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Endoplasmic Reticulum Stress Contributes to Mitochondrial Exhaustion of CD8⁺ T cells

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Abstract

Tumor antigen-specific T cells rapidly lose energy and effector function in tumors. The cellular mechanisms by which energy loss and inhibition of effector function occur in tumor infiltrating lymphocytes (TILs) are ill-defined, and methods to identify tumor-antigen-specific TILs that experience such stress are unknown. Processes upstream of the mitochondria guide cell-intrinsic energy depletion. We hypothesized that a mechanism of T cell-intrinsic energy consumption was the process of oxidative protein folding and disulfide-bond formation that takes place in the endoplasmic reticulum (ER) guided by protein kinase R-like endoplasmic reticulum kinase (PERK) and downstream PERK axis target ER oxidoreductase 1 (ERO1 α). To test this hypothesis, we created TCR transgenic mice with a T cell-specific PERK gene deletion (OT1⁺Lck^{cre} +PERK^{f/f}, PERK KO). We found that PERK KO and T cells that were pharmacologically inhibited by PERK or ERO1 α maintained reserve energy and exhibited a protein profile consistent with reduced oxidative stress. These T cell groups displayed superior tumor control compared to T effectors. We identified a biomarker of ER-induced mitochondrial exhaustion in T cells as mitochondrial reactive oxygen species (mtROS), and found that PD-1⁺ tumor antigen-specific CD8⁺ TILs express mtROS. *In vivo* treatment with a PERK inhibitor abrogated mtROS in PD-1⁺ CD8⁺ TILs and bolstered CD8⁺ TIL viability. Combination therapy enabled 100% survival and 71% tumor clearance in a sarcoma mouse model. Our data identify the ER as a regulator of T cell energetics and indicate that ER elements are effective targets to improve cancer immunotherapy.

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K.L. and K.E.H. performed all experiments and aided in manuscript preparation. M.E. aided in manuscript preparation and intellectual development of the work. Z.W and L.L. guided clinical data and provided translational input. J.E.T. designed experiments, analyzed data, and prepared manuscript.

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Keywords

T cell; PERK; ER Stress; Metabolism; PD-1

INTRODUCTION

In response to antigen, T cells undergo rapid expansion, engaging in up to 15-20 cell divisions, and proliferation is characterized by differentiation to an effector phenotype from a naïve state (1). To support the bioenergetic demands of growth and proliferation, naïve T cells that primarily engage oxidative phosphorylation (OXPHOS) shift to generate adenosine triphosphate (ATP) via increased OXPHOS and aerobic glycolysis as they become functional effector cells (2,3). Highly differentiated effector T cells rely primarily on glycolysis and are characterized by a loss of mitochondrial integrity (4) that likely accounts for their inability to effectively continue OXPHOS. The cell-intrinsic mechanisms that consume metabolic energy and impart mitochondrial exhaustion in T cells are unknown.

Programmed cell death protein 1 (PD-1) is expressed on highly differentiated effector T cells that have become exhausted due to chronic antigen exposure (5). In cancers, programmed death-ligand 1 (PD-L1) is expressed by tumor cells to further impair anti-tumor effector function of PD-1⁺ T cells (6). Checkpoint blockade therapy is a means to reinvigorate effector function of exhausted T cells by inhibition of the PD-1-PD-L1 interaction (7). Unfortunately, anti-PD-1 therapy remains ineffective for the majority of cancer patients due to initial lack of response or loss of durable responses (8-10). In early-stage exhaustion, PD-1⁺ T cells show diminished OXPHOS and glycolysis, and long-term chronically exhausted PD-1^{high} T cells exhibit a dependence on glycolysis due to dysfunctional mitochondria (11). T cell-intrinsic factors that drive PD-1⁺ CD8⁺ tumor infiltrating lymphocyte (TIL) metabolic exhaustion in response to tumor antigen are unknown, and simple methods to characterize the metabolic state of PD-1⁺ tumor infiltrating lymphocytes (TILs) in patient tumors are ill-defined.

T cell expansion initiated by antigen recognition requires T effector cells to greatly increase new protein synthesis and initiate post-translational modifications (12). Imbalances of unfolded and misfolded proteins are detected by endoplasmic reticulum (ER) stress sensors inositol-requiring enzyme-1 (IRE1 α), protein kinase R-like endoplasmic reticulum kinase (PERK), and activating transcription factor 6 (ATF6) (13). The acute arm of the stress response aims to alleviate the burden of unfolded or misfolded proteins in the ER to restore proteostasis. In the face of insurmountable stress, the unfolded protein response (UPR) initiates cell death. PERK mediates the terminal UPR through regulation of transcription factors activating transcription factor 4 (ATF4) and C/EBP α homologous protein (CHOP) which induce downstream target ER oxidoreductase 1 (ERO1 α) (14-17). As a catalyst of disulfide bond formation in the ER, ERO1 α facilitates oxidation-reduction reactions (Redox). Excess ERO1 α produced in response to chronic protein folding induces accumulation of reactive oxygen species (ROS) and impairs survival (15,16). The PERK-specific cell stress response has not been measured in T cells or applied to the rubric of anti-tumor immunity.

Here we report that the PERK axis contributes to ERO1 α activation in T effector cells. We find that this axis consumes metabolic energy in T cells and drives a protein profile synonymous with oxidative stress. We show that accumulation of mitochondrial ROS (mtROS) is a hallmark of ER-induced mitochondrial exhaustion, connecting ER biology to metabolic function in T cells. Memory T cells show reduced expression of mtROS and ERO1 α compared to T effectors. PERK KO, and PERK, or ERO1 α inhibitor-treated T cells exhibited superior tumor control compared to T effectors. Tumor antigen-specific PD-1⁺ CD8⁺ TILs acquire mtROS in tumors. In sarcoma patients and a sarcoma mouse model, we find that high proportions of PD-1⁺ CD8⁺ TILs express mtROS. *In vivo* treatment with a PERK inhibitor reduced mtROS in PD-1⁺ CD8⁺ TILs, increased tumor clearance, and extended animal survival in combination with anti-PD-1 therapy.

MATERIALS & METHODS

Mice

T cell-specific deletion of PERK on a C57BL/6J background was accomplished by cross of PERK^{loxP} (Eif2ak3^{tm1.2Drc/J}) mice with OT-1 (C57BL/6-Tg(Tcr α Tcr β)1100Mjb/J) or Lck-Cre (B6.Cg-Tg(Lck-icre)3779Nik/J) mice to obtain OT-1xPERK^{f/f} and LckCrexPERK^{f/w} mice, respectively. These lines were crossed to obtain OT1⁺Lckcre⁺PERK^{f/f} mice and OT1⁺Lckcre⁻PERK^{f/f} littermate controls. Lck-Cre, PERK^{loxP}, OT-1, C57BL/6J, Ly5.2 (B6.SJL-Ptprc^a Pepc^b/BoyJ), and P-mel (B6.CgThy1^a/CyTg(Tcr α Tcr β)8Rest/J) mice were obtained from the Jackson Laboratory. All animal experiments were approved by the Medical University of South Carolina (MUSC) Institutional Animal Care and Use Committee and the Division of Laboratory Animal Resources at MUSC maintained all mice.

Human Samples

This work was determined by MUSC Institutional Review Board to be exempt under protocols Pro00055941 & Pro00055960. Normal donor patients undergoing routine non-cancer-associated surgery (Pro00055941) or patients undergoing surgical removal of high grade deep pleomorphic undifferentiated sarcomas (Pro00055960) granted consent under MUSC Biorepository surgical consent forms. Tissue samples were de-identified. Studies were conducted in accordance with the Declaration of Helsinki, International Ethical Guidelines for Biomedical Research Involving Human Subjects (CIOMS), Belmont Report, or U.S. Common Rule. Blood (8 mL) was collected in EDTA coated tubes and PBMC were isolated via Histopaque-1077 centrifugation (Sigma). Sarcoma tissue was collected on ice and immediately cut into 2mm³ pieces and dissociated to a single cell suspension using Human Tumor Dissociation Kit and gentleMACS dissociator (Miltenyi Biotec) according to manufacturer's protocol.

RT-PCR and Immunoblot analysis

RNA was isolated with RNeasy Mini Kit (QIAGEN) and single-strand cDNA was made with High Capacity RNA-to-cDNA Kit (Applied Biosystems, Thermo Fisher Scientific). Taqman gene expression assays (Applied Biosystems, Thermo Fisher) were used to perform real-time PCR using the StepOnePlus Real-Time PCR system (Applied Biosystems, Thermo Fisher). Gene expression for PERK (*Eif2ak3*), ATF4 (*Atf4*), CHOP (*Ddit3*), and ERO1 α

(*Ero1*) were normalized to *Gapdh*. For immunoblots cell lysates were prepared in RIPA buffer (Sigma-Aldrich) and probed with PERK (Cell Signaling Technology), ERO1 α (Santa Cruz Biotechnology) antibodies or β -actin (Cell Signaling Technology) loading control.

Cell Sort, FACS Staining & Analysis

Fluorochrome-conjugated monoclonal antibodies and respective isotype controls listed in Supplementary Table S1 were purchased from ThermoFisher (Ebioscience). Extracellular stains were performed in PBS supplemented with 2% FBS. IFN- γ was probed after 4-6 hours of cell restimulation with Cell Stimulation Cocktail (eBioscience) and Golgi Plug Protein Transport Inhibitor (eBioscience). Foxp3/Transcription Factor Intracellular Staining Buffer Set was used according to manufacturer's protocol (eBioscience). Mitochondrial reactive oxygen species (mtROS) were measured with MitoSOX Red Mitochondrial Superoxide Indicator (3 μ M) loaded at 37°C for 30 minutes in RT PBS. Extracellular stains were added post dye incubation. For Annexin staining, MitoSOX dye loaded cells were washed and stained for using Annexin V-FITC Apoptosis Detection Kit (eBioscience) according to manufacturers' protocol. Samples were run directly on a BD Accuri C6 flow cytometer. For FACS sorting of mtROS⁺ and mtROS⁻ cell fractions, samples were stained with MitoSOX Red Mitochondrial Superoxide Indicator and CD8 and sorted on a FACS Aria IIu (BD Biosciences). Further analysis was performed post-sort for confirmation of population purity. For RNA *ex vivo* gene analysis, lymphocytes were isolated via Histopaque gradient (Sigma) and CD8⁺ T cell Isolation Kit (Miltenyi) was used to obtain >90% purity from spleens and tumors.

Cell lines

MCA-205-OVA (Gift of William Redmond) and MC38 (Kerafast Inc.) cell lines were maintained in DMEM 10% FBS. B16F1-OVA and B16F10 (Gifts of Mark Rubinstein) were maintained in RPMI complete T cell media. Cell lines were last determined to be mycoplasma free in March 2017 (MCA-205-OVA), January 2018 (MC38), 2012 (B16F1-OVA, B16F10). Cell lines were not authenticated in the past year. All growth media were supplemented with Plasmocin mycoplasma prophylactic (Invivogen). All cell lines were passaged three times prior to *in vivo* tumor inoculation, and a fresh vial of cells was prepared for individual experiments.

T cell activation and treatment

Spleens from OT-1 mice were dissociated and RBC lysis was performed using ACK Lysing (Thermo-Fisher Scientific) to achieve a single cell suspension. T cells were activated and expanded for indicated time points from total splenocytes incubated with 1 μ g/mL OVA 257-264 (Invivogen) or hgp100 25-33 (GenScript) peptide in complete T cell media (200U rhIL-2, NCI). Cells were washed and media was changed after 3 days of initial activation and expansion. For inhibitor treatments, PERK (100nM, GSK2606414, Tocris) or ERO1 α inhibitors (10 μ M, EN460, Cayman Chemical) were incubated with splenocytes for 10 min prior to introduction of peptide and re-introduced at the d3 cell split. For central memory T cell development, IL-2 was replaced in T cell media with rhIL-15 (50ng/mL, Shenandoah) at the day 3 cell split.

Metabolic & Proteomic Analysis

Oxygen consumption rate (OCR) was measured in non-buffered RS media supplemented with HEPES under basal conditions and in response to 1 μ M oligomycin, 1.5 μ M FCCP, and 2 μ M rotenone + 1 μ M Antimycin A using the XFe96 Extracellular Flux Analyzer (Seahorse Bioscience). Cell-Tak (Corning) was used for T-cell adherence.

Liquid Chromatography-Tandem Mass Spectrometry—Proteomics were performed by Bioproximity, LLC. Samples were prepared for digestion using the suspension-trapping (S-trap, Protifi) method. Digested peptides were collected by centrifugation. Peptides were eluted with 80% acetonitrile, 5% ammonium hydroxide and lyophilized in a SpeedVac (Thermo Savant) to remove volatile components. Digestion mixtures were analyzed by UHPLC-MS/MS. LC was performed on an Easy-nLC 1000 UHPLC system (Thermo) interfaced to a quadrupole-Orbitrap mass spectrometer (Q-Exactive HF-X, Thermo Fisher) via nano-electrospray ionization using a source with an integrated column heater (Thermo Easy Spray source).

Data Processing and Library Searching—Tandem mass spectra were searched using X!Tandem and Open Mass Spectrometry Search Algorithm (OMSSA), requiring expectation value scores of 0.01 or better to be considered a match. Protein intensity values were calculated using OpenMS to measure the area under the curve of identified peptides. Searches were performed on Amazon Web Services-based cluster compute instances using the Proteome Cluster interface which builds species- and genus-specific protein sequence libraries monthly from current UniProtKB distributions. Gene annotations were obtained from Ensembl Release 93 database, Mouse genes (v93GRCm38.p6) data set through the BioMart website. Official Gene Ontology categories to annotate mouse proteins based on corresponding gene symbols were used.

Tumor mouse models and *In vivo* treatments

For transfer of 7-day expanded OT-1⁺ or PERK KO T cells, C57BL/6 mice were implanted subcutaneously (s.c.) with 2.5 $\times 10^5$ B16F1-OVA tumor cells and 5 $\times 10^5$ T cells were infused via tail vein to 5Gy irradiated mice after 7 days of tumor growth. For transfer of 7-day expanded Pmel or Pmel inhibitor-treated T cells, C57BL/6 mice were implanted subcutaneously (s.c.) with 2.5 $\times 10^5$ B16F10 tumor cells and 2 $\times 10^6$ T cells were infused via tail vein to 5Gy irradiated mice after 7 days of tumor growth. Tumor growth was measured every other day for 3 weeks. For tracking acquisition of mtROS/PD-1⁺ status in tumor antigen-specific CD8⁺ TILs, 2.5 $\times 10^5$ MCA-205-OVA were implanted s.c. to Ly5.2 mice. Naïve OT-1⁺ T cells were obtained via Mouse CD8⁺ T Cell Isolation Kit (Miltenyi Biotec), and 1 $\times 10^6$ CD8⁺ T cells were transferred via tail vein on day 7 of tumor growth. After 7 days of *in vivo* expansion, tumor draining lymph nodes (TDLNs) and tumors were harvested. Tumors were processed to single cell suspension using Mouse Tumor Dissociation Kit (Miltenyi Biotec) according to manufacturer's protocol. For combination therapy experiments, 2.5 $\times 10^5$ MCA-205-OVA were injected s.c. to C57BL/6 mice and tumors were established for 7 days. 50mg/kg PERK Inhibitor (GSK2606414, GlaxoSmithKline) or vehicle was administered twice daily via oral gavage as a suspension of 0.5% hydroxypropylmethyl cellulose + 0.1% Tween-80 in water. 200 μ g Anti-PD-1

(RMP1-14) or RatIgG2a isotype control (2A3) (Bio X Cell) was administered every 4 days after 12 days of initial tumor growth. For CD8 α cell depletion, 100 μ g Anti-CD8 α (53-6.7, Bio X Cell) was administered three times per week after 5 days of tumor growth.

RESULTS

PERK contributes to activation of chronic ER stress in T effector cells

Previously we found that ER activation contributed to mitochondrial stimulation in CD4 $^{+}$ T cells through inositol 1,4,5-triphosphate receptor (IP(3)R)-mediated Ca $^{2+}$ signaling. Inhibition of IP(3)R shifted T cell fate and promoted cell persistence in tumors (18). Continuous disruptions in Ca $^{2+}$ homeostasis impair proper protein folding and a high burden of misfolded proteins induces chronic stress on the ER. In the face of irresolvable stress, PERK integrates signals from Ca $^{2+}$ binding protein BiP/grp78 to initiate the terminal UPR through activation of transcription factors ATF4 and CHOP. A key downstream target of ATF4/CHOP is ERO1 α . As a facilitator of Redox reactions, excessive ERO1 α activity can induce oxidative stress and impair cell survival as cells lose capability to scavenge ROS through anti-oxidant systems (17)(19,20). We asked how the ER stress sensor PERK and the terminal UPR were regulated in differentiating T effector cells. We found that gene expression of PERK (*Eif2ak3*), ATF4 (*Atf4*), CHOP (*Ddit3*), and ERO1 α (*Ero1l*) were significantly increased over the course of T cell activation and differentiation (Fig. 1A-D). PERK protein content increased in T cells and peaked after 5 days of expansion. ERO1 α protein increased abundance as T cells differentiated (Fig. 1E). PERK gene expression peaked 7 days post activation, but protein expression was reduced after the 5-day time point. Our data point out a discrepancy between gene and protein regulation of PERK in T cells and indicate that further study of how PERK is post-transcriptionally regulated is needed.

To measure the role of PERK in induction of the terminal UPR in T cells, we created T cell receptor (TCR) transgenic T cell-specific conditional knock out mice (OT1 $^{+}$ Lckcre $^{+}$ PERK $^{f/f}$, PERK KO). We measured genes associated with the chronic PERK response; *Atf4*, *Ddit3*, and *Ero1l* in naïve and 7-day expanded WT and PERK KO T cells. As expected, *Eif2ak3*, *Atf4*, *Ddit3*, and *Ero1l* were significantly reduced in PERK KO T cells (Figs. 1F-I). In line with these data, PERK and ERO1 α proteins were decreased in PERK KO T cells (Fig. 1J). A longer exposure for PERK was performed to convey absence of PERK protein. These data reveal a role for chronic ER stress as a component of differentiating T effector cells.

PERK axis consumes energy in CD8 $^{+}$ T effector cells

Increased spare respiratory capacity (SRC) is a property associated with superior anti-tumor function of T cells (21). The molecular processes that consume T cell metabolic energy are not well understood. Redox reactions for protein folding and disulfide bond formation in the ER are energy consumptive processes that integrate ER activation with mitochondrial bioenergetics (17,19,22). We measured oxygen consumption rates (OCR) in WT and PERK KO T cells and found that PERK KO T cells maintained superior SRC compared to WT cells. To assess a potential contribution of ERO1 α to this phenomenon, we treated T cells with a target-specific PERK (PERK I) (23) or ERO1 α inhibitor (ERO1 I) (24) across the

course of differentiation and expansion. We found that PERK I, or ERO1 I-treated T cells preserved metabolic energy compared to WT controls (Fig. 2A-C). Loss of SRC is associated with a reduction in T cell effector function (25). We measured capacity to secrete IFN- γ in the abovementioned T cell groups, and found that PERK KO, PERK I, or ERO1 I T cells had increased IFN- γ secretion compared to WT controls (Fig. 2D). Inhibitor treatments did not significantly reduce cell numbers and experimental T cell groups exhibited increased viability compared to respective WT controls (Supplementary Fig. S1A-B).

Activation of chronic PERK UPR mediated by ATF4/CHOP-ERO1 α signaling has previously been implicated to drive protein synthesis, consume cellular energy, and contribute to death through oxidative cell stress (16,17,19). To gain insight into how PERK and downstream target ERO1 α shape T effector cells, we assayed the proteomes of T effector, PERK KO, and ERO1 α I-treated T cells. Shotgun liquid chromatography-tandem mass spectrometry (LC-MS/MS) was used to assess the top 100 proteins with greatest intensity expression in T effectors compared to PERK KO or ERO1 α I-treated T cells (Supplementary Table S2). User-defined categories were used to qualify proteins into biologically functional groups. Proteomics revealed protein groups associated with energy and metabolism, redox reactions and oxidative stress/ DNA damage with increased expression in T effectors compared to PERK KO and ERO1 α I-treated T cell groups (Fig. 2E-F). Data are represented as fold increased expression values in T effectors compared to PERK KO or ERO1 α I-treated T cell groups. Of user-defined categories the greatest overlap in PERK KO and ERO1 α -treated T cells was in the “ER transport/ cell stress” group (n=6 proteins). 3/7 of redox-associated proteins overlapped as highly expressed in T effectors compared to both PERK KO and ERO1 α I-treated T cells, highlighting lactate dehydrogenases, Thioredoxins, and Glutathione-S-transferases as protein families of potential relevance. Further analysis to validate these protein pathways to elucidate the role of Redox and oxidative stress in T cells is warranted.

Mitochondrial ROS are a hallmark of mitochondrial exhaustion in T cells

Persistent ERO1 α activation is bioenergetically costly to cells and OXPHOS may be stimulated in efforts to replenish depleted ATP stores (19). We wished to establish a biomarker of energy loss and aging in T cells that may be associated with chronic stress on the ER consistent with pathogenic ERO1 α activation. We reasoned that persistent mitochondrial activation through continued OXPHOS would result in accumulation of mitochondrial reactive oxygen species (mtROS) in T cells. The live cell dye MitoSOX Red detects the superoxide anion, a precursor to the majority of cellular ROS, in mitochondrial membranes and is detectable by flow cytometry (26). Under normal physiological conditions, detoxification of superoxide occurs through superoxide dismutase-mediated conversion to hydrogen peroxide (27). However, excess ROS is associated with disease and aging (28). We measured mtROS in OT-1⁺ T cells over the course of T effector cell differentiation. As naïve T cells lost CD62L expression, a measure of stemness, mtROS accumulated in T cell mitochondria (Fig. 3A). Human CD8⁺ T cells underwent the same pattern over the course of 3 weeks of *in vitro* expansion (Fig. 3B).

To determine whether mtROS accumulation was a hallmark of energy loss in T cells, we FACS sorted day 3 and day 5 mtROS⁻ and mtROS⁺ CD8⁺ T cell subsets. The lowest 25% mtROS⁻ and highest 25% mtROS⁺ cells were obtained (Fig. 3C) and SRC among groups was measured. Accumulation of mtROS indicated energy loss in T cells (Fig. 3D). We next measured the capacity for IFN- γ secretion among day 5 and 7 expanded mtROS⁻ and mtROS⁺ T cell groups. We found that IFN- γ secretion underwent a similar pattern to SRC among sorted T cell populations (Fig. 3E). In day 7 cells, we asked whether mtROS⁺ may indicate early stage apoptosis. We found that ~8% of CD8⁺ mtROS^{high} T cells harvested on day 7 were in early stage apoptosis (Fig. 3F). This was only detectable in day 7 samples, not in cells harvested at earlier time points. Our data show that mtROS can be used as a hallmark of metabolic energy loss and aging in T cells.

PERK axis contributes to mitochondrial exhaustion of T effector cells

Through ERO1 α activation, oxidative protein folding in the stressed ER has been linked to generation of cellular ROS and inhibition of cell survival (19). However, the specific effects of the PERK axis on mitochondrial activation have not been assessed. Given the maintenance of SRC and diminished oxidative stress-associated proteomic signature in PERK KO and ERO1 α I T cells (Fig. 2), we reasoned that these T cell groups may show reduced mitochondrial activation and low mtROS accumulation. We found that mtROS was significantly diminished in PERK I, ERO1 I, and PERK KO T cells compared to T effector controls (Fig. 4A). Indeed, in the abovementioned T cell groups, maintained expression of CD62L was a mark of reduced cell aging (Fig. 4B).

Reacquisition (29) or continued expression of CD62L has been associated with memory T cell formation (25,30). We developed memory T cells *in vitro* with IL-15 cytokine conditioning and found that memory T cells expressed reduced mtROS compared to T effector controls (Fig. 4C). These data are consistent with maintenance SRC of memory cells (25). We asked if memory T cells experience reduced activation of the chronic PERK axis. We found that *Atf4*, *Ddit3*, and *ERO11* genes were reduced in memory T cells compared to T effectors (Fig. 4D). As expected, ERO1 α protein expression was increased in T effectors, and ERO1 α protein was not highly expressed in memory T cells. Gene expression of *Eif2ak3* was not significantly different between T effector and T memory cells, but protein expression of PERK was reduced (Fig. 4D-E). These data, similar to data obtained in Fig. 1, may suggest post-transcriptional regulation of PERK in T cells. Together, the data indicate that ER programs may impact T cell lineage fate.

PERK and ERO1 α hinder T cell-mediated tumor control

Memory T cells promote durable anti-tumor responses (30). We used adoptive T cell therapy mouse models to measure how PERK and ERO1 α impact T cell-specific tumor control. Mice bearing B16F1-OVA-expressing tumors were left untreated or treated with OT-1 (T eff) or PERK KO T cells and tumor control was measured every other day for 3 weeks. OT-1-PERK KO T cells exhibited significantly greater tumor control compared to T effectors (Fig. 5A). Survival was significantly extended in tumor-bearing mice treated with PERK KO T cells (Fig. 5B). Although PERK can be acutely protective, the PERK axis can also impair T cell-mediated anti-tumor immunity. To further test how the PERK axis contributes to T cell-

specific tumor control, P-mel T cells-conditioned with PERK I or ERO1 α I were transferred to mice bearing pathogenic B16F10 melanomas and tumor growth was monitored for 3 weeks. Both inhibitor treatments of T cells yielded significant increases in tumor control compared to T effectors (Fig. 5C-D).

Tumor antigen-specific PD-1⁺ CD8⁺ TILs exhibit mitochondrial exhaustion

We next asked how the terminal PERK UPR is expressed among endogenous CD8⁺ TILs. CD8⁺ cells were sorted from spleens and tumors of mice bearing immunogenic MCA-OVA sarcomas and gene expression was measured between splenocytes and TILs. The chronic PERK signaling axis was significantly upregulated in CD8⁺ TILs compared to splenic-matched controls (Fig. 6A). We next asked whether mtROS accumulated in CD8⁺ TILs cells in mice bearing immunogenic tumors. In multiple tumor models, CD8⁺ TILs accrued mtROS compared to T cells in tumor draining lymph nodes (TDLNs). To expand this finding, we asked whether mtROS was a property of exhausted T cells in tumors. We measured co-expression of mtROS and PD-1 in CD8⁺ T cells from TDLNs and tumors of mice bearing MCA-205-OVA sarcomas or MC-38 colon carcinomas. In both mouse models mtROS⁺ cells were localized to the PD-1⁺ compartment and mtROS expression was significantly increased in TILs compared to TDLNs (Fig. 6B-C). In agreement with gene expression data, splenocytes from MCA-205-OVA tumor-bearing mice also expressed reduced mtROS/PD-1⁺ CD8⁺ T cells compared to TILs (Supplementary Fig. S2A).

PD-1⁺ TILs comprise a pool of T cells enriched for tumor antigen specificity (31) and tumor-specific T cells home to and proliferate in tumors within 7 days of adoptive transfer (32). We reasoned that metabolically exhausted TILs would be in the tumor antigen-specific T cell compartment. To address this hypothesis, we transferred naïve OT-1⁺ T cells to mice bearing 7 day-established MCA-205-OVA sarcomas and measured mtROS/PD-1⁺ co-expression among endogenous (CD45.1) and transferred (CD45.2) T cells recovered from tumors after 7 days of expansion (Fig. 6D). ~70% of transferred CD45.2 CD8⁺ TILs co-expressed mtROS/PD-1 compared to ~20-30% in the endogenous polyclonal pool of TILs, and this expression could be visualized in TDLNs (Fig. 6E). Our data agree with reports that PD-1⁺ T cells experience mitochondrial depolarization (33), and shed light on the molecular events within T cells that contribute to mitochondrial exhaustion.

PERK inhibition improves anti-PD-1 therapy

We wished to determine whether mtROS⁺ CD8⁺ TILs could be identified in patients. Bone and soft tissue sarcomas are immunogenic human tumor types, but have shown poor responsiveness to anti-PD-1 and combination checkpoint blockade therapies (34). We obtained tumor tissue from patients undergoing routine surgical removal of high grade deep pleomorphic undifferentiated (HGD PU) sarcomas. These patients had not received radiation and had not undergone recent chemotherapy. Dissociation and analysis of CD8⁺ TILs from fresh HGD PU sarcomas showed a CD8⁺ T cell infiltrate in all tumors and FACS analysis of PBMC versus autologous TILs showed a significant increase in mtROS/PD-1⁺ CD8⁺ T cells in tumors (Fig. 7A). Our data show that PERK inhibition overcomes mitochondrial exhaustion in T cells *in vitro* (Fig. 4). MCA-205-OVA tumor-bearing mice were treated with PERK I for 7 days during the time period when T cells become functionally exhausted. Day

14 harvest of TILs showed that PERK I-treated PD-1⁺ CD8⁺ TILs expressed significantly reduced mtROS compared to vehicle treated controls (Fig. 7B). In line with these data, PERK I treated mice exhibited increased absolute numbers of CD8⁺ T cells in tumors (Fig. 7C). mtROS⁺ TILs comprise the pool of highly activated TILs measured by CD44 (Supplementary Fig. S2B). A reduction in mtROS⁺ TILs was concordant with reduced CD44⁺ expression among PERK I-treated TILs in both MCA-205-OVA and MC-38 tumor models (Supplementary Fig. S2C). These data suggest that PERK I effects on CD8⁺ TILs were not due to increased activation associated with reduced formation of immunosuppressive TIL populations or reduced tumor growth. *In vivo* PERK I treatment augmented anti-PD-1 therapy as 5/7 combination therapy treated mice achieved complete response compared to 2/7 mice treated with anti-PD-1 therapy alone. Depletions of CD8⁺ cells over the course of therapy demonstrated that CD8⁺ cells were necessary for combination therapy effect (Fig. 7D-E). Combination therapy-treated mice exhibited 100% survival compared to ~28% survival in the anti-PD-1 therapy condition (Fig. 7F). Together, our data indicate that targeting ER stress in combination with traditional immunotherapy may improve responses in patients.

DISCUSSION

Works by several groups have brought the ER to the forefront of programming pro- and anti-tumor immunity (35). ROS in the tumor microenvironment drive MDSC cell death through an ER stress-mediated mechanism. The shortened lifespan of MDSCs promotes their continued expansion in bone marrow (36). In multiple human tumor types, MDSCs in peripheral blood directly correlate with low overall survival rates in patients (37). Similarly, the tumor microenvironment appears to impair tumor-associated dendritic cell (tDC) antigen-presentation, and antigen presentation is enhanced by impairing the IRE1 α -stress pathway in tDCs (38). Lastly, the role of ER stress, and in particular PERK, in augmenting tumor cell development is well defined (39). Together, these data indicate that targeting ER stress pathways in cancer patients may be a formidable strategy to promote anti-tumor immune function and impair immune suppression and tumor cell growth.

Few reports have focused on the role of ER stress in control of T cell anti-tumor immunity, and little attention has been paid to the role of the ER in defining T cell biology. Our previous work found that inhibition of ER activation improved T cell mitochondrial function and aided anti-tumor immunity (18). Our proteomic screen yielded increased expression of multiple proteins associated with ER-mitochondrial crosstalk enriched in T effectors compared to PERK KO T cells. A second report found that inhibition of IRE1 α stress signaling led to memory T cell development in response to acute infection (40). These data agree with our data presented here that uncover a role for ER stress signaling a response that affects T cell differentiation and effector cell maturation.

Modulation of ER stress elements alone was able to impact mitochondrial bioenergetics and enhance T cell-specific tumor control. Mechanistically, acute PERK-specific ER stress is protective to cells through momentary attenuation of protein synthesis mediated by phosphorylation of eukaryotic translation initiation factor 2A (EIF2 α) (13). The chronic PERK axis drives protein synthesis, energy consumption, oxidative cell stress and death

through activation of transcription factors ATF4 and CHOP (16,17). Though PERK gene and protein expression were reduced in our PERK KO T cells, we do not discount the possibility of remnant PERK expression that may have conferred the protective effect of the acute PERK response combined with reduced lethal effects from the terminal UPR. Based on our findings, T cells present a paradigm in which to study the effect of biologically relevant chronic ER stress on cell development and death. Our data show that activation of ERO1 α in rapidly dividing T effectors/TILs governs a cell-intrinsic mechanism that drives energy loss and oxidative stress. How the PERK-mediated transcription factors control expression of ERO1 α in T cells remains unknown. This question needs to be addressed due to potential implications for cancer immunotherapy (41,42).

Based on the durability of anti-tumor immunity associated with memory T cells *in vivo* (4,21,25,43), we note that modulation of the PERK pathway could promote *in vivo* T cell durability and contribute to long-term efficacy of anti-PD-1 therapy. Multiple potential benefits of targeting chronic ER stress have been identified in modulation of pro and anti-tumor immunity (35). Development of novel ER stress axis inhibitors that may circumvent previous toxicities associated with inhibitors is on the horizon (44-48). Future research that defines the role of ER stress in T cell biology is needed. Work that measures how tumor microenvironments drive cell stress signaling that impacts T cell anti-tumor function is essential.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. PERK contributes to chronic ER stress in C8⁺ T effector cells.

Naïve WT OT-1⁺ CD8⁺ T cells were activated and expanded with cognate peptide and harvested at indicated time points. (A-D) PERK (*Eif2ak3*), ATF4 (*Atf4*), CHOP (*Ddit3*), and ERO1α (*Ero1l*) gene expression were measured by qPCR and (E) PERK and ERO1α proteins measured by immunoblot (5μg, 2min). Data from 4 biological replicates are quantified and represented as SEM; results from student's *t* test performed for naïve versus day 7 T cells are displayed. Experiments were repeated with four different WT animals and immunoblot is representative. Naïve PERK KO (OT-1xLck-Cre⁺xPERK^{f/f}) and littermate controls (OT-1xLck-Cre⁻xPERK^{f/f}) or 7-day expanded T cells were harvested. (F-I) PERK (*Eif2ak3*), ATF4 (*Atf4*), CHOP (*Ddit3*), and ERO1α (*Ero1l*) gene expression measured by qPCR and (J) immunoblot (5μg, 1min) for PERK and ERO1α proteins. PERK 8min exposure is shown to convey lack of protein expression. Data from three WT and littermate pairs are quantified and represented as SEM, student's *t* test. Experiments repeated twice and immunoblot data are representative of four independent experiments. **p*<0.05, ***p*<0.01, ****p*<0.001.

Figure 2. PERK axis impacts CD8⁺ T effector profiles.

Representative oxygen consumption rate (OCR) trace and quantification of spare respiratory capacity (SRC) from day 7 (A) PERK KO or littermate controls or (B) WT and PERK I or (C) WT and ERO1 I-treated OT-1⁺ CD8⁺ T cells measured via Seahorse Bioanalysis. Spare respiratory capacity (SRC) calculated as the difference between initial OCR rate and the maximal OCR rates achieved after FCCP uncoupling. Data are quantified and represented as SEM, students *t* test performed for each condition versus control T cells. Experiments were repeated at least 3 times. (D) IFN- γ production from day 7 WT, PERK I, or ERO1 I-treated OT-1⁺ T cells and WT (littermate) and PERK KO CD8⁺ T cells. Data from four biological replicates are quantified and represented as SEM, students *t* test performed for each condition versus WT cells. Individual experiments were repeated 3 times. * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$. Gene symbol and expression intensity of proteins identified by LC-MS/MS-based shotgun proteomics extracted from the top 100 proteins with greatest enrichment in WT OT-1⁺ (T eff) compared to (E) PERK KO or (F) ERO1 α I-treated T cells. Biologically functional groups of energy & metabolism, ER transport/ cell stress, oxidative stress/ DNA damage and Redox are shown. Heat maps represent fold increased intensity of proteins from average value of three replicates in each T cell group. *Acly* (+212.98) in Teff versus PERK KO T cells is represented numerically.

Figure 3. Mitochondrial reactive oxygen species signify mitochondrial exhaustion.

Representative FACS plots of (A) Naïve WT OT-1⁺ CD8⁺ T cells activated and expanded with cognate peptide or (B) human PBMC expanded in high dose IL-2 (3000U/mL) and CD8⁺ T cells FACS stained at indicated time points. FACS gates are set from fluorescence minus one controls. Data points represent quantification of five individual mice or human samples and are represented as SEM, students *t* test performed for each time point versus T₀ control. Experiments repeated twice. (C) Representative FACS plot with gating from FACS sorts of mtROS/ CD8⁺ T cells. Lowest 25% - and highest 25% + mtROS populations were collected and (D) Representative oxygen consumption rate (OCR) trace and quantification of spare respiratory capacity (SRC) from sorted populations at indicated time points measured via Seahorse Bioanalysis. Spare respiratory capacity (SRC) calculated as the difference between initial OCR rate and the maximal OCR rates achieved after FCCP uncoupling. Data are quantified and represented as SEM, students *t* test performed for each condition versus control T cells. (E) IFN- γ production from sorted subsets at indicated time points. Post-sort purity was >97%. (F) Representative FACS plot and quantification of mtROS-Annexin co-staining on day 7 WT OT-1⁺ T cells. FACS gates are set from fluorescence minus one controls. Data are quantified and represented as SEM, students *t* test. Individual experiments repeated three times. * *p*<0.05, *****p*<0.0001.

Figure 4. PERK axis drives mitochondrial exhaustion and is impaired in memory T cells.

Representative FACS plots and quantification of day 7 WT, PERK I, ERO1 I-treated and WT (littermate) and PERK KO OT-1⁺ CD8⁺ T cells probed for (A) mtROS or (B) CD62L expression. Bar graphs from 4 biological replicates are quantified and represented as SEM, Students *t* test performed for each condition versus control. FACS gates are set from fluorescence minus one controls. Experiments were repeated at least three times. T effector (Teff) or memory (Tmem) cells were developed and harvested. (C) Representative FACS plot and quantification of mtROS expression (D) quantification of PERK (*Eif2ak3*), ATF4 (*Atf4*), CHOP (*Ddit3*), and ERO1α (*Ero1I*) gene and (E) immunoblot for PERK and ERO1α proteins. FACS quantification from four biological replicates. Experiment repeated 3 times. Gene expression bar graphs represent average of 3 separate experiments and are shown as SEM, Teff values are expressed relative to respective Tmem values set to 1. Immunoblot is representative data from 3 experiments. ***p*<0.01, ****p*<0.001, *****p*<0.0001.

Figure 5. Inhibition of PERK axis augments T cell-specific tumor control

(A) Individual graphs of mice bearing 7-day B16F1-OVA tumors left untreated (n=5) or treated intravenously with 5 × 10⁵ 7-day expanded OT-1⁺ (Teff) (n=8) or PERK KO (n=7) T cells. Tumor size recorded every other day for 3 weeks. Lines represent individual mice. (B) Survival to 45 days or tumor size of 400mm² was recorded, Log-rank test, ***p* < 0.01 survival proportions of mice treated with Teff (12%) versus PERK KO T cells (86%) (C) Mice bearing 7-day B16F10 melanomas were treated intravenously with 2 × 10⁶ 7-day expanded Pmel (Teff) or Pmel T cells developed in the presence of (B) PERK inhibitor (PERK I T) or (D) ERO1α inhibitor (ERO1α I T). Tumor size recorded every other day for 3 weeks. Lines represent individual mice. Linear regression of Teff versus PERK KO or inhibitor-treated T cell groups, n=5-6 mice per group, *****p* < 0.0001. Experiments repeated twice.

Figure 6. Tumor antigen-specific PD-1⁺ CD8⁺ TILs experience mitochondrial exhaustion.

CD8⁺ cells were sorted from spleens and tumors of mice bearing 14-day MCA-205-OVA tumors and qPCR was performed to quantify (A) PERK (*Eif2ak3*), ATF4 (*Atf4*), CHOP (*Ddit3*), and ERO1α (*Ero1l*) gene expression. Bar graphs represent averages of 4 mice per group and are shown as SEM, experiment repeated twice. Representative FACS plots and quantification of mtROS/PD-1⁺ CD8⁺ populations in tumor draining lymph nodes (TDLNs) and tumors (tumor infiltrating lymphocytes (TILs)) harvested from mice bearing 14-day (B) MCA-205-OVA sarcomas or (C) MC38 colon carcinomas. Populations represent gating from CD8⁺/CD45⁺ lymphocytes and quadrants are set from PD-1 isotype control expression. Bar graphs represent 4-5 mice per group and are shown as SEM. Individual experiments were repeated 3 times. (D) 1×10⁶ naïve CD45.2 OT-1⁺ T cells were transferred via tail vein to CD45.1 C57BL/6 mice bearing 7 day established s.c. MCA-205-OVA sarcomas. Tumors were harvested 7 days post transfer. (E) Representative FACS plot overlay and quantification of mtROS/PD-1 co-staining from CD45.1 (gray) or CD45.2 (black) CD8⁺ cells in TDLNs and tumors. Gates are set from isotype control data. Bar graphs represent 4 mice per group and are shown as SEM. Individual experiments repeated twice. Students *t* test, ***p*<0.01, *****p*<0.0001.

Figure 7. PERK inhibition reduces CD8⁺ TIL mtROS and augments anti-PD-1 therapy.

(A) Representative FACS plot and quantification of mtROS/PD-1⁺ CD8⁺ T cells from PBMC and tumor of three patients with pleomorphic undifferentiated high grade deep (PU HGD) sarcoma. Gates are set from isotype controls. PERK inhibitor (PERK I) or vehicle control was administered for 1 week (days 7-14) to mice bearing MCA-205-OVA sarcomas. (B) Representative FACS plots and quantification of mtROS/PD-1 TILs gated from CD45⁺/CD8⁺ populations. Gates are set from isotype control data. (C) Absolute number of CD45⁺/PI⁻/CD8⁺ TILs calculated per gram of tumor weight. Bar graphs represent 4-5 mice per group and are shown as SEM. Individual experiments repeated twice. Students *t* test, **p*<0.05, ****p*<0.001. PERK I or vehicle control was administered beginning after 7 days of tumor growth to mice bearing MCA-205-OVA sarcomas and anti-PD-1 or isotype antibody was administered on day 12 and every four days thereafter. Anti-CD8 was administered every 2-3 days beginning 5 days after tumor inoculation. (D) Composite and (E) individual graphs of tumor growth measured every other day for 40 days with complete response (CR) listed per group, composite data represented as SEM. Linear regression of combination measured against anti-PD-1 therapy, *****p*<0.0001. (F) Survival to 41 days or tumor size of 200mm² was recorded, Log-rank test, ***p*<0.01 survival proportions of anti-PD-1 therapy (28%) versus combination therapy (100%). Combination experiment repeated twice, anti-CD8 depletion condition performed once.