



Combined sublethal irradiation and agonist anti-CD40 enhance donor T cell accumulation and control of autochthonous murine pancreatic tumors

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Abstract

Tumor-reactive T lymphocytes can promote the regression of established tumors. However, their efficacy is often limited by immunosuppressive mechanisms that block T cell accumulation or function. ACT provides the opportunity to ameliorate immune suppression prior to transfer of tumor-reactive T cells to improve the therapeutic benefit. We evaluated the combination of lymphodepleting whole body irradiation (WBI) and agonist anti-CD40 (α CD40) antibody on control of established autochthonous murine neuroendocrine pancreatic tumors following the transfer of naïve tumor-specific CD8 T cells. Sublethal WBI had little impact on disease outcome but did promote T cell persistence in the lymphoid organs. Host conditioning with α CD40, an approach known to enhance APC function and T cell expansion, transiently increased donor T cell accumulation in the lymphoid organs and pancreas, but failed to control tumor progression. In contrast, combined WBI and α CD40 prolonged T cell proliferation and dramatically enhanced accumulation of donor T cells in both the lymphoid organs and pancreas. This dual conditioning approach also promoted high levels of inflammation in the pancreas and tumor, induced histological regression of established tumors, and extended the lifespan of treated mice. Prolonged survival was entirely dependent upon adoptive transfer, but only partially dependent upon IFN γ production by donor T cells. Our results identify the novel combination of two clinically relevant host conditioning approaches that synergize to overcome immune suppression and drive strong tumor-specific T cell accumulation within well-established tumors.

Keywords CD8 T cell · CD40 agonist antibody · Immunotherapy · Transgenic mice · Pancreatic neuroendocrine cancer

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Abbreviations

α CD40	Anti-CD40
gB	Glycoprotein B
GKO	Interferon gamma knockout
Gy	Gray
PLN	Pancreaticoduodenal lymph node
RT4	Rip1-Tag4
T Ag	T antigen
TetI	H-2D ^b /I tetramer
TRAMP	Transgenic adenocarcinoma of the mouse prostate
WBI	Whole body irradiation

Introduction

The presence of tumor-infiltrating T cells is associated with a positive prognosis in several cancers and correlates with the success of some immune-based therapies [1, 2].

However, tumor-reactive T cells are susceptible to mechanisms that suppress their function in the TME, and in the case of non-inflamed tumors, are excluded from the TME entirely [3]. ACT provides the opportunity to modify the immunosuppressive host environment prior to introduction of tumor-reactive T cells and can generate durable regression of established cancer in both experimental mouse models and cancer patients [4]. Host conditioning regimens that modify immune suppression are closely linked to successful ACT. In particular, nonmyeloablative lymphodepletion dramatically improves the success of ACT-based therapies, most often consisting of chemotherapy in patients [5, 6] and effectively reproduced by WBI in mouse models [5]. Additional approaches to target immune suppression during ACT include administration of antibodies that enhance immune function or block immune checkpoint inhibitory pathways [7–11], as well as the use of adjuvants and immunization to improve T cell expansion [12, 13].

For solid tumors, ACT with *in vitro* expanded tumor-infiltrating T lymphocytes in combination with lymphodepletion has a high success rate in metastatic melanoma patients [14], but a gap remains in our understanding of the efficacy of this approach for other tumor types. In mice, host conditioning with WBI prior to ACT induces lymphodepletion, which can increase donor cell access to survival cytokines and eliminate regulatory T cells [5]. WBI can also promote normalization of the tumor vasculature [15, 16], enhance host APC function [17, 18], and increase type I IFN production and innate sensing of tumors [19, 20]. Our group previously demonstrated that WBI increased the magnitude and duration of the donor CD8 T cell response, resulting in regression and durable control of established autochthonous SV40 T antigen (T Ag)-induced brain tumors [7, 21]. This approach also was beneficial in the immunosuppressive TRansgenic Adenocarcinoma of the Mouse Prostate (TRAMP) model, resulting in regression of established lesions and CD8 T cell persistence [22]. However, combining the beneficial effects of host lymphodepletion with other activators of T cell immunity may provide a more potent anti-tumor response that could broaden the success of T cell-based therapies.

We previously demonstrated that agonist α CD40 administration dramatically enhanced initial CD8 T cell priming against established T Ag-induced tumors, and resulted in either prolonged tumor control or tumor regression [7, 8, 23]. Increased T cell accumulation *in vivo* is consistent with the proposed mechanism of α CD40-enhanced antigen presentation and delivery of co-stimulatory and cytokine signals by professional APCs [24–26]. In addition, α CD40 reverses the accumulation of suppressive myeloid cells, facilitating T cell-based anti-tumor immunity [27, 28]. Whether host lymphodepletion can be combined with agonist α CD40 to mediate more effective control of established tumors is unknown.

To address this question, we utilized the Rip1-Tag4 (RT4) model of neuroendocrine pancreatic cancer that undergoes multistage carcinogenesis as a result of T Ag expression from the rat insulin II promoter [29]. T Ag expression in the β cells beginning at 5 weeks of age [30] leads to widespread islet hyperplasia and progression to insulinomas by 3 and 6 months of age, respectively [29]. All mice succumb to tumor progression at an average of 263 days of age [30]. Once T Ag is expressed in the pancreas, peripheral CD8 T cell tolerance manifests and abrogates the ability of immunization to block tumor progression [30, 31]. Adoptively transferred naïve tumor-specific T cells are efficiently activated in tumor-bearing RT4 mice, but are rapidly deleted and fail to alter tumor progression [31]. Administration of agonist α CD40 antibody can enhance the accumulation of T Ag-specific donor T cells within both the peripheral lymphoid organs and tumors of RT4 mice. However, these T cells are rapidly eliminated, and treatment had only a transient impact on tumor progression [32]. Thus, this model provides a challenging setting to test the hypothesis that the novel combination of host lymphodepletion and agonist α CD40 can improve ACT-mediated immunotherapy by promoting the expansion and accumulation of functional tumor-specific donor T cells.

Materials and methods

Mice

RT4 mice [30] on the C57BL/6J background were maintained in specific pathogen-free barrier housing in the Penn State College of Medicine animal vivarium. Both male and female hemizygous mice were used for all experiments. Therapy was initiated at 6 months of age, when mice have established neoplasia [32]. TCR-I mice on the C57BL/6J background have been described previously [8], and are available from the Jackson Laboratory [B6.Cg-Tg(TcraY1,TcrbY1)416Tev/J]. TCR-I mice were used between the ages of 8–12 weeks.

Cell lines and reagents

B6/WT-19 cells that express full-length, wild-type SV40 T Ag were originally derived by Tevethia, and have been described previously [33]. Processing and short-term culture of single-cell lymphocyte suspensions from mice was performed in RPMI 1640 medium supplemented with 2% fetal bovine serum, 100 U/ml penicillin, 100 μ g/ml streptomycin, 2 mM L-glutamine, 10 mM HEPES, and 50 μ M 2-ME.

Adoptive immunotherapy

6-month old RT4 mice were exposed to 4 Gray (Gy) whole body γ -irradiation using a ^{60}Co -source Gamma-Cell 220 irradiator (Nordion International) 1 day before adoptive transfer or injected intraperitoneally with 100 μg αCD40 agonist monoclonal antibody (clone FGK45; BioXCell) 1 day before and 1 day following adoptive transfer. Alternatively, the two conditioning approaches were combined with mice receiving WBI 6–8 h prior to the first dose of αCD40 . RT4 mice were intravenously injected with 1×10^6 TCR-I cells in PBS. TCR-I cell purity was determined by flow cytometric detection with Site I-specific MHC tetramer, and ranged from 30 to 45% of the bulk spleen and LN single-cell suspension. For proliferation experiments, 5 μM CFSE in 0.1% bovine serum albumin in PBS was used to label cells prior to transfer [31]. Blood glucose levels were measured every 7–10 days in peripheral blood from the tail vein using a TRUE2go glucose meter (NIPRO Diagnostics).

Processing, staining and flow cytometry analyses

Red blood cell-depleted single-cell suspensions from spleens and LNs were obtained as previously described [34]. Pancreata were dissected from the mouse, and enzymatically digested with a master mix of 1% FBS, 1 mg/ml collagenase (Life Technologies) and 50 U/ml DNase I (Roche) in RPMI 1640 complete media with rocking at 37 °C for 15–30 min. Large particulates were removed by passing the cell suspension through wire mesh.

Aliquots of 2×10^5 pancreatic or LN cells or 2×10^6 splenocytes were stained with commercially available antibodies and PE-labeled H-2D^b/I tetramer (TetI) [35] prior to analysis on a FACSCanto II, LSRII or LSR Fortessa (BD Biosciences) in the Penn State Hershey Flow Cytometry Core Facility. Samples were fixed with 2% paraformaldehyde or stained with 7-aminoactinomycin D (7-AAD) prior to analysis. Data were analyzed using FlowJo software (FlowJo, LLC). Antibodies used include: CD45.2-V500 (clone 104), CD8-V450 (clone 53-6.7), CD8-BV786 (clone 53-6.7), CD8-PE (clone 53-6.7), CD90.1-APC (clone HIS51), CD90.1-PerCP Cy5.5 (clone HIS51), CD44-FITC (clone IM7), CD44-Alexa fluor700 (clone IM7), CD62L-APC α 780 (clone MEL-14), CD62L-APC-Cy7 (clone MEL-14), KLRG1-PE Cy7 (clone 2F1), PD-1-FITC (clone J43), CD127-V450 (clone SB/199), Ki67-PE (clone SolA15). Antibodies were purchased from BD Biosciences or eBioscience. Donor T cells were identified as 7-AAD^{neg} CD45.2 + CD8 + and then CD90.1 + and/or TetI+.

Intracellular cytokine staining

2×10^6 cells from single-cell suspensions were incubated at 37 °C in RPMI 1640 complete media containing 2% FBS, 1 $\mu\text{g}/\text{ml}$ brefeldin A and 1 μM Site I peptide ($_{206}\text{SAIN-NYAQKL}_{215}$) or H-2K^b/HSV glycoprotein B (gB) peptide ($_{498}\text{SSIEFARL}_{505}$). Following a 4-h incubation, cells were stained for CD8 and CD90.1. The Cytofix/Cytoperm kit (BD Pharmingen) was used to permeabilize and intracellularly stain cells with IFN γ -FITC (clone XMG1.2) and TNF α -PE (clone MP6-XT22) antibodies per the manufacturer's instructions. Site I-reactive T cells was calculated by subtracting the proportion of gB peptide responders in parallel samples.

Histology

Mice were euthanized by CO₂ asphyxiation and perfused with 10 ml of PBS followed by 10 ml of 10% neutral buffered formalin. The pancreas was dissected from the mouse, and immersion fixed in 10% neutral buffered formalin for 24 h followed by transfer into 70% ethanol for at least 24 h. Pancreata were embedded in paraffin, and sections stained with H&E or Masson's trichrome for blinded scoring by a board-certified veterinary pathologist. Images were collected using an Olympus BX51 microscope fitted with an Olympus DP71 digital camera and cellSens Standard 1.12 imaging software (Olympus). Briefly, severity of pancreatic lesions (pancreatitis) was quantified as previously described [36]: 0, no pathological changes; 1, minimal infiltration of periductal tissue with leukocytes but no parenchymal destruction; 2, moderate periductal infiltration with leukocytes associated with beginning parenchymal destruction; 3, severe periductal inflammation and/or more extended parenchymal destruction; 4, diffuse leukocyte infiltrates, destruction of acini and (partial) replacement by adipose tissue. Inflammation in the pancreatic islets (isletitis) was scored as follows: 0, no significant inflammation; 1, scattered low numbers of mononuclear leukocytes; 2, multifocal moderate numbers of mononuclear leukocytes; 3, diffuse and large numbers of mononuclear leukocytes. Hyperplastic islets were identified as described [29, 37].

Statistical analyses

Two-sided Student's *t* test, one-way ANOVA or two-way ANOVA were used to compare the outcomes of different treatment groups. In all figures, graphical data represent the mean \pm SEM. Kaplan–Meier survival curves and log-rank were used to determine differences in survival outcome. All statistical analyses were performed with the significance level set to 0.05 using GraphPad Prism software (v5.0f or higher, San Diego, CA).

Results

Dual conditioning of RT4 mice with WBI and anti-CD40 delays tumor progression following ACT

To evaluate the role of combined lymphodepletion and α CD40 (dual conditioning) on ACT-mediated control of tumor progression, groups of 6-month old RT4 mice were administered WBI and/or agonist α CD40 with transfer of naïve TCR-I T cells (Fig. 1a), specific for the H-2D^b-restricted T Ag site I determinant. Tumor progression was monitored by the measurement of blood glucose which decreases due to increased insulin production by insulinomas [32]. Neither TCR-I cell transfer alone nor in combination with either α CD40 or WBI conditioning was able to delay progression of established tumors (Fig. 1b). However, dual conditioning with ACT (WBI + α CD40 + TCR-I) delayed tumor progression, with blood glucose levels remaining near normal for approximately 80 days post treatment in most animals. This effect required donor TCR-I cells.

Control of tumor progression in dual-conditioned RT4 mice receiving ACT resulted in a highly significant extension of life span compared to all other groups (Fig. 1c; median of 350 days). No other treatment had a significant impact on survival. Three mice from the dual-conditioned + ACT group were killed at 332 days and observed to contain advanced insulinomas, consistent with tumor progression at this late time point (unpublished observations). These data demonstrate that α CD40 and WBI conditioning synergize to improve ACT-mediated control of pancreatic tumors, while the individual approaches had no impact.

Dual conditioning with ACT reduces overall tumor burden

We evaluated the impact of each conditioning regimen on inflammation within the pancreas 7 days post ACT. Blinded scoring of H&E-stained pancreas sections revealed that WBI + ACT failed to induce inflammation in the periductal region and acini of the pancreas (pancreatitis) or within the pancreatic islets (isletitis) (Fig. 2a). α CD40 alone induced general pancreatitis that was reduced when mice also received WBI, suggesting that irradiation suppressed or reduced the endogenous inflammatory cells (Fig. 2a; Supplementary Fig. 1c, d). In contrast, administration of α CD40 + ACT promoted both pancreatitis and isletitis (Fig. 2a; Supplementary Fig. 1a) with the pancreatitis mainly observed peritumorally and adjacent to the LNs (unpublished observations). Mice receiving dual conditioning with ACT displayed a similar high degree of

pancreatitis and isletitis (Fig. 2a; Supplementary Fig. 1b). In this case, however, inflammation was observed both throughout the pancreas and within the tumor (Fig. 2b), indicating a more uniform level of inflammation than observed in α CD40 + ACT treated mice. These results demonstrate that α CD40 conditioning triggers inflammation within the pancreas but donor T cells are required for inflammation within the islets and tumors, while WBI + α CD40 + TCR-I produced more uniform inflammation than α CD40 + ACT.

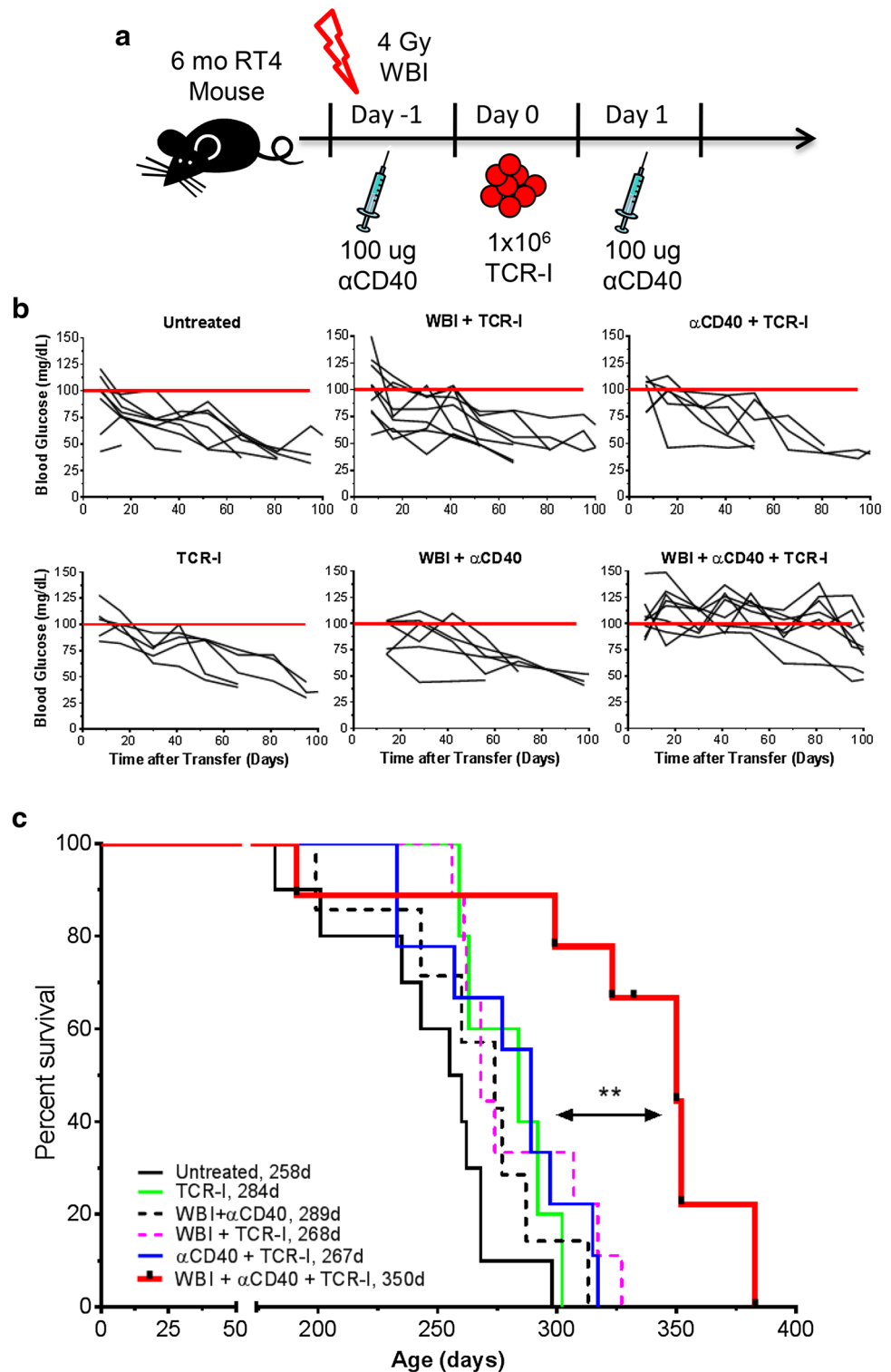
By 21 days post ACT, insulinomas in dual-conditioned mice showed significant loss of cellularity by H&E staining, consistent with tumor regression (Fig. 2e). Regressed tumors were replaced with coarse fibrosis as indicated by positive (blue) staining in Masson's trichrome-stained sections (Fig. 2h). No other treatment produced tumor regression and H&E stained sections revealed well-developed insulinomas (Fig. 2c, d, f, g and unpublished observations). Only dual-conditioned mice receiving ACT exhibited a subset of tumor-free mice 70 days post treatment (Fig. 2i) while at least one tumor was identified in all surviving mice in other treatment groups. In addition, only WBI + α CD40 + TCR-I led to a significantly reduced percentage of hyperplastic islets (Fig. 2j). These results indicate that dual conditioning, but neither single conditioning regimen, significantly reduced the tumor burden following ACT.

Dual conditioning increases donor T cell accumulation in the lymphoid organs and tumor-bearing pancreas

Donor cell accumulation in the tumor-draining pancreaticoduodenal LN (PLN) was assessed to define the impact of each conditioning regimen on initial T cell priming and subsequent accumulation. Individually, WBI and α CD40 each had a small impact on the frequency of donor TCR-I cells in the PLN at day 5, followed by contraction at day 7 (Fig. 3a, b). In contrast, dual conditioning dramatically increased the frequency of TCR-I cells, achieving peak levels at day 7. This increase was statistically significant compared to all other groups at days 5, 7 and 10, and donor T cells remained readily detectable in dual-conditioned mice at day 21. In contrast, total CD8 T cells were only significantly increased at day 7 in dual-conditioned mice (Supplementary Fig. 2).

We evaluated donor T cell proliferation using both CFSE dilution and Ki67 staining. By day 3 post transfer, almost all CFSE-labeled donor T cells in the PLN had undergone multiple rounds of cell division regardless of host conditioning (Fig. 3c). CFSE signal was almost completely eliminated by day 5 in all treatment groups (Fig. 3c), despite differences in T cell accumulation at this early time point (Fig. 3a). To evaluate donor T cell proliferation beyond day 5, recovered donor cells were stained for Ki67. T cells from dual-conditioned mice maintained a significantly higher proportion

Fig. 1 Dual conditioning plus TCR-I transfer delays tumor progression and increases the survival of RT4 mice. **a** Treatment schema. **b** Peripheral blood glucose levels of individual mice (black lines). The red line indicates 100 dI/ml (normal). **c** Kaplan–Meier survival analysis. Median survival (days) is listed beside each group in the key. **b, c** $N=5-10$ /group; WBI + α CD40 + TCR-I vs. all other treatment groups; *** $p < 0.001$ by log-rank test in **c**



of proliferating cells 7 days after transfer (Fig. 3d). Sustained proliferation corresponded with higher accumulation of donor T cells at days 7 and 10 in the dual-conditioned group (Fig. 3a). These results indicate that sufficient antigen is available to prime tumor-specific T cells in the PLN independent of host conditioning, while dual conditioning

promotes continued proliferation and accumulation of donor T cells.

We also quantified T cells in the spleen as an indication of systemic immunity. Donor T cell accumulation was not improved by WBI, but was initially increased with α CD40 (Fig. 4a). TCR-I cells in α CD40 treated mice exhibited

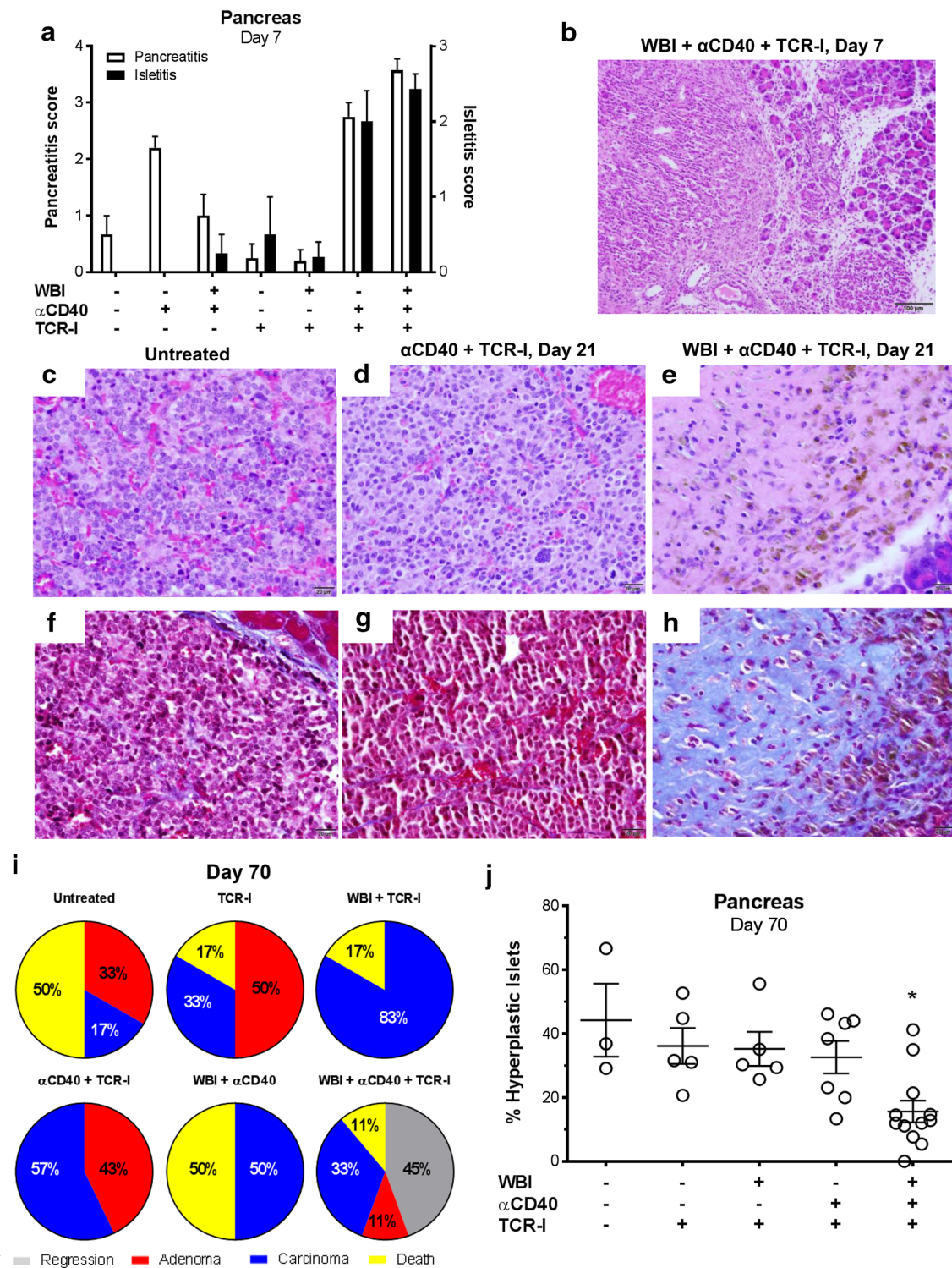


Fig. 2 Dual conditioning with ACT reduces disease burden in neoplastic islets. **a** Inflammation scores in the periductal and acinar regions (pancreatitis) and islets (isletitis) from H&E-stained sections of whole pancreata harvested 7 days after the indicated treatments. *N* = 4–6/group. **b** Representative H&E-stained section showing inflammation within a pancreatic carcinoma and adjacent pancreatic acinar compartment. **c–h** Representative H&E (**c–e**) and Masson’s trichrome-stained (**f–h**) tumors harvested from untreated (**c, f**) or RT4

mice that received anti-CD40+TCR-I cells (**d, g**) or dual conditioning+TCR-I cells (**e, h**). **i** Highest disease stage observed 70 days after treatment, *N* = 6–9/group. **j** Percentage hyperplastic islets in whole pancreas sections from mice shown in **i** that survived to day 70 post treatment. Each point represents an individual mouse. *N* = 5–12/group; WBI+ α CD40+TCR-I vs. all other treatment groups, **p* < 0.05 by one-way ANOVA with Dunnett’s post-test

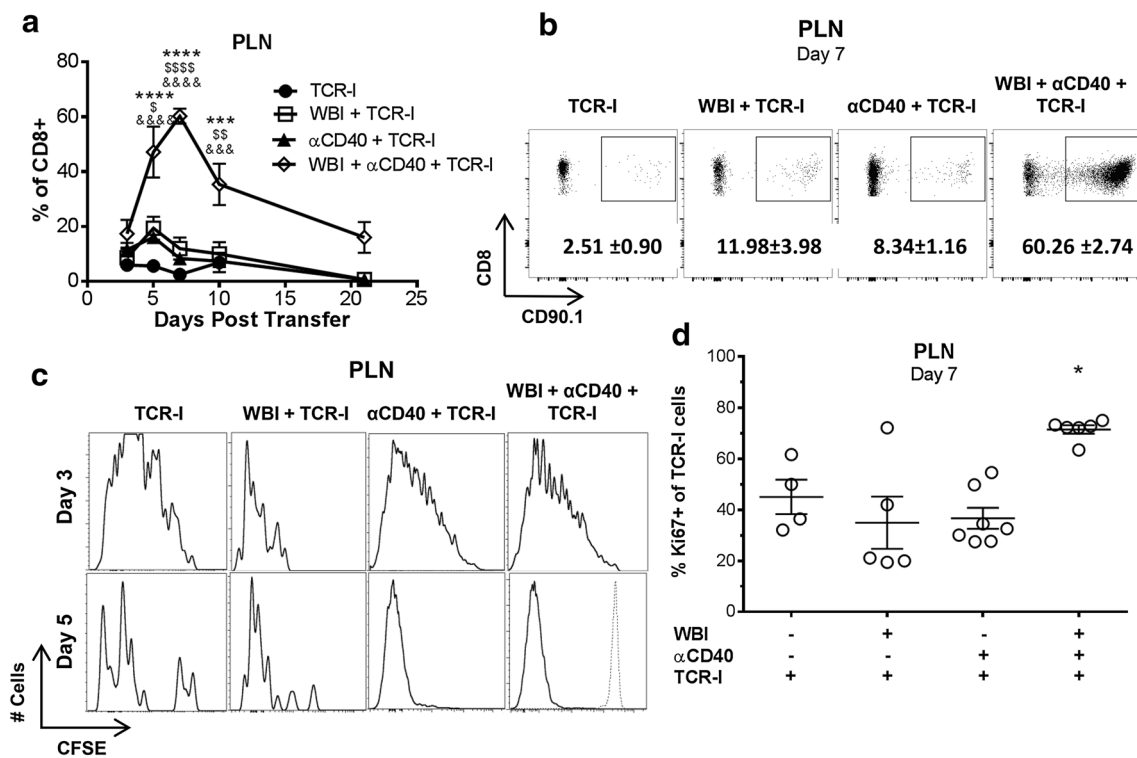


Fig. 3 Dual conditioning prolongs donor T cell proliferation and increases accumulation in the PLN. **a** Percentage of CD90.1+ or TetI+TCR-I cells accumulating in the PLN over time. *N*=6–18/group (days 3–10), 2–3/group (days 14, 21): WBI+αCD40+TCR-I vs. TCR-I (asterisks), vs. WBI+TCR-I (dollar symbol), vs. αCD40+TCR-I (ampersand); 1 digit *p*<0.05, 2 digits *p*<0.01, 3 digits *p*<0.001, 4 digits *p*<0.0001 by two-way ANOVA with Bon-

ferroni corrections. **b** Representative flow cytometry panels from **a**. Numbers indicate the mean percentage ± SEM of CD8⁺ cells. **c** CFSE staining of CD90.1+TCR-I cells in the PLN 3 and 5 days post treatment. **d** Ki67 staining of CD90.1+TCR-I cells in the PLN 7 days post treatment. *N*=4–6/group; WBI+αCD40+TCR-I vs. all other groups, ***p*<0.01 by one-way ANOVA with Dunnett’s post-test

peak accumulation at days 5–7, and contracted to baseline levels within 2 weeks. In contrast, accumulation of TCR-I cells in dual-conditioned mice peaked 10 days post ACT, and achieved significantly higher total cells at days 7 and 10 compared to all other treatments. This increase in total donor T cell accumulation was mirrored by a proportional increase among splenic CD8 T cells (Fig. 4b). T cells from dual-conditioned mice contracted approximately threefold by day 14, but remained above the levels observed with other treatments (Fig. 4a). These results indicate that systemic accumulation of donor T cells is most effectively increased by dual conditioning.

As found in the lymphoid organs, the number of TCR-I cells infiltrating the pancreas was not significantly enhanced by WBI, but αCD40 produced a small, transient donor cell accumulation 7 days after transfer (Fig. 4c, d). Importantly, dual conditioning increased peak accumulation of TCR-I T cells in the pancreas at day 7 above that achieved in all other groups (Fig. 4c). TCR-I T cells contracted after day 7 with kinetics similar to that observed in the PLN rather than continuing to accumulate as observed in the spleen. Donor

T cells recovered from spleen (Fig. 5a–c) and pancreas (Fig. 5d–f) on day 7 did not significantly vary in expression of phenotypic surface markers including CD62L and PD-1. A high, yet similar proportion of donor T cells recovered from the spleen and pancreas of treated mice expressed PD-1, regardless of the type of treatment. Splenic T cells recovered from dual-conditioned mice showed a minor but statistically significant increase in the proportion of KLRG1+ cells (Fig. 5a). Thus, no major phenotypic differences were observed despite the dramatic increase in T cell accumulation following dual conditioning.

Donor T cells in the pancreas remained at low but detectable numbers up to 21 days post ACT in dual-conditioned mice (Fig. 4c: average 5595 cells/pancreas). While TCR-I T cells were not detected at later time points in the pancreas (unpublished observations), donor T cells persisted at low levels in the spleen up to 70 days post ACT in a subset of both dual- and WBI-conditioned mice, but not in αCD40- or ACT only mice (Fig. 4e). Persisting T cells showed some features consistent with memory T cell formation such as CD62L expression but few cells expressed CD127

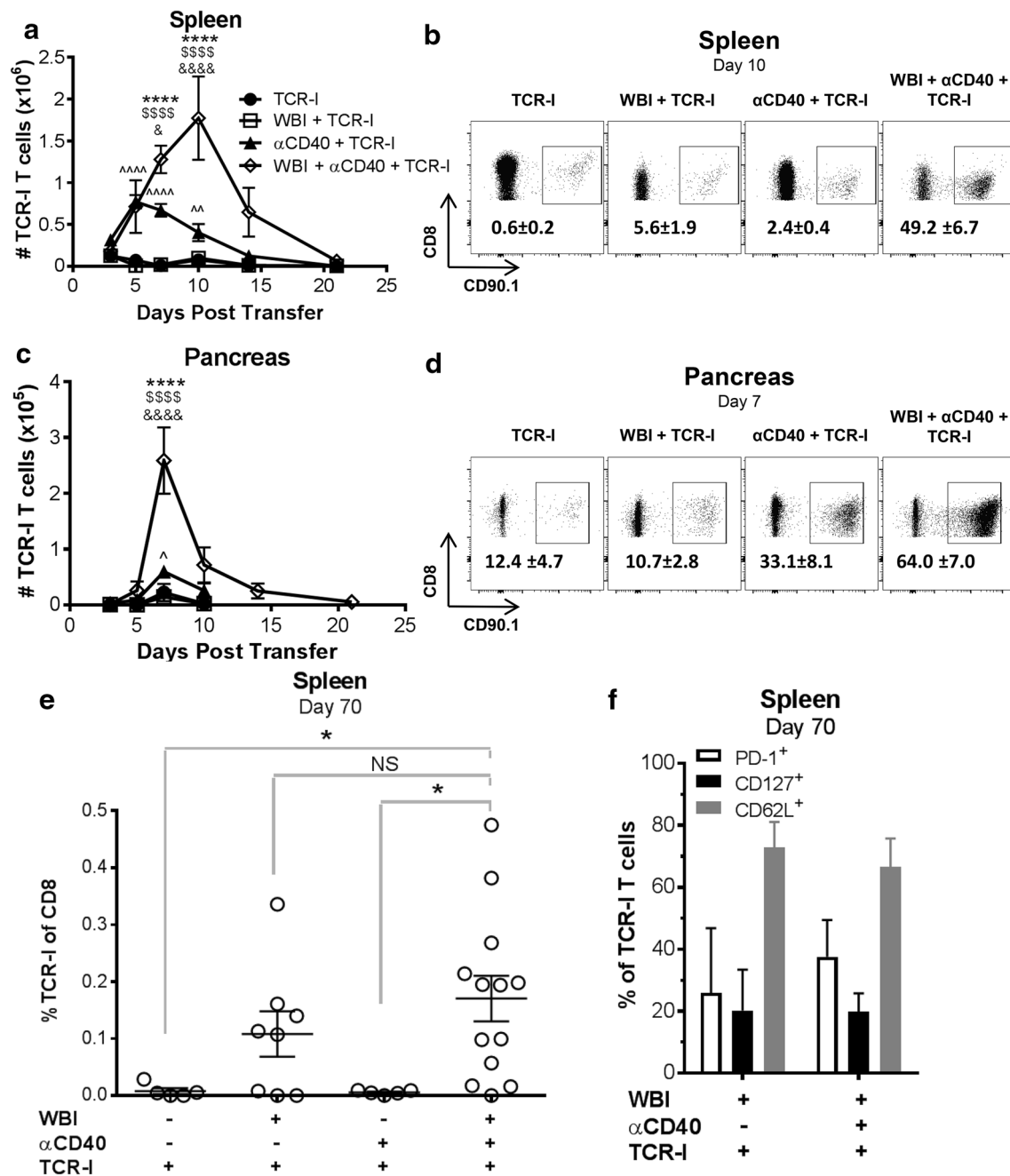


Fig. 4 Dual conditioning enhances accumulation of TCR-I cells in the spleen and pancreas. **a, b** Absolute numbers of CD90.1+ or TetI+TCR-I cells in the spleen (**a**) and pancreas (**c**) were determined by flow cytometry. $N=6-18$ mice/group (days 3–10), 2–10/group (days 14, 21). WBI+ α CD40+TCR-I vs. TCR-I (asterisk), vs. WBI+TCR-I (dollar symbol), vs. α CD40+TCR-I (ampersand); α CD40+TCR-I vs. TCR-I alone (hat symbol). 1 digit $p < 0.05$, 2 digits $p < 0.01$, 4 digits $p < 0.0001$ by two-way ANOVA with Bonferroni corrections. **b, d** representative flow cytometry plots for

TCR-I T cells recovered from spleen (**b**) and pancreas (**d**). Numbers inside the dot plots represent the mean percentage of each group \pm SE of CD8+ cells identified as TCR-I cells. **e** TCR-I T cells recovered from the spleen 70 days post treatment. $N=5-13$ /group. WBI+ α CD40+TCR-I vs. indicated group, * $p < 0.05$ by one-way ANOVA with Dunnett's post-test, NS not significant. **f** Proportion of donor TCR-I T cells expressing the indicated surface markers 70 days post treatment. $N=2-7$ /group. No significant differences by Student's t test

or PD-1 (Fig. 4f). These results indicate that α CD40 modestly improved donor T cell accumulation in the spleen and pancreas of tumor-bearing RT4 mice while WBI promoted

residual T cell persistence. Dual conditioning prolonged T cell expansion and dramatically increased acute T cell accumulation.

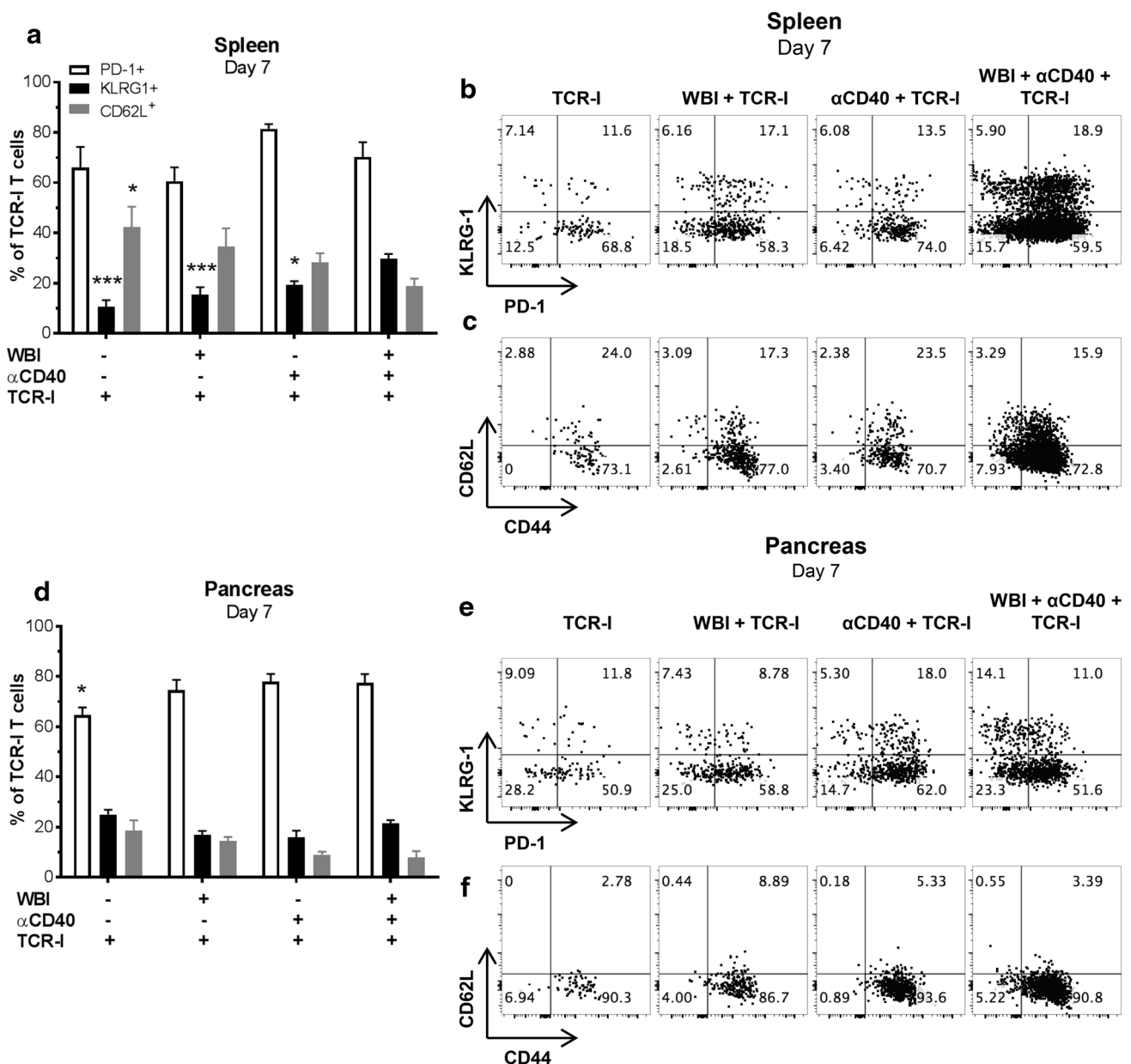


Fig. 5 Phenotype of donor T cells recovered from RT4 mice. **a, d** expression of surface markers on CD8+CD90.1+ donor cells 7 days post treatment. $N=7-12$ /group. WBI+ α CD40+TCR-I vs. indicated

group; *digit $p < 0.05$, ***digits $p < 0.001$ by one-way ANOVA with Dunnett's post-test. **b, c, e, f** representative flow cytometry plots for TCR-I T cells recovered from the spleen (**b, c**) and pancreas (**e, f**)

IFN γ production by donor T cells modestly impacts survival

We next evaluated T cell functions that may be associated with control of tumor progression including production of IFN γ and TNF α . Low proportions of donor T cells from mice treated with ACT alone or ACT with α CD40 or WBI produced these cytokines, while a significantly increased proportion of donor cells produced IFN γ and TNF α in dual-conditioned mice (Fig. 6a). Previous investigations have demonstrated that donor T cells control tumor

progression through IFN γ -dependent tumor cell killing and cell cycle arrest [21, 38–41]. To assess the role of IFN γ in the anti-tumor response in this system, we transferred naive IFN γ -deficient TCR-I donor cells (TCR-IxGKO) into dual-conditioned RT4 mice. TCR-IxGKO T cells accumulated to similar frequencies as wild-type TCR-I T cells in both the lymphoid organs and pancreas of dual-conditioned mice (Fig. 6b). TCR-IxGKO donor T cells recovered at day 7 were predominantly PD-1⁺ and CD62L^{lo} but did not vary significantly in phenotype from wild-type TCR-I T cells (Fig. 6c-e). Thus, loss of IFN γ did not negatively impact T

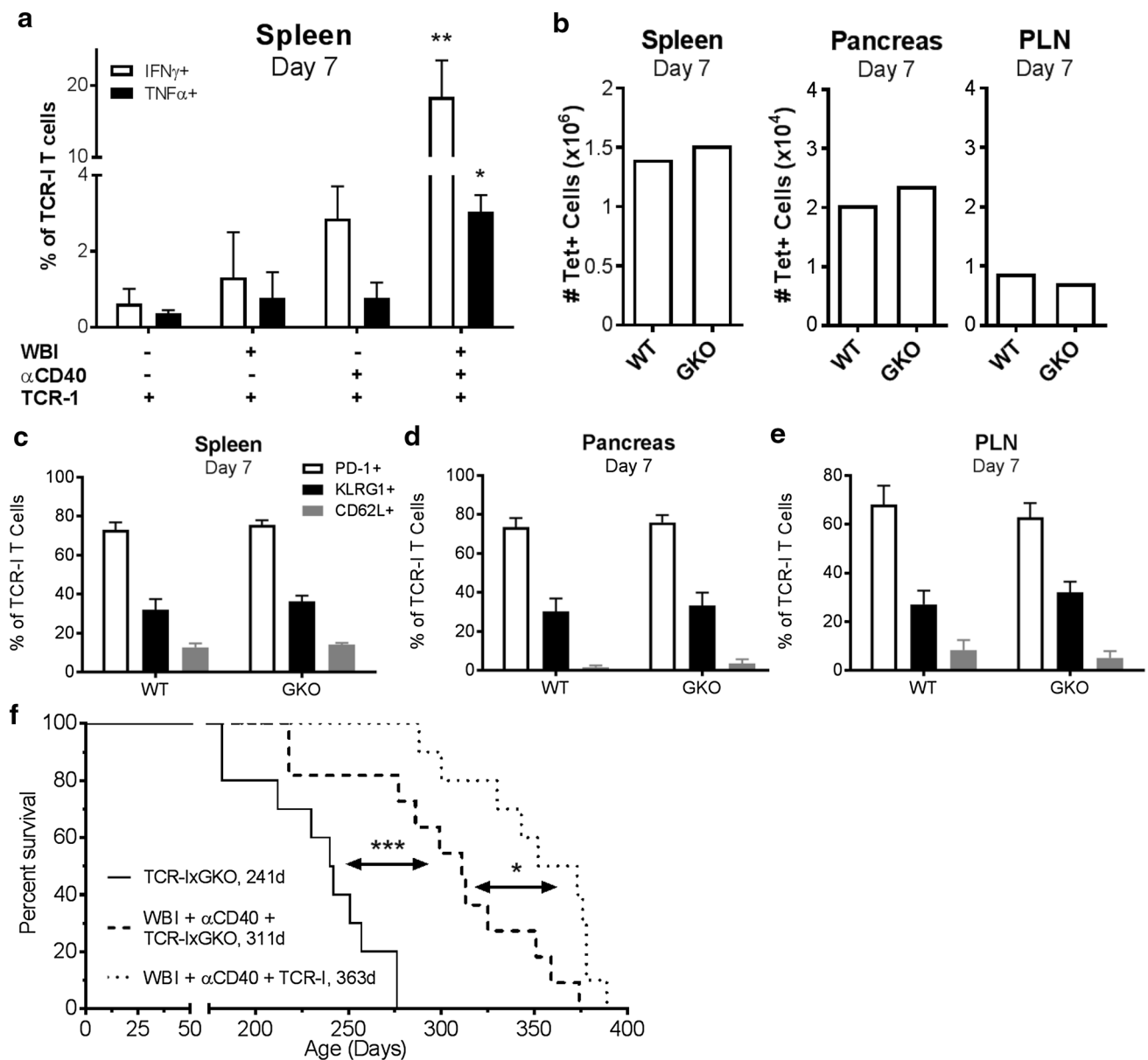


Fig. 6 IFN γ -producing donor cells are required to achieve maximum control of tumor progression. **a** IFN γ - and TNF α -production by TCR-I donor cells measured by intracellular cytokine staining. $N=3$ /group. WBI + α CD40 + TCR-I vs. all other groups; * $p < 0.05$, ** $p < 0.01$ by one-way ANOVA with Dunnett's post-test. **b–f** RT4 mice received dual conditioning and ACT of wild-type TCR-I or

TCR-IxGKO cells. **b** Total TCR-I donor cells 7 days post treatment. $N=6-7$ /group. No significant differences by Student's t test. **c–e** Surface marker expression on TCR-I cells recovered from the spleen (**c**), pancreas (**d**) and PLN (**e**). $N=3-7$ /group. No significant differences by Student's t test. **f** Survival analysis with median survival (days) indicated. $N=10$ /group. * $p < 0.05$, *** $p < 0.001$ by log-rank test

cell accumulation or alter differentiation in dual-conditioned RT4 mice.

In addition, TCR-IxGKO donor T cells mediated a highly significant increase in survival of RT4 mice following dual conditioning compared to mice that received TCR-IxGKO ACT alone (Fig. 6f). However, this increase was significantly less than achieved with wild-type TCR-I cells. These data indicate that IFN γ production by donor T cells plays a modest role in control of tumor progression, but

suggest that alternative donor T cell effector mechanisms are likely to promote anti-tumor immunity in this system.

Discussion

Approaches that promote T cell accumulation within tumors are likely to improve the response rate to immune-based therapies, particularly for tumors that effectively exclude T

cells [3]. We demonstrate that combining two distinct conditioning approaches, WBI and agonist α CD40, improves ACT. These regimens function synergistically to produce a more potent anti-tumor response than either achieves alone. Our results show that α CD40 promotes T cell accumulation in the lymphoid organs and pancreas, although this inflammatory effect was not sufficient to control tumor progression. WBI dramatically improved acute accumulation of tumor-specific T cells within the pancreas when combined with α CD40, consistent with the more potent impact of this combined regimen on tumor progression.

Agonist α CD40 has been shown to license APCs to effectively initiate cytotoxic CD8 T cell responses in tolerizing environments [24, 42]. While increased α CD40-induced donor T cell accumulation could be explained by improved tumor antigen presentation, we did not directly measure the quantitative effects of α CD40 on antigen presentation. However, our results do show that sufficient tumor antigen was constitutively presented to activate almost all of the donor T cells within the draining LNs regardless of treatment (Fig. 3c). Thus, improved T cell accumulation within the lymphoid tissues of α CD40-treated mice may be explained by a qualitatively better signal, such as improved costimulatory signals or altered cytokines provided during T cell differentiation [43, 44]. Addition of WBI to α CD40 conditioning may prolong the period of effective tumor antigen presentation resulting in extended donor T cell proliferation in the PLN (Fig. 3d). These effects could be mediated through radiation-induced immunogenic tumor cell death [45] or further licensing of APCs [18, 19].

A second role for α CD40 may be to induce a pro-inflammatory state that promotes immune cell infiltration into the pancreas. Clinically, α CD40 administration is associated with cytokine release syndrome [46] and triggering of CD40 on human endothelial cells promotes increased levels of adhesion molecules and production of pro-inflammatory cytokines [47]. In mice, intraperitoneal delivery of α CD40 promotes an acute hepatitis and accumulation of myeloid cells in tumor-bearing and tumor-free hosts [48]. Thus, α CD40 may play a key role in facilitating T cell entry into the pancreas, which is further augmented by WBI. In the related Rip1-Tag5 model, irradiation transformed the irregular insulinoma vasculature into a phenotypically normal capillary network to facilitate T cell infiltration [15]. Radiation up-regulated expression of adhesion molecules on tumor blood vessels, correlating with T cell extravasation into the tumor [15, 49]. These changes appear to be dependent on the development of an inflammatory microenvironment, and may be mediated through M1-skewing of tumor-associated macrophages [16]. Here we found that WBI + ACT did not promote T cell accumulation or pancreatic inflammation, while the

combination of α CD40 + WBI + ACT promoted extensive T cell accumulation and inflammation throughout the pancreas and tumors. A similar effect was recently found in mice with pancreatic ductal adenocarcinomas, where the combination of chemotherapy and α CD40 dramatically improved T cell-dependent control of tumor progression [50]. Intriguingly, that response was dependent on the presence of Batf3 + DCs, critical for antigen cross-presentation, and was associated with decreased regulatory T cells and improved T cell effector function in the TME. Indeed the presence of DCs in the TME may be essential for recruitment of donor T cells [51]. The nature of the inflammatory cells recruited into the pancreas by either α CD40 alone or dual conditioning and their role in T cell recruitment in RT4 mice remains to be determined.

We also demonstrate that WBI extended the persistence of a small population of donor T cells (Fig. 4e). However, the level of T cell persistence was not improved by dual conditioning suggesting that the increased magnitude of acute T cell accumulation did not increase long-term T cell persistence in this tumor model. Whether additional interventions prior to T cell contraction might improve T cell persistence remains to be determined. Nonetheless, WBI prevented the complete loss of donor T cells observed with α CD40 conditioning [7], offsetting a major limitation to α CD40 conditioning in the setting of ACT. This combination may represent a general approach to prolong donor T cell persistence. Our previous studies in other models also demonstrated the capacity for WBI conditioning to extend survival of donor T cells [21, 22]. The lymphodepleting effect of both WBI and chemotherapy is associated with creating sufficient space and access to survival cytokines in tumor-bearing recipients to promote differentiation and persistence of memory-like T cells [52, 53]. WBI was sufficient to promote donor T cell persistence in RT4 mice, but the addition of α CD40 was required to produce a population of donor T cells capable of exerting a measurable therapeutic effect. In addition, only dual conditioning promoted an increase in the proportion of IFN γ and TNF α -producing donor T cells. Taken together, these data suggest that effective tumor control required high-level acute donor T cell accumulation and effector T cell differentiation that was only effectively accomplished in the setting of dual conditioning.

Our findings are consistent with previous studies in other tumor models showing that IFN γ is important for elimination of tumor antigen-expressing cells and the tumor stroma [38, 39, 54]. IFN γ produced by T cells in the TME slows tumor growth by arresting tumor cells in the G1/G0 phase of the cell cycle [40, 41]. However, we demonstrate that IFN γ -deficient TCR-I T cells maintained a significant capacity to control tumor progression, suggesting that additional effector mechanisms (e.g., Fas-FasL function, cytotoxicity) may contribute to the anti-tumor response observed in

dual-conditioned RT4 mice. Indeed, sublethal irradiation can increase Fas expression on tumor cells [55], potentially rendering insulinomas in dual-conditioned mice more sensitive to Fas-mediated apoptosis than tumors in α CD40-only conditioned mice. Our studies do not rule out a role for host-cell derived IFN γ in the observed anti-tumor effect, although our data clearly demonstrate that ACT is required for dual conditioning to control tumor progression. Further studies are needed to determine the specific contributions of IFN γ -dependent and -independent components of donor T cell activity involved in RT4 tumor regression and stasis.

These results have the potential to be applied clinically using patient TILs and autologous T cells reprogrammed with chimeric antigen receptors or cloned TCRs. The individual conditioning approaches have previously been applied in patients; WBI administered with ACT in the setting of metastatic melanoma [4], and α CD40 administered without ACT in the settings of pancreatic ductal adenocarcinoma and metastatic melanoma [27]. Combining these two approaches with ACT in cancer patients could have potential toxicities as α CD40-induced acute hepatitis in mice has been observed. How WBI may impact the α CD40-induced inflammatory response remains unknown [48]. While we observed no toxicity in our studies either in the target organ or at other sites, more in-depth studies are required to assess potential toxicities. In summary, we found that combining distinct host conditioning regimens with ACT produced immune-enhancing effects beyond those predicted using each individual approach, allowing for effective therapy against established tumors.

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Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All animal studies were approved by the Penn State Hershey Institutional Animal Care and Use Committee (Protocol #47088) and were performed in accordance with recommendations in the Guide for the Care and Use of Laboratory Animals.

Animal source Mice were bred in specific pathogen-free barrier housing in the Penn State College of Medicine animal vivarium. RT4 mice on the C57BL/6J background were maintained as a homozygous line and bred with C57BL/6J mice to produce hemizygous RT4 mice for experiments. Hemizygous TCR-I mice were bred to homozygous B6.PL-Thy1a/CyJ females (the Jackson Laboratory) to generate CD90.1⁺ donor T cells. TCR-I mice on the IFN γ -knockout background (TCR-IxGKO) were derived by backcrossing TCR-I mice to homozygous B6.129S7-*Irfng*^{tm1Ts}/J mice from the Jackson Laboratory.

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