell function *in vitro* as well as their antitumor activity *in vivo*. Similarly, genetic modification of OVA-specific T cells to overexpress Hvcn1, a selective H⁺ outward current mediator that prevents cell acidification, significantly improved T cell function *in vitro*, even at low pH conditions. The adoptive transfer of OVA-specific T cells overexpressing Hvcn1 exerted a better antitumor activity in B16OVA tumor-bearing mice. Hvcn1 overexpression also improved the antitumor activity of CAR T cells specific for Glypican 3 (GPC3) in mice bearing PM299L-GPC3 tumors. Our results suggest that preventing intracellular acidification by regulating the expression of acidifier ion channels such as Ae2 or alkalinizer channels like Hvcn1 in tumor-specific lymphocytes enhances their antitumor response by making them more resistant to the acidic TME.


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CONTACT Flor Navarro  fnavarron@unav.es; jjlasarte@unav.es; Immunology and Immunotherapy Program, University of Navarra, IdiSNA, Pamplona, Spain; Juan José Lasarte Immunology and Immunotherapy Program, University of Navarra, IdiSNA, Pamplona, Spain

*These authors share senior authorship.

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Introduction

Adoptive cell transfer (ACT) immunotherapy is one of the most promising advanced therapies for the treatment of cancer. Once adoptively transferred T cells reach the tumor site, the major challenge they face is overcoming the hostile TME and the multiple mechanisms the tumor can elicit to avoid immune-mediated elimination. Tumors are characterized by having insufficient blood flow, which results in a hypoxic microenvironment and cancer cell metabolic shift from oxidative phosphorylation to glycolysis (Warburg effect).^{1,2} The accumulation of glycolytic products such as lactate and protons in the TME causes the acidification of the cancer cell extracellular milieu.³ Indeed, extracellular pH (pH_e) in tumors can be as low as 5.6, although the most common values recorded are between 6.4 and 6.8 compared to healthy tissue, which maintains its pH around 7–7.4.⁴ This acidic pH_e directly inhibits $CD8^+$ and $CD4^+$ T cell function and T cell mediated antitumor immunity resulting in immune-escape.^{5,6} Previous studies showed that lowering the environmental pH to 6–6.5 induced a $CD8^+$ T anergic state, which is characterized by impairment of cytotoxic ability, and reduced cytokine production and CD25 expression. This anergic state can be reversed by buffering pH to neutral values.⁷ Exposing $CD8^+$ T cells to lactic acid caused a significant decrease in their proliferation, cytokine production, lytic activity, and tumor infiltration.⁵ Furthermore, raising the TME pH by the administration of sodium bicarbonate (NaBi) was shown to enhance anti-tumor immune responses in mice and humans.^{8,9} These data indicate that the negative effect the acidic TME has on infiltrating lymphocytes can be controlled and even reversed by basifying the TME.

Although T cells are highly sensitive to acidic pH, they have mechanisms to resist intracellular pH (pH_i) acidification and achieve acid-base homeostasis. To recover from intracellular acidification or alkalinization, cells are equipped with acid extruders and acid loaders to maintain the pH_i within a narrow physiological range that is generally ~ 7.2 . Notably, cancer cells exploit several proton pumps and transporters to neutralize metabolic acid loading and achieve higher pH_i values than normal cells, which promotes cell proliferation and evasion of apoptosis, therefore favoring tumor progression.^{10,11} Accordingly, physiological pH sensors hold promise as targets in cancer therapeutics.^{12,13}

They include the family of carbonic anhydrases (CAs),¹⁴ the vacuolar-type H^+ -ATPase proton pump,¹⁵ anion exchangers such as AE1 (SLC4A1)¹⁶ and AE2 (SLC4A2),¹⁷ Na^+ / HCO_3^- co-transporters such as SLC4A4,¹⁸ and the Na^+ / H^+ exchanger 1 NHE1 (SLC9A1).¹⁹ Among the SLC4 family of HCO_3^- transporters, the Na^+ -independent Cl^- / HCO_3^- anion exchanger 2 (AE2, SLC4A2) is considered a master acid loader in many cell types.^{20,21} Under physiological conditions, AE2 favors the extrusion of intracellular HCO_3^- in exchange for extracellular Cl^- , resulting in an acid load. Previous studies showed that mice carrying a targeted deletion of Ae2 ($Ae2a,b^-/-$ mice) have

lymphocytes with abnormal pH_i values, which eventually leads to an abnormal state of T cell activation and autoimmunity.^{22–24} We also found that AE2 inhibition with a synthetic peptide improved effector T cell functions *in vitro*.²⁵ These data prompted us to investigate the role of the pH acidifier AE2 as a potential target for tumor immunotherapy. On the other hand, the voltage-gated H^+ channel (Hv1) encoded by the *Hvcn1* gene is a highly selective H^+ extruder that avoids cell acidification and depolarization.²⁶ Hv1 can restore cytoplasmic pH in seconds after heavy acid loads in different cell types including neurons, neutrophils, macrophages, and epithelial cells, among others.^{27–30} Moreover, in breast and colorectal cancers, Hv1 expression was correlated with worse prognosis, and the pharmacological inhibition of Hv1 caused the acidification of the tumor cell pH_i and consequently decreased tumor proliferation and migration.^{13,31} Hv1 was also shown to be necessary to regulate the pH_i of Jurkat cells.¹² In this scenario, we hypothesized that over-expression of Hv1 or AE2 silencing in T cells could facilitate the alkalinization of their pH_i even in the acidic TME and accordingly enhance the antitumor activity. These genetic modifications could be of interest in adoptive T cell therapies based on T cell receptor (TCR) transgenic T cells or in chimeric antigen receptor expressing T cells (CAR T cells).

Materials and methods

Cell lines, mice and reagents

B16OVA (ATCC, American Type Culture Collection), LLCOVA (a gift from Dr Daniel Ajona, CIMA, Universidad de Navarra), PM299L (kindly provided by Dr Lujambio, Mount Sinai, NY, USA) were cultured in RPMI medium (Gibco) supplemented with 10% FBS, antibiotics, 2 mM glutamine and 50 μ M β -mercaptoethanol. Platinum Ecotropic cells (platE) (ATCC) were cultured in DMEM medium supplemented with 10% FBS and the selection antibiotics puromycin (100 μ g/mL) and blasticidin (10 μ g/mL).

The PM299L cell line was genetically modified to over-express human glypican-3 (GPC3). PM299L cells were transduced with a pcDNA3.1 of hGPC3 using Lipofectamine 2000. GPC3⁺ cells were sorted via FACS using an anti-hGPC3-APC antibody (RD Systems) and maintained in culture with 0.5 mg/mL of G418 antibiotic. These hGPC3-expressing PM299L tumor cells are referred as PM299L-GPC3.

Female C57B/16 mice were purchased from Harlan Laboratories. CD45.1 (B6.SJL-PtprcaPep3b/BoyJ) (CD45.1), LSL-Cas9-GFP (B6;129-Gt(ROSA)26Sortm1(CAG-cas9*-EGFP)Fezh/J) and OT-1 (C57B/16-Tg[Tcr α /Tcr β]110Mjb/J) transgenic mice were purchased from Jackson Laboratories (Bar Harbor, ME). OT-1 mice were crossed with CD45.1 mice at our facility to obtain homozygous CD45.1 mice. OT-1 \times CD45.1 mice were crossed with LSLCas9GFP to obtain homozygous OT1/LSLCas9GFP mice. All mice were housed under specific pathogen-free conditions, and the

animal protocols were approved by the Ethics Committee of Animal Experimentation at the University of Navarra and The Local Authority for the use of laboratory animals (protocol R-131-16 GN).

DIDS (4,4'-Diisothiocyanostilbene-2,2'-disulfonic Acid) was obtained from SIGMA (309795) and diluted to 50 mg/mL or 100 mM in 0.1 M KHCO₃. The solution was aliquoted and stored at -20° degrees until experimental use.

Mouse and human T cell purification

Mouse CD4⁺ and CD8⁺ T cells were obtained from the spleens and lymph nodes of C57Bl/6 mice. To obtain single-cell suspensions of T cells, organs were first homogenized through a cell strainer in ACK lysis buffer and passed through a magnetic column labeled with negative selection microbeads (Miltenyi) following manufacturers' instructions. CD8a⁺ and CD4⁺ T cell isolation kits (Miltenyi, Refs 130-104-075 and 130-104-454) were used to isolate mouse T cells. The same procedure was performed to purify OT-1 CD8⁺ T cells from CD45.1 OT-1 mice. Purified lymphocytes were cultured in complete RPMI medium containing 10% FBS.

Human peripheral blood mononuclear cells (PBMCs) were isolated from healthy donor's buffy coats by a centrifugation gradient in Ficoll-paque (GH Healthcare) and then, CD4⁺ and CD8⁺ T cells were purified using negative selection microbeads through a magnetic column. CD8a⁺ and CD4⁺ T cell isolation kits (Miltenyi 130-096-495, and 130-096-533) were used. Human T cells were cultured in Ex Vivo serum free medium (Gibco) containing 10% human serum AB (Sigma). The study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Institutional Ethics Committee of the Universidad de Navarra (protocol R-131.16 (Ref 2019.162)).

Adjusting the pH of culture media

The pH of RPMI or Ex Vivo media was modified by adding different volumes of HEPES buffer (1 mM) pH 4.5 until the desired pH was reached. While adding HEPES pH 4.5, the pH of the complete media was measured using a pH-meter Basic20⁺ (CRISON). 6.5, 6.8, 7 and 7.4 pH media was prepared this way.

Retrovirus production and lymphocyte transduction

Ae2 silencing via CRISPR/Cas9: In order to silence the expression of Ae2, a retrovirus expression plasmid pRubiG containing CRE-Thy1.1 was modified to also contain an anti-murine Ae2-specific sgRNA obtained using CHOPCHOP software (sgRNA forward: AGACTGGAGGAGCTGAAGACAG, sgRNA reverse: CTGTCTTCAGCTCCTCCAGTCT) or left unmodified as control to infect OT1/LSLCas9 GFP CD8⁺ T cells. sgRNA efficiency at modifying Ae2 expression was measured by INDEL % using TIDE software. The CD8⁺ T cells that

were modified by the Ae2 sgRNA were sorted based on Thy1.1⁺ and GFP⁺ expression and are referred to from now on as Ae2 KO.

Hvcn1 overexpression: In order to overexpress Hvcn1, the mSCV-IRES-Hvcn1-Thy1.1 vector was created using Benchling and synthesized by GeneScript. The mSCV-IRES-Thy1.1 vector was used as a control to infect OT-1 CD8⁺ T cells. Hvcn1-overexpressing or control CD8⁺ T cells were sorted based on Thy1.1 expression.

CAR RV generation: We prepared a human GPC3 CAR containing the antihuman GPC3 ScFv (purified from GC33 hybridoma) and the murine 4-1BB-CD3ζ endodomains linked through a F2A self-cleaving peptide sequence to eGFP. This expression cassette was cloned in a pRubiG retroviral vector (produced by GeneScript) to produce GPC3 CAR T cells. CD8⁺ T cells were co-infected with pRubiG expressing GPC3 CAR and mSCV-IRES-Hvcn1-Thy1.1 retroviral vectors. Hvcn1⁺ GPC3 CAR T cells and GPC3 CAR T cells were purified by FACs sorting based on the expression of Thy1.1⁺GFP⁺ or GFP⁺ alone, respectively.

The PlatE cell line was used for retrovirus production. PlatE packaging cells were transfected with 5 µg of retroviral plasmids in combination with 2.5 µg pCL-Eco plasmid DNA using Lipofectamine 2000 (ThermoFisher Scientific, MA, USA). Retroviral supernatants were collected at 48 and 72 h after transfection. CD8⁺ T cells were isolated from OT-1 or OT1/LSLCas9 GFP spleens and purified by magnetic negative selection. Then, CD8⁺ T cells were activated with dynabeads CD3/CD28 at a 1:2 bead:T cell ratio for 24 h, at a 10⁶ cells/mL density in 12-well plates in RPMI complete media containing 100 IU/mL recombinant human interleukin-2 (rhIL-2). Twenty-four hours later, CD8⁺ T cells were collected and resuspended in retroviral supernatants with 100 IU/mL rhIL-2 and 10 µg/mL protamine sulfate (Sigma) and spun at 2000xg at 32°C for 90 min in 12 well plates. Infection was repeated 1 d later using 72-h supernatant. After infection, lymphocytes were cultured in complete RPMI medium with IL-2 until day 5 when they were used for functional *in vitro* or *in vivo* assays.

Tumor interstitial fluid (TIF) isolation and analysis

B16OVA tumors were harvested in cold saline and washed by placing them on a 10 µm cell strainer over a 50 mL conical tube and centrifuged at 50xg for 5 min. Then, tumors were punctured to break the tumor capsule and spun down at 400xg for 10 min. TIF was transferred to a 1.5 mL Eppendorf tube and spun down at 6000 rpm for 10 min before analyzing the pH and lactate levels using a blood gas analyzer (RapidPoint 500, Siemens). Serum obtained from tumor bearing mice was used as control.

Functional activity of engineered lymphocytes

The functional activity of transduced (1) Ae2 KO CD8⁺ T cells and their corresponding pRubiG control (Ctrl) or (2) Hvcn1-overexpressing and Ctrl Thy1.1 CD8⁺ T cells was measured by *in vitro* experiments. First, genetically modified OT-1 CD8⁺ T cells were stimulated with SIINFEKL peptide at different

concentrations from suboptimal (0.01 ng/ml) to optimal (1 ng/ml) concentrations in complete RPMI medium or RPMI medium at different pH (6.5, 6.8, 7, 7.4) for 48 h. The number of IFN γ or IL-2 producing cells was analyzed by ELISA. Briefly, culture supernatants were collected and diluted 1:10 in Assay Diluent (PBS + 10% FBS) and cultured in triplicate for 2 h in anti-IFN γ or anti-IL-2 antibody coated plates. Then, the ELISAs were developed according to manufacturer's instructions (BD Biosciences). T cells were cultured with SIINFEKL at different pH on anti-IFN γ coated ELISPOT plates for 18 h before developing the plates according to the manufacturer's instructions (BD Biosciences). The number of spot forming cells was automatically counted by an ELISPOT reader (CTL, Germany). Proliferation of genetically modified T cells was measured by ^3H -thymidine incorporation (0.5 μCi per well) as previously described.³²

Adoptive T cell transfer

C57Bl/6 mice (6 to 12 weeks of age) were injected with 5×10^5 B16-OVA melanoma cells or with 1×10^6 LLCOVA lung cancer cells subcutaneously. Seven days later, mice were sublethally irradiated (total body irradiation) with 500 cGy and received 5×10^6 CD8 $^+$ T cells from OT1/LSLCas9GFP or OT-I mice retrovirally transduced to express pRubiG or AE2 sgRNA or Thy1.1 or Hvcn1-Thy1.1 respectively. Modified CD8 $^+$ T cells were purified by flow sorting of GFP $^+$ Thy1.1 $^+$ cells before ACT experiments. The perpendicular diameters of the tumors were subsequently measured with a caliper. Mice were sacrificed when a tumor diameter reached a value greater than 2 cm. Tumor volume was estimated using the formula $1/2(\text{Length} \times \text{Width}^2)$. For characterization experiments, B16OVA tumor-bearing mice (expressing CD45.2 allele) were treated with genetically modified CD8 $^+$ T cells (5×10^6) from OT-IxCD45.1 mice and 7 d later, mice were sacrificed to analyze the presence of transferred cells into the tumors by flow cytometry. There was no exclusion of animals in the analyses.

For CAR T cell immunotherapy experiments, C57Bl/6 mice were challenged with 1×10^6 PM299L-GPC3 cells subcutaneously in 100 μL matrigel (Merck), and 7 d later, when the tumor is 5–7 mm in diameter, mice were sublethally irradiated (total body irradiation) with 500 cGy and received immunotherapy with 5×10^6 Hvcn1-overexpressing CD8 $^+$ T cells, GPC3-CART cells, or Hvcn1-overexpressing GPC3 CAR T cells injected intravenously. Twenty-thousand IU/mouse of rhIL-2 were injected intraperitoneally once a day for 4 d after ACT. Tumor diameters were measured using calipers, and tumor volume was estimated using the formula $1/2(\text{Length} \times \text{Width}^2)$.

Measurement of SIINFEKL-specific IFN γ producing T cells after ACT

To evaluate the behavior of the genetically modified T cells *in vivo*, tumor-bearing mice were sacrificed 7 d after T cell infusion, and IFN γ producing T cells were determined by

ELISPOT (BD Biosciences) following the manufacturer's instructions. Briefly, splenocytes (1×10^5 /well) were stimulated with or without 1–10 $\mu\text{g}/\text{ml}$ SIINFEKL peptide. After 1 d of culture, the number of spot-forming cells was enumerated with an automated ELISPOT reader (CTL, Aalen, Germany).

Flow cytometry

Excised tumors were digested with 400 U/mL collagenase D and 50 $\mu\text{g}/\text{mL}$ DNase-(Roche) for 20 min at 37°C. After washing with PBS, red blood cells were lysed by ACK buffer (Sigma). Spleens were passed through a cell strainer in PBS. For functional analyses, cells were stimulated with the SIINFEKL peptide (1–10 $\mu\text{g}/\text{mL}$) in the presence of GolgiStop and GolgiPlug (BD Biosciences). After 5 h, cells were incubated with Zombie NIR Fixable viability dye (BioLegend). Subsequently, they were stained with fluorochrome-conjugated mAbs against CD45.1-FITC (A20), CD45.2-APC(104), CD8-PerCp-Cy5.5. (53–6.7), and CD4-Brilliant Violet 510 (RM4-5), CD25-PeCy7(3C7), CD62L-Brilliant Violet 421 (MEL-14), CD44-PerCp-Cy5.5. (IM7), F4/80- Brilliant Violet 421 (BM8), Ly6C-PerCp-Cy5.5. (HK1.4), CD11b-PeCy7(M1/70), CD11c-PE(N418) (BioLegend) and Ly6G-FITC(1A8) (BD Biosciences) in the presence of purified Fc block anti-CD16/32 mAb to prevent nonspecific binding. For intracellular and intranuclear staining, cells were fixed and permeabilized with the BD Fixation/Perm buffer (BD Biosciences) or the Foxp3 Transcription Factor Staining Buffer Kit (eBioSciences) following the manufacturers' instructions and then stained with anti-IFN γ -PE(XMG1.2), anti-TNF α -PeCy7(16A8) (BioLegend), anti-Granzyme B-Brilliant Violet 421 (GB11) and anti-FoxP3-FITC(FJK16 F) (Invitrogen). Samples were acquired on a FACSCanto-II cytometer (BD Biosciences). Data were analyzed using FlowJo software (TreeStar).

Hvcn1 overexpression on the CD8 $^+$ T cells' membrane was detected via flow cytometry using an anti-Hvcn1 polyclonal antibody (Invitrogen, PA5-77462) at a 1:25 dilution followed by a goat-anti-rabbit-APC secondary antibody (Abcam, ab150083) at a 1:2000 dilution.

Intracellular pH measurement

The pHrodo Red AM fluorogenic probe (ThermoFisher) was used for pH measurement. First, human or mouse T cells were plated on 96 well plates at a 100,000 cell/well concentration and washed with HBSS (Gibco). Then, cells were loaded with pHrodo Red AM diluted in a PowerLoad concentrate according to manufacturer's instructions and incubated at 37° for 30 min. Cells were washed with HBSS and then cultured in different pH media with indicated treatments for 6 h. pH measurements based on pHrodo Ex/Em were taken by flow cytometry in the PE-A channel in a Cytoflex Lx instrument (Beckman Coulter).

The pH_i values were estimated from calibration curves plotted for each sample by using pHrodo Red AM-loaded T cells incubated in solutions containing 10 μ M nigericin and valinomycin at different pH (4.5, 5.5, 6.5, and 7.5).

RNA isolation and quantitative real time polymerase time reaction (qRT-PCR)

Total RNA isolation from Ae2 KOCD8⁺ and CD4⁺ and their corresponding control T cells (1×10^6 cells) that had been activated with anti-CD3/CD28 Dynabeads for 0, 2, 6, or 24 h were carried out using the Maxwell RSC simplyRNA Tissue kit (Promega). After retrotranscription (Invitrogen), the expression of target genes was measured by a real-time PCR reaction using specific primers for Ae2 (Forward: GCTAAGATTTGGCCATGAGC, Reverse: CGGTGGTATTCAAAGTCTTCC), Ae1 (Forward: TTCAGAAACCACACTGGTGC, Reverse: TACAGTCCAGAAAGCTCTCC) and the iQ SYBR Green Supermix (Bio-Rad, Hercules, CA). GAPDH (Forward: TGCACCACCAACTGCTTA, Reverse: GGATGCAGGGATGATGTTTC) was used to normalize gene expression. mRNA values were represented by the formula: $2^{-\Delta Ct}$, where ΔCt indicates the difference in the threshold cycle between GAPDH and target genes.

Statistical analysis

Data are represented as averages \pm standard error of the mean (SEM). Student's *t* tests were used for statistical comparisons between two groups of normally distributed variables, and one-way or two-way ANOVA and subsequent Tukey's post-hoc test were used for comparisons between more than two groups. For tumor growth, data were analyzed using nonlinear third-order polynomial (cubic) regression curves. Kaplan–Meier survival curves were evaluated for statistical significance with the Log-rank Mantel–Cox test. For all tests, a *p* value $< .05$ was considered statistically significant. GraphPad PRISM software was used for statistical analysis.

Results

Pharmacological inhibition of anion exchangers enhances mouse and human T cell function

DIDS is a pharmacological inhibitor of anion exchangers (Ae)³³ such as the chloride-bicarbonate exchanger Ae2, the most widely expressed member of the SLC4 family that contributes to the regulation of T cell pH_i by extruding HCO_3^- and loading Cl^- into the cytoplasm in response to alkaline signals.³⁴ Previous studies showed that treatment of T lymphocytes with DIDS *in vitro* results in progressive intracellular alkalinization and increased TNF release.³⁵ Thus, we first wanted to evaluate by flow cytometry the alkalinizing effect of DIDS treatment on the pH_i of T cells incubated in culture medium buffered at different pH (6.8, 7.0 and 7.4 pH units). To this end, murine C57BL/6CD8⁺ and CD4⁺ or OT-1CD8⁺ T cells were labeled with the pH-

sensitized dye pHrodo Red-AM and stimulated with anti-CD3 beads or SIINFEKL, respectively, for 6 h in the presence/absence of 50 μ M DIDS and analyzed by flow cytometry to estimate the pH_i as described in the methods section (Supplementary Fig 1A, B, C). As expected, the results obtained from these experiments confirmed the DIDS-mediated alkalinizing effect on the pH_i of T cells. Similar results were obtained on human CD4⁺ and CD8⁺ T cells stimulated with anti-CD3/CD28 beads (Supplementary Fig 1D, E). In all cases, with the exception of CD4⁺ T cells cultured at high pH values (pH 7.4), DIDS treatment had a significant alkalinizing effect on the pH_i of T cells.

To study the effect of DIDS on the functionality of T cells, mouse and human CD4⁺ and CD8⁺ were activated for 48 h using anti-CD3/CD28 beads in the absence or presence of DIDS. DIDS treatment significantly enhanced IL-2 and IFN γ secretion as well as proliferation of mouse (Figure 1a,b) and human (Figure 1c,d) CD8⁺ and CD4⁺ T cells in a dose-dependent manner.

Since CD8⁺ T cells critically rely on the cell acidifier Ae2 for pH_i homeostasis after mitogenic stimulation,²³ we decided to investigate if DIDS could improve CD8⁺ T cell function in physiological and acidic pH conditions. OT-1CD8⁺ T cells isolated from the spleen of OT-1 mice, and human CD8⁺ T cells purified from the peripheral blood of healthy donors were activated with their cognate Ag SIINFEKL peptide or with anti-human CD3/CD28 beads, respectively, at different pH (from 6.5 to 7.4 units) for 48 h in the presence of DIDS. It was observed that DIDS treatment was able to significantly increase IL-2 and IFN γ secretion of mouse OT-1CD8⁺ T cells (Figure 2a,b) and human CD8⁺ T cells (Figure 2c, d) at all tested pH (6.5, 6.8, 7 and 7.4). Overall, these data indicate that pharmacological inhibition of Ae2 can enhance the function of CD8⁺ T cells by regulating their physiological pH_i through alkalinization even under acidic pH conditions.

Pharmacological inhibition of anion exchangers enhances the endogenous T cell antitumor immune response

After finding that DIDS was able to enhance T cell function *in vitro* even at low pH, we hypothesized that DIDS treatment might also improve antitumor T cell immune responses by enhancing T cell function in the acidic TME. First, we measured the acidity of the B16OVA TME. Mice were challenged with B16OVA, and then the TIF was extracted, and the pH and lactate concentration were measured in comparison to serum of these mice. B16OVA TIF had a significantly more acidic pH and a higher lactate concentration than the serum of the animals (Figure 3a). In order to test if DIDS can enhance antitumor T cell immune responses in the acidic TME, we challenged mice with B16OVA melanoma cells and treated them daily with an intraperitoneal administration of 100 μ M DIDS or PBS alone for 15 d (Figure 3b). We found that tumor growth was significantly delayed in DIDS-treated mice compared to control mice (treated with PBS) (*p* $< .0001$) (Figure 3c,d). A similar trend of delayed tumor growth was observed in

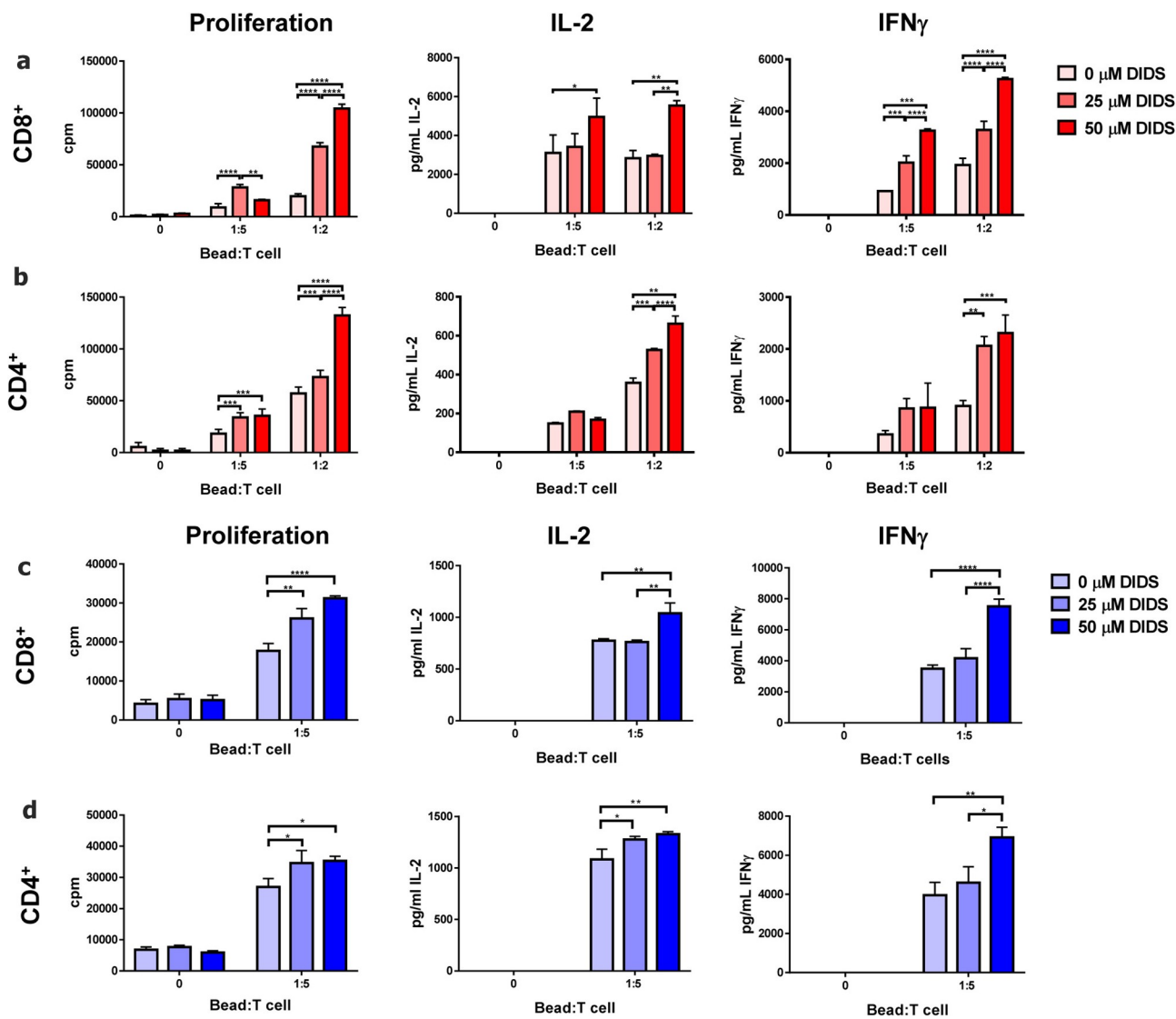


Figure 1. DIDS enhances T cell function. Murine and human CD4⁺ and CD8⁺ T cells purified from splenocytes or healthy donor PBMCs respectively were activated using anti-CD3/CD28 dynabeads and treated with 0, 25, or 50 μ M DIDS in unmodified RPMI (pH 7.4). After 48 h, their proliferation, IL-2 and IFN γ secretion were measured. DIDS treatment enhanced proliferation, IL-2 and IFN γ secretion of murine (a) CD4⁺ and (b) CD8⁺ T cells, and human (c) CD4⁺ and (d) CD8⁺ T cells. $n = 4$. Data represent mean \pm SEM. Analyzed using 2-way-ANOVA with a post hoc Tukey test. **** = $p < .0001$, *** = $p < .001$, ** = $p < .01$, * = $p < .05$.

mice bearing Lewis lung carcinoma LLCOVA tumors after treatment with DIDS, although in this case statistical significance was not reached ($p = .08$) (Supplementary Fig 2). Interestingly, the DIDS-induced tumor growth delay was not observed in immunodeficient mice (NGS) bearing B16OVA or LLCOVA tumors (Supplementary Fig 3), indicating that DIDS treatment specifically triggers an increase of the antitumor efficacy of the immune cell component.

To characterize the immunomodulatory effects of DIDS treatment on immunocompetent mice bearing tumors, we studied the phenotype of tumor-infiltrating lymphocytes (TILs) at day 15 after DIDS treatment, when tumor weight was significantly lower in DIDS-treated mice compared to control mice (Figure 3e). We found significantly more CD8⁺ and CD4⁺ T cells infiltrating the tumor in DIDS-treated mice compared to control mice (Figure 3f,g) ($p < .05$). Moreover, CD8⁺ TILs from DIDS-treated mice

secreted significantly more TNF α (Figure 3h), ($p < .05$), whereas CD4⁺ TILs from DIDS-treated mice secreted significantly more IFN γ than those from control mice (Figure 3i) ($p < .05$). The number of CD8⁺ and CD4⁺ T cells was also significantly higher in the tumor draining lymph nodes (DLN) of DIDS-treated mice compared to control mice (Figure 3j,k) ($p < .05$). Similarly, significantly more IFN γ -producing Ag-specific T cells were found in the spleen of DIDS-treated mice compared to control mice when stimulated with SIINFEKL (Figure 3l), ($p < .05$). Overall, these data indicate that DIDS treatment enhances the T cell antitumor immune response.

To ensure that DIDS treatment was not directly acting on the tumor cells, we performed a proliferation assay in which different numbers of B16OVA and LLCOVA tumor cells were treated with 100 μ M DIDS (Supplementary Fig 4a). Complementary, 10,000 tumor cells were treated

with decreasing concentrations of DIDS (from 200 μM to 0.2 μM) (Supplementary Fig 4b). No significant differences of tumor cell proliferation were observed except for a slight decrease in proliferation when B16OVA were treated with 200 μM DIDS.

DIDS treatment enhances the T cell antitumor immune responses of adoptive cell therapy

Next, we wanted to assess if pharmacological inhibition of Aes using DIDS could enhance the CD8^+ T cell antitumor immune response during ACT immunotherapy. Thus, mice were challenged with B16OVA melanoma cells and 7 d later, when tumors reached 5 mm in diameter, they were given ACT immunotherapy with OT-1 CD8^+ T cells and subsequently treated intraperitoneally with 100 μM DIDS or PBS for 15 d daily (Figure 4a). DIDS treatment alone slightly delayed tumor growth as expected from previous

experiments, but it did not significantly increase overall survival (Figure 4b–d). ACT immunotherapy with OT-1 CD8^+ T cells delayed tumor growth and increased overall survival, and more importantly, the efficacy of ACT immunotherapy was further enhanced when combined with DIDS treatment (Figure 4b–d). ACT immunotherapy combined with DIDS significantly increased overall survival compared to ACT immunotherapy alone (Figure 4d) ($p < .05$). Similar results were obtained when mice challenged with LLCOVA tumor cells were treated with ACT alone or a combination ACT immunotherapy and DIDS (Supplementary Fig 2) ($p < .01$).

The analysis of the tumor infiltrates at day 15 post-DIDS treatment revealed a significant increase in the absolute number of OT-1 CD8^+ T cells infiltrating the tumor in mice treated with DIDS and ACT combination therapy compared to mice that received ACT alone (Figure 4e). These tumor infiltrating OT-1 CD8^+ T cells were

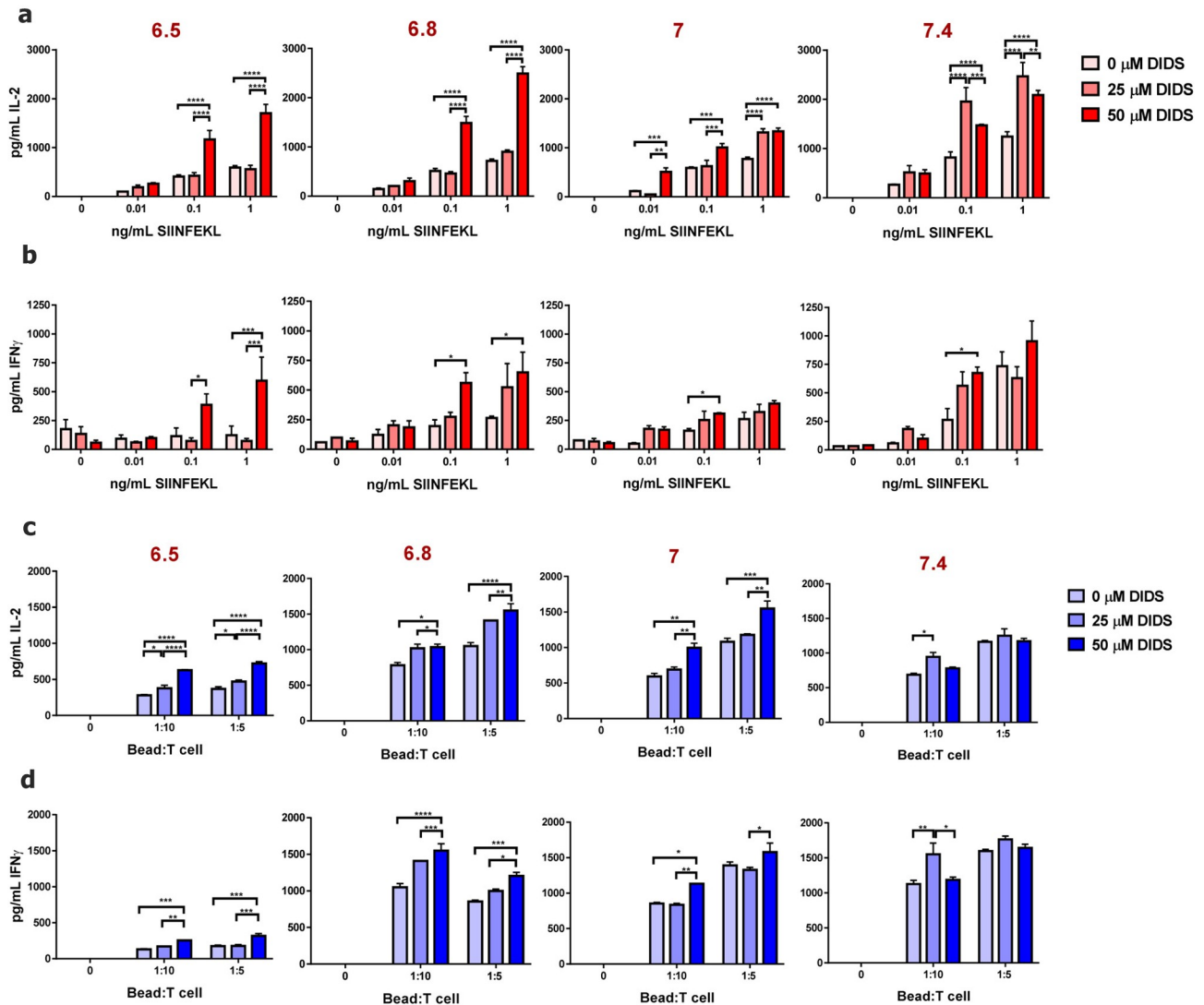


Figure 2. DIDS enhances CD8^+ T cell function even at acidic pH. (a, b) Mouse OT-1 CD8^+ T cells and (c, d) human CD8^+ T cells were purified and activated with SIINFEKL cognate Ag or anti-CD3/CD28 dynabeads respectively at different pH (6.5, 6.8, 7, and 7.4) for 48 h. DIDS treatment enhanced the secretion of (A, C) IL-2 and (B, D) IFN γ of CD8^+ T cells even at acidic pH. $n = 4$. Data represent mean \pm SEM. Analyzed using 2-way-ANOVA with a post hoc Tukey test. **** = $p < 0.0001$, *** = $p < .001$, ** = $p < .01$, * = $p < .05$.

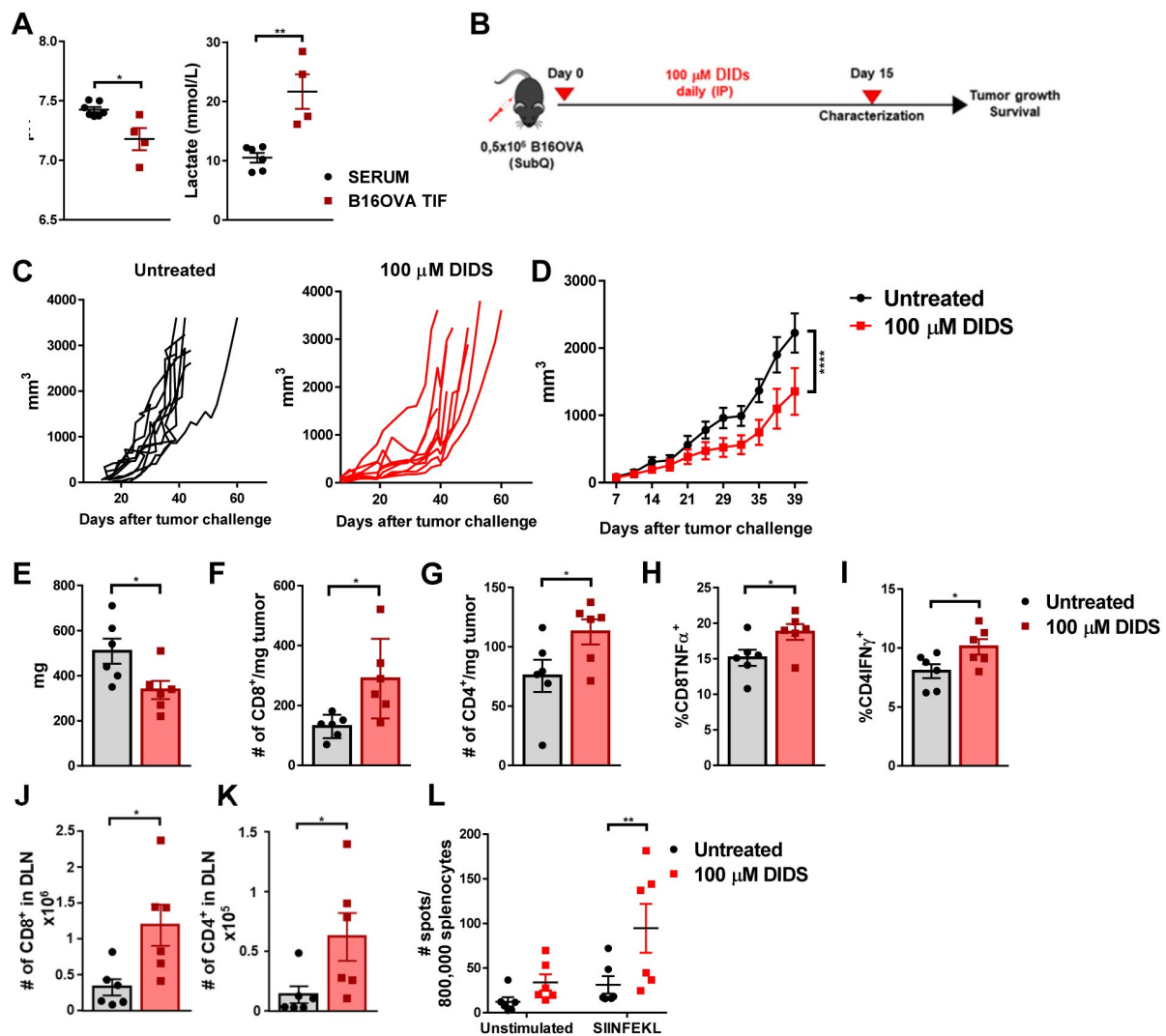


Figure 3. DIDS treatment enhances the endogenous T cell antitumor immune response. A) pH and lactate concentration in the tumor interstitial fluid (TIF) and serum of B16OVA tumor bearing mice. B) C57B/6 mice were challenged with 0.5×10^6 B16OVA melanoma cells subcutaneously and treated intraperitoneally with 100 μM DIDS or PBS for 15 d. C, D) Tumor growth was delayed in DIDS-treated mice compared to mice that received PBS. E) At day 15, tumors from DIDS-treated mice were significantly smaller than those that received PBS. Significantly more F, J) CD8⁺ and G, K) CD4⁺ T cells were found infiltrating the tumor and in the draining lymph nodes (DLN) of DIDS-treated mice compared to control mice. A higher percentage of tumor infiltrating CD8⁺ T cells from DIDS-treated mice expressed H) TNFα after being stimulated *ex vivo* with 10 μg/mL SIINFEKL for 5 h. I) Higher percentage of CD4⁺ TILs expressed IFNγ in DIDS-treated mice compared to control mice. L) Significantly more SIINFEKL Ag-specific T cells were found in the spleen of mice that received DIDS treatment compared to control mice. n = 6. Data analyzed with student *t* tests. ***p* < .01, **p* < .05.

significantly more differentiated into effector memory (EffM) T cells (CD62L⁻CD44⁺) in mice treated with DIDS and ACT compared to mice treated with ACT alone, which had a higher percentage of naïve TILs (CD62L⁺CD44⁻) (Figure 4f). Equivalent percentages of central memory (CM) T cells (CD62L⁺CD44⁺) were observed in both groups (Figure 4f). Mice treated with the combination of DIDS and ACT had significantly higher percentages of TILs producing Granzyme B (Figure 4g), and IFNγ and TNFα simultaneously (Figure 4h) when activated *ex vivo* with SIINFEKL compared to TILs from mice that received ACT alone. Gating strategies for these experiments are represented in Supplementary Fig 5a, b. Overall, these data indicate that the pharmacological blockade of the Ae family of Cl⁻/HCO₃⁻ transporters using DIDS enhances the efficacy of ACT immunotherapy by increasing the functional capacities of CD8⁺ T cells.

Additionally, we also analyzed the endogenous tumor infiltrate in these mice. The absolute number of endogenous (CD45.2⁺) CD8⁺ T cells infiltrating the tumor was significantly higher in mice treated with the combination of DIDS and ACT compared to the ones that received ACT alone (Supplementary Fig 6a). No significant differences were observed in the numbers of tumor infiltrating CD4⁺ T cells, T_{regs} (CD4⁺CD25⁺FoxP3⁺), DCs (CD11c^{high}), tumor associated macrophages (CD11c⁻CD11b⁺F480⁺), or monocytic myeloid-derived suppressor cells (CD11c⁻CD11b⁺Ly6c⁺Ly6g⁻) between groups of mice treated with OT-1 T cells alone or with OT-1 T cells + DIDS (Supplementary Fig 6b, c, D, E). Representative gating strategies for these data are presented in (Supplementary Fig 5c, d). These data indicate that DIDS treatment mainly affects the CD8⁺ TIL population, enhancing their numbers and antitumor function.

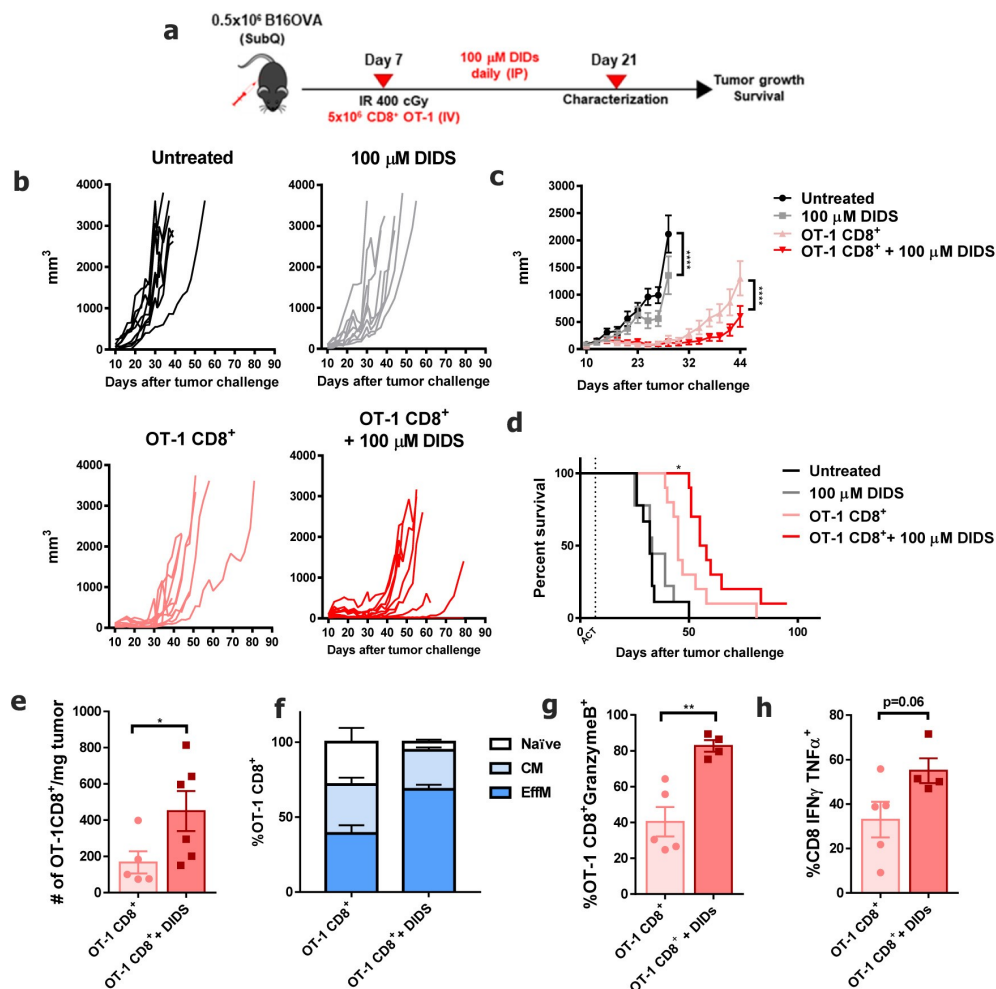


Figure 4. DIDS treatment enhances the T cell antitumor immune response during ACT immunotherapy. A) C57B/16 mice were challenged with 0.5×10^6 B16OVA melanoma cells and 7 d after mice were irradiated and received ACT of 5×10^6 OT-1CD8⁺ T cells intravenously. Groups were treated with 100 μ M DIDS or PBS alone intraperitoneally for 15 d after immunotherapy. B, C) DIDS monotherapy delayed tumor growth, and ACT immunotherapy combined with DIDS delayed tumor growth significantly more than immunotherapy alone as well as increased the D) overall survival. A significantly higher number of E) OT-1CD8⁺ T cells were found infiltrating the tumor of DIDS-treated mice, these cells were significantly more effector memory (EffM) than those of mice that received ACT alone F). A significantly higher percentage of DIDS-treated CD8⁺ OT-1 TILs showed expression of Granzyme B G), and IFN γ and TNF α simultaneously H) when stimulated with 10 μ g/mL SIINFEKL *ex vivo* for 5 h compared to OT-1 T cells from untreated mice. $n = 6$. Data analyzed with student's *t*-test. ** $p < .01$, * $p < .05$.

Genetic silencing of Ae2 in CD8⁺ T cells enhances T cell function in acidic pH and improves their antitumor efficacy *in vivo*

Pharmacological inhibition of Ae using DIDS-enhanced T cell function and antitumor immunity. While these results were interesting due to CD8⁺ T cells depending on Ae2 for Cl⁻/HCO₃⁻ transport, DIDS is a pan inhibitor of the SLC4 family, and to our knowledge, there is no information on its potential use or toxicity in humans.

Therefore, we aimed to evaluate the impact of silencing Ae2 expression on CD8⁺ T cell function at different pH levels and antitumor immunity. We first studied the effect of Ae2 silencing on T cell function by using the CRISPR-Cas9 technology. We engineered a pRubiG retroviral (RV) vector encoding chimeric single guide RNAs (sgRNA) containing a short sequence homologous to Ae2 exon 4, the recombinase CRE, and Thy1.1 as a reporter protein to evaluate transduction efficiency and to purify transduced

cells. The pRubiG RV expressing only CRE and Thy1.1 was used as a control. These vectors were used to transduce CD8⁺ T cells from CRE-dependent CD45.1. OT1/LSL Cas9 GFP Cas9 knock-in mice. OT1/LSL Cas9 GFP mice are genetically modified to constitutively express the endonuclease Cas9 and GFP under the ubiquitous CAG promoter interrupted by a loxP-stop(33 polyA signal)-loxP(LSL) cassette to render Cas9 expression inducible by CRE.³⁶ Using this system, successfully transduced CD8⁺ T cells were identified and sorted based on Thy1.1 and GFP expression. Three different sgRNAs were tested for their cleavage efficacy. The best sgRNA was #3 with an average 34.6% INDELS, so we used this sgRNA for the characterization experiments. First, we characterized the effect of Ae2 silencing on the pHi of T cells after TCR stimulation in culture medium buffered at different pH. We confirmed that, under all tested pH conditions, Ae2 silencing on CD8⁺ T cells resulted in a more alkaline pHi after TCR stimulation (Supplementary Fig 7a) similar to the results obtained with its pharmacological inhibition. Notably, OT-1CD8⁺ T cells

infected with Ae2 sgRNA#3 (AE2 KO) proliferated significantly more and produced significantly more IL-2 and IFN γ (Figure 5a–c) in response to SIINFEKL cognate Ag stimulation even at acidic pH 6.5 compared to control T cells. When Ae2 KOCD8⁺ T cells were co-cultured with B16OVA, they were able to lyse tumor cells significantly more than control T cells (Figure 5d). Overall, these data indicate that genetic deletion of Ae2 improves CD8⁺ T cell function at acidic pH levels and improves their cytotoxicity making CD8⁺ T cells more resistant to acidic conditions.

To ensure that the deletion of Ae2 expression in T cells was not inducing a compensatory mechanism through other AE family members, we measured the mRNA activity of Ae1 in Ctrl and Ae2 KOCD8⁺ and CD4⁺ T cells activated overtime. Ae1 expression was not detected on CD8⁺ T cells as previously reported in Ae2 KO mice.²³ In CD4⁺ T cells, the expression of Ae1 was increased at 2 and 6 h after stimulation, but there were no differences in its expression between Ctrl and Ae2 KO T cells (Supplementary Fig 8).

The effect of Ae2 genetic deletion was then tested *in vivo* during ACT immunotherapy using Ae2 KO or control OT-1CD8⁺ T cells against B16OVA. Mice bearing subcutaneous

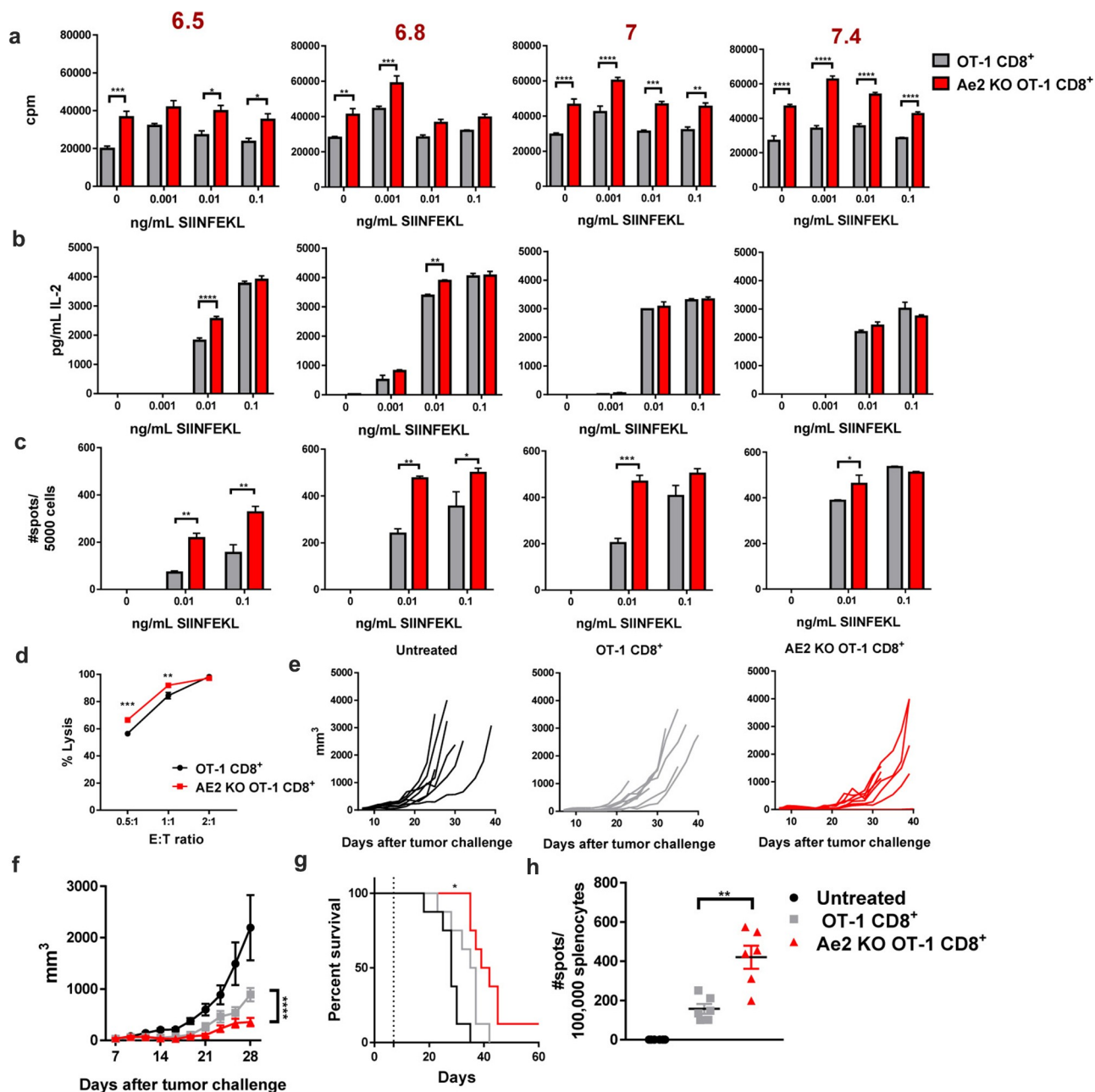


Figure 5. AE2 silencing enhances CD8⁺ T cell function and antitumor immunity. AE2 KOCD8⁺ T cells A) proliferated, and secreted significantly more B) IL-2, and C) IFN γ than CtrlCD8⁺ T cells in response to SIINFEKL cognate Ag stimulation even at acidic pH 6.5. D) AE2 KOCD8⁺ T cells were able to lyse B16OVA cells significantly more than Ctrl OT-1CD8⁺ T cells at different effector:tumor ratios. E, F) AE2 KOCD8⁺ T cells delayed tumor growth significantly more than Ctrl T cells during ACT immunotherapy, and G) improved the overall survival of mice. H) Significantly more Ag-specific T cells were found on the spleen of mice that received Ae2 KO T cells than Ctrl T cells. n = 4. Data represent mean \pm SEM. Analyzed using 2-way-ANOVA with a posthoc Tukey test. **** = p < .0001, *** = p < .001, ** = p < .01, * = p < .05.

B16OVA tumors were treated with Ae2 KO OT-1CD8⁺ T cells or with unmodified control OT-1CD8⁺ T cells. Tumor growth was significantly delayed, and overall survival was improved in mice that received ACT of Ae2 KO OT-1CD8⁺ T cells compared to control OT-1CD8⁺ T cells (Figure 5e–g) ($p < .0001$ and $p < .05$, respectively). In a parallel experiment, on day 21 after tumor challenge and 14 d after ACT, mice were sacrificed to study the functionality of the transferred CD8⁺ cells. We found significantly more IFN γ -producing T cells in the spleens of mice that received Ae2 KO CD8⁺ T cells compared to control T cells in response to SIINFEKL (Figure 5h). Overall, these data indicate that silencing the expression of Ae2 in CD8⁺ T cells can enhance the efficacy of ACT immunotherapy.

Hvcn1 overexpression in CD8⁺ T cells enhances their function in acidic pH and improves the efficacy of T cell based ACT immunotherapy

An alternative way to silencing AE2 to avoid lymphocyte acidification could be the overexpression of hydrogen channels to maintain a basic intracellular pH, even in extracellular acid conditions. One of these basifier channels is Hvcn1. In order to overexpress Hvcn1 in CD8⁺ T cells, we modified the MSCV-IRES-Thy1.1-based RV vector (kindly provided by Dr A. Rao) to express murine Hvcn1 (MSCV-Hvcn1-IRES-Thy1.1). OT-1CD8⁺ T cells were retrovirally transduced and purified based on Thy1.1 expression. Then, Hvcn1 expression was measured via flow cytometry. It was observed that RV transduced T cells overexpressed Hvcn1 compared to control T cells that received the empty MSCV-IRES-Thy1.1 RV (Supplementary Fig. 9). To evaluate the functionality of the overexpressed Hvcn1 in the T cell pH_i, CD8⁺ T cells transduced with Hvcn1 expressing RV or with the control were stimulated with anti-CD3 beads in culture medium buffered at different pH. As occurred for Ae2 silencing, Hvcn1 overexpression on CD8⁺ T cells allowed a more alkaline pH_i after TCR stimulation (Supplementary Fig 7b). Importantly, Hvcn1 overexpressing CD8⁺ T cells proliferated more intensely (Figure 6a) and secreted significantly more IL-2 (Figure 6b) and IFN γ as measured by ELISA and ELISPOT (Figure 6c,d) than control CD8⁺ T cells in response to the SIINFEKL peptide even at acidic pH 6.5. Indeed, the greatest differences were observed in the most unfavorable pH conditions tested for lymphocyte functionality (pH 6.5), where proliferation, IL-2 and IFN- γ production were significantly higher in the Hvcn1 OT-1CD8⁺ T cells compared to control T cells. When Hvcn1-overexpressing OT-1CD8⁺ T cells were co-cultured with B16OVA, they were able to lyse tumor cells significantly more than control T cells (Figure 6e), indicating significantly higher cytotoxic potential. These data indicate that overexpressing Hvcn1 on CD8⁺ T cells enhances their function in acidic pH, putatively making CD8⁺ T cells more resistant to intratumor acidic conditions.

Next, we evaluated the effect of Hvcn1 overexpression on CD8⁺ T cells *in vivo* during ACT immunotherapy using Hvcn1-overexpressing or control OT-1CD8⁺ T cells in mice bearing B16OVA tumors. As was the case with Ae2 KO CD8⁺ OT-1 T cells, tumor growth was significantly delayed, and overall survival was slightly improved (two mice were cured, 12.5%) in mice treated with Hvcn1-CD8⁺OT-1 T cells, when

compared to mice receiving control OT-1CD8⁺ T cells (Figure 6f–h). In addition, a significantly higher number of OT-1CD8⁺ T cells were found infiltrating the tumor of mice treated with Hvcn1-CD8⁺OT-1 T cells compared to control OT-1CD8⁺ T cells (Figure 6i). We found significantly more IFN γ -producing T cells in the spleens of mice that received Hvcn1-overexpressing CD8⁺ T cells compared to control T cells in response to SIINFEKL (Figure 6j). Overall, these data indicate that increasing the expression of the H⁺ channel Hvcn1 on CD8⁺ T cells can enhance the efficacy of ACT immunotherapy and the antitumor function of transferred T cells.

Hvcn1 overexpression enhances the efficacy of CAR T cell immunotherapy

To study if Hvcn1 overexpression can also improve the efficacy of CAR T cell-based therapy, we combined these strategies by overexpressing Hvcn1 in CAR T cells against human GPC3. The antitumor efficacy of the GPC3 CAR T cells was tested in mice bearing PM299L-GPC3-tumors. Thus, mice were treated with 5×10^6 Hvcn1⁺ GPC3 CAR T cells or GPC3 CAR T cells purified by FACs sorting as described in methods. Hvcn1⁺ T cells were also used as control. GPC3 CAR T cells that overexpressed Hvcn1 delayed tumor growth significantly more efficiently than control GPC3 CAR T cells increasing overall survival (Figure 7a,b). These data indicate that overexpression of Hvcn1 can enhance CAR T cell immunotherapy.

Discussion

Tumor acidity is one of the key driving forces that renders the TME an immunosuppressive milieu for antitumor immune cells. The large amounts of protons and lactate produced by tumors as a consequence of anaerobic glycolysis lead to extracellular acidification (pH 6.2–6.8) which has a suppressive effect on T cell function.^{5,6} Adoptive cell therapies based on *ex vivo* expanded tumor-infiltrating lymphocytes and genetically modified T cells to express tumor-specific chimeric receptors can be affected by this hostile microenvironment. As other groups have previously described,^{37,38} we found that a pH as low as 6.5 led to impaired proliferation and production of IFN γ and IL-2 in response to TCR stimulation in both murine and human CD4⁺ and CD8⁺ T cells. These differences are even more pronounced when the strength of TCR stimulation is suboptimal (0.01–0.1 ng/ml of SIINFEKL peptide for murine OT-1CD8⁺ T cells, and an antiCD3/CD28 beads 1:10 ratio), suggesting that acidity might raise the activation threshold in T cells. The acidic pH-induced impairment of T cell function was significantly overcome by DIDS, a pharmacological inhibitor of the Cl⁻/HCO₃⁻ exchangers (AEs). The alkalinizing effect of DIDS on T cell pH_i might explain its capacity to improve T cell proliferation and function even at low pH conditions.

Despite the lack of data on the use of DIDS *in vivo* and regardless of the fact that its targets, the AEs, are widely expressed in many cell types, we assessed its potential immune-enhancing effect in tumor-bearing mice. We previously confirmed that DIDS had no effect on the growth of tumor cell lines B16OVA or LLCOVA *in vitro*. However,

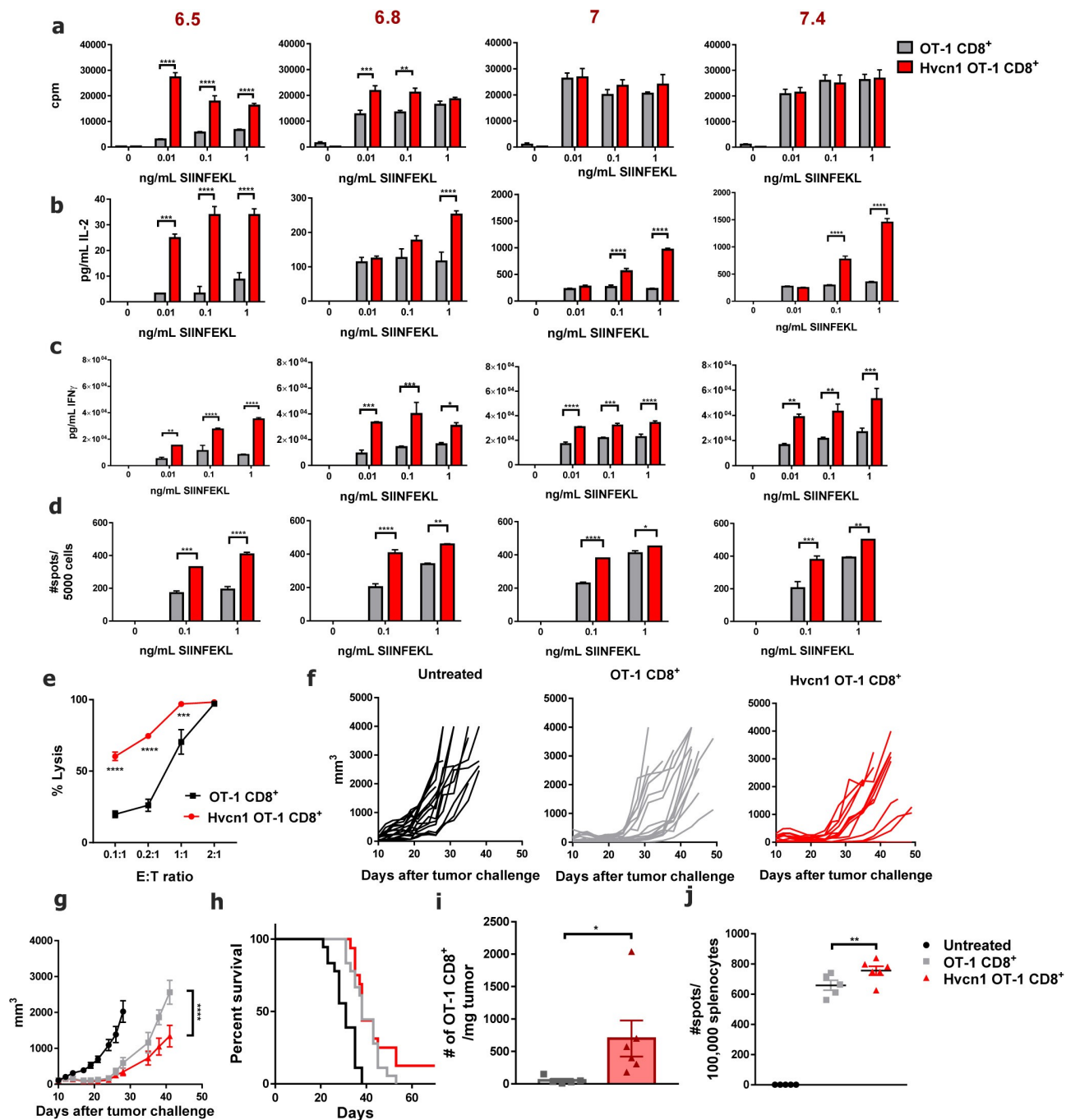


Figure 6. Hvcn1 overexpression enhances CD8⁺ T cell function and antitumor immunity. Hvcn1 overexpressing OT-1CD8⁺ T cells A) proliferated, and secreted B) IL-2, and C, D) IFN γ significantly more than CtrlCD8⁺ T cells in response to SIINFEKL cognate Ag even at acidic pH. E) Hvcn1-overexpressing OT-1CD8⁺ T cells were able to lyse B16OVA melanoma cells significantly better than CtrlCD8⁺ T cells at different effector:tumor (E:T) cell ratios. C57B/6 mice were challenged with B16OVA and 7 d after, they were given ACT with 5×10^6 Hvcn1-overexpressing or Ctrl OT-1CD8⁺ T cells intravenously. F, G) Tumor growth was significantly delayed by ACT immunotherapy with Hvcn1-overexpressing CD8⁺ T cells compared to CtrlCD8⁺ T cells. H) Overall survival was increased by ACT immunotherapy with Hvcn1-overexpressing OT-1CD8⁺ T cells compared to CtrlCD8⁺ T cells even though significance was not achieved. Significantly more Ag-specific T cells were found I) infiltrating the tumor and J) in the spleens of mice that received ACT with Hvcn1-overexpressing OT-1CD8⁺ compared to Ctrl T cells. $n = 4$. Data represent mean \pm SEM. Analyzed using 2-way-ANOVA with Tukey's post hoc. **** = $p < .0001$, *** = $p < .001$, ** = $p < .01$, * = $p < .05$. *In vivo* data show two experiments combined, $n = 16$.

administration of DIDS to tumor-bearing mice resulted in a significant delay in tumor growth. This *in vivo* effect was not observed when the tumor was implanted in NSG mice, indicating that the impact of DIDS on the immune system was relevant for the antitumor effect. DIDS treatment enhanced the endogenous T cell antitumor immune response, with a significant increase in the numbers of tumor-specific CD8⁺ and CD4⁺ T cells infiltrating the

tumor and present in the tumor draining lymph nodes and the spleen. This beneficial effect of DIDS was also observed in combination with adoptive T cell transfer immunotherapy of antitumor CD8⁺ T cells. Mice treated with DIDS after ACT with OT-1CD8⁺ T cells showed a significant delay in tumor growth, and an increase in the overall survival of mice bearing B16OVA or LLCOVA tumors. This improvement in survival was concomitant to

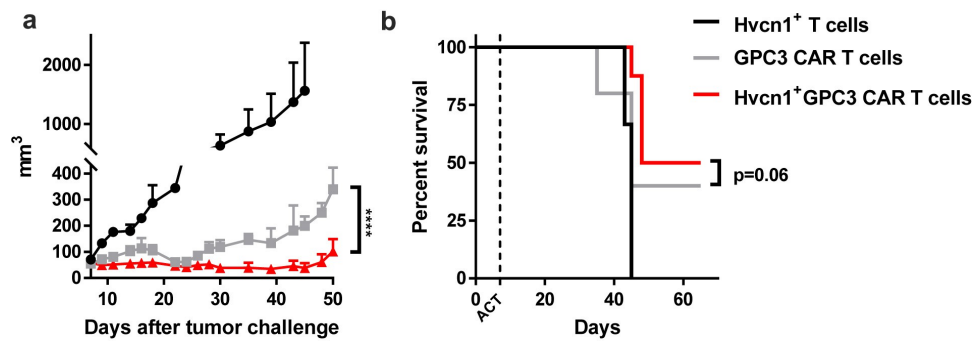


Figure 7. Hvcn1 overexpression improves the efficacy of CAR T cell immunotherapy. C57BL/6 mice were challenged subcutaneously with 1×10^6 PM299L-GPC3 embedded in matrigel and 7 d later they were irradiated and treated with 5×10^6 CD8⁺ T cells overexpressing Hvcn1, GPC3 CAR T cells or Hvcn1 overexpressing GPC3 CAR T cells. A) Tumor growth and B) survival were measured overtime. $n = 6-8$. **** = $p < .0001$.

an increase in the number of CD8⁺ T cells expressing granzyme B, IFN γ and TNF α suggesting that intratumoral pH acidification of transferred T cells impairs their antitumor activity. These results are in line with recent reports showing that neutralizing tumor acidity with bicarbonate monotherapy or in combination with other immunotherapies improved antitumor responses in murine models.⁸ There are other alternative pharmacologic approaches that could indirectly lead to the control of intracellular acidification. Potassium-sparing diuretics (amiloride) for metabolic alkalosis compensation,³⁹ and the use of inhibitors for carbonic anhydrase IX⁴⁰ or lactate dehydrogenase (LDH)⁴¹ are being considered to improve antitumor therapies. However, the efficacy of these agents to neutralize tumor pH or their effects on antitumor immunotherapy is not known. Other pathways associated with the control of pH_i that are also under consideration for intervention include the use of inhibitors of monocarboxylate transporters (MCT) (reviewed in⁴²), the vacuolar-type H⁺-ATPase proton pump,¹⁵ the sodium-hydrogen exchanger 1 (NHE-1)⁴³ or Ae2.²⁵ In preliminary experiments, we studied CD8⁺ T cell function in the presence of MCT inhibitors (AR-C155858, α -cyano-4-hydroxycinnamic acid, AZD3965 and diclofenac sodium salt) that impair lactate transport. We could observe an improvement on T cell proliferation in the presence of diclofenac sodium salt (at 0.01 and 0.1 mM) after TCR stimulation, but not with the other inhibitors (not shown). A recent report showed that diclofenac lowers lactate secretion of tumor cells and improves anti-PD1-induced T cell killing *in vitro* delaying tumor growth *in vivo*.⁴⁴ However, other works have reported an inhibition of IFN- γ production and T cell proliferation in the presence of diclofenac during T cell activation.⁴⁵ It has been also reported that sodium lactate, can either enhance or impair T cell proliferation depending on the dose,⁴⁶ suggesting a complex role for lactate during T cell activation. Further studies are needed to determine the role of MCT inhibitors in lactate transport across the membrane, the pH_i and consequently on T cell function.

Moreover, the use of inhibitors for enzymes, ion channels and anion exchangers that are ubiquitously expressed in different cell types may pose toxicity problems that must be carefully considered. For these reasons, we planned to

directly genetically modify lymphocytes to provide them with the tools to resist TME acidification and preserve their antitumor activity while minimizing the risk for treatment-related toxicities.

Based on the beneficial effect of DIDS treatment *in vivo*, we focused on the Ae2 anion exchanger. Previous studies reported that Ae2-deficient mice exhibit marked splenomegaly, expansion of the CD8⁺ T cell compartment, and an increase in the production of cytokines, which favor the development of autoimmunity.²⁴ It was also reported that Ae2 deficiency favors the alkalization of CD8⁺ T cells after TCR stimulation enhancing cell proliferation and IL-2 production.²³ Although those mice were whole-body knockouts for Ae2, these data highlight the relevance of Ae2 for CD8⁺ T cells to maintain pH_i homeostasis and modulate immune responses. These experiments prompted us to evaluate the effect of Ae2 silencing on the antitumor activity of Ag-specific CD8⁺ T cells. Using CRISPR/Cas9 technology we generated OT-1CD8⁺ T cells lacking Ae2. As observed *in vitro* with DIDS treatment,¹⁵ Ae2-deficient OT-1CD8⁺ T cells proliferated better than Ae2 proficient T cells and produced more IL-2 and IFN γ upon TCR stimulation with the SIINFEKL peptide. This improvement was observed at all pH levels tested, including the acidic pH 6.5 condition. When injected into tumor-bearing mice, Ae2-silenced OT-1CD8⁺ T cells delayed tumor growth more efficiently than Ae2-expressing T cells. When we isolated these cells from the spleen of tumor-bearing mice, we found higher numbers of SIINFEKL-specific T cells in mice treated with OT-1CD8⁺ T cells lacking Ae2. These results may indicate that Ae2 silencing makes CD8⁺ T cells more resistant to acidic conditions.

As an alternative to the silencing of the acidifier anion exchangers, we also tested the possibility of maintaining a more basic pH_i by overexpressing proton extruder channels. Proton pump inhibitors (PPI) have been used to reduce proton extrusion by tumor cells and smooth the acidification of TME while inducing tumor cell apoptosis and inhibition of tumor growth.⁴⁷⁻⁵¹ These studies elegantly described the effects of inhibiting acid extrusion in tumor cells but did not characterize the effects of PPIs on tumor infiltrating lymphocytes except for macrophages.⁵⁰ These PPIs might involve the risk of inducing T cell apoptosis as was described for the inhibition of HVCN1 in Jurkat T cells.¹² On the other hand, there are serious concerns about the increased cancer risk associated

with the long-term use of PPIs (reviewed in⁵²). In our study, we considered a different alternative to protect the T lymphocytes from excessive intracellular acidification in the TME by exploiting the basifying effect that these proton pumps can exert. For this reason, we genetically modified T cells to overexpress Hvcn1. We found that overexpression of Hvcn1 in lymphocytes increases their proliferative and cytokine-producing capacities even under acidic pH culture conditions. Notably, we observed that at pH as low as 6.5, where unmodified OT-1CD8⁺ T lymphocytes do not respond appropriately to antigenic stimulation with the SIINFEKL peptide, Hvcn1-overexpressing OT-1CD8⁺ T cells have a high functional activity, similar to that observed at physiological pH for unmodified T lymphocytes. These results suggest that Hvcn1 protects the cells from excessive acidification facilitating the TCR signaling that, as was previously described, is impaired by the acidic pH leading to an anergic state.⁷ When mice bearing B16OVA tumors were treated with these Hvcn1-expressing CD8⁺ OT-1 T cells, we found a significant delay in tumor growth, although only 12.5% of mice were able to totally reject the tumor and survive. This is particularly relevant in a model in which adoptive T cell transfer experiments are not very efficient because OVA antigen expression is rapidly lost due to immunopressure.^{53,54} Interestingly, Hvcn1 overexpression improved the antitumor efficacy of GPC3 CAR-T cells in a murine model of hepatocellular carcinoma, suggesting that the control of excessive pH acidification can also have a positive effect in CART cell-based therapies for solid tumors.

In conclusion, we have found that silencing of the acidifier Ae2 or the overexpression of the proton channel Hvcn1 may offer an advantage for lymphocytes thus improving their functionality in unfavorable acidic pH conditions present in the TME and might constitute an alternative for the improvement of cancer therapies based in the adoptive cell transfer of T lymphocytes.

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Author contributions statement

Conceptualization, F.N, T.L. and J.J.L.; Methodology, F.N, N.C, C.M-O., T.L., M.G., P.S., D.L.L., D.R., N.V., J.R, R-M. and J.J.L.; Investigation: F.N, N.C., T.L. and J.J.L.; Writing – Original Draft, F.N. and J.J.L.; Writing – Review & Editing, all authors; Funding Acquisition, T.L., F.P, and J.J.L.; Supervision, T.L. and J.J.L.

Disclosure statement













No potential conflict of interest was reported by the author(s).

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ORCID

Flor Navarro  <http://orcid.org/0000-0001-5250-8437>
 Noelia Casares  <http://orcid.org/0000-0003-3817-6434>
 Celia Martín-Otal  <http://orcid.org/0000-0002-5442-4496>
 Aritz Lasarte-Cia  <http://orcid.org/0000-0003-4758-7542>
 Patricia Sarrión  <http://orcid.org/0000-0002-3497-0481>
 David Reparaz  <http://orcid.org/0000-0003-2706-6870>
 Nerea Varo  <http://orcid.org/0000-0002-2362-9472>
 Juan Roberto Rodríguez-Madoz  <http://orcid.org/0000-0003-4327-1866>
 Felipe Prosper  <http://orcid.org/0000-0001-6115-8790>
 Sandra Hervás-Stubbs  <http://orcid.org/0000-0003-3391-1516>
 Teresa Lozano  <http://orcid.org/0000-0001-7122-5475>
 Juan José Lasarte  <http://orcid.org/0000-0003-1641-3881>

Data availability statement

The data that support the findings of this study are available from the corresponding author [F.N and J.J.L.], upon reasonable request.

References

- Vaupel P, Hockel M. Blood supply, oxygenation status and metabolic microenvironment of breast cancers: characterization and therapeutic relevance. *Int J Oncol.* 2000;17(5):869–879. doi:10.3892/ijo.17.5.869.
- Vaupel P. Physiological properties of malignant tumours. *NMR Biomed.* 1992;5(5):220–225. doi:10.1002/nbm.1940050505.
- Gatenby RA, Gillies RJ. Why do cancers have high aerobic glycolysis? *Nat Rev Cancer.* 2004;4(11):891–899. doi:10.1038/nrc1478.
- Griffiths JR. Are cancer cells acidic? *Br J Cancer.* 1991;64(3):425–427. doi:10.1038/bjc.1991.326.
- Fischer K, Hoffmann P, Voelkl S, Meidenbauer N, Ammer J, Edinger M, Gottfried E, Schwarz S, Rothe G, Hoves S, et al. Inhibitory effect of tumor cell-derived lactic acid on human T cells. *Blood.* 2007;109(9):3812–3819. doi:10.1182/blood-2006-07-035972.
- Mendler AN, Hu B, Prinz PU, Kreutz M, Gottfried E, Noessner E. Tumor lactic acidosis suppresses CTL function by inhibition of p38 and JNK/c-Jun activation. *Int J Cancer.* 2012;131(3):633–640. doi:10.1002/ijc.26410.
- Calcinotto A, Filipazzi P, Grioni M, Iero M, De Milito A, Ricupito A, Cova A, Canese R, Jachetti E, Rossetti M, et al. Modulation of microenvironment acidity reverses anergy in human and murine tumor-infiltrating T lymphocytes. *Cancer Res.* 2012;72(11):2746–2756. doi:10.1158/0008-5472.CAN-11-1272.
- Pilon-Thomas S, Kodumudi KN, El-Kenawi AE, Russell S, Weber AM, Luddy K, Damaghi M, Wojtkowiak JW, Mulé JJ, Ibrahim-Hashim A, et al. Neutralization of tumor acidity improves antitumor responses to immunotherapy. *Cancer Res.* 2016;76(6):1381–1390. doi:10.1158/0008-5472.CAN-15-1743.
- Uhl FM, Chen S, O'Sullivan D, Edwards-Hicks J, Richter G, Haring E, Andrieux G, Halbach S, Apostolova P, Büscher J, et al. Metabolic reprogramming of donor T cells enhances graft-versus-leukemia effects in mice and humans. *Sci Transl Med.* 2020;12(567). doi:10.1126/scitranslmed.abb8969.
- Parks SK, Chiche J, Pouyssegur J. pH control mechanisms of tumor survival and growth. *J Cell Physiol.* 2011;226(2):299–308. doi:10.1002/jcp.22400.

11. Webb BA, Chimenti M, Jacobson MP, Barber DL. Dysregulated pH: a perfect storm for cancer progression. *Nat Rev Cancer*. 2011;11(9):671–677. doi:10.1038/nrc3110.
12. Asuaje A, Smaldini P, Martin P, Enrique N, Orłowski A, Aiello EA, Docena G, Milesi V. The inhibition of voltage-gated H⁺ channel (HVCN1) induces acidification of leukemic Jurkat T cells promoting cell death by apoptosis. *Pflugers Arch*. 2017;469(2):251–261. doi:10.1007/s00424-016-1928-0.
13. Wang Y, Wu X, Li Q, Zhang S, Li SJ. Human voltage-gated proton channel hvl1: a new potential biomarker for diagnosis and prognosis of colorectal cancer. *PLoS One*. 2013;8(8):e70550. doi:10.1371/journal.pone.0070550.
14. Mboge MY, Mahon BP, McKenna R, Frost S. Carbonic anhydrases: role in pH control and cancer. *Metabolites*. 2018;8(1):19. doi:10.3390/metabo8010019.
15. De Milito A, Canese R, Marino ML, Borghi M, Iero M, Villa A, Venturi G, Lozupone F, Iessi E, Logozzi M, et al. pH-dependent antitumor activity of proton pump inhibitors against human melanoma is mediated by inhibition of tumor-acidity. *Int J Cancer*. 2010;127(1):207–219. doi:10.1002/ijc.25009.
16. Shiozaki A, Kudou M, Ichikawa D, Shimizu H, Arita T, Kosuga T, Konishi H, Komatsu S, Fujiwara H, Okamoto K, et al. Expression and role of anion exchanger 1 in esophageal squamous cell carcinoma. *Oncotarget*. 2017;8(11):17921–17935. doi:10.18632/oncotarget.14900.
17. Zhang LJ, Lu R, Song YN, Zhu J-Y, Xia W, Zhang M, Shao Z-Y, Huang Y, Zhou Y, Zhang H, et al. Knockdown of anion exchanger 2 suppressed the growth of ovarian cancer cells via mTOR/p70S6K1 signaling. *Sci Rep*. 2017;7(1):6362. doi:10.1038/s41598-017-06472-w.
18. Parks SK, Pouyssegur J. The Na⁺/HCO₃⁻ co-transporter SLC4A4 plays a role in growth and migration of colon and breast cancer cells. *J Cell Physiol*. 2015;230(8):1954–1963. doi:10.1002/jcp.24930.
19. Amith SR, Fliegel L. Regulation of the Na⁺/H⁺ exchanger (NHE1) in breast cancer metastasis. *Cancer Res*. 2013;73(4):1259–1264. doi:10.1158/0008-5472.CAN-12-4031.
20. Alper SL. Molecular physiology of SLC4 anion exchangers. *Exp Physiol*. 2006;91(1):153–161. doi:10.1113/expphysiol.2005.031765.
21. Romero MF. Molecular pathophysiology of SLC4 bicarbonate transporters. *Curr Opin Nephrol Hypertens*. 2005;14(5):495–501. doi:10.1097/01.mnh.0000168333.01831.2c.
22. Concepcion AR, Salas JT, Saez E, Sarvide S, Ferrer A, Portu A, Uriarte I, Hervás-Stubbs S, Oude Elferink RPJ, Prieto J, et al. CD8⁺ T cells undergo activation and programmed death-1 repression in the liver of aged Ae2a,b^{-/-} mice favoring autoimmune cholangitis. *Oncotarget*. 2015;6(30):28588–28606. doi:10.18632/oncotarget.5665.
23. Concepcion AR, Salas JT, Sarvide S, Saez E, Ferrer A, López M, Portu A, Banales JM, Hervás-Stubbs S, Oude Elferink RPJ, et al. Anion exchanger 2 is critical for CD8⁺ T cells to maintain pH homeostasis and modulate immune responses. *Eur J Immunol*. 2014;44(5):1341–1351. doi:10.1002/eji.201344218.
24. Salas JT, Banales JM, Sarvide S, Recalde S, Ferrer A, Uriarte I, Oude Elferink RPJ, Prieto J, Medina JF. Ae2a,b-deficient mice develop antimitochondrial antibodies and other features resembling primary biliary cirrhosis. *Gastroenterology*. 2008;134(5):1482–1493. doi:10.1053/j.gastro.2008.02.020.
25. Celay J, Lozano T, Concepcion AR, Beltrán E, Rudilla F, García-Barchino MJ, Robles EF, Rabal O, de Miguel I, Panizo C, et al. Targeting the anion exchanger 2 with specific peptides as a new therapeutic approach in B lymphoid neoplasms. *Haematologica*. 2018;103(6):1065–1072. doi:10.3324/haematol.2017.175687.
26. Capasso M, DeCoursey TE, Dyer MJ. pH regulation and beyond: unanticipated functions for the voltage-gated proton channel-HVCN1. *Trends Cell Biol*. 2011;21(1):20–28. doi:10.1016/j.tcb.2010.09.006.
27. Seredenina T, Demaurex N, Krause KH. Voltage-gated proton channels as novel drug targets: from NADPH oxidase regulation to sperm biology. *Antioxid Redox Signal*. 2015;23(5):490–513. doi:10.1089/ars.2013.5806.
28. DeCoursey TE, Cherny VV. Potential, pH, and arachidonate gate hydrogen ion currents in human neutrophils. *Biophys J*. 1993;65(4):1590–1598. doi:10.1016/S0006-3495(93)81198-6.
29. Kapus A, Romanek R, Grinstein S. Arachidonic acid stimulates the plasma membrane H⁺ conductance of macrophages. *J Biol Chem*. 1994;269(7):4736–4745. doi:10.1016/S0021-9258(17)37606-8.
30. Murphy R, Cherny VV, Morgan D, DeCoursey TE. Voltage-gated proton channels help regulate pH_i in rat alveolar epithelium. *Am J Physiol Lung Cell Mol Physiol*. 2005;288(2):L398–408. doi:10.1152/ajplung.00299.2004.
31. Wang Y, Li SJ, Wu X, Che Y, Li Q. Clinicopathological and biological significance of human voltage-gated proton channel Hvl1 protein overexpression in breast cancer. *J Biol Chem*. 2012;287(17):13877–13888. doi:10.1074/jbc.M112.345280.
32. Casares N, Rudilla F, Arribillaga L, Llopiz D, Riezu-Boj JJ, Lozano T, López-Sagasetta J, Guembe L, Sarobe P, Prieto J, et al. A peptide inhibitor of FOXP3 impairs regulatory T cell activity and improves vaccine efficacy in mice. *J Immunol*. 2010;185(9):5150–5159. doi:10.4049/jimmunol.1001114.
33. Jessen F, Sjöholm C, Hoffmann EK. Identification of the anion exchange protein of Ehrlich cells: a kinetic analysis of the inhibitory effects of 4,4'-diisothiocyanato-2,2'-stilbene-disulfonic acid (DIDS) and labeling of membrane proteins with 3H-DIDS. *J Membr Biol*. 1986;92(3):195–205. doi:10.1007/BF01869388.
34. Romero MF, Chen AP, Parker MD, Boron WF. The SLC4 family of bicarbonate (HCO₃⁻) transporters. *Mol Aspects Med*. 2013;34(2–3):159–182. doi:10.1016/j.mam.2012.10.008.
35. Qian C, Diez J, Larrea E, Garcandiá A, Arrazola A, Civeira MP, Medina JF, et al. The stilbene disulfonic acid DIDS stimulates the production of TNF- α in human lymphocytes. *Biochem Biophys Res Commun*. 1992;189(3):1268–1274. doi:10.1016/0006-291x(92)90210-c.
36. Platt RJ, Chen S, Zhou Y, Yim M, Swiech L, Kempton H, Dahlman J, Parnas O, Eisenhaure T, Jovanovic M, et al. CRISPR-Cas9 knockin mice for genome editing and cancer modeling. *Cell*. 2014;159(2):440–455. doi:10.1016/j.cell.2014.09.014.
37. Bosticardo M, Ariotti S, Losana G, Bernabei P, Forni G, Novelli F. Biased activation of human T lymphocytes due to low extracellular pH is antagonized by B7/CD28 costimulation. *Eur J Immunol*. 2001;31(9):2829–2838. doi:10.1002/1521-4141(200109)31:9<2829::AID-IMMU2829>3.0.CO;2-U.
38. Carswell KS, Papoutsakis ET. Extracellular pH affects the proliferation of cultured human T cells and their expression of the interleukin-2 receptor. *J Immunother*. 2000;23(6):669–674. doi:10.1097/00002371-200011000-00008.
39. Poon AC, Inkol JM, Luu AK, Mutsaers AJ. Effects of the potassium-sparing diuretic amiloride on chemotherapy response in canine osteosarcoma cells. *J Vet Intern Med*. 2019;33(2):800–811. doi:10.1111/jvim.15382.
40. van Kuijk SJ, Gieling RG, Niemans R, Lieuwes NG, Biemans R, Telfer BA, Haenen GRMM, Yaromina A, Lambin P, Dubois LJ, et al. The sulfamate small molecule CAIX inhibitor S4 modulates doxorubicin efficacy. *PLoS One*. 2016;11(8):e0161040. doi:10.1371/journal.pone.0161040.
41. Oshima N, Ishida R, Beebe K, Brender JR, Yamamoto K, Urban D, Rai G, Johnson MS, Benavides G, et al. Dynamic Imaging of LDH inhibition in tumors reveals rapid in vivo metabolic rewiring and vulnerability to combination therapy. *Cell Rep*. 2020;30(6):1798–1810 e4. doi:10.1016/j.celrep.2020.01.039.
42. Payen VL, Mina E, Van Hee VF, Porporato PE, Sonveaux P. Monocarboxylate transporters in cancer. *Mol Metab*. 2020;33:48–66. doi:10.1016/j.molmet.2019.07.006.

43. Guan X, Hasan MN, Begum G, Kohanbash G, Carney KE, Pigott VM, Persson AI, Castro MG, Jia W, Sun D, et al. Blockade of Na/H exchanger stimulates glioma tumor immunogenicity and enhances combinatorial TMZ and anti-PD-1 therapy. *Cell Death Dis.* 2018;9(10):1010. doi:10.1038/s41419-018-1062-3.
44. Renner K, Bruss C, Schnell A, Koehl G, Becker HM, Fante M, Menevse A-N, Kauer N, Blazquez R, Hacker L, et al. Restricting glycolysis preserves T cell effector functions and augments checkpoint-therapy. *Cell Rep.* 2019;29(1):135–150 e9. doi:10.1016/j.celrep.2019.08.068.
45. Chirasani SR, Leukel Q P, Gottfried E, Hochrein J, Stadler K, Neumann B, Oefner PJ, Gronwald W, Bogdahn U, Hau P, et al. Diclofenac inhibits lactate formation and efficiently counteracts local immune suppression in a murine glioma model. *Int J Cancer.* 2013;132(4):843–853. doi:10.1002/ijc.27712.
46. Ostroukhova M, Goplen N, Karim MZ, Michalec L, Guo L, Liang, Alam R. The role of low-level lactate production in airway inflammation in asthma. *Am J Physiol Lung Cell Mol Physiol.* 2012;302(3):L300–7. doi:10.1152/ajplung.00221.2011.
47. Chen M, Zou X, Luo H, Cao J, Zhang X, Zhang B, Liu W. Effects and mechanisms of proton pump inhibitors as a novel chemosensitizer on human gastric adenocarcinoma (SGC7901) cells. *Cell Biol Int.* 2009;33(9):1008–1019. doi:10.1016/j.cellbi.2009.05.004.
48. Kim YJ, Lee JS, Hong KS, Chung JW, Kim JH, Hahm KB. Novel application of proton pump inhibitor for the prevention of colitis-induced colorectal carcinogenesis beyond acid suppression. *Cancer Prev Res (Phila).* 2010;3(8):963–974. doi:10.1158/1940-6207.CAPR-10-0033.
49. Morimura T, Fujita K, Akita M, Nagashima M, Satomi A. The proton pump inhibitor inhibits cell growth and induces apoptosis in human hepatoblastoma. *Pediatr Surg Int.* 2008;24(10):1087–1094. doi:10.1007/s00383-008-2229-2.
50. Vishvakarma NK, Singh SM. Immunopotentiating effect of proton pump inhibitor pantoprazole in lymphoma-bearing murine host: implication in antitumor activation of tumor-associated macrophages. *Immunol Lett.* 2010;134(1):83–92. doi:10.1016/j.imlet.2010.09.002.
51. Yeo M, Kim DK, Kim YB, Oh TY, Lee JE, Cho SW, Kim HC, et al. Selective induction of apoptosis with proton pump inhibitor in gastric cancer cells. *Clin Cancer Res.* 2004;10(24):8687–8696. doi:10.1158/1078-0432.CCR-04-1065.
52. Suissa S, Suissa A. Proton-pump inhibitors and increased gastric cancer risk: time-related biases. *Gut.* 2018;67(12):2228–2229. doi:10.1136/gutjnl-2017-315729.
53. Goldberger O, Volovitz I, Machlenkin A, Vadai E, Tzevoval E, Eisenbach L. Exuberated numbers of tumor-specific T cells result in tumorescape. *Cancer Res.* 2008;68(9):3450–3457. doi:10.1158/0008-5472.CAN-07-5006.
54. Lozano T, Chocarro S, Martin C, Lasarte-Cia A, Del Valle C, Gorraiz M, Sarrion P, et al. Genetic modification of CD8(+) T cells to express EGFR: potential application for adoptive T cell therapies. *Front Immunol.* 2019;10:2990. doi:10.3389/fimmu.2019.02990.