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ORIGINAL RESEARCH

Mesothelin-targeting T cells bearing a novel T cell receptor fusion construct (TRuC) exhibit potent antitumor efficacy against solid tumors

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ABSTRACT

T cell Receptor (TCR) Fusion Construct (TRuC®) T cells harness all signaling subunits of the TCR to activate T cells and eliminate tumor cells, with minimal release of cytokines. While adoptive cell therapy with chimeric antigen receptor (CAR)-T cells has shown unprecedented clinical efficacy against B-cell malignancies, monotherapy with CAR-T cells has suboptimal clinical efficacy against solid tumors, probably because of the artificial signaling properties of the CAR. TRuC-T cells may address the suboptimal efficacy of existing CAR-T therapies for solid tumors. Here, we report that mesothelin (MSLN)-specific TRuC-T cells (referred to as TC-210 T cells) potently kill MSLN+ tumor cells *in vitro* and efficiently eradicate MSLN+ mesothelioma, lung, and ovarian cancers in xenograft mouse tumor models. When benchmarked against MSLN-targeted BBζ CAR-T cells (MSLN-BBζ CAR-T cells), TC-210 T cells show an overall comparable level of efficacy; however, TC-210 T cells consistently show faster tumor rejection kinetics that are associated with earlier intratumoral accumulation and earlier signs of activation. Furthermore, *in vitro* and *ex vivo* metabolic profiling suggests TC-210 T cells have lower glycolytic activity and higher mitochondrial metabolism than MSLN-BBζ CAR-T cells. These data highlight TC-210 T cells as a promising cell therapy for treating MSLN-expressing cancers. The differentiated profile from CAR-T cells may translate into better efficacy and safety of TRuC-T cells for solid tumors.

Introduction

The T cell receptor (TCR) is an intricate, octameric signaling complex that triggers a tightly regulated T cell signaling cascade that controls cell migration, cytokine release, cytotoxicity, survival, proliferation, and differentiation.

T cell receptor fusion construct (TRuC) T cells harness all signaling subunits of the TCR to activate T cells independently of peptide-major histocompatibility complex (MHC).[1–3] TRuC-T cells are endowed with a fusion protein comprising a tumor-antigen binder tethered to full-length CD3ε or γ subunits. Like natural CD3 subunits, the TRuC is assembled into the TCR and reprograms T cells to kill tumor cells expressing the cognate surface antigen.[1] CD19-targeted TRuC-T cells potently eliminate tumors in mouse models, while releasing fewer cytokines than existing CD19-targeted chimeric antigen receptor (CAR)-T cells, without the need for an additional co-stimulatory domain.[1]

Existing CAR-T therapies can cure certain cancer types, such as B-cell malignancies,[4–9] but are less effective in solid tumors. CAR-T cells transiently expressing mesothelin (MSLN) – an antigen strongly expressed by solid tumor cells and more weakly or natively expressed by normal mesothelial cells[10] – exhibited suboptimal persistence and efficacy in one patient with malignant pleural mesothelioma and one patient with pancreatic cancer.[11] Likewise, in a study that enrolled

patients with malignant pleural mesothelioma, ovarian cancer, or pancreatic ductal adenocarcinoma, CAR-T cells stably expressing the CAR yielded stable disease as the best observed response.[12]

This apparent suboptimal effectiveness might be because the signaling properties of the CAR are significantly altered relative to the native TCR. Insufficient or aberrant signaling by CARs, which solely utilize the CD3ζ chain of the TCR complex fused to one or more co-stimulatory domains, may limit T cell activity and clinical efficacy in solid tumors. Furthermore, inadequate accumulation and proliferation of CAR-T cells have been linked to extrinsic immunosuppressive factors in the solid tumor microenvironment (TME), such as anti-inflammatory cytokines, co-inhibitory ligand expression, and the presence of immunosuppressive cells.[13] Patients with glioblastoma treated with EGFR variant III-CAR-T cells had increased expression of PD-L1, IDO, and TGFβ, and higher regulatory T cell infiltration than pre-treatment specimens in *in situ* evaluation.[14,15] However, a more recent trial of patients with malignant pleural cancer observed a 63% objective response rate by combining MSLN-targeted CAR-T cells with checkpoint inhibitors, without on-target/off-tumor toxicities.[16]

Given that TRuC-T cells better leverage native TCR signaling, they might address the suboptimal efficacy of existing CAR-T therapies for solid tumors, such as those expressing

MSLN. Herein, we demonstrate that MSLN-targeted TRuC-T cells ("TC-210 T cells") show potent activity *in vitro* and eliminate MSLN-expressing tumors in mice. Compared with CAR-T cells, TC-210 T cells were more efficacious, accumulated more rapidly in the TME, and released fewer cytokines. TRuC-T cells are thus promising to treat solid tumors, and clinical testing of TC-210 was initiated in 2019 (NCT03907852).

Material and methods

Full methods are described in the supplemental material (see **Supplemental Methods**) and summarized herein.

Generating MSLN-targeted TRuC and CAR-T cells

The MSLN (membrane-proximal)-targeted ε-TRuC was generated by tethering the sequence of the MSLN-specific single-domain antibody (MH1) to the TCR CD3ε subunit via a flexible linker (GGGGSx3). MSLN-targeted CARs bearing the MH1 binder (MSLN-CAR) were generated by fusing the MH1 sequence to the 41BB-CD3ζ CAR construct sequence.[17] We selected a BBζ CAR construct for comparisons because most CAR-T cell therapies being developed to treat solid tumors use the 4–1BB domain.[18] The constructs were cloned into lentiviral transfer plasmids.

Lentiviruses were prepared by transient transfection of HEK293 suspension cells with packaging plasmids and the TRuC or CAR lentiviral transfer plasmids. Supernatants were collected 48 hours post-transfection, centrifuged, filtered, and precipitated. Clarified supernatants were resuspended in TexMACS medium supplemented with 3% human AB serum and stored at –80°C until use.

On day 0, primary human T cells were isolated by magnetic bead separation using anti-CD4 and anti-CD8 microbeads. T cells were activated using Human T Cell TransAct at a 1:1 ratio and cultured in TexMACS medium with 3% human AB serum, 12.5 ng/mL human IL-7, and 12.5 ng/mL human IL-15. T cells were transduced with the respective lentiviral vectors on day 1 and harvested on day 10.

Flow cytometry analysis

Live human T cells were identified by Live/Dead Zombie UV and anti-human CD3 (HIT3a), anti-human CD4 (RPA-T4) and anti-human CD8 (SK3) staining. Transduction efficiency was measured as the proportion of TRuC+ or CAR+ cells, using a biotinylated MonoRab™ Rabbit anti-camelid heavy chain-only antibodies (VHH) antibody followed by streptavidin-APC.

Tumor cell lines and mouse xenograft models

Four tumor cell lines were used across experiments: nonsmall cell lung cancer cell line A549, mesothelioma cell line MSTO-211H, ovarian adenocarcinoma cell line OVCAR3 (which expresses MSLN), and ovarian adenocarcinoma cell line C30 (which does not express MSLN). Parental A549 and MSTO-211H cells were transduced with lentiviral vectors encoding both the full-length human MSLN and firefly luciferase (Luc) genes to generate MSLN+ and Luc+ tumor cell lines ("A549-M-Luc" and "MSTO-M-Luc"). OVCAR3 and C30 cells were transduced with a lentiviral vector encoding luciferase to generate OVCAR3-Luc and C30-Luc cells, respectively.

For subcutaneous xenograft models, 10^6 MSTO-M-Luc cells or 10^6 A549-M-Luc cells were injected subcutaneously in the dorsal hind flank of NOD scid gamma (NSG) mice. For the intraperitoneal xenograft model, 10^7 OVCAR3-Luc cells were resuspended in sterile RPMI-1640 with 10% fetal bovine serum (FBS) and injected intraperitoneally (200 μL/mouse).

In vivo efficacy of engineered T cells

Engineered T cells – MSLN-BBζ CAR and TC-210 – were tested in the OVCAR3-Luc intraperitoneal model and MSTO-M-Luc subcutaneous model. A second study was carried out with the MSTO-M-Luc and the A549-M-Luc subcutaneous models. Tumor volume was monitored twice weekly by caliper measurement. Tumor growth was monitored twice weekly by bioluminescence imaging using an IVIS instrument.

T cells were injected, at the indicated doses, into the tail vein of mice with established tumors. To compare CAR and TC-210 T cells, differences in T cell transduction rates were adjusted to the lowest transduction rate of all constructs by adding nontransduced (NT) T cells.

T cell accumulation in tumors

Mice were euthanized at the indicated timepoints, and MSTO-M-Luc tumors and spleens were excised and weighed. A portion of the tumor and spleen was collected into 10% buffered formalin. Tissues were transferred to 70% ethanol after 24–72 hours followed by standard immunohistochemistry (IHC) staining against human CD7.

T cell cytokine release and tumor cell cytotoxicity

To assess the effects of engineered T cells *in vitro*, Luc-expressing tumor cells were plated in a 96-well plate at 10,000 cells/well, and T cells were added at the desired effector-to-target ratios. After a 24-hour co-culture, 50% of the supernatant was removed for cytokine analysis using a custom 4-plex Mesoscale Discovery kit. Cell viability was determined using the Bright-Glo™ Luciferase Assay System.

Human cytokines in co-culture supernatant and mouse plasma were measured using the Luminex-based MILLIPLEX MAP Human CD8+ T Cell Magnetic Bead Panel Premixed 17 Plex–Immunology Multiplex Assay. Culture supernatants were collected after 24 hours of co-culture and stored at –80°C until analysis.

Gene expression profiling of tumor-infiltrating T cells

Tumor samples were collected for selected *in vivo* studies at the indicated time points after T cell injection and dissociated using a human tumor dissociation kit. CD3+ tumor-infiltrating leukocytes were isolated, counted, and lysed. Gene expression in cell lysates was assessed using the pre-designed

Human nCounter CAR-T Characterization panel and the nCounter SPRINT profiler instrument. Three biological replicates were analyzed at each timepoint. Data were analyzed with the nCounter Advanced Analysis 2.0 plugin of the nSolver Software (Nanostring Technologies, Version 4) following default procedures, with *TRAC* and *CD3E* added to the internal housekeeping genes to normalize T cell count. Differentially expressed genes between TC-210 and MSLN-BBζ CAR-T cells were identified based on a fold change of >2 or <0.5 and a P-value of <0.01.

Metabolic profiling of engineered T cells

For *in vitro* analysis, T cells were thawed and activated with 10 μg/mL MSLN for 24 hours. Cells were then expanded in fresh culture media (complete RPMI + 10% serum) supplemented with 200 U/mL human IL-2 for 72 hours, pulsed with 2-NDBG, stained for surface markers with MitoTracker Deep Red, and analyzed by flow cytometry. Basal oxygen consumption rates (OCRs) and extracellular acidification rates (ECARs) were measured using a Seahorse XFe96 Bioanalyzer.

For *ex vivo* metabolic flow cytometry assays, single-cell suspensions from tumors (2×10^7 cells) were resuspended in fluorescence activated cell sorting buffer with 5 mM 2-NBD-Glucose and anti-VHH, and incubated for 30 minutes at 37°C. Cells were washed and then incubated with 250 nM MitoSpy-Orange and anti-human CD3, CD4, and CD8 for 20 minutes at 37°C.

Statistical analysis

In vitro experiments were performed with three technical replicates. *Ex vivo* experiments were performed with three biological replicates. For *in vivo* studies, treatment groups were compared using a two-way repeated-measures analysis of variance (ANOVA), followed by Bonferroni's posttest. Plasma cytokine levels were compared using Dunnett's test with two-way ANOVA. For *in vitro* metabolism studies, and for *ex vivo* IHC and flow cytometry studies, groups were compared using an unpaired Student t-test. Statistical significance was systematically defined as P< 0.05.

Study approval

Animal study protocols were approved by the Institutional Animal Care and Use Committees of the study facilities. Abpro (Burlington, MA) performed the studies in Figure 2. TCR[2] Therapeutics performed the other animal studies at the Charles River Accelerator and Development Lab (CRADL™) (Cambridge, MA).

Results

Mesothelin-targeted TRuC-T cells kill MSLN-expressing cells and produce cytokines

An MSLN-targeted ε-TRuC was designed by fusing the MSLN-specific single-domain antibody (MH1) to the N-terminus of the invariant CD3ε subunit of the TCR (Figure 1a). After transduction of T cells with a lentiviral vector encoding the MSLN ε-TRuC, cells were expanded for 9 days. Across three donors, TRuC+ cells were comprised of 20.9–30.5% CD4+ cells and 4.59–9.21% CD8+ cells (Figure 1b). Within CD4+ cells, 53.50–75.68% expressed the TRuC, whereas 27.25–36.54% of CD8+ expressed it (data not shown), suggesting the lentivirus has preferential tropism for CD4+ cells.

In a 24-hour tumor-cell lysis assay, TC-210 T cells efficiently killed tumor cells expressing high MSLN levels, including MSTO-M-Luc mesothelioma cells (Figure 1c), A549-M-Luc cells (**Figure S1A and S1B**), and OVCAR3-Luc cells with native MSLN expression (**Figure S1A and S1B**). MSLN-negative C30 cells were spared from killing, confirming the antigen-dependent cytotoxicity of TC-210 (Figure 1c). Of note, MSTO-M-Luc cells, OVCAR3-Luc cells, and A549-M-Luc cells expressed varying levels of co-stimulatory ligands (**Figure S1C**). TC-210 T cells proliferated only in response to MSLN+ MSTO-M-Luc cells (Figure 1d). Likewise, TC-210 T cells, but not NT cells, secreted robust amounts of IFN-γ, IL-2, GM-CSF, and TNF-α when exposed to MSTO-M-Luc cells, but not when exposed to MSLN- C30-Luc cells (Figure 1e).

TC-210 T cells eradicate MSLN-expressing solid tumors in vivo

We first tested the anti-tumor activity of TC-210 T cells in the OVCAR3-Luc xenograft model, which naturally expresses MSLN (**Figure S1A**). NSG mice with established intraperitoneal OVCAR3-Luc tumors received a single infusion of phosphate-buffered saline vehicle, NT cells (as a control), or TC-210 T cells with a transduction rate of approximately 40%. At day 9 post-injection, TC-210 T cells significantly decreased the mean tumor burden compared with vehicle and NT controls (Figure 2a). Specifically, tumors were undetectable in 6 of 7 animals by day 13 post TC-210 infusion, and mice remained tumor-free by day 27 (i.e., the end of the observation period) (Figure 2b). In one mouse, a small tumor remained after initial regression but was otherwise well controlled (Figure 2b).

We next compared the anti-tumor activity of TC-210 T cells against MSLN-targeted BBζ CAR-T cells in a xenograft model of A549-M-Luc cells overexpressing MSLN. The MSLN-BBζ CAR-T cells used the same MH1 binder as the MSLN ε-TRuC T cells and were similarly developed. NSG mice subcutaneously engrafted with A549-M-Luc cells were intravenously infused with 2×10^6 TC-210 T cells or MSLN-BBζ CAR-T cells (transduction rate: ~20%). TC-210 or MSLN-BBζ CAR-T cells eliminated tumors in all animals; however, TC-210 T cells cleared tumors slightly faster than MSLN-BBζ CAR-T cells (Figure 2c). Tumors in TC-210-treated mice started to regress earlier than those in MSLN-BBζ CAR-T-treated mice.

In both TC-210- and MSLN-BBζ CAR-T-treated mice, elevated plasma levels of GM-CSF, IFN-γ, IL-2, IL-4, IL-5 and granzymes A and B were observed during active tumor clearance; however, cytokine levels were significantly lower in TC-210-treated mice than in MSLN-BBζ-CAR-T cell-treated mice (Figure 2d). In addition, most cytokines in MSLN-BBζ CAR-T cell-treated animals were elevated for longer before returning to baseline levels; some remained elevated throughout the observation period.
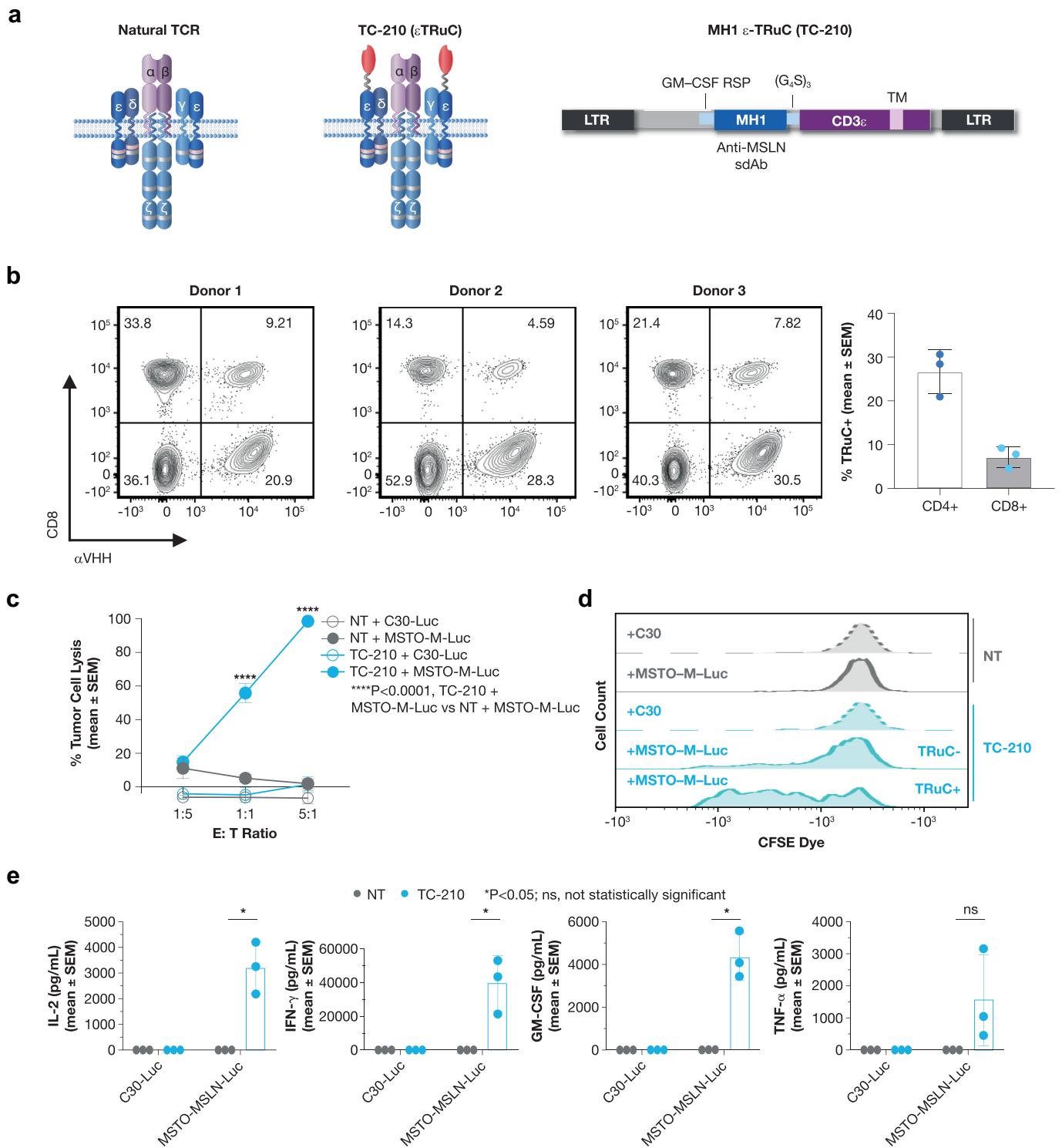
Figure 1. TC-210 T cells potently kill antigen-dependent tumor and release cytokines. (a): The natural TCR complex and MSLNε-TRuC-TCR complex. (b): Flow-cytometry-based detection of TRuCs at the cell surface. (c): Killing of MSLN-M-Luc tumor cells by TC-210 T cells across E:T ratios. (d): Proliferation of TC-210 TRuC or NT T cells by dye dilution after co-culture with MSTO-M-Luc cells. (e): Cytokines levels secreted during the tumor cell killing assay in c. E:T = effector-to-target; GM-CSF = granulocyte-macrophage colony-stimulating factor; IFN = interferon; IL = interleukin; Luc = luciferase; MSLN = mesothelin; MSTO = mesothelioma; NT = nontransduced; TCR = T cell receptor; TNF = tumor necrosis factor; TRuC = T cell receptor fusion construct; VHH = variable heavy-chain antibody fragment.

The lower production of cytokines by TC-210 T cells was confirmed in the MSTO-M-Luc mesothelioma xenograft model. In the MSTO-M-Luc model, TC-210 T cells induced a more rapid regression of MSTO-M-Luc tumors (**Figure S2A**), while releasing fewer cytokines than MSLN-BBζ CAR-T cells (except for granzyme and perforin) (**Figure S2B**).

Rapid eradication of tumors by TC-210 T cells is associated with efficient intratumoral accumulation

We then compared the kinetics of the accumulation of TC-210 T cells and MLSN-BBζ CAR-T cells in MSTO-M-Luc tumors, including various *ex vivo* endpoints. TC-210 T and
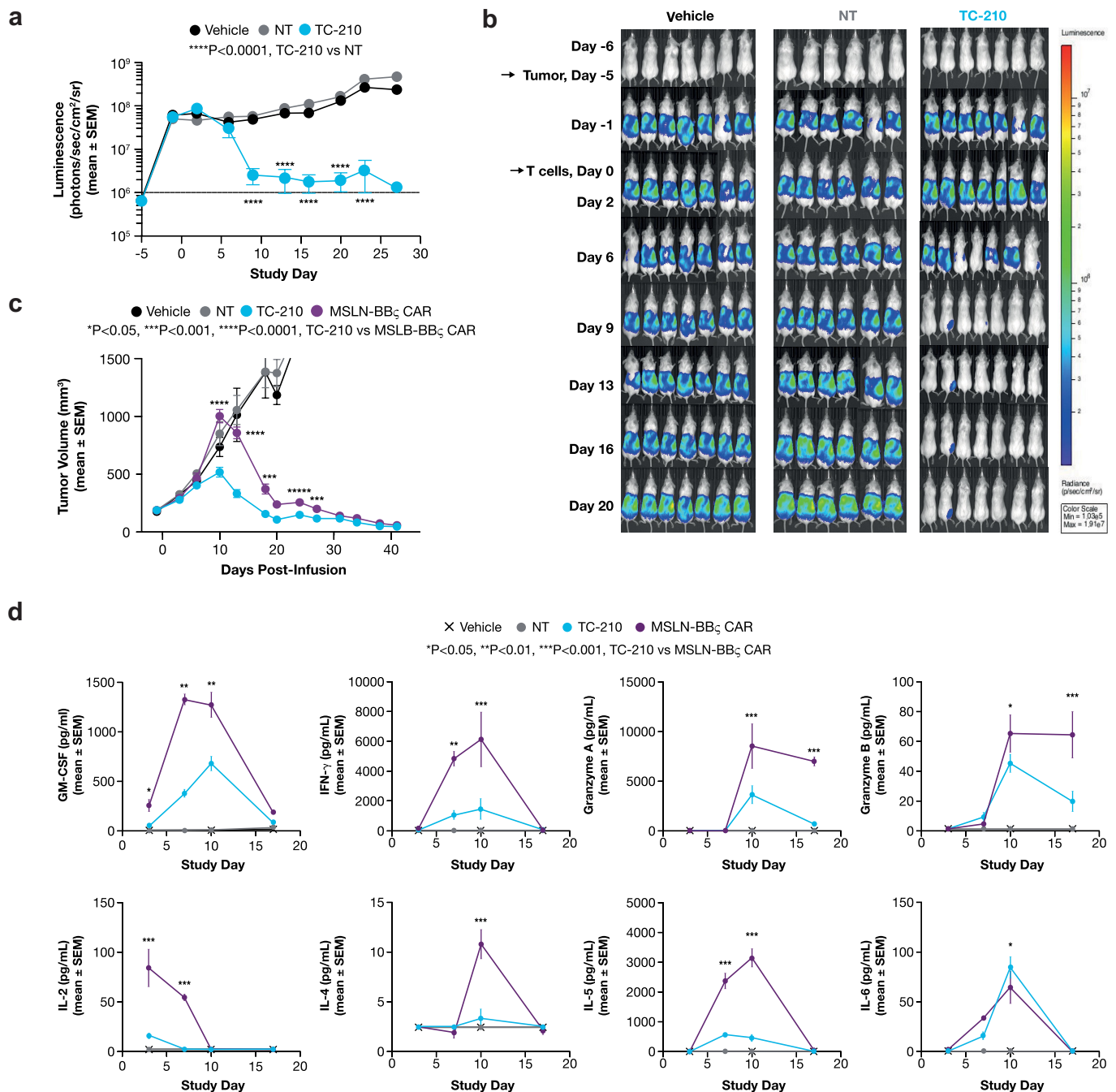
Figure 2. TC-210 T cells eradicate OVCAR3-M-Luc and A549-M-Luc solid tumors in mice, with lower systemic cytokine release than CAR-T cells. (a): Tumor burden (measured by BLI) of NSG mice intraperitoneally injected on day – 6 with OVCAR3-Luc cells and, on study day 0, and treated infused with vehicle (10% FBS medium), NT, or TC-210 T cells. Dashed line indicates baseline BLI measure on Day – 6. (b): Whole-mouse luminescence over time. (c): Tumor volume of NSG mice bearing subcutaneous A549-M-Luc tumors (n = 10 mice/group) and treated with vehicle, nontransduced (NT) control, TC-210, or MSLN-BBζ-CAR-T cells. (d): Release of human cytokines in the plasma of A549-M-Luc tumor bearing mice (n = 5 mice per time point) treated as in Panel a. BLI = bioluminescence imaging; CAR = chimeric antigen receptor; FBS = fetal bovine serum; GM-CSF = granulocyte-macrophage colony-stimulating factor; IFN = interferon; IL = interleukin; Luc = luciferase; MSLN = mesothelin; NT = nontransduced.

CAR-T cells exhibited comparable transduction efficiency (**Figure S3A**) and *in vitro* cytotoxicity (**Figure S3B**). Consistent with earlier findings, and despite similar *in vitro* activity, tumor cells were rejected significantly faster with TC-210 T cells than with MLSN-BBζ CAR-T cells (Figure 3a). Both the onset of tumor regression (TC-210: day 10, MLSN-BBζ CAR-T: day 13) and tumor eradication (TC-210: day 20, MLSN-BBζ CAR-T: day 27) occurred earlier with TC-210 T cells.

By day 7 post-infusion, significantly more T cells had accumulated in the tumors of TC-210 treated mice than in MLSN-BBζ CAR-T treated mice (Figures 3b, c). This trend was maintained – albeit not statistically significant – on day 10 (Figure 3b, c). Consistent with the IHC results, TC-210-treated tumors contained significantly more human CD3+ T cells than MLSN-BBζ CAR-T cells or NT cells on days 7 and 10 post-infusion (Figure 3d). The number of tumor-infiltrating TC-210 T cells was also significantly higher than that of tumor-
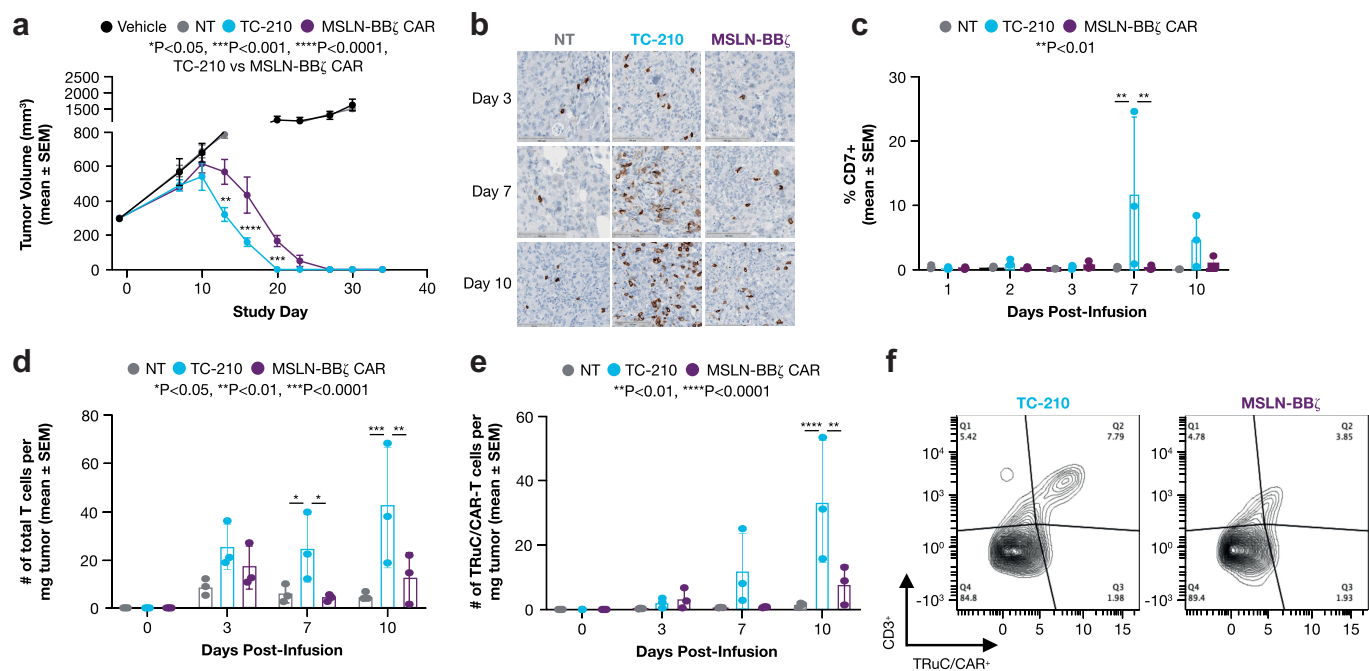
Figure 3. Rapid eradication of tumors by TC-210 T cells is associated with efficient tumor accumulation. (a): Tumor volume of MSTO-M-Luc tumor-bearing NSG mice (n = 5 mice/group) treated with vehicle, NT control, TC-210 T cells, or MSLN-BBζ CAR-T cells. (b): IHC-based detection of tumor-infiltrating CD7+ human T cells (n = 3 mice/group/time point). (c): HALO® imaging analysis of T cell infiltration. (d): Flow cytometry-based quantification of total intratumoral CD3+ cells. Data are reported as mean ± SEM. (e): Flow cytometry-based quantification of CD3+ TC-210/CAR+ cells. (f): Representative contour plots of TRuC/CAR expression on CD3+ intratumoral T cells. CAR = chimeric antigen receptor; IHC = immunohistochemical; Luc = luciferase; MSLN = mesothelin; NT = nontransduced.

infiltrating CAR-T cells at day 10 post-infusion (Figure 3e). Notably, TRuC was more highly expressed than CAR on tumor-infiltrating T cells (figure 3f).

TC-210 T cells are activated earlier than MSLN-BBζ CAR-T cells in the tumor microenvironment

Tumor-infiltrating TC-210 T cells and MSLN-BBζ CAR-T cells were further analyzed using Nanostring technology to profile gene expression. On day 7, TC-210 T cells showed higher expression of several genes associated with T cell activation and effector function, including *CD69, IL12RB1, IL-21R, CXCR3, CXCR4, FASLG, TNF, GZMA,* and *GZMB*, suggesting TC-210 T cells were more activated at this time point than MSLN-BBζ CAR-T cells (Figures 4a **and S4**). In addition to *CXCR3* and *CXCR4*, expression of other chemokine and chemokine receptor genes (i.e., *CXCL9, CCL5* and *CCR6)* was higher in TC-210 T than in MSLN-BBζ-CAR-T cells. The TCR alpha/beta subunits (i.e., *TRBV1-4, TRAV34,* and *TRBC1*) and the TCR signaling regulators *PTPRC* (which encodes the CD45 phosphatase) and *SHD21A* (which encodes the SLAM-associated protein [SAP]) were also more highly expressed in TC-210 T cells than in MSLN-BBζ CAR-T cells. On day 10, the expression of genes involved in T cell activation/exhaustion (i.e., *HAVRC2, TIGIT, LAG-3, ICOS,* and *CTLA4*) was higher in TC-210 T than in MSLN-BBζ CAR-T cells (Figures 4a **and S4**). *FOXO1*, a transcription factor that opposes terminal T cell differentiation,[19] was also more highly expressed in TC-210 T cells than in MSLN-BBζ-CAR-T cells.

To allow for a sufficient density of T cells to support phenotypic analysis, tumors were harvested 10 days after T cell infusion. Given the limited number of CD8+ CAR-T cells,

comparative analysis focused on the CD4+ subset. Consistent with the gene profiling results, more TC-210 T cells than CAR-T cells expressed the early activation marker CD69, with a trend toward a higher cell-surface density, but fewer expressed CD25 (Figure 4b). Compared with MSLN-BBζ-CAR-T cells, tumor-infiltrating TC-210 T cells showed a higher cell surface expression of the exhaustion markers TIGIT and LAG-3 (Figure 4c). The PD-1 receptor followed the same trend, whereas TIM-3 surface expression was similar between TC-210 T cells and MSLN-BBζ-CAR-T cells.

TC-210 T cells are shifted toward mitochondrial metabolism

On day 7, the *HK2* gene, which encodes the glucose metabolism regulator hexokinase II, was significantly less expressed in intratumoral TC-210 T cells than in CAR-T cells (Figure 4a). To evaluate the metabolic profiles of TC-210 T and CAR T cells, NT, TC-210 T cells, and MSLN-BBζ CAR-T cells were stimulated for 4 days in MSLN-coated plates and analyzed by *in vitro* extracellular flux analysis. Representative traces for OCR and ECAR are depicted in Figure 5a. There was no appreciable difference in basal oxidative metabolism between TC-210 T and MSLN-BBζ CAR-T cells; however, spare respiratory capacity was higher in TC-210 T cells (Figure 5b), suggesting an increase in mitochondrial content. ECAR was lower in TC-210 T cells, indicating a lower glycolytic activity than in MSLN-BBζ CAR-T cells.

Consistent with *in vitro* findings, intratumoral CD4+ TC-210 T cells showed significantly lower glucose uptake than CD4 + MSLN-BBζ CAR-T cells; CD8 + T cells followed a similar trend (Figure 5c). The mitochondrial mass was significantly
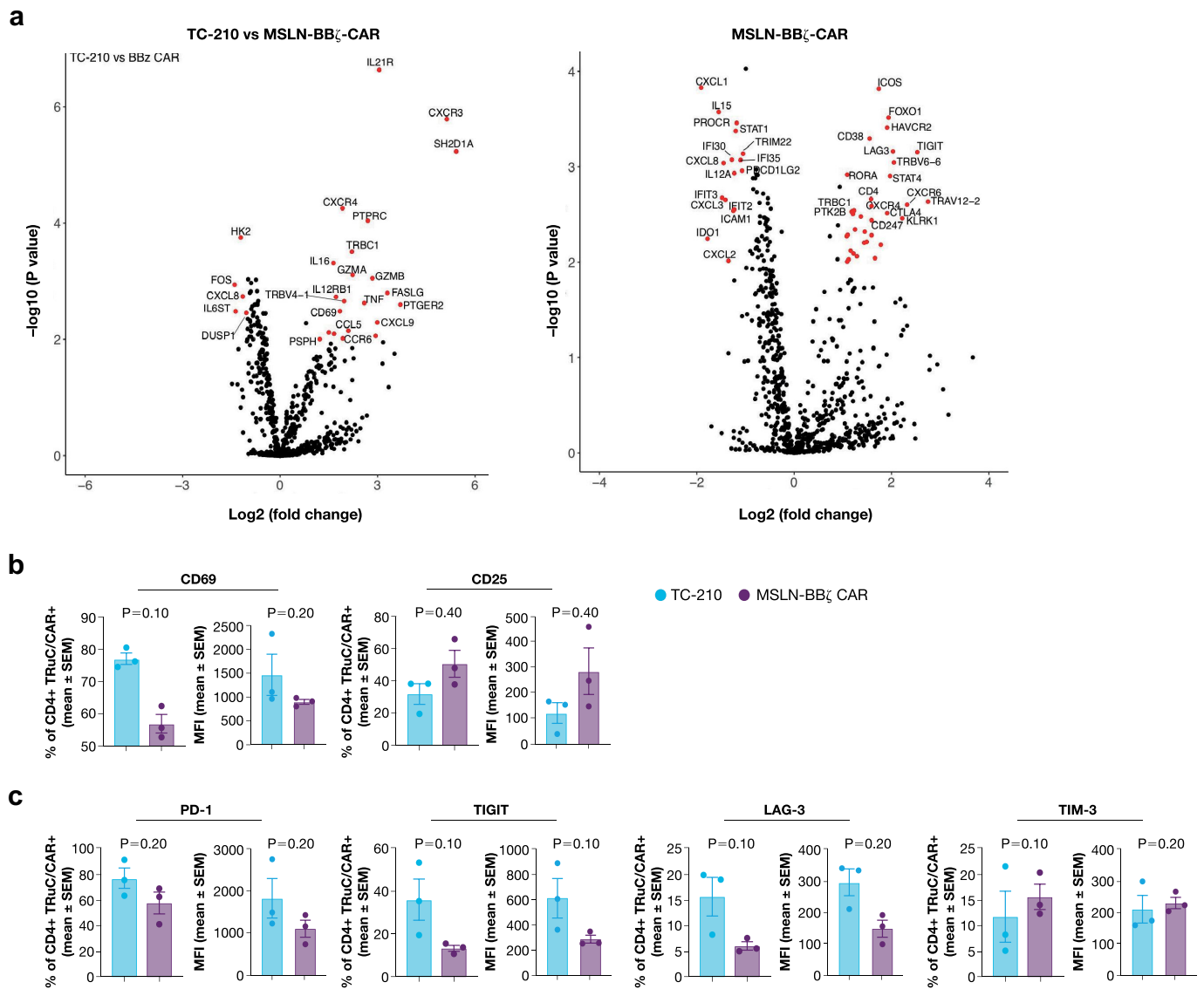
Figure 4. Intratumoral TC-210 T cells display higher activation than MSLN-BBζ-CAR T cells and express TCR regulatory genes. Tumors collected from the same satellite cohorts described in Figure 3 were further analyzed. (a): Differentially expressed genes (from a Nanostring gene expression panel) in human T cells isolated at days 7 and 10 from MSTO-M-Luc tumors. (b): Expression of activation markers and chemokine receptors in human T cells isolated from dissociated MSTO-M-Luc tumors collected on study day 10. (c): T cell exhaustion markers on study day 10 detected by flow cytometry. 2-NDBG = 2-NDB-glucose; CAR = chimeric antigen receptor; Luc = luciferase; MFI = mean fluorescence intensity; MSLN = mesothelin; TCR = T cell receptor; TRuC = T cell receptor fusion construct.

higher in CD8+ TC-210 T cells than in CD8+ MSLN-BBζ CAR-T cells, indicating that tumor-infiltrating TC-210 T cells are shifted toward oxidative phosphorylation (Figure 5c). A similar trend – albeit not statistically significant – was observed for CD4+ cells.

Discussion

We evaluated TC-210, a TRuC-T cell therapy for MSLN-expressing solid tumors. As previously shown for CD19-targeted ε-TRuCs,[1] MSLN-targeted ε-TRuCs were efficiently integrated into the TCR and expressed on the T cell surface, thus redirecting T cell cytotoxicity and cytokine production toward MSLN-expressing tumors.

Our findings demonstrate that TC-210 T cells recognize and efficiently kill tumor cells in an antigen-dependent manner. TC-210 T cells displayed potent cytotoxicity and robust

proliferation without covalently linked co-stimulation, which likely reflects their ability to leverage the full signaling capacity of the TCR .[1] The anti-tumor activity of TC-210 T cells may be further potentiated by the fact that the tumor cell lines herein used natively expressed one or more co-stimulatory ligands.

TC-210 T cells consistently eradicated various types of large, well-established tumors in *in vivo* experiments. Mice treated with TC-210 T cells or CAR-T cells (that expressed the same MSLN binder) eradicated the tumor xenograft; however, tumor rejection was consistently faster with TC-210 T cells. This difference may be partially driven by the higher levels of transgene expression on tumor-infiltrating TC-210 T cells than CARs. Such differential transgene expression may be because TRuCs are less prone to downregulation than CARs upon antigen encounter.[20,21]

Interestingly, TC-210 T cells were more efficient in controlling early tumor growth than CAR-T cells despite releasing
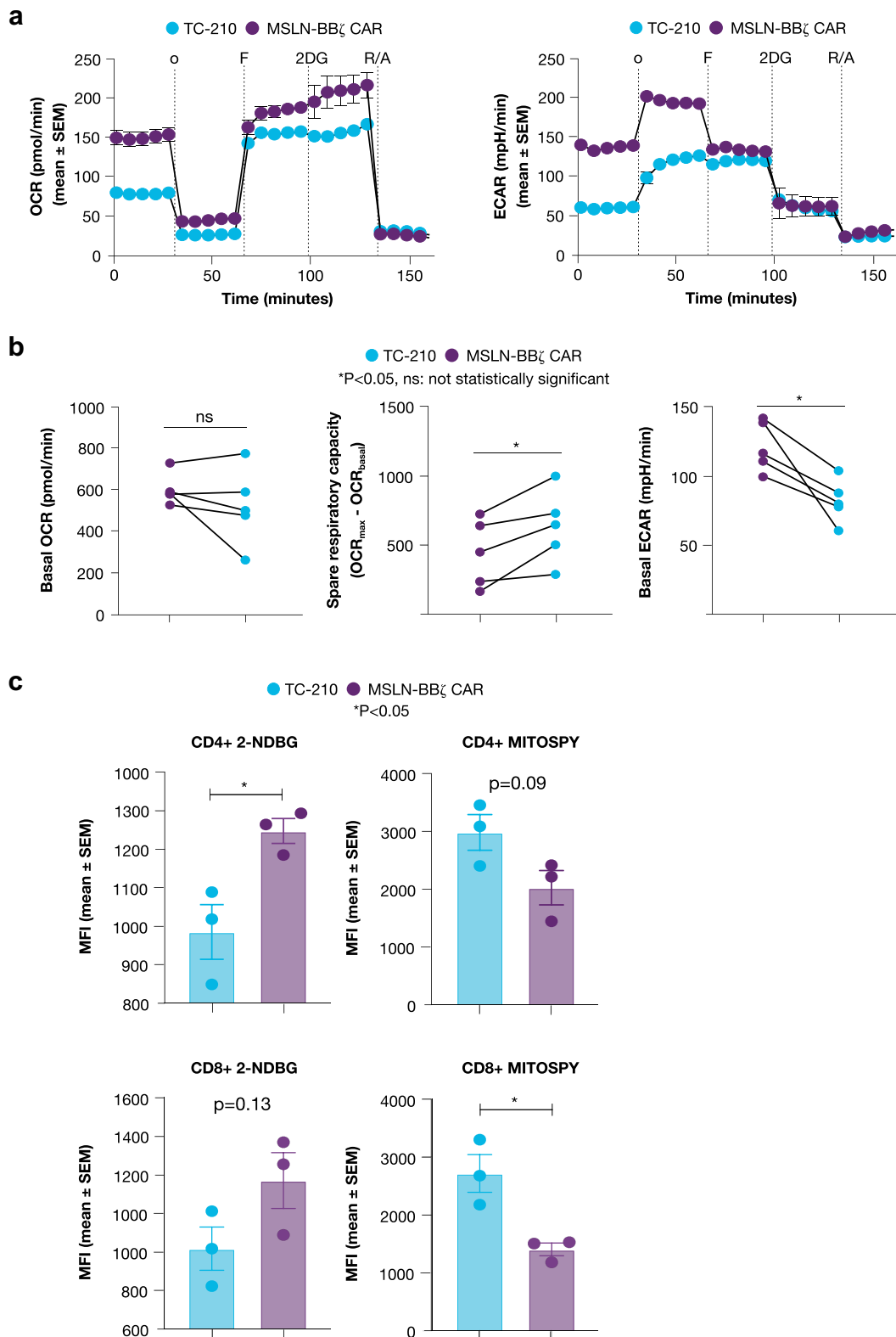
Figure 5. TC-210 T cells display a more favorable metabolic profile than CAR T cells. (a): Representative OCR and ECAR traces of TC-210 T cells and MSLN-BBζ -CAR-T cells measured after a 4-day stimulation with plate bound MSLN-Fc antigen. (b): Comparison of basal OCR, SRC, and basal ECAR measurements in TC-210 T and MSLN-BBζ - CAR-T cells from five normal donors. (c): Glucose uptake (2-NDBG pulse) and mitochondrial mass (MitoSpy staining) in tumor-infiltrating TC-210 T cells and MSLN-BBζ - CAR-T cells collected from three MSTO-M-Luc tumor-bearing mice on day 10 post-infusion, measured by ex vivo flow cytometry analysis. 2-NDBG = 2-NDB-glucose; CAR = chimeric antigen receptor; ECAR = extracellular acidification rate; MFI = mean fluorescence intensity; MSLN = mesothelin; Luc = luciferase; OCR = oxygen consumption rate; SRC = spare respiratory capacity.

lower levels of cytokines and cytotoxic factors into the circulation. Probable differences in immune synapse formation and associated signaling could explain this observation. It is known that CARs form a nonclassical synapse and the quality of synapse formation has been proposed as a predictor of CAR efficacy.[22,23] By coopting the full TCR, the immune synapse formed by a TRuC is presumably more native-like, potentially leading to a better tuned activation signal and a more tightly regulated polarization and release of cytotoxic granules. Future studies of TRuC signaling and synapse formation are warranted.

In addition, more TC-210 T cells than CAR-T cells were present in the TME of MSTO-M-Luc tumors, which might reflect better migration capacity of TC-210 T cells into solid tumors and/or superior fitness within the TME, thus improving intratumoral expansion and persistence. In a pleural mesothelioma xenograft model, MSLN-targeted CAR-T cells suboptimally infiltrated the tumor after systemic administration, which could be overcome by regional cell delivery.[24] Other groups have expressed chemokine receptors or chemokines in CAR-T cells to enhance T cell homing and address this issue.[25–28] Although the mechanism is not fully elucidated, the *CXCR3* and *CXCR4* chemokine receptors were more highly expressed in tumor-infiltrating TC-210 T cells than in CAR-T cells. Should this be confirmed, it could enhance tumor homing and tumor retention, thus explaining differences in tumor rejection kinetics.

Additionally, and consistent with earlier CD19-targeted TRuC-T cell studies,[1] TC-210 T cells produced fewer systemic cytokines than CAR-T cells in tumor-bearing mice. Excess production of inflammatory cytokines by CAR-T cells is associated with cytokine release syndrome (CRS) in patients with hematological malignancies.[8,29–32] Lower cytokine release might translate into an improved safety profile; however, further research is necessary to draw such conclusions. Unlike second-generation CARs, TRuCs do not contain an ectopic co-stimulatory domain, which may explain this lower cytokine release.

Taken together, the early rejection of xenograft tumors and the reduced production of cytokines suggest that ε-TRuC T cells deliver a well-tuned activation signal that efficiently eliminates tumors *in vivo*. These findings are consistent with prior observations for CD19-targeted ε-TRuC-T cells, which potently killed tumor cells while releasing fewer cytokines than CAR-T cells.[1] Of note, TCR regulatory factors such as CD45 and SAP were upregulated in TC-210 T cells, suggesting that TRuC T cells not only drive T cell activation but also invoke the natural TCR-intrinsic feedback mechanisms that tune the T cell response.[33] *In vitro* stimulation assays and mouse xenograft models provide mechanistic insights, but are poor predictors of CRS. Therefore, it remains unclear whether the lower levels of cytokine produced by TC-210 T cells improve the safety profile.

Intratumoral T cells compete with tumor cells for metabolic resources, such as glucose and amino acids, limiting their proliferation and effector functions.[34] TC-210 T cells had a lower (*in vitro*) ECAR than CAR-T cells, consistent with reduced glycolytic activity. This possibility is further supported by the elevated glucose uptake of tumor-infiltrating CAR-T cells in tumor-bearing mice and the lower expression of *HK2* by tumor-infiltrating TC-210 cells, which encodes hexokinase 2 – the first enzyme in the glycolytic pathway. This differential reliance on glycolysis is likely due to differences in the designs of TRuCs and CARs, and their associated signaling properties. Given that TC-210 T cells show a lower glycolytic activity than MSLN-BBζ-CAR-T cells and a higher mitochondrial load, their metabolic profile may favor long-lived memory cell differentiation.[35] Additionally, intratumoral TC-210 T cells displayed higher cell-surface levels of some T cell activation/exhaustion markers, including LAG-3 and TIGIT, suggesting a more efficient activation of TC-210 T cells within the TME. Notably, expression of the gene encoding TOX, a transcription factor associated with T cell exhaustion,[36] was not increased in TC-210 cells, suggesting a state of activation rather than exhaustion. Although not explored in our study, TRuC signaling through the TCR might activate a metabolic program that favors mitochondrial metabolism, which may enhance T cell memory formation, effector function, and fitness within the hostile metabolic conditions of the TME. Our study did not explore the mechanism underlying the rapid accumulation of TC-210 T cells in the TME, but the gene expression and metabolic profiles of TC-210 T cells are hypothesis-generating for plausible mechanisms.

Adoptive cell therapy with CAR-T cells offers unprecedented clinical efficacy against B-cell malignancies;[4–9] however, CAR-T cell monotherapy has suboptimal clinical activity against solid tumors, possibly due to the inefficient homing and low persistence of engineered T cells. This limitation may be partially attributed to the artificial signaling properties of the CAR. We engineered MSLN-targeted TRuC-T cells ("TC-210 T cells") that use the full signaling capacity of the TCR. Like existing CAR-T cells, TRuC-T cells kill cancer cells in a human leukocyte antigen (HLA)-independent manner, thus bypassing a major tumor resistance mechanism, i.e., the downregulation of HLA class I and antigen processing.[37–39] We show that the use of TC-210 T cells is a promising treatment for solid tumors overexpressing MSLN, with TC-210 T cells eradicating MSLN+ tumors of various histologies. TC-210 T cells more rapidly infiltrated and rejected tumors while producing less pro-inflammatory cytokines than second-generation MSLN-targeted CAR-T cells. Future research on the persistence and memory phenotype of MSLN-targeted TRuC-T cells compared with CAR-T cells is warranted. Based on these promising preclinical findings, TC-210 is now being studied in a human clinical trial (NCT03907852) and has shown clinical activity in patients with refractory MSLN-expressing solid tumors[40].

Disclosure statement

Funding

Data availability statement

The authors confirm that the data supporting the findings of this study are available within the article and/or its supplementary materials.

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