Cancer Immunology, Immunotherapy (2019) 68:1805–1817
https://doi.org/10.1007/s00262-019-02397-7

ORIGINAL ARTICLE

Antisense targeting of CD47 enhances human cytotoxic T-cell activity and increases survival of mice bearing B16 melanoma when combined with anti-CTLA4 and tumor irradiation

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Received: 3 April 2018 / Accepted: 14 September 2019 / Published online: 18 October 2019

Abstract

Antibodies targeting the T-cell immune checkpoint cytotoxic T-lymphocyte antigen-4 (CTLA4) enhance the effectiveness of radiotherapy for melanoma patients, but many remain resistant. To further improve response rates, we explored combining anti-CTLA4 blockade with antisense suppression of CD47, an inhibitory receptor on T cells that limit T-cell receptor signaling and killing of irradiated target cells. Human melanoma data from The Cancer Genome Atlas revealed positive correlations between CD47 mRNA expression and expression of T-cell regulators including CTLA4 and its counter receptors CD80 and CD86. Antisense suppression of CD47 on human T cells in vitro using a translational blocking morpholino (CD47 m) alone or combined with anti-CTLA4 enhanced antigen-dependent killing of irradiated melanoma cells. Correspondingly, the treatment of locally irradiated B16F10 melanomas in C57BL/6 mice using combined blockade of CD47 and CTLA4 significantly increased the survival of mice relative to either treatment alone. CD47 m alone or in combination with anti-CTLA4 increased $CD3^+$ T-cell infiltration in irradiated tumors. Anti-CTLA4 also increased $CD3^+$ and $CD8^+$ T-cell infiltration as well as markers of NK cells in non-irradiated tumors. Anti-CTLA4 combined with CD47 m resulted in the greatest increase in intratumoral granzyme B, interferon-γ, and NK-cell marker mRNA expression. These data suggest that combining CTLA4 and CD47 blockade could provide a survival benefit by enhancing adaptive T- and NK-cell immunity in irradiated tumors.

Keywords CD47 · Immunotherapy · Melanoma · Phosphorodiamidate morpholino oligomer · Thrombospondin-1 · SIRP-α

Abbreviation

APCs	Antigen-presenting cells
ATCC	American Type Culture Collection
CD47 m	CD47-translational blocking morpholino
CTLA4	Cytotoxic T-lymphocyte-associated protein 4
MDSCs	Myeloid-derived suppressor cells
NCI	National Cancer Institute
NY-ESO-1	Cancer/testis antigen 1B
PD-1	Programmed death-1
RT	Radiation therapy
SIRPα	Signal-regulatory protein-α
TCGA	The Cancer Genome Atlas

Anthony L. Schwartz and Pulak R. Nath contributed equally to this work.

The authors of this paper report on their T-cell assays transparently and comprehensively as per field-wide consensus, allowing the community a full understanding and interpretation of presented data as well as a comparison of data between groups. The electronic supplementary materials of this publication include a MIATA checklist. For more details, see http://miataproject.org/.

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s00262-019-02397-7) contains supplementary material, which is available to authorized users.

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Introduction

Engagement of CD80 and/or CD86 on antigen-presenting cells (APCs) with CD28 on T cells co-stimulates T-cell proliferation and enhances metabolism and survival factors. Conversely, cytotoxic T-lymphocyte antigen-4 (CTLA4) induced on activated T cells functions as an immune checkpoint by competing with CD28 for binding CD86/CD80 and negatively regulates T-cell activation [1]. Inhibition of

cell-cycle progression, T-cell activation, and cyclin activity are results of CTLA-4 ligation [2]. CTLA-4 is also expressed on various cancers, and therapeutic blockade can limit tumor progression [3–5]. Although many durable responses and improved survival have been reported using anti-CTLA4 (Ipilimumab), many patients do not respond to this therapy alone. However, response rates for some cancers, including melanomas, can be increased by combining checkpoint inhibitors with radiation therapy (RT) [6–8].

CD47 is a newly described innate and adaptive immune checkpoint. Blockade of CD47 has shown efficacy in various preclinical models and is currently in Phase I/II trials [9–13]. CD47 is widely expressed on mammalian cells, but more highly expressed in some human tumors [10]. CD47 is a signaling receptor for thrombospondin-1, which regulates growth factor receptor signaling, cell fate, viability, and responses to stress [14, 15]. Binding of thrombospondin-1 to CD47 in human and murine T cells modulates their differentiation and inhibits TCR signaling and H_2S biosynthesis required for optimal T-cell activation [16–21]. Antisense or antibody blocking of this inhibitory CD47 on murine T cells in vitro enhances their antigen-dependent killing of irradiated tumor cells in vitro [11]. Thus, the thrombospondin-1/CD47 axis is a T-cell immune checkpoint.

CD47 on tumor cells interacts with its counter-receptor SIRPα on macrophages and elicits a "don't eat me" signal [22], thus also serving as an innate immune checkpoint to limit phagocytosis of tumor cells and presentation of tumor antigens to T cells [12, 23]. We recently identified an additional thrombospondin-1-dependent immune checkpoint function for CD47 on NK cells and demonstrated that CD47 in the tumor microenvironment limits growth of B16 melanoma associated with impaired NK-cell activation [24, 25]. Conversely, treating B16 tumors with the CD47-blocking antibody miap301 delayed tumor growth and was associated with enhanced intratumoral infiltration of $IFN\gamma^+$ and granzyme B^+ NK cells [25]. Thus, blocking CD47 can enhance tumor cell clearance by activating several innate and adaptive immune pathways [10–12, 26]. Irradiation combined with CD47 blockade further delayed tumor progression in several immune-competent syngeneic murine models including the relatively weakly immunogenic B16 melanoma [11, 13].

The CD47 therapeutics currently in clinical trials block CD47 binding to SIRPα [27], but preclinical studies indicate that additional agents targeting the thrombospondin-1/CD47 interaction may have additional therapeutic benefits by directly activating NK and T cells [11, 25, 28]. Here, we evaluate potential additive effects of combining CTLA4 and CD47 immune checkpoint inhibitors on tumor-specific human cytotoxic T cells and in a murine melanoma model. CD47 mRNA expression in human melanomas correlates with that of the CTLA4 counter receptors CD80 and CD86. Blocking CD47 alone or in combination with blocking CTLA-4 enhances antigen-dependent T-cell killing of irradiated melanoma cells in vitro. Using a syngeneic subcutaneous melanoma model in immune-competent mice, we show that local tumor irradiation at an optimal dose for enhancing anti-tumor immunity [29] in combination with anti-CTLA4 (αCTLA4) and a CD47 translation-blocking morpholino (CD47 m) significantly prolongs survival when compared to RT and αCTLA4 alone. Increased markers of T- and NK-cell invasion and activation combined with histological evidence of necrosis and $CD3^+$ T-cell infiltration further suggest that combining CD47 and CTLA4 immune checkpoint inhibitors may increase survival of melanoma patients in a clinical setting.

Methods

B16 melanoma model

C57BL/6 mice were injected with 1×10^6 B16F10 melanoma cells into the right hind limb to induce tumor growth. Twenty-four hours prior to RT, CD47m groups received an IP injection of 10 µM CD47 morpholino in 750 µl of PBS. In the αCTLA4 groups, mice received 100 µg of CTLA4 blocking antibody (BioXcell) IP on the day of radiation and at 3, 6, and 9 days thereafter. Once tumors reached a mean volume of 100 mm^3, hind limbs were treated with 10 Gy local irradiation utilizing a Therapax DXT300 X-ray Irradiator (Pantak, Inc.) using 2.0 mm A1 filtration (300 kV/10 mA) at a dose rate of 2.53 Gy/min. Tumor size was measured every 3 days using calipers and calculated by $[width^2 \times length]/2$. Total tumor mass was determined at the end of the study. Mice were euthanized per the protocol when tumors reached approximately 2.5 cm^3.

CD47 correlation with gene expression and survival in human cancers

The Cancer Genome Atlas (TCGA) tumor RNAseq expression data from 469 melanoma patients in the Skin Cutaneous Melanoma TCGA Provisional data set were analyzed using cBioPortal tools as described [30, 31]. Coexpression analysis was performed to identify T-cell-related genes that correlate with elevated mRNA expression of CD47. To assess correlation between gene expression and survival, tumors were grouped into high and low expressions using the mean expression as cutoff. TCGA Kaplan–Meier survival curves were prepared in cBioPortal to determine log-rank test p values.

Gene expression

Tumors were homogenized, and RNA was isolated using the NucleoSpin RNA kit (Takara/Clontech Laboratories), and cDNA was synthesized using SensiFAST cDNA kit (Bioline). Real-time PCR using primers listed in Supplemental Table 1 was performed using SYBR Green detection with a BioRad CFX96 thermal cycler as previously described [32]. Beta-2-microglobulin (B2M) and beta actin primers were used for normalization to calculate ΔCt values.

Flow cytometry

Five mice per group were sacrificed by euthanizing with CO_2 inhalation. Spleens and tumors were isolated in ice-cold FACS buffer (PBS containing 1% BSA and 0.01% NaN_3) and were mechanically disrupted to generate single-cell suspensions. Tumor tissues were cut into small pieces and enzymatically dissociated with Collagenase/Dispase (Roche, 1 mg/ml) and DNase 1 (Sigma, 100 µg/ml). Red blood cells were lysed with ACK lysis buffer (Lonza), and single-cell suspensions of cells were prepared by straining through a 70 µm mesh.

Single-cell suspensions from spleen and tumor were stained in FACS buffer on ice for 30 min for the indicated cell surface markers following Fc block: CD45.2 (104), CD4 (RM4–5), CD8 (53-6.7), CD11b (M1/70), Gr1 (RB6-8C5), NK1.1 (PK136), and NKp46 (29A1.4). Biotinylated antibodies against B220 (RA3-6B2), CD19 (eBioD3), CD3 (17A2), Gr1 (RB6-8C5), CD11c (HL3), and Ter119 (TER-119) were used to prepare the Lineage-cocktail (Lin). Cells were then washed and re-stained with Streptavidin APC eFluor 780. All the antibodies were purchased from eBioscience. Cells were finally incubated on ice with 500 ng/ml DAPI for 5 min before acquiring on a flow cytometer. FACS Fortessa (BD) was used for cell acquisition and the flow cytometry data were analyzed with FlowJo 10.1 (TreeStar). Absolute cell counts for CD8 + and Gr1 + CD11b + isolated from spleens are depicted in Supplemental Fig. 6b.

Histology

Tumors were harvested at time of sacrifice when they reached 2.5 cm^3, fixed in 10% formalin, and embedded in paraffin. Sections were stained with hematoxylin–eosin according to standard procedures. Immunohistochemistry for CD3 (BioRad, MCA1477, 1:100) was performed at the National Cancer Institute core facility at Fredrick. Necrosis, hemorrhage, and T-cell infiltrates were assessed by a pathologist who was blinded to treatment groups. Slides were scanned with the Aperio ScanScope XT (Leica Biosystems, Buffalo Grove, IL) at 40x.

The extent of tumor necrosis was evaluated as a histopathological surrogate to assess treatment response. Aperio's "Positive Pixel Count" algorithm (version 9, Supplemental Table 2) was employed to quantify areas of viable and necrotic tumor. Markup images were carefully compared to H&E stained slides to ensure valid demarkation of viable and necrotic tumor areas. Areas of interest were carefully outlined to avoid the quantification of non-neoplastic surrounding tissues. The ratio of necrotic tumor to viable tumor was calculated and reported as a percentage.

Infiltrating $CD3^+$ T cells and total tumor cells per slide were counted with Aperio's proprietary "Nuclear" algorithm (version 9; Supplemental Table 3). The ratio of infiltrating $CD3^+$ T cells to viable tumor cells ($n = 3$ per group) was calculated and reported as a percentage. The entire tissue section for each replicate was evaluated using an automated scanning microscope and imager.

Cytotoxic T-lymphocyte assay

Human $CD8^+$ T cells from two donors engineered to recognize the melanoma antigen NY-ESO-1 were obtained and generated as previously described [33]. The T cells were expanded in RPMI-1640 medium containing penicillin, streptomycin, L-glutamine, 12.5 mM HEPES, 10% fetal bovine serum, and 300 U/ml rIL-2 at day 1 [35]. On day 2, Dynabeads Human T-Activator CD3/CD28 (ThermoFisher) at a bead/cell ratio of 1:1 were combined and cultured for 6 days. On day 7, the Dynabeads were removed, and cells were allowed to rest for 1–3 days, and the cycle was then repeated until the required number of cells was obtained. SK23 melanoma cell line transduced via retrovirus containing NY-ESO-1 expressing vectors (NY-ESO-1^+SK23) and control cells (NY-ESO-1^-SK23) were cultured in RPMI-1640 supplemented with 10% fetal bovine serum. For the CTL assay, T cells were cultured with/without CD47 morpholino (1–10 µM) for 24 h prior to addition to target cells. Irradiated (10 Gy) or non-irradiated SK23 (± NY-ESO-1) cells were labeled with Indium-111. Human αCTLA-4 (0.5 µg/ml, Ipilimumab) was added to indicated wells and co-cultured with T cells at various effector:target (E:T) ratios. After incubation overnight at 37 °C, released radioactivity in supernatant was determined utilizing the Wizard-2 2470 Automatic Gamma Counter (PerkinElmer). Cytotoxicity was calculated as percent specific lysis = [(experimental cpm − spontaneous cpm)/(total cpm − spontaneous cpm)] × 100. Triplicates are shown as mean ± SE. T-cell assay results were reported following MIATA guidelines [34].

Results

CD47 mRNA expression in human melanomas is positively correlated with T-cell functional markers

Elevated CD47 expression in some solid tumors and hematologic malignancies correlates with a poorer prognosis, which may involve its protection of tumor cells from innate immune surveillance [10, 35, 36]. However, our recent analysis of CD47 mRNA expression data in TCGA for a set of 461 melanomas with RNAseq and survival data demonstrated that increased CD47 mRNA expression was dose-dependently correlated with longer overall survival and disease-free survival [25]. However, CD47 expression is not correlated with melanoma disease or tumor stage or with their *NRAS* of *BRAF* mutation status (Supplemental Fig. 1a–c). As expected for this analysis, NRAS and BRAF mutations were mutually exclusive [37]. The TCGA data do not differentiate elevated CD47 expression in tumor cells from increased expression in the tumor microenvironment, but further analysis of human TCGA data combined with mouse model data indicated that CD47 on NK cells regulates their differentiation and activation, and the protective role of high CD47 in melanomas is associated with increased NK-cell recruitment and activation [25].

Because CD47 is also a well-documented inhibitory signaling receptor in T cells [15–21], we further analyzed human melanoma RNAseq data in the TCGA database to explore potential relationships between CD47 mRNA expression and expression of markers of T-cell infiltration and function. CD47 mRNA expression was positively correlated with that of CD8A, CD8B, CD4, and FOXP3, suggesting increased CD4, CD8, and Treg infiltration in high CD47 tumors (Fig. 1a). Consistent with the report that cMyc positively regulates expression of CD47 and PD-L1 [38], PD-L1 expression was strongly correlated with that of CD47 ($p = 1.8 \times 10^{-24}$), and expression of its counter receptor PD-1 was also positively correlated with CD47 ($p = 7.5 \times 10^{-12}$). Expression of the inhibitory receptor CTLA4 was positively correlated with CD47 expression ($p = 7.6 \times 10^{-10}$), but much stronger positive correlations were observed for the CTLA4 counter receptors CD86 and CD80 ($p = 4.7 \times 10^{-20}$ and 5.3×10^{-25}, respectively) and the inducible T-cell costimulatory receptor ICOS, which is enhanced by therapeutic blockade of CTLA4 [39] (Fig. 1a–c).

Consistent with the positive correlation between CD47 mRNA expression and overall survival [25], elevated expression of *CD80* and *CD86* with a mean cutoff was associated with significantly increased overall survival for the melanoma patients (148 months versus 64 months median survival, p value 3×10^{-5}, Supplemental Fig. 2b). Expression of mRNA encoding the T-cell activation markers CD69 and interferon-γ and the lytic effectors granzyme A (GZMA) and granzyme B (GZMB) were also positively correlated with CD47 mRNA expression, suggesting that the protective effect of high CD47 in melanomas also involves increased CTL activity (Supplemental Fig. 2b). This suggested that increased T-cell coactivation via CD28 [20, 40, 41] may contribute to the positive association between high CD47 expression and overall survival, and checkpoint inhibitors targeting CTLA4 could overcome inhibition of T-cell immunity by its coincident over-expression in melanomas.

a

Gene	Correlation with CD47 mRNA expression	
	Spearman	P-value
CD4	0.27	2.51e-9
CD8A	0.36	1.79e-15
CD8B	0.33	1.41e-13
FOXP3	0.23	4.46e-7
CD274 (PD-L1)	0.45	1.79e-24
PDCD1 (PD-1)	0.31	7.53e-12
CD28	0.34	2.02e-14
CTLA4	0.28	7.55e-10
CD80	0.45	5.33e-25
CD86	0.41	4.66e-20
ICOS	0.43	2.70e-22

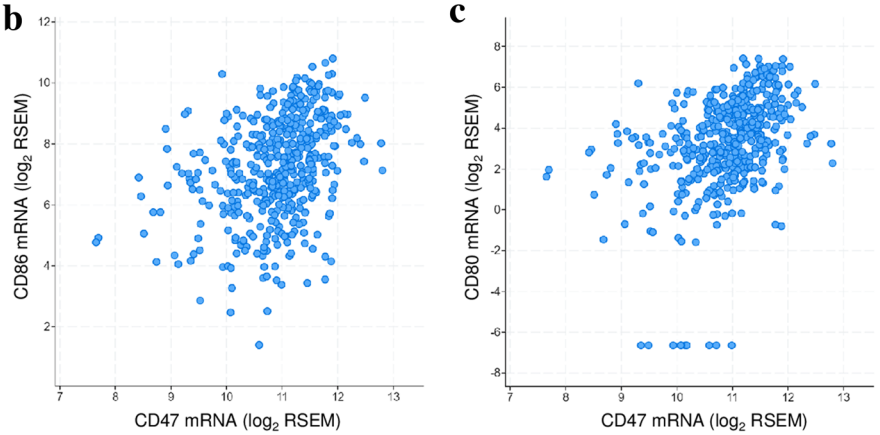

Fig. 1 CD47 expression is associated with altered survival and immune gene expression in human melanomas. **a** Correlation of CD47 mRNA with expression of T-cell-related genes in human melanomas (*Spearman scores > 0.3 and $p < 0.05$). **b**, **c** Positive correlation of CD47 mRNA expression determined by RNAseq analysis with that of the CTLA4 counter receptors CD86 and CD80 in human melanoma tumors in the TCGA database. Scatter plots represent $\log_2$(mRNA expression) for the indicated genes calculated using RSEM [64]

CD47 m and Ipilumimab directly increase specific T-cell killing of human melanoma cells

Because CD47 limits antigen-dependent killing of murine fibrosarcoma cells by murine CD8 T cells [11], we investigated direct effects of CD47 blockade on human T-cell cytolytic activity towards human melanoma cells (SK23–NY-ESO-1^{+}) using human T cells from two donors that were transduced with a recombinant T-cell receptor specific for the antigen NY-ESO-1. Antigen-independent killing of non-transduced SK23 cells was minimal, not altered by CD47 m or anti-CTLA4 (Ipilumimab) treatments, and not increased by irradiation of the target cells (Fig. 2a, b, e, f). For both donors, optimal responses to treatment were observed at an effector to target ratio of 10:1 (Supplemental Fig. 3). For donor A, treatment with 1 µM CD47 m or 1 µg/ml αCTLA4 alone or combined did not significantly alter antigen-dependent killing of non-irradiated SK23-ESO cells (Fig. 2c). However, antigen-dependent killing of irradiated SK23-ESO cells was significantly increased by treatment with CD47 m, Ipilimumab, or the combination compared to the untreated group (Fig. 2d). Using a second donor, preliminary experiments indicated an optimal dose of 10 µM for CD47 m, and this concentration significantly increased killing of non-irradiated SK23-ESO cells (Fig. 2g). Ipilimumab treatment alone did not significantly increase antigen-dependent killing of irradiated SK23-ESO cells, but CD47 m treatment significantly increased killing when used alone or in combination with αCTLA4 (Fig. 2h). These data extend our previous demonstration that CD47 blockade directly enhances antigen-dependent murine CTL killing [11] to human CTLs and establishes CD47 as an immune checkpoint on human cytotoxic T cells.

Irradiation combined with targeting CD47 and CTLA4 increases survival and tumor necrosis

The strong correlations between CD47 mRNA expression and expression of the CD28 counter receptors CD86 and CD80 suggested that therapeutic blockade of CD47 and CTLA4 could enhance CD28 co-stimulation and thereby be more effective in melanomas than either treatment alone. We evaluated combining CD47 blockade with αCTLA4 therapy in a B16F10 melanoma model in immune-competent C57BL/6 mice based on our previous evidence that CD47 m enhances its response to RT in a CD8 T-cell-dependent

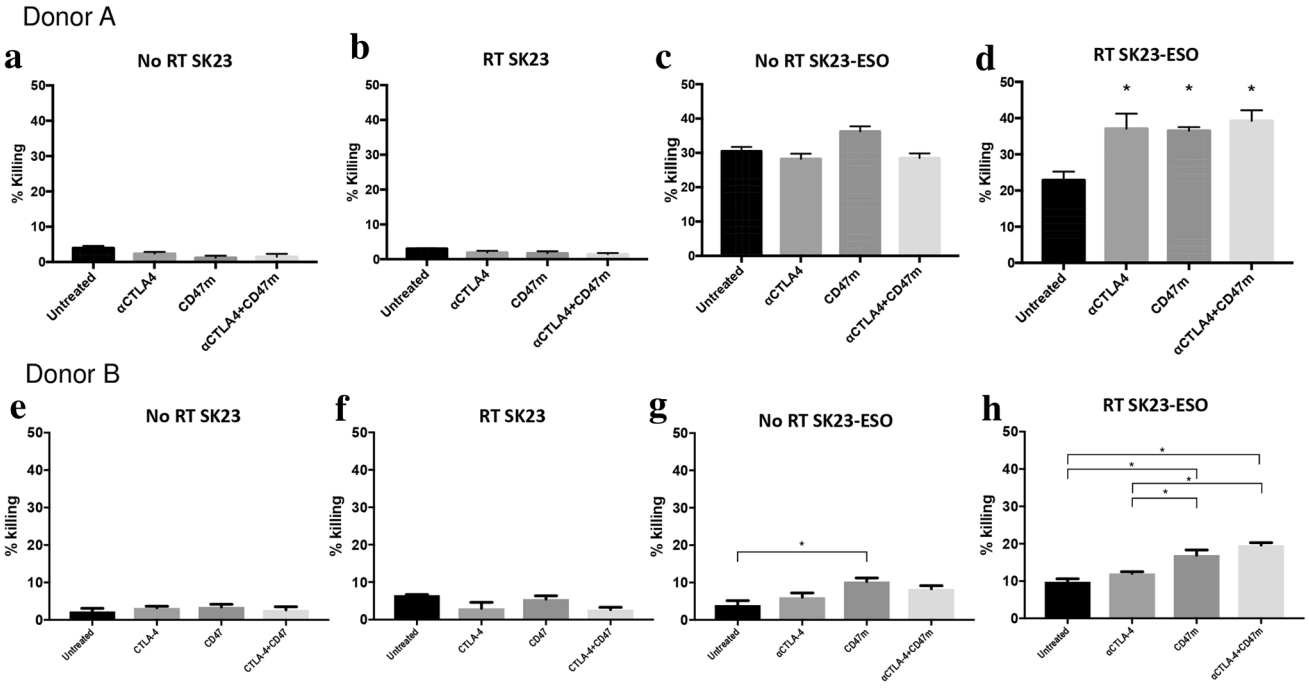

Fig. 2 T-cell killing of irradiated and non-irradiated human melanoma cells. Antigen-independent (**a**, **b**) and antigen-specific (**c**, **d**) human T-cell killing of irradiated (**b**, **d**) or non-irradiated human NY-ESO-1^{-} SK23 and NY-ESO-1^{+} SK23 melanoma cells (**a**, **c**) was assessed by ^{111}In release at an E:T radio of 10:1. Anti-CTLA4, CD47 m (1 µM), and combination treatment with RT resulted in significantly increased killing of melanoma cells by donor A (**d**, *p value determined by a one-way ANOVA, *$p < 0.001$). No significant differences in antigen-dependent killing was observed in the non-RT groups. Antigen-independent killing of NY-ESO-1^{-} SK23 cells was minimal and not enhanced by RT (**a**, **b**, $N = 3$). Using a second donor, 10 µM CD47 m was used and resulted in significantly increased tumor killing of non-irradiated SK-23-ESO cells (**g**) and RT cells treated with CD47 m alone or in combination with αCTLA4 (**h**, one-way ANOVA, $p < 0.001$). No differences were observed for the antigen-independent target cells (**e**, **f**)

manner and evidence that αCTLA4 improves survival in the B16F10 model in a CD8 T-cell- and NK-cell-dependent manner [42]. Tumor-bearing mice were treated with CD47 morpholino (CD47 m) using conditions documented to decrease CD47 expression in tumors and isolated CD8 T cells [11], local RT, and/or αCTLA4 (Fig. 3a). Consistent with our previous reports and a study combining a CD47 nanobody with αCTLA4 [43], treatment with morpholino alone, or CD47 m + αCTLA4 did not significantly delay B16F10 tumor growth or improve survival, and tumor irradiation alone resulted in the expected regrowth delay, but no mice with long-term survival (Fig. 3b) [11, 13]. Consistent with the previous murine model and human data [44], RT combined with αCTLA4 further delayed tumor growth and resulted in 23% long-term survival after 80 days. Long-term survival was further increased to 50% at 80 days ($p = 0.001$) with prior CD47 m treatment and αCTLA4 therapy following RT (Fig. 3b). Regression without regrowth was observed only in the groups treated with RT combined with αCTLA4 or combined with αCTLA4/CD47 m (Fig. 3c).

Histopathological and computer-aided quantification of necrosis of harvested B16F10 melanoma tumors (5 sections per group) revealed increased tumor necrosis following αCTLA4/CD47 m combination therapy with and without irradiation (Fig. 4). Untreated mice showed mostly viable tumor with minimal necrosis that increased slightly following treatment with CD47 m or αCTLA4 alone. All irradiated groups showed significantly increased necrosis over non-irradiated groups. The triple combination of RT, αCTLA4, and CD47 m resulted in widespread tumor necrosis indicating much greater tumor cell death than in all other groups. The triple combination exhibited 10% more necrosis than either single treatment paired with RT (Fig. 4b). The total number of tumor cells present reflects this trend (Fig. 4c). Immunohistochemical staining for CD3 revealed that treatments with αCTLA4 only, RT/CD47 m, and RT/αCTLA4/CD47 m groups increased T-cell infiltration (Fig. 5a). Residual viable tumor cells adjacent to intact capillaries were surrounded by immune infiltrates (Fig. 5a).

αCTLA4/CD47 m combination therapy effects on infiltrating immune cells

Effects of treatment on immune-cell tumor infiltration were assessed using flow cytometry, and expression of granzyme B was determined using RT-qPCR (Fig. 5b). Granzyme B,

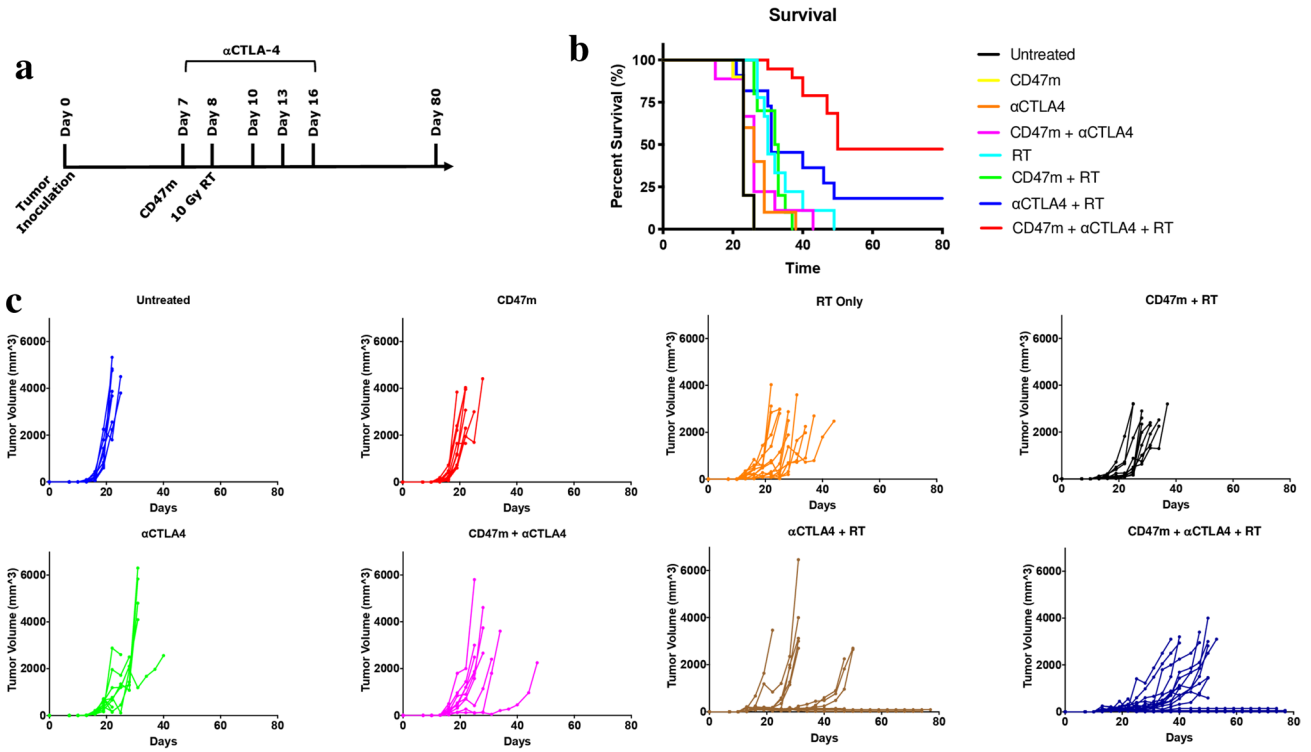

Fig. 3 Survival and tumor growth of tumor-bearing mice treated with RT, CD47 m, αCTLA4 and/or αCTLA4/CD47 m. **a** Treatment timeline of the experiment. **b** Kaplan–Meier survival plot for mice receiving the indicated treatments. After approximately 40 days, 100% of mice in all groups except RT combined with αCTLA4 or αCTLA4/CD47 m combo had succumbed to disease. The αCTLA4/CD47 m RT combination resulted in a significant increase in survival when compared to the RT αCTLA4 group (*$p < 0.05$, $n = 15$ per group). **c** Spider plots of tumor volume for the indicated treatment groups

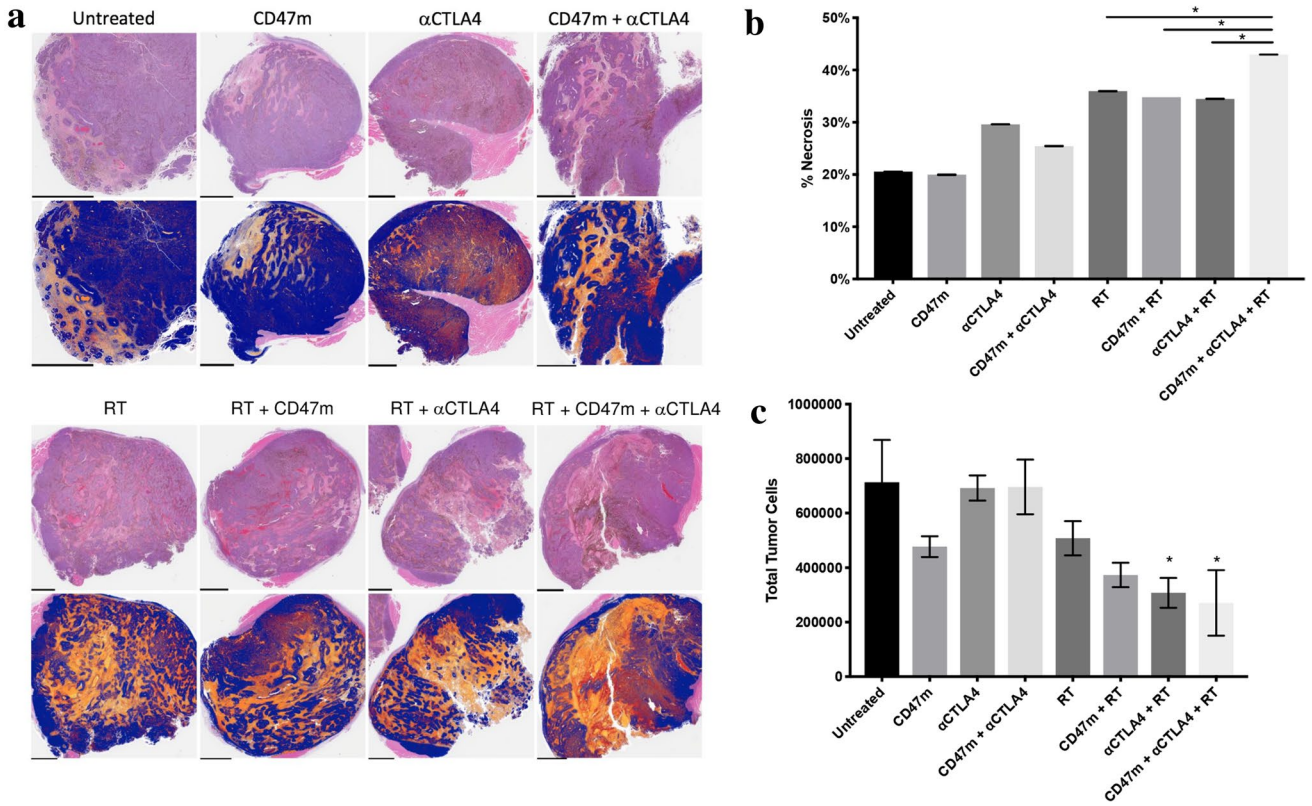

Fig. 4 Quantitative histologic analysis of tumors in treated mice. **a** Representative low magnification images of B16F10 melanoma tumors harvested from immune-competent C57Bl/6 mice that received treatments as indicated. Hematoxylin–eosin stained sections (top row) showing variable extend of tumor necrosis (eosinophilic/light pink areas). Markup images (bottom row) calculated using a positive pixel count algorithm provide colored overlay images indicating areas of viable tumor (blue), necrosis (yellow and orange), and melanin pigment (red, scale bar = 2 mm). **b** Quantification of necrosis (*$p < 0.05$, $n = 4$ per group). **c** Quantification of total number of tumor cells in the imaged section. The combination of RT, CD47 m and αCTLA4 resulted in abundant tumor necrosis and decrease number cell number indicating greater treatment response than the untreated group (*$p < 0.05$, $n = 4$ per group)

a marker of cytotoxic $CD8^+$ T and NK cells, significantly increased in the non-irradiated animals after αCTLA4 blockade and more with the combination of αCTLA4 and CD47 morpholino, suggesting an enhanced cytotoxic response. In the irradiated animals, αCTLA4 alone increased granzyme B more than in the non-irradiated animals. The combination of CD47 morpholino and αCTLA4 in the irradiated group further extended this effect. The ratio of $CD3^+$ T cells to tumor cells was increased in both the RT CD47 and RT αCTLA4/CD47 morpholino, but decreased in the RT + αCTLA4 group (Fig. 5c).

Because CD47 blockade can enhance the anti-tumor $CD8^+$ T-cell immune response both directly and indirectly [11, 12, 23], we further evaluated the effect of the αCTLA4/CD47 morpholino combination therapy on splenic and tumor-associated immune cells. Although no difference was observed in the frequency of $CD8^+$ cells within the spleens of the different treatment groups (Fig. 6b, supplemental Figs. 4, 6), a significant increase of the infiltrating $CD8^+$ T cells was evident within the tumors of combination-treated groups, compared to the untreated control (Fig. 6c, Supplemental Figs. 4, 6). Total myeloid cells assessed as $CD11b^+$ were moderately increased in spleen only following CD47 m treatment (Supplemental Fig. 7a). In tumors, the percent $CD11b^+$ cells were significantly increased by RT and further by RT + CD47 m, but not in irradiated tumors of mice treated with aCTLA4 (Supplemental Fig. 7b), consistent with our previous report that CD47 m treatment increased $CD68^+$ macrophage recruitment into irradiated tumors [13]. Myeloid-derived suppressor cells (MDSCs) are associated with poor patient outcomes and reduced anti-tumor immune response primarily T-cell inhibition [45, 46]. Concomitant decreases in the frequency of MDSCs were observed in both spleen and tumor of the combination-treated groups, compared to the untreated control (Fig. 6d, e, Supplemental Figs. 5, 6). These results show an increase in T cells and a decrease in immune suppressor cells within the tumor that could drive the increase in survival and tumor necrosis of the triple combination therapy.

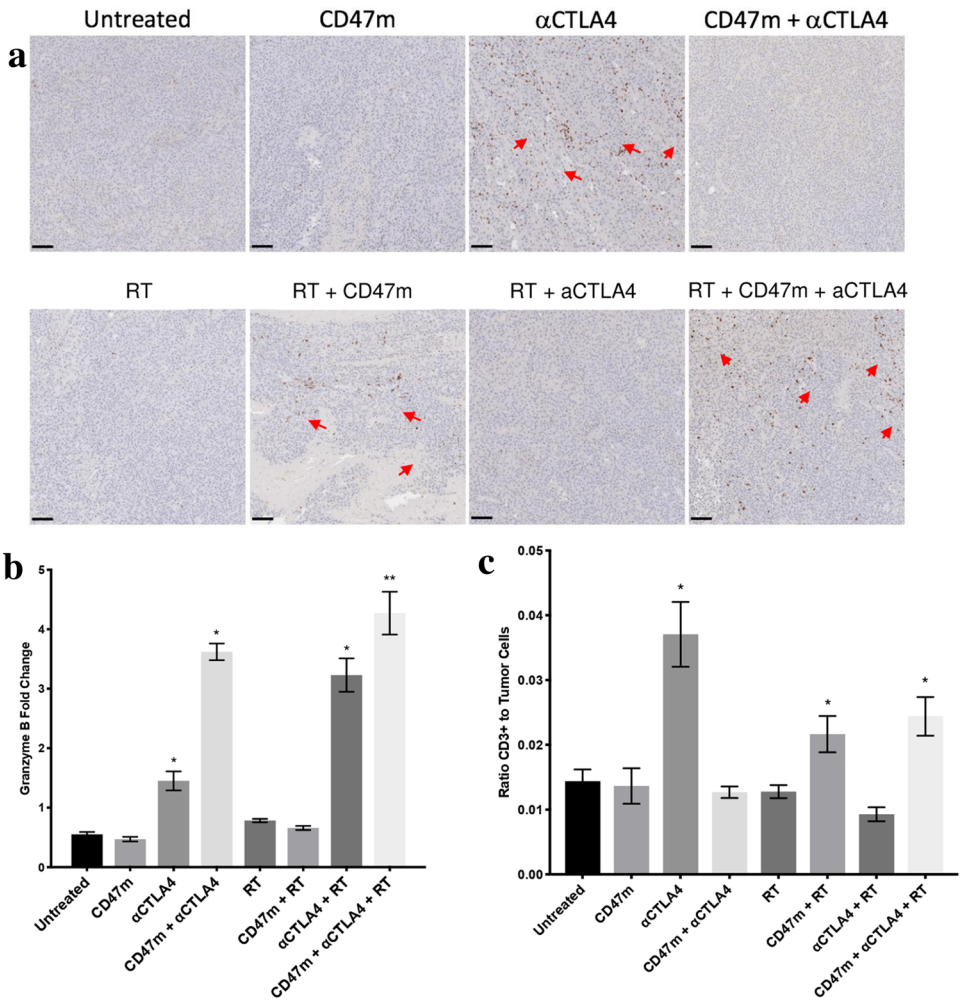

Fig. 5 Immunohistochemistry staining of $CD3^+$ cells in B16F10 tumors sections. **a** Anti-CD3 staining of tumor sections indicates that αCTLA4 treatment alone, CD47 m combined with RT, or αCTLA4/CD47 m combined with RT significantly increases the number of tumor-infiltrating $CD3^+$ cells (representative arrows). **b** RT-qPCR of Granzyme B mRNA in B16F10 tumors treated with RT in combination with CD47 morpholino and CTLA4 antibody. Granzyme B expression in the group treated with RT with the CD47 m and αCTLA4 treatment is increased to a further extent than without RT ($*p < 0.05$, $n = 5$). **c** Quantification of the ratio of $CD3^+$ cells to tumor cells ($*p < 0.01$, $n = 3$ per group)

CD47 m and αCTLA4 have additive effects on intratumoral NK-cell recruitment and activation

Although blockade of CD47 on murine and human CD8 T cells directly increases tumor cell killing [11], TCGA data and mouse models demonstrated that NK-cell function in melanomas is also regulated by altering CD47 signaling [25]. Furthermore, the increased intratumoral granzyme B mRNA in non-irradiated combination-treated tumors versus either treatment alone (Fig. 5b) did not coincide with a significant increase in $CD8^+$ T cells in those tumors (Fig. 6c). Flow cytometry data from tumors treated as indicated in Fig. 6a and harvested at day 20 showed a significant increase in infiltrating leukocytes expressing the common antigen CD45.2 only in mice treated with the combination (Fig. 6f), and a parallel increase in the percent of CD45.2 + cells that express NK1.1 and NKp46 suggested that NK cells are a source of this granzyme B (Fig. 6f). However, the percentage of $CD45.2^+$ cells that express NK1.1 and NKp46 was decreased in all irradiated groups (Supplemental Fig. 7c). The highest percentage of leukocytes expressing the NK-cell-specific antigens NK1.1 (CD161) and NKp46 was in the combo-treated group (Fig. 6f). Levels of *Klrb1c* mRNA, which encodes the NK-cell-specific receptor NK1.1 and *Ncr1* mRNA, encoding the natural cytotoxicity triggering receptor-1/NKp46, were significantly induced in the combo-treated group (Fig. 6g), validating the higher infiltration of NK cells within the tumor. Combination of CTLA-4/CD47 m treatment significantly upregulated mRNA levels for transcription factors included Eomes, T-bet, and Gata3 as well as the effector molecules granzyme B and interferon-γ but not RORγt (Fig. 6g). Eomes in combination with T-bet drive the induction of granzymes and IFNγ in NK cells as well as in cytotoxic T cells [47]. These data suggest enhanced NK-cell infiltration and/or activation within the tumor in combo-treated animals, subsequently promoting tumor necrosis when the tumors express damage markers induced by therapeutic irradiation. However, our analysis at day 20 indicates that these NK cells do not persist in the tumors 12 days after irradiation.

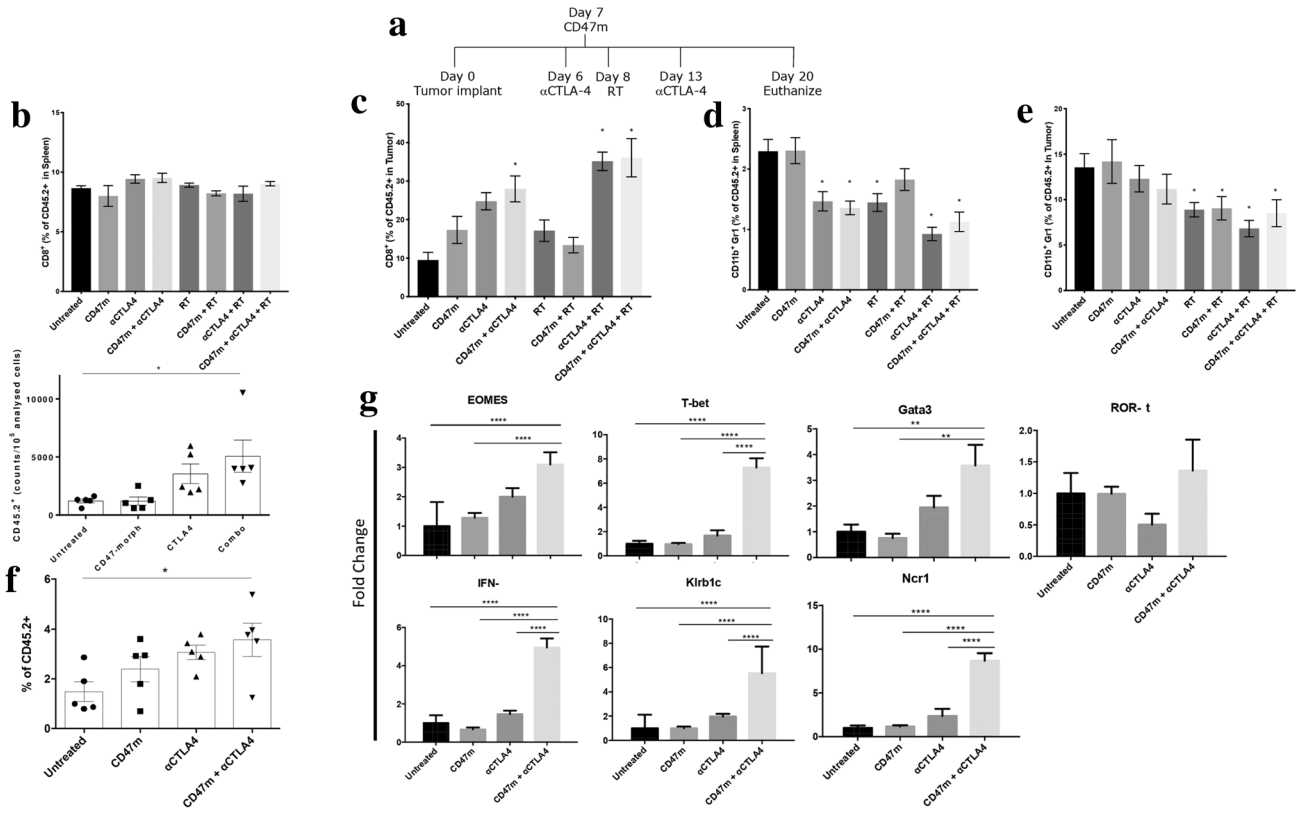

Fig. 6 Flow cytometry analysis of immune cells and gene expression in tumors or spleens from treated mice. **a** Treatment timeline for characterization of tumor-infiltrating leukocytes in tumors of mice receiving RT (day 8), CD47 morpholino, anti-CTLA4, or a combination treatment. Immune cells isolated from tumors or spleen of tumor-bearing mice at day 20 were stained for CD45.2, CD4 and CD8, CD11b and Gr1 to quantify $CD8^+$ cells as a percent of live $CD45.2^+$ cells from the spleens (**b**) or tumors (**c**). More $CD8^+$ T cells infiltrated into the tumors of mice treated with a combination of irradiation, CD47 morpholino and anti-CTLA4 antibody, whereas the abundance of $CD8^+$ cells in spleen was unchanged. $CD11b^+Gr1^+$ cells quantified as a percent of live CD45.2 from the spleen (**d**) or tumor (**e**) were decreased in the spleens of the aCTLA4 and combination therapies as compared to untreated control. In tumors, $CD11b^+Gr1^+$ cells were decreased in all the RT groups. ($N=5$, *p value determined by a one-way ANOVA, *$p<0.05$). Characterization of tumor-infiltrating leukocytes in tumors of mice receiving CD47 morpholino, anti-CTLA4, or a combination treatment. **f** Flow cytometry analysis of tumor-infiltrating cell counts expressing the leukocyte common antigen CD45.2 and infiltrating $CD45.2^+$ cells expressing the NK-cell-specific antigens NK1.1 (CD161) and NKp46. **g** Gene expression assessed by real-time PCR in tumors from mice treated as indicated. Upregulation of the Th2 transcription factor GATA3 is consistent with increased intratumoral T cells with the combination of CD47 morpholino and anti-CTLA4 treatment, and induction of Eomes, encoding the transcription factor eomesodermin, is consistent with increased intratumoral $CD8^+$ effector T cells with the combination treatment, but upregulation of Eomes in combination with upregulation of Klrb1c, which encodes NK1.1, and Ncr1, encoding natural cytotoxicity triggering receptor 1, suggests that the granzyme B-expressing effector cells in tumors receiving the combination treatment belong to the NK-cell lineage ($N=5$, *p value determined by a one-way ANOVA, *$p<0.05$)

Discussion

Immune checkpoint blockade therapeutics targeting PD-1 and CTLA4 are effective for melanoma patients, but a large subset fail to respond [48]. Combining checkpoint blockade with radiotherapy increases the effectiveness of both therapies in a subset of these patients [48]. CD47 is another potential therapeutic target that has shown promise in preclinical studies when used in combination with RT or cytotoxic chemotherapy treatment regimens [11, 49]. In contrast to most cancers, CD47 mRNA expression is positively correlated with increased overall survival for melanoma patients [25]. Because the premise for the current CD47-SIRPα-targeted therapeutics is that elevated CD47 protects tumor cells from phagocytic clearance, melanoma may not be an appropriate cancer for such therapeutics if the decreased expression is localized to melanoma cells in these tumors. In contrast, antisense suppression of CD47 or genetic deletion of CD47 in the tumor microenvironment enhances growth delay of various cancers including melanoma in conjunction with radiation or chemotherapy [13, 32, 50–52]. Therefore, the decreased survival of melanoma patients with lower tumor CD47 mRNA expression is not inconsistent with achieving a therapeutic benefit by further

suppression of CD47 expression in the tumor microenvironment that enhances anti-tumor immunity. Further investigation is needed to determine whether response rates to Ipilumimab treatment correlate with CD47 expression in melanoma patients, which might be expected based on the correlations in the TCGA data with both activators and inhibitors this pathway.

Combining CD47 blockade with anti-PD-L1 treatment extended survival of mice bearing syngeneic B16 melanomas [53], but a CD47 nanobody that blocks its interaction with SIRPα did not enhance the activity of αCTLA4 in the B16 melanoma model [43]. It is not known whether that nanobody would block inhibitory CD47 signaling in T cells. Another recent study showed extended survival after combining anti-PD-1, anti-CTLA4, and anti-CD47 compared to anti-PD-1 and anti-CTLA4 alone or anti-CD47 alone in a syngeneic esophageal squamous carcinoma model [54]. However, the combination of anti-CTLA4 with anti-CD47 was not evaluated in that study. We hypothesized that inhibitory CTLA4 signaling may limit the ability of CD47 blockage as a single therapeutic to enhance the anti-tumor immune response and survival in irradiated melanomas. In our previous studies using the same mouse melanoma model, combining CD47 m and RT significantly delayed B16 melanoma growth [11, 13], but the present data revealed that this is not sufficient to significantly increase overall survival. However, combined blockade of CTLA4 and CD47 along with RT significantly increased overall survival compared to CTLA4 blockade and RT alone. The increased survival correlated with an increase in tumor necrosis with the combinatorial approach. In vitro data demonstrated that CD47 m and αCTLA4 have similar activities to directly increase antigen-dependent T-cell killing of irradiated human melanoma cells. Therefore, CD47 is a functional immune checkpoint on human cytotoxic T cells that limits their anti-tumor activity. Taken together, these data show that CD47 morpholino and RT in combination with CTLA4 blockade augment each other to improve their therapeutic potential.

Previous studies attributed the beneficial effects of CD47 therapy to overcoming the "don't eat me" signal mediated by SIRPα that inhibits macrophage-mediated phagocytosis of tumor cells that express elevated CD47 [10, 26]. Blocking the CD47/SIRPα interaction can induce tumor phagocytosis and polarize M1 macrophages. The CD47 antibodies and recombinant SIRPα in ongoing clinical trials are designed to target this pathway. However, the similar growth kinetics of B16 tumors in immune-competent C57Bl/6 mice and SIRPα-mutant mice lacking any "don't eat me" signaling in macrophages clearly demonstrated that the complete absence of this signal is not sufficient to induce immune rejection of syngeneic melanoma in an immune-competent host [55]. Similarly, the CD47 antibody miap301 potently inhibits CD47–SIRPα binding, but did not inhibit growth of MT1A2 mouse breast tumors in an immune-competent host [35, 56], and a CD47/SIRPα-blocking antibody that could not engage Fc receptors did not impair B16 tumor growth in C57Bl/6 mice [53]. Therefore, blockade of the "don't eat me" signal is generally not sufficient to elicit immune rejection of syngeneic tumors in mice and, specifically, of the relatively weakly immunogenic B16 melanoma.

By limiting signaling induced by its ligand thrombospondin-1, antisense suppression of CD47 protects T cells against DNA damaging therapies including RT by activation of autophagy pathways and globally stabilizing metabolic pathways [32, 57]. Thus, antisense suppression of CD47 in combination with RT leads to enhanced anti-tumor adaptive immune response [11, 13, 32]. The increased sensitivity of B16 melanomas grown in either *Thbs1*$^{-/-}$ or *Cd47*$^{-/-}$ mice to RT provides direct genetic evidence that CD47 signaling in the tumor microenvironment, rather on tumor cells, limits immunogenic tumor death induced by RT. In addition to cytoprotection of T cells from damage by irradiation [32, 58], blocking thrombospondin-1 signaling through CD47 increases the activation of T cells in response to T-cell receptor engagement [20, 59]. This results in increased antigen-dependent killing of target tumor cells by CD8^{+} T cells and was previously associated with increased intratumoral expression of granzyme B in syngeneic melanoma and fibrosarcoma tumor models [11]. The present data extend the benefits of antisense blockade of CD47 enhancing the anti-tumor activity of human cytotoxic T cells.

The present data show enhanced granzyme B expression in non-irradiated tumors when CD47 and CTLA4 blockade are combined that is not associated with increased CD8 T-cell infiltration. NK cells also have rapid and potent anti-tumor effector properties [60]. CD47 positively correlates with NK-cell signature gene expression in human melanomas [25], suggesting that intratumoral CD47 positively regulates NK-cell infiltration or activation. The inhibitory receptor CTLA4 is also induced in activated NK cells [61], and a CD47 antibody that blocks SIRPα and thrombospondin-1 binding increased NK-cell killing of head-and-neck squamous cell carcinoma [28] and the abundance of IFNγ^{+} and granzyme B^{+} NK cells infiltrating B16 tumors in mice [25]. NK cells are not known to express SIRPα, but we found that the observed killing could result from blocking inhibition of thrombospondin-1/CD47 signaling in the NK cells [25]. Therefore, benefits of the combined blockade of CTLA4 and CD47 may include promoting NK-mediated tumor cell cytotoxicity. However, because the NK-cell numbers decrease following RT, the survival advantage of combinatorial blockade of CTLA4 and CD47 m with RT may be mediated primarily by the observed increases in T-cell infiltration and a decrease in suppressive MDSCs.

The CD47 antibodies and recombinant SIRPα decoys in ongoing clinical trials and in preclinical development

are tailored to inhibit CD47/SIRPα rather than CD47/thrombospondin-1 interactions [62, 63]. SIRPα-selective inhibitors may be more effective in combination with the approved CTLA4 antibody Ipilimumab for treating melanoma patients when the single checkpoint blockade is ineffective. However, the ability to block thrombospondin-1/CD47 signaling on T cells in the tumor microenvironment is critical for radioprotection of tumor-associated immune cells and directly enhancing the adaptive immune response [11, 18, 19]. Therefore, the CD47 morpholino, which inhibits all CD47-dependent pathways by reducing CD47 expression, may offer advantages when combined with Ipilimumab over the CD47 therapeutics currently in clinical trials for treating melanoma and other immunogenic cancers.

Acknowledgements We would like to thank the staff of NCI's Pathology/Histotechnology Laboratory at Frederick for performing immunohistochemical staining for CD3.

Author contributions Anthony L. Schwartz, Pulak R. Nath, Michael Allgauer, Elizabeth C. Lessey-Morillon, Lisa A. Ridnour, John M. Sipes, and Yves M. Morillon performed experiments described herein. Zhiya Yu and Nicholas P. Restifo prepared ESO-specific human T-cell lines. Anthony L. Schwartz and David D. Roberts planned experiment design and wrote the paper.

Funding This work was supported by the Intramural Research Program of the NIH/National Cancer Institute.

Compliance with ethical standards

Conflict of interest Anthony L. Schwartz is the Chief Executive Officer and shareholder of Morphiex Biotherapeutics which holds licensing rights to the CD47 Morpholino. All other authors of this paper declare no conflicts of interest.

Ethical approval Animal Studies: The B16 melanoma animal model was carried out under approved protocols (Protocol #LP-026, January 2016) following the guidelines of the National Cancer Institute's Animal Care and Use Committee.

Informed consent Human T-cell studies: TCGA data were obtained under informed consent as described (https://cancergenome.nih.gov/abouttcga/policies/informedconsent). No new human materials for T-cell experiments were obtained, thus did not require consent or IRB review.

Animal source C57BL/6 mice were obtained from Jackson Laboratories (Bar Harbor, ME).

Cell line authentication B16F10 (CRL-6475) mouse melanoma cell line was purchased from ATCC and were authenticated at the Frederick National Laboratory for Cancer Research (Frederick, MD). The origin of the T-cell lines was previously published, described and provided by the Surgery Branch, Center for Cancer Research, National Cancer Institute [33].

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