Thioredoxin-1 improves the immuno-metabolic phenotype of anti-tumor T cells

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ABSTRACT
Adoptive transfer of tumor epitope reactive T cells has emerged as a promising strategy to control tumor growth. However, chronically stimulated T cells expanded for adoptive cell transfer (ACT) are susceptible to cells death in an oxidative tumor microenvironment. Since oxidation of cell surface thiols (c-SH) also alters protein functionality, we hypothesized that increasing the levels of thioredoxin (Trx), an anti-oxidant molecule facilitating reduction of proteins through cysteine thiol-disulfide exchange, in T cells will promote their sustained anti-tumor function. Using pre-melanosome protein (Pmel)-Trx1 transgenic mouse-derived splenic T cells, flow cytometry, and gene expression analysis, here we observed that higher Trx expression inversely correlated with ROS, and susceptibility to TCR restimulation or oxidation mediated cell death. These Trx1 overexpressing T cells exhibited a cluster of differentiation 62L^hi (CD62L^hi) central memory-like (Tcm) phenotype with reduced glucose uptake (2-NBDG^lo) and decreased effector function (IFN\(\gamma\)\(^lo\)). Further, culturing tumor reactive T cells in the presence of recombinant Trx increased the dependence of T cells on mitochondrial metabolism and improved tumor control. We conclude that strategies for increasing the anti-oxidant capacity of anti-tumor T cells modulate their immune-metabolic phenotype leading to improved immunotherapeutic control of established tumors.

INTRODUCTION
Adoptive T cell therapy (ACT) is a promising approach for treating patients with advanced malignancies (1). However, quantitative or qualitative decrease of transferred anti-tumor T cells in tumor bearing host results in tumor recurrence, leaving a substantial room for improvement. Successful approaches to program T cells towards a central memory (Tcm) or stem cell memory (Tscm) phenotype or by blocking the mTOR, the Akt, or the glycolytic pathways has led to increased persistence and exhibit better tumor control (2). Therefore, understanding mechanisms
that are central to Tcm or Tscm phenotype has potential to improve the effectiveness of ACT.

It has been widely recognized that Reduction-Oxidation (redox) responses occurring at the intra- and extra-cellular levels are important in regulating several biological processes (3). Recent studies have also shown importance of redox-mediated regulation of immune response (4,5). We have previously observed that reactive oxygen species (ROS) contribute to T cell re-stimulation induced cell death (6), and T cells with higher cell surface thiol (c-SH) expression exhibit improved immunotherapeutic control of established melanomas (5). Therefore, we hypothesized that c-SH\textsuperscript{hi} is a biomarker for T cells with increased persistence and mechanisms that results in c-SH\textsuperscript{hi} phenotype would lead to improved anti-tumor activity in vitro and in vivo. Since thioredoxin enzyme system (thioredoxin (Trx), thioredoxin reductase, NADPH) has the capacity to reduce oxidized thiols and contribute to cellular redox balance (7), we determined in Trx-1 over expression alters T cell phenotype and function. We show in here that T cells overexpressing thiol regulating molecule thioredoxin (Trx) are not only c-SH\textsuperscript{hi}, but also provide unique immune-metabolic phenotype akin to the memory T cells. This leads to improved persistence of anti-tumor T cells in an oxidative tumor microenvironment. We believe that this strategy to generate robust “anti-tumor T cells” by targeting redox status of a T cells will be of high translational significance in ACT.

RESULTS

Trx transgenic T cells exhibit increased thiols, reduced susceptibility to tumor microenvironment (TME) mediated oxidative stress, and Tcm/Tscm phenotype. Trx is a class of 12 kDa ubiquitous redox proteins found primarily in the cytosol. Trx possess a catalytically active di-thiol function in a Cys-Gly-Pro-Cys motif and are present in all organisms. Biomolecules with redox-active sulfhydryl (-SH) functions are necessary for the
maintenance of mildly reductive cellular environments to counteract oxidative stress, and for the execution of redox reactions for metabolism and detoxification (10). We recently bred melanoma epitope gp100 reactive TCR bearing transgenic mouse Pmel with Thioredoxin1 (Trx1)-transgenic mouse, in which human Trx1 is systemically over expressed under control of the β-actin promoter (11), to obtain Pmel-Trx mouse. **Fig. S1A** shows successful generation of the Pmel-Trx mice. The gel picture shows the characterization of the Pmel-Trx mice. While Pmel mice showed gp100 TCRα (600 bp) and TCRβ (500 bp) in *lane 1*, Trx expression was undetectable in *lane 2*. Similarly, the Trx-Tg mouse does not show any Pmel TCRαβ expression (*lane 3*), but is positive for Trx (*lane 4*). Further, the Pmel-Trx mouse shows expression of Pmel TCRαβ (*lane 5*), and Trx (*lane 6*). A comparison of thymus showed a reduction in the CD8 single positive (SP), increase in CD4+ SP and CD4+CD8+ double positive cells (**Fig. S1B**). A FACS based comparison also showed increased Trx expression in class I epitope gp100 reactive Pmel-Trx CD8+ T cells, than that observed in Pmel CD8+ T cells (**Fig. 1A**). The high expression of Trx also correlated with increased expression of cell surface thiols (c-SH) in splenic Pmel-Trx T cells as compared to T cells from Pmel mice (**Fig. 1B**). The c-SH staining was done using the alexa-fluor labeled maleimide dye (Invitrogen), as previously reported (5). The increased antioxidant thiol levels also inversely correlated to the ROS levels in the activated T cells as measured by DCFDA (**Fig. 1C**), and less cell death mediated by oxidant injury using H2O2 (**Fig. 1D**), or activation induced cell death induced by TCR restimulation (**Fig. 1E**). Importantly, the levels of receptor-interacting protein kinase (RIPK)-1 and RIPK-3, which are known to regulate oxidative stress and AICD leading to necroptosis (12) were also reduced in activated T cells overexpressing Trx (**Fig. 1F**, and **Fig. S1C**).

In order to confirm the functional advantage of Trx overexpression on T cells, activated congeneric Pmel or Pmel-Trx Tg T
cells were transferred *i.p.* into the C57BL/6 mice with EL4 ascites. The analysis of Vβ13*+* T cells retrieved after 24 hr. from ascites showed reduced 8-hydroxy guanine [8-OHdG], and reduced nitrotyrosine (marker for ROS/RNS stress) (13) in Pmel-Trx, as compared to Pmel cells alone (Fig. 1G). This established that increased Trx levels indeed protect the T cells in oxidative tumor microenvironment.

Since a recent study has shown that T stem cell-like memory (Tscm) cells may reside within the Tcm group (14), we evaluated if Trx overexpressing T cells from Pmel-Trx mouse have increased Tscm phenotype, which could be responsible for better tumor control. We found that Tscm fraction, which can be tracked by gating on CD8*CD62L*hiCD44loSca-1*hiCD122*hi (15), was indeed greater in Pmel-Trx T cells (Fig. 2A). The increased ‘stemness’ in Pmel-Trx T cells also correlated with significantly high expression of stem cell genes as *Lef1*, *Oct4*, *β-catenin*, and *Bcl6* (Fig. 2B). Thus, our data confirms that Trx indeed increases “stemness” phenotype in Trx overexpressing T cells.

In order to determine the trafficking ability and capacity of Trx-Tg T cells to establish memory *in vivo*, we activated both Pmel and Pmel-Trx mouse derived splenic T cells with melanoma epitope gp100 for three days and transferred them *i.v.* into Rag1*−/−* mice. After 25 days of initial transfer of T cell, the recipient mice were subcutaneously injected with murine melanoma B16-F10 cells. Then transgenic T cells were tracked after 5 days of tumor injection in different lymphoid and non-lymphoid organs. We observed that Pmel-Trx T cells exhibited higher recall response to tumor challenge as compared to Pmel control T cells, which was evident by its significantly increased expansion in each organ (Fig. 2C). Additionally, the Pmel-Trx T cells tracked from the spleen of recipient mice also showed enhanced cytokine secretion upon restimulation, as compared to the Pmel T cells (Fig. 2D). This indicates that Trx overexpression renders T cells with a functional memory phenotype.
Increased Trx expression alters T cell signaling. To determine if any differences exist in TCR signaling between the Pmel and Pmel-Trx cells we determined the phosphorylation levels of key signaling molecules AKT, JNK and STAT5. Our data in Fig. 3A show reduced phosphorylation levels of AKT, JNK, and ERK. Given the importance of STAT5 involvement in assessing a T cell response to the cytokine microenvironment that shapes its function (16), we determined the pSTAT5 in Pmel-Trx T cells. We observed that Pmel-Trx T cells have reduced upregulation of pSTAT5 as compared to the Pmel T cells (Fig. 3B). This also corresponded to the reduced ability of Pmel-Trx T cells to secrete cytokine IFN-γ (33% by Pmel-Trx vs. 59% by Pmel) (Fig. 3C), which is shown to dependent upon glucose availability (17). Using fluorescence glucose analog 2NBDG we observed that Pmel-Trx T cells exhibit lower glucose uptake as compared to the Pmel T cells alone (Fig. 3D). A real-time PCR analysis confirmed that activated Pmel-Trx T cells exhibit reduced expression of glucose transporter Glut1, and lesser expression of key glycolytic molecule hexokinase II (HKII) (Fig. 3E). However, the transcription factor associated with mitochondria (TFAM) and ND4 was upregulated in Pmel-Trx T cells. This indicated that the Trx overexpressing T cells are more dependent upon mitochondrial oxidative phosphorylation, than glycolysis for their energy demands. A seahorse based metabolic flux analysis confirmed that antigen Pmel-Trx T cells activated with cognate antigen for three days exhibit higher basal oxygen consumption rate (OCR), and the possessed enhanced spare respiratory capacity (SRC) than the Pmel T cells alone (Fig. 3F). Overall, these differences established that OCR/ECAR ratio was higher in Pmel-Trx as compared to Pmel (Fig. 3F, right panel). Mitochondrial fatty acid oxidation (FAO) has been shown to modulate OXPHOS in memory T cells with high SRC and CPT1a, a rate-limiting enzyme that regulates the entry of fatty acid from cytosol to mitochondria (18). However,
we observed that inhibition of CPT1a using etomoxir did not deplete OCR and SRC in Trx-Tg T cells. Thus, it is possible that other pathway, as glutaminolysis, is involved in shaping phenotype of Trx-Tg T cells.

**Comprehensive metabolic profiling of activated Pmel vs. Pmel-Trx shows distinct metabolites in Pmel-Trx T cells.**

Since commitment of the T cells to different metabolic pathways has been shown to result in differential fate of the T cells (19), we utilized the services of a commercial vendor Metabolon Inc. (NC) to quantify the differences in metabolites accumulated within the TCR activated Pmel vs. Pmel-Trx T cells. For this purpose 10 million activated Pmel and Pmel-Trx T cells were sorted and the pellets were frozen as per the protocol before overnight shipping, for analysis that was done using the Gas Chromatography-Mass Spectroscopy (GC-MS). The principle component analysis in **Fig. 4A** summarizes the degree of differences between metabolites in Pmel vs. Pmel-Trx T cells, whereas **Fig. 4B-D** shows the heat maps of key metabolic pathways comparing the metabolites between Pmel vs. Pmel-Trx T cells. Specifically, the Pmel-Trx T cells exhibited higher metabolites related to pentose phosphate pathway (PPP) and tricarboxylic acid (TCA) cycle. While a number of amino acids were found to be upregulated in Pmel-Trx T cells, the noticeable were the ones that have been shown to be involved in life-span extension, *i.e.* serine, proline, or histidine (20). Importantly, Trx are characterized by the presence of three conserved prolines, with one located between the catalytic cysteine residues of the -Cys-Gly-Pro-Cys-motif. Proline is also the key residue that determines the reducing power of Trx and replacing it by a serine or a threonine has a dramatic effect on the redox and stability properties of the protein (20). Thus, we believe that the thioredoxin over expression in T cells potentiates the reductive phenotype. This could be attributed to increased usage of PPP pathway (as evident by increased NADPH), and accumulation of the alpha-ketoglutarate (α-KG). A recent study showed that α-KG
blocks ATP synthase and lowers ATP levels in the cells with longer lifespan, and aids in stem cell differentiation (21).

**Increased glutamine uptake by Trx overexpressing T cells imprints unique metabolic advantage.** The observation that Trx-T cells exhibit elevated levels of metabolite α-KG, that is also replenished by anaplerotic reactions using glutamine and enters into the mitochondrial citric acid cycle, led us to hypothesize that differential glutamine levels in Trx overexpressing T cells may be responsible for their increased persistence in tumor microenvironment. It has also been shown that while glutamine helps differentiation of T cells to effector phenotype (22), the deficiency of glutamine can result in formation of Treg’s (22). To establish the role of glutamine dependence of Pmel-Trx T cells, we used the tracer studies by incubating the three-day TCR activated T cells with L-[3,4-3H (N)] glutamine (0.5 mCi) for 5 min at room temperature, and incorporation per cell was measured as counts per minute (CPM) as detailed earlier (22). Our data shows that Pmel-Trx T cells have increased glutamine uptake as compared to the Pmel T cells (Fig. 5A), and Pmel T cells when activated in presence of recombinant Trx (rTrx) also display high glutamine uptake. Since, increased levels of glutamine leading to higher α-KG accumulation can be either due to increased activity of glutamine synthetase, or increased transportation of glutamine (due to transporters), or higher degree of glutaminolysis (*i.e.* degradation of available glutamine), we determined if the contribution of each of these pathways by determining the mRNA expression of these molecules in activated Pmel, Pmel-Trx and Pmel+rTrx groups (Fig. 5B). We observed that the genes associated with glutamine uptake (*e.g.*, glutamine transporters Slc1a5, Slc3a2, Slc7a5, xCT), and its catabolism (Glud1, Gls1, Gfpt) were considerably higher in Pmel-Trx and Pmel+rTrx T cells as compared to Pmel T cells alone. While the solute carrier family members (Slc1a5, Slc3a2, Slc7a5) facilitate exchange of glutamine (and other neutral, or branched-chain and aromatic amino acids) (23),
Glud1 (glutamate dehydrogenase), is a mitochondrial matrix enzyme that catalyzes the oxidative deamination of glutamate to alpha-ketoglutarate and ammonia (24). Glud1 plays a key role in glutamine anaplerosis by producing alpha-ketoglutarate, an important intermediate in the tricarboxylic acid cycle. Similarly, Gls1 (glutaminase I) is an aminohydrolase enzyme that generates glutamate from glutamine. Further, flow-cytometry analysis confirmed the increased expression of xCT (SLC7A11), the antiporter that plays an antioxidant role by exporting glutamate for cysteine (Fig. 5C). However, we observed the lower level of free enzyme in glutamine pathway (Fig. 5C), which indicates active utilization of glutamine and elevated glutaminolysis in Trx-T cells. The enhanced expression of glutaminolysis enzymes in Pmel-Trx T cells was supported by the increased accumulation of the key metabolites glutamine, glutamate, and \( \alpha \)-KG (Fig. 5D).

Further, to determine if glutamine catabolized \( \alpha \)-KG (which supports energy production through TCA cycle anaplerosis) is responsible for memory phenotype exhibited by Pmel-Trx T cells, we used cell permeable octyl-\( \alpha \)-KG (Cayman, stock # CAS 876150) in cell culture. Our data shows that Pmel T cells activated and expanded for 72 hr. with octyl-\( \alpha \)-KG results in an increased fraction of cells co-expressing CD44\(^{lo}\)CD62L\(^{+}\), (7.43% in untreated vs. 23.9 % in treated in lower right quadrant), indicating an increase in Pmel cells with a Tscm phenotype (Fig. 5E), and exhibit decreased glucose uptake (\( \approx \)80% lower mean intensity of the fluorescence glucose uptake) (Fig. 5F). It has also been shown that many chromatin modifying enzymes including Jumonji C domain-containing histone demethylases and Tet DNA demethylases are sensitive to \( \alpha \)-KG levels (25). To further address the role of Trx in mediating epigenetic modification we used a qPCR array analysis for epigenetic modifiers (cat # PAMM-085A, Qiagen). Our data in Fig. 5G shows that the expression of Hdac11 was lower, whereas enzymes involved in modifying methylation (as
PRMT1), and chromatin structure (as Setd family) were increased in in Trx-Tg T cells. Next, a comprehensive RNA analysis revealed that Trx transgenic T cells differentially expressed 1689 genes. Table 1 and Fig. S2 shows the iPathway guide analysis (Advaita Bioinformatics) for key biological pathway and genes that were significantly different in Trx overexpressing T cells. This data indicates that Trx overexpression renders unique metabolic phenotype to T cells, which in turn could also modify epigenetic pathway molecules and boost its anti-tumor potential.

Pmel-Trx T cells secrete Trx upon antigen stimulation, and recombinant Trx reprograms Pmel T cells ex vivo: To get an insight into the mechanism that may be responsible for the phenotypic differences observed between the antigen activated gp100 epitope reactive Pmel and Pmel-Trx T cells, we determined if Pmel-Trx secreted Trx upon antigen restimulation that in-turn affected the functional outcome. We observed that as compared to Pmel T cells, Pmel-Trx T cells upon overnight activation with cognate antigen do secrete significantly high amount (≥1000 pg/ml) of Trx in the culture supernatant (Fig. 6A). In order to determine how secreted Trx would have modulated the Pmel T cells, we used recombinant Trx during antigen induced in vitro Pmel activation and compared the phenotype with untreated Pmel and Pmel-Trx T cells. We observed that T cells loose Trx with every cell division (Fig. 6B), as has been linked with cellular aging (26), and incorporating rTrx during T cell activation is non-toxic as it does not hamper the cell division (Fig. 6C), but leads to increased expression of Trx and iGSH (Fig. 6D, 6E). It has also been shown recently that Trx1-mediated reduction of Cys130 and Cys174 is essential for AMPK function, and decreased Trx levels could lead to oxidation of Cys130 and Cys174 by inducing aggregation that prevents its activation and phosphorylation by AMPK kinases (27). Given the role of AMPK in T cell memory, and in regulating T cell metabolic adaptation for effector responses in vivo (28), we next
compared AMPK levels between different T cells. Our data in shows that Pmel-Trx and rTrx treated Pmel T cells exhibit increased pAMPK compared to activated Pmel T cells (Fig. 6F), and supports that maintaining high Trx levels on T cells is essential for “metabolic fitness” of T cells. Since, crosstalk between AMPK and mTOR is known to regulate energy balance (29), we next determined if rTrx would modulate phosphorylation of S6, a downstream molecule in mTOR pathway. We observed that Trx concentration present during the activation of T cells inversely correlated to pS6 levels, as its expression decreased with increasing concentration of rTrx (Fig. 6G). The ability of rTRx to downregulate pS6 is intriguing, since another anti-oxidant intracellular glutathione has been shown to increase mTOR activity recently (30). We also observed that while Trx and L-NAC treatment reduced pS6, GSH increased pS6 (Fig. S3A), along with a concomitant increase in Trx (Fig. S3B). Importantly, qPCR analysis for ‘stemness’ genes showed up-regulation of the Lef1 and Tcf7 genes in Pmel T cells that were activated in presence of recombinant Trx (Fig. 6H).

While it has been reported that regulatory T cells exhibit higher Trx levels (31), when using rTrx in an iTreg generation protocol, we did not observe any increase in the quantity or suppressive quality of the iTreg’s generated (Fig. S3C). Additionally, compared to cognate antigen activated Pmel T cells (light blue overlay), Pmel T cells cultured with recombinant Trx for three days (orange overlay) exhibit reduced cell death as measured by Annexin V levels (Fig. 6I), and glucose uptake (similar to Pmel-Trx T cells in dark blue overlay) (Fig. 6J) upon TCR re-stimulation with cognate antigen (hgp100). Thus, this data indicates that restoring high Trx levels on immediate effector T cells could be important to render the memory phenotype with unique differentiation program (as high AMPK, low glucose uptake) (28).

Further, we determined if the strategy to activate and expand in presence of rTrx ex vivo would render tumor epitope reactive T cells with robust anti-tumor
property. Our data shows that gp100 reactive effector T cells generated in presence of rTrx do survive longer in vivo after adoptive transfer in a C57BL/6 host bearing subcutaneous B16-F10 murine melanoma and lead to much improved tumor control (Fig. 6K). Further, a tracking experiment done to compare any quantitative and qualitative differences between the adoptively transferred Pmel and Pmel+rTrx treated T cells showed that higher number of gp100 TCR Vβ13+ T cells were obtained from the tumors, draining lymph nodes, spleen and blood of the mice were ACT was performed with Pmel+rTrx T cells (Fig. 6L). Importantly, the increased numbers of Vβ13+ T cells tracked in Pmel+rTrx group also showed higher ability to secrete effector cytokine IFNγ upon restimulation (Fig. 6M, Fig. S3D), and express enhanced levels of granzyme B (Fig. 6N, Fig. S3E). The frequency of Treg’s from these sites was also not found to be any different between the two groups (Fig. S3F). This data shows that rTrx cultured T cells could keep their functional phenotype in vivo, and similar strategies could be employed in clinical scenario where TCR transduced T cells or chimeric antigen receptor (CAR) transduced patient T cells could be reprogrammed to improve their anti-tumor function.

**Human T cells engineered to express Trx exhibit enhanced anti-oxidant levels and central memory phenotype.** T cells from human patients are being used for adoptive immunotherapy approaches after engineering them with tumor reactive T cell receptors (TCR) or chimeric antigen receptors (CARs). In order to determine if our strategy to increase anti-oxidant property of T cells will render human T cells with altered phenotype (as observed in mouse studies) we generated a human melanoma epitope tyrosinase reactive TIL1383I retroviral construct with human Trx inserted to it. For this purpose we synthesized the gene construct (at Genscript) with a Trx gene flanked by Bsp119i restriction sites and then cloned it into the original Samen/1383I-34t vector. The clones were screened for correct
orientation (Fig. S4A). The retroviral supernatant was used to transduce the activated human T cells with either TIL1383I TCR or TIL1383I-TCR+Trx. Fig. S4B shows that retroviral construct with Trx could be used to generate tyrosinase epitope reactive T cells with transduction efficiency of 40% or more. Fig. S4C shows that majority of the expanded cells exhibited CD62L^CD44^CD45RA^ Tscm phenotype (14,32). Importantly, engineering Trx on human T cells also resulted in reducing the glycolytic commitment as observed by lower glucose uptake in 2NBDG assay (Fig. S4D). Further, upon overnight TCR re-stimulation with cognate antigen the TIL1383I-Trx transduced T cells showed less cell death as indicated by higher mitochondrial membrane potential (Fig. S4E), and reduced NO accumulation (Fig. S4F). A qPCR based analysis also showed that TIL1383I-Trx transduced T cells express significantly higher level of ‘stemness’ genes as compared to the TIL1383I TCR transduced T cells (Fig. S4G, p<0.005). This data establishes that the antitumor effector T cells can be programmed ex vivo for increasing anti-oxidant phenotype that could translate to better tumor control in vivo.

DISCUSSION

It has long been known that lymphocytes require a reducing milieu for optimal activation/proliferation (33). It has been shown that T lymphocytes are defective in cystine uptake and require exogenous thiols for activation and function (33). As the functional group of the amino acid cysteine, the thiol (-SH) group plays a very important role in biology (34). Recently, oxidative cysteine modifications have emerged as a central mechanism for dynamic post-translational regulation of almost all major protein classes, and correlate with many disease states (35). Certain proteins in which the redox state of cysteine residues are modified (termed ‘redox sensors’), seem to be involved in the initial and direct regulation of signaling molecules in response to ROS. Such ‘redox sensors’ commonly possess highly conserved free cysteine (Cys) residues of
which the -SH functional groups are the most important direct cellular targets or ‘sensors’ of ROS (36). A number of ‘redox sensors’ have been identified that participate in many important biological functions, some of which are crucial molecules modulating stem cell self-renewal and differentiation, including HIF-1α, FoxOs, APE1/Ref-1, Nrf2, AMPK, p38 and p53 (37). In addition, glutamate (Glu) and anti-Trx-inactivating antibodies inhibit antigen-dependent T lymphocyte proliferation (33). In T lymphocytes, intracellular GSH is critical for the proliferative response to mitogens or antigens (38). However, lymphocytes lack an efficient system of Cys2 import, whereas they easily take up free thiols (39). Therefore, to sustain lymphocyte activation and proliferation, exogenous thiols must somehow be generated in the microenvironment of an immune response. Extracellular thioredoxin (Trx) has been proposed to exert a synergistic activity on the mitogen- or cytokine-induced proliferation of lymphocytes (40). The importance of thioredoxin reductase in the last step of nucleotide biosynthesis has also been described recently, where it donates reducing equivalents to ribonucleotide reductase to overcome DNA damage response (41). We show here that increasing the Trx in tumor microenvironment by using Trx over-expressing T cells that secrete Trx or by using rTrx during ex vivo programming maintains the reducing environment and leads to long-term T cell anti-tumor function in vivo.

Our data indicates the functional differences between the CD8+ T cells obtained from the Pmel and Pmel-Trx mice, likely due to the protein thiol alterations that remain unknown at this time. Oxidation of thiol (-SH) groups is a post-translational modification that regulates numerous processes, including differentiation, cellular proliferation and apoptosis (35). We hypothesize that “number of free reduced vs. oxidized thiols present on signaling molecules could lead to differences in their functionality, and thus dictate the effector T
cells vs. memory T cell phenotype”. Our data shows that the difference observed between the Pmel and Pmel-Trx T cells is exclusively due to Trx, since addition of rTrx also leads to similar phenotype as observed when using T cells over expressing Trx. We observed the reduction in mTOR when T cells where incubated with L-NAC, and rTRx, there was an increase in pS6 in presence of GSH. While the role of GSH in upregulating mTOR has been shown recently (30), it is likely that increased c-SH due to rTRx and L-NAC resulted in lower pS6 levels that we reported earlier (5). An earlier study done using T cells from systemic lupus erythematosus patients also showed that T-cell dysfunction in patients was controlled by orally administering L-NAC that resulted in inhibition of mTOR (42). Given the role of oxidative stress signaling and use of mTOR inhibitor rapamycin and other anti-oxidant to promote anti-aging (43), one would expect that Trx treatment should reduce mTOR activation, as observed by us. The growing evidence that Trx and GSH target different cysteines in the proteome could also support the existence of non-redundant functions observed by us (44).

The commitment to different metabolic pathways could lead to differences in level of intrinsic metabolites in a cell, which could be important in regulating various signaling pathways (45). Our preliminary data quantifying the metabolite levels between TCR activated Pmel vs. Pmel-Trx T cells show distinct profile in thioredoxin over expressing T cells. The activated Pmel-Trx cells exhibit increased level of pentose phosphate pathway (PPP) metabolites that contribute to nucleotide precursors and helps regenerate the reducing agent NADPH, which can contribute to ROS scavenging. In addition, the tricarboxylic acid cycle (TCA, also known as Krebs’s cycle) metabolite alpha-ketoglutarate (α-KG) was also found to be significantly elevated in the Pmel-Trx cells. The role of α-KG, also produced by deamination of glutamate, in the detoxification of ROS has only recently begun to be appreciated (46). This keto-acid
neutralizes ROS in an NADPH-independent manner with the concomitant formation of the succinate and CO₂. In addition, α-KG has also been shown to extend the lifespan of adult *C. elegans* (21). This study showed that α-KG inhibits ATP synthase leading to reduced ATP content, decreased oxygen consumption, and is dependent on the target of rapamycin (TOR) downstream. Further, the role of metabolite α-KG has also been shown in maintaining the pluripotency of the embryonic stem cells (25). It has been shown that many chromatin modifying enzymes including Jumonji C domain-containing histone demethylases and Tet DNA demethylases are sensitive to α-KG levels (25). This study also showed that embryonic stem cells cultured in glutamine-free media had high ratio of tri- to mono-methylation on H3K9, H3K27, H3K36 and H4K20 and the addition of α-KG reversed these changes (25). Thus, high levels of the small metabolite α-KG in cells promote naive pluripotency by suppressing the accumulation of repressive histone modifications and DNA methylation.

Further, Trx overexpressing T cells also showed differences epigenetic modifiers as compared to normal T cells. The reduced expression of HDAC11 was observed in Trx-T cells, which has been recently shown enhance the T cell effector function and enhanced alloreactivity in a murine model (47). We also observed increased expression of Protein arginine N-methyltransferase (PRMT1), which is the main enzyme that mediates monomethylation and asymmetric dimethylation of histone H4 'Arg-4' (H4R3me1 and H4R3me2a, respectively), a specific tag for epigenetic transcriptional activation. Importantly, it has been shown to methylate Foxo1 and retain it in the nucleus leading to increased transcriptional activity (48). Similarly, we also observed an increase in expression of the SET domain-containing proteins that play a vital role in regulating gene expression during development through modifications in chromatin structure (49). We observed that
histone methyltransferase SETD1B, which specifically methylates 'Lys-4' of histone H3, was elevated in the Trx-Tg T cells. Similarly, Setd5 expression was also increased in Trx-T cells. Setd5-deficient embryonic stem cells have impaired cellular proliferation, increased apoptosis, and defective cell cycle progression (50). Additionally, Setd7, histone methyltransferase that specifically monomethylates 'Lys-4' of histone H3, was increased in Trx-T cells. This has also been shown to have methyltransferase activity toward non-histone proteins such as p53/TP53 and TAF10 (51). Thus, it is likely that Trx mediated differences in metabolic pathways that lead to difference in accumulation of metabolites (such as α-KG) may have led to epigenetic reprogramming of T cells resulting in sustained tumor control and memory generation.

The increased glutamine uptake due to enhanced expression of anti-porter xCT in Pmel-Trx T cells or in presence of rTrx could be an important feature that renders potent anti-tumor control, a mechanism that has been shown to promote tumor growth where ambient glutamine indirectly supports environmental cystine acquisition via the xCT antiporter (52). The observation that Trx overexpressing T cells exhibit increased glutamine uptake also implies that this amino acid may have contributed towards the programming of Pmel-Trx effectors for enhanced anti-tumor phenotype. It has also been shown that while glutamine helps differentiation of T cells to effector phenotype (22), and the deficiency of glutamine can result in formation of Treg's (22). Importantly, higher glutamine levels in Pmel-Trx T cells would have led to down-regulation of CD95 and CD95L expression, and up-regulation of memory marker CD45RO and Bcl-2 expression as has been shown earlier (53). Thus, the results presented here in support the role of Trx in regulating redox status of adoptively transferred T cells, and that Trx mediated "anti-oxidant help" in vivo may be important in generating long-lived anti-tumor memory T cells in the oxidative tumor microenvironment. We believe that the strategy to generate anti-tumor memory T
cells using Trx will have great translational significance in the field of cancer immunotherapy.

MATERIALS AND METHODS

Mice

C57BL/6, B6-Thy1.1 (B6.PL-Thy1a/CyJ) and Pmel mice were obtained from Jackson Laboratory (Bar Harbor, ME). Animals were maintained in pathogen-free facilities and experimental procedures were approved by Institutional Animal Care and Use Committees of Medical University of South Carolina, Charleston.

Reagents and cell lines

Penicillin, streptomycin, glucose-free RPMI-1640, and Iscove’s Modified Dulbecco’s Medium (IMDM) were purchased from Life Technologies, Grand Island, NY. FBS was procured from Atlanta Biologicals, Atlanta. hgp10025-33 peptide (KVPRNQDW) peptide was purchased from GenScript (Piscataway, NJ). Recombinant IL2 (rIL2) was procured from NCI, Biological Resources Branch. Fluorochrome conjugated anti-mouse CD8 (53-6.7), CD71 (RI7217), CD25 (PC61), CD69 (H1.2F3), CD44 (IM7), CD62L (MEL-14), Sca1 (D7), and anti-human CD8 (SK1), CD44 (IM7), CD62L (DREG-56), CD28 (CD28.2) were purchased from Biolegend, San Diego, CA. Anti-mouse Vβ13 (MR12-3) was procured from BD Biosciences, San Jose, CA. Anti-human Vβ12 was from Thermo Scientific (Rockford, IL). Anti-mouse pS6 conjugated with Alexa647, pAkt (S473) conjugated with PE and pFoxo1 (S256) were purchased from Cell Signaling Technology, Danvers, MA. Anti-mouse CD3 (145-2C11), and CD28 (37.51) were purchased from BioXcell, West Lebanon, NH. B16-F10 melanoma (Cat # CRL-6475) was obtained from ATCC, Manassas, VA. PBMCs from healthy donors were obtained from a commercial vendor, Research Blood Components, LLC, after institutional approval by the Human Investigation Review Board.

Cell Culture. Splenocytes from Pmel or Pmel-Trx mice were activated with using
hgp100<sub>25-33</sub> peptide (KVPRNQDW, 1μg/ml) for three days. rIL2 (100U/ml) was added during T cell activation. In some cases Pmel splenic T cells were activated with cognate antigen in presence of rTrx. Complete IMDM media supplemented with 10% FCS, 4 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 5 μM beta-mercaptoethanol was used for T cell culture. Where indicated purified CD4<sup>+</sup> T cells were differentiated to induced regulatory T cells using the standard protocol as published recently (8).

**Adoptive T cell protocol.** Mouse melanoma tumor (B16-F10) was maintained in complete IMDM. On day 9<sup>th</sup> following B16-F10 cell inoculation, the C57BL/6 recipient mice were injected intraperitoneally (<i>i.p.</i>) with cyclophosphamide (CTX) at 4 mg/mice. After 24 h of CTX injection, tumor-bearing C57BL/6 were either kept untreated or adoptively transferred with three–day-activated pMel cells (1 × 10<sup>6</sup>/mouse). Recipient mice were given IL2 (50,000 U/mouse; <i>i.p</i>) for 3 consecutive days after ACT.

**Flow cytometry.** Detailed protocol for staining of cell surface molecules and intracellular proteins has been described earlier (8). Samples were acquired on LSRFortessa and analysed with FlowJo software (Tree Star, OR).

**Real-time quantitative-PCR array.** Total RNA was isolated from pellets of the indicated T cell subsets (2 x 10<sup>6</sup> cells) using Trizol reagent (Life Technologies, Grand Island, NY). cDNA was generated from 1 μg total RNA using iScript cDNA Synthesis Kit (BioRad, Hercules, CA). SYBR Green incorporation quantitative real-time PCR was performed using a SYBR Green mix (Biorad, Hercules, CA) in the CFX96 Detection System (BioRad, Hercules, CA). All array data has been deposited in the EBI ArrayExpress Database accession number E-MTAB-7571.

**Activation-induced T cell death.** Three-day-activated T cells from either Pmel or
Pmel-Trx mice were either left untreated or re-stimulated overnight with cognate antigen gp100. Apoptosis was measured by Annexin V (BD Biosciences, San Jose, CA) vs. 7AAD staining according to the manufacturer’s protocol, followed by flow cytometry. Data were analysed with FlowJo software (Tree Star, OR).

**Glucose uptake, oxygen consumption and glycolytic flux.** Glucose uptake by activated T cells were determined by incubating cells with 100 µM 2NBDG (Cayman Chemical, Ann Arbor, MI) for 30 minutes before measuring fluorescence by flow cytometry. Extracellular acidification rate (ECAR) was evaluated as described earlier (8).

**Statistical analysis.** All data reported are the arithmetic mean from three independent experiments performed in triplicate ± standard deviation (SD) unless stated otherwise. The unpaired Student’s t-test was used to evaluate the significance of differences observed between groups, accepting $p < 0.05$ as a threshold of significance. Data analyses were performed using the Prism software (GraphPad, San Diego, CA). Analysis of tumor size (mm$^2$) was performed using R statistical software version 3.2.3 and SAS version 9.4. Tumor size at each time point was measured relative to tumor size at the day of T cell transfer (day 9) to adjust for differences between animals at baseline. We transformed resulting fold-change (FC) values using a log base 2 transformation to achieve approximate normality. Using maximum likelihood, we fit linear mixed effects regression models of log$_2$ FC as a function of experimental group, time (as a continuous variable), group-by-time interaction and mouse-specific random effects to account for the correlation among measures obtained from the same animal over time. We evaluated the functional form of time in each model, and considered non-linear transformations as appropriate based on fractional polynomials (9). Group comparisons were performed using model-based linear contrasts, and were restricted
to time points at which data were available from at least two animals per group.

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FIGURE LEGENDS

Figure 1: Trx overexpressing transgenic T cells exhibit higher anti-oxidant capacity and reduced susceptibility to oxidative stress. Splenic T cells from Pmel and Pmel-Trx T cells were activated with gp100 peptide antigen for three days in presence of IL2 (50 IU/ml) before using to determine: A) Trx expression staining on the Pmel and Pmel-Trx T cells. B) Expression of cell surface thiols (c-SH) using alexa-fluor labeled maleimide dye. C) Intracellular reactive oxygen species accumulation (H2O2) by DCFDA. D) Annexin V levels after overnight culture in presence of 50 µM of exogenous H2O2. E) Annexin V levels 4 hr after restimulation with cognate antigen. F). Cell in E were stained intracellularly using fluochrome conjugated anti-RIPK3 antibody. G) Three day activated Pmel and Pmel-Trx splenocytes were transferred i.p. to the EL4 ascitis established for 14 days in C57BL/6 mice. The T cells were retrieved after 24 hrs. and oxidative stress markers 8-OHdG (left panel) and nitrotyrosine (right panel) were determined in Vβ13 gated CD8+ T cells. Mean fluorescence intensity (MFI) from 2 independent experiments is represented as bar diagram. The bar diagram adjacent to each panel represents the cumulative data of mean fluorescence intensity (MFI) from 3-5 independent experiments *p value < 0.05, **p value <0.01.

Figure 2: Trx overexpressing T cells exhibit enhanced Tscm phenotype and recall response. A) Three day antigen activated gp100 TCR specific splenic T cells from Pmel and Pmel-Trx mouse were gated on CD44loCD62L+ and evaluated for CD122 and Sca1 expression. B) RNA prepared from activated Pmel and Pmel-Trx T cells was used to determine expression of stem cell related genes. N=3. C) Three day activated Pmel and Pmel-Trx transgenic T cells (1×10^6 cells/mouse) were adoptively transferred into Rag1−/− mice. After 25 days of T cell transferred mice were subcutaneously injected with B16-F10 solid tumors (0.25 × 10^6 cells/mouse). Upper panel shows representative flow cytometric
analysis done to determine the percentage of TCR transgenic T cells retrieved from spleen, lymph nodes, blood, lung, liver after 5 days of tumor injection. *Lower panel* is the cumulative data from different mice.  

D) Splenocytes from (C) were stimulated overnight with hgp100 antigen before being analyzed for intracellular signature of IFNγ.  

*Figure 3: Cell signaling and function of Pmel-Trx T cells.* Pmel and Pmel-Trx derived splenic T cells were activated for three days with cognate antigen and used for determining:  

A) Phosphorylation levels of key signaling molecules by intracellular staining with phospho-Abs (from BD) as per the manufacturers protocol.  

B) Cells were left in IL2 (50 IU) for 15 minutes and 60 minutes before staining with fluorochrome conjugated antibody for phopho-STAT5.  

C) Intracellular IFNγ staining after reactivation with cognate antigen overnight. Adjacent bar diagram represents the cumulative data for the percentage of Vβ13+CD8+ cells producing IFNγ from 3 independent experiments.  

D) Glucose uptake (using 2NBDG) after antigen restimulation for 4 hrs. Adjacent bar diagram represents the cumulative data of MFI from 3 independent experiments.  

E) qPCR analysis of various genes in three day activated Pmel and Pmel-Trx cells. Data are representative of 3 independent experiments.  

F) Oxygen consumption rate (OCR) was measured using Seahorse flux analyzer in presence or absence of etomoxir (200 µM) followed by the addition of indicated mitochondrial inhibitors in 3 day activated WT or Trx over expressing T cells in the left panel. Graphs in the right panel represents Maximum OCR (maximum rate measured after FCCP injection), spare respiratory capacity (SRC, maximum respiration-basal respiration) and OCR/ECAR ratio. Results are representative of 3 independent experiments with similar observation.  

*Figure 4: Metabolic profiling of Pmel-Trx T cells.* Pmel and Pmel-Trx T cells were activated with the cognate antigen for three
days and used for quantifying intracellular metabolites using mass spectrometry. A) The Principal Component Analysis (PCA) shows distribution of the metabolites. B-D) Relative amount of metabolites evaluated between the triplicate Pmel and Pmel-Trx T cells are segregated into different metabolic pathways and shown in heat maps.

**Figure 5:** Increased glutamine dependence of Trx overexpressing T cells. Pmel and Pmel-Trx T cells activated using cognate peptide antigen for three days were used to: A) determine uptake of radiolabeled glutamine that was measured in count per minute (CPM) and presented as mean of 4 replicate samples from 3 repeat experiments; B) obtain RNA and determine the expression of various glutaminolysis associated genes using qPCR. The data presented is from 3 experiments. C) Cell surface expression of amino acid transporter xCT and glutaminolytic enzymes (upper panel). Bar diagrams (lower panel) represent the cumulative data of MFI from 3 independent experiments. *p<0.05, **p< 0.01.

D) Activated Pmel and Pmel-Trx cells were used to measure intracellular metabolites using mass spectrometry. E) Splenic T cells from C57BL/6 mouse were activated with anti-CD3/28 (each 2µg/ml) either in absence or presence of α-KG for three days, after which: Tscm phenotype was evaluated using CD62L and CD44 cell surface expression. F) The cells in E) were also used for determining glucose uptake using 2NBDG. Representative data from one of three experiments with similar results is presented. G) RNA was used to determine the expression of genes involved in chromatin modification Enz using mouse epigenetic chromatin modification enzymes PCR array (Qiagen).

**Figure 6:** Exogenous Trx renders increased anti-oxidant capacity, altered signaling and improved anti-tumor property to T cells. A) Three day activated gp100 epitope reactive splenic T cells from Pmel and Pmel-Trx mouse were re-stimulated overnight with cognate antigen,
and supernatant was evaluated for secreted Trx by ELISA as per protocol. N=3 **p<0.005. B) Pmel T cells were labeled with CFSE and stimulated with cognate antigen gp100 for three days. The expression of Trx was then determined using flurochrome conjugated antibody by gating on the cells in different phase of division. C) CFSE labeled Pmel T cells were stimulated with cognate antigen in absence or presence of rTRx at various doses. D) Pmel T cells activated with cognate antigen in presence of rTrx (5 µg/ml) were used at day 3 to determine the expression of Trx. Cells in D were also used for determining: E) iGSH, and F) pAMPK. G) Pmel T cells stimulated with cognate antigen in absence or presence of rTRx at various doses were used to determine pS6 levels using flurochrome conjugated antibody. H) Three day activated Pmel and Pmel-Trx T cells were used to prepare RNA for determining stemness genes using qPCR. I) Three day TCR activated T cells were used for determining Annexin V upregulation after 4 hr of TCR restimulation with cognate antigen hgp100 or non-cognate ova peptide. J) Cell in I were used for glucose uptake using 2NBDG. Numerical values in each plot represent mean fluorescence intensity. *p <0.05, **p<0.01. K) Melanoma epitope gp100 reactive T cells obtained from Pmel TCR transgenic mouse were activated with cognate antigen either in presence or absence of rTrx for three days before being adoptively transferred to the immunocompetent C57BL/6 recipient mice with ten day subcutaneously established murine melanoma B16-F10. Tumor measurements from two different experiments where Pmel and Pmel+rTrx were transferred to a minimum of nine recipient mice were compiled for this presentation. *** p value < 0.01 obtained at last time point of the tumor measurement before the experiment was terminated. L) C57BL/6 mice (n=3 mice/group) were inoculated (s.c) with 0.25×10^6 B16-F10 melanoma cells for 14 days, after which mice were adoptively transferred with 1×10^6 three day gp100 activated Pmel T cells, Pmel cells treated with rTrx (10 µg/ml).
After 12 days of T cells transfer, lymphocytes were retrieved from the excised tumor and the indicated lymphoid organs. Upper panel shows FACS plot for TCR transgenic T cell determined by staining for Vβ13 expression. Lower panel shows cumulative data. Retrieved lymphocytes were stimulated overnight with αCD3 (2 µg/ml) and αCD28 antibody (5 µg/ml) before staining with fluochrome-conjugated antibodies to determine: M) intracellular IFNγ level, and N) Granzyme B (GzmB) level. N=3. *p<0.05, **p<0.005.
Table 1: List of biological pathways significantly altered in Trx overexpressing T cells.

| Biological Pathways                                      | p-value         |
|-----------------------------------------------------------|-----------------|
| Cysteine and methionine metabolism                        | 0.000292547     |
| TNF signaling pathway                                     | 0.001535877     |
| Tight junction                                            | 0.001581198     |
| Regulation of actin cytoskeleton                          | 0.002066769     |
| Leukocyte transendothelial migration                      | 0.002511068     |
| Chemical carcinogenesis                                   | 0.002823408     |
| MicroRNAs in cancer                                       | 0.004175701     |
| PI3K-Akt signaling pathway                                | 0.008407723     |
| Sphingolipid signaling pathway                            | 0.008425501     |
| Cytokine-cytokine receptor interaction                    | 0.031239851     |
| Chemokine signaling pathway                               | 0.03186533      |
| Antigen processing and presentation                       | 0.033102383     |
| NF-kappa B signaling pathway                              | 0.035012555     |
| MAPK signaling pathway                                    | 0.037652869     |
| T cell receptor signaling pathway                         | 0.04559592      |
| mTOR signaling pathway                                    | 0.046443821     |
| Alanine, aspartate and glutamate metabolism               | 0.046562088     |
Figure 1

A) Pmel (CD8^+Vβ13^+)

B) + H_2O_2

C) + hgp100

D) % Annexin^+ 7AAD^+ cells

E) % Annexin^+ 7AAD^+ cells

F) RIPK3 (MFI)

G) 8OHdG (MFI), Nitrotyrosine (MFI)

---

WT
Trx-Tg

* p < 0.05
** p < 0.01
Figure 2

A) CD8^+V_13^+ gated

B) Fold Change

Figure 2
Figure 3

A) 

B) 

C) 

D) 

E) 

F)
Figure 4

A) 

WT Trx-Tg

Amino acid metabolism
N-acetylaspartate (NAA)
pyroglutamine*
imidazole lactate

\[ 0.19 \] 2.92

B) 

WT Trx-Tg

Comp.2 [22.92%]

Comp.1 [49.83%]

Pentose phosphate pathway
ribose 1-phosphate
ribulose/xylose 5-phosphate
ribonate

Fructose/Mannose/Galactose Metabolism
mannitol

TCA Cycle
alpha-ketoglutarate
succinate

Oxidative phosphorylation
acetylphosphate
phosphate

\[ 0.08 \] 3.41

C) 

WT Trx-Tg

Lipids
valerate
heptanoate (7:0)
deocarnitine

\[ 0.18 \] 2.52

D) 

WT Trx-Tg

glycerophosphoinositol*
palmitoyl-oleoyl-glycerophosphoglycerol (1)*
oleyl-linoeloyl-glycerophosphocholine (1)*
apamitoyl-oleoyl-glycerophosphoglycerol (2)*
stearoyl-oleoyl-glycerophosphoserine (1)*
stearoyl-linoeloyl-glycerophosphoethanolamine (1)*
steiroyl-arachidonyl-glycerophosphoserine (1)*
1-linoeloylglycerophospholinositol*
palmitoyl sphingomyelin
Figure 6

A. Thioredoxin1 (Pg/ml)

B. Isotype

C. CFSE

D. Trx (MFI)

E. IGS (MFI)

F. pAMPK (MFI)

G. cytokine analysis

H. Fold Change

I. Annexin V

J. 2NBDG

K. Fold change

L. Flow cytometry analysis

M. % of Vβ13+ CD8+

N. % of GzmB+ Vβ13+

Pmel + rTrx1 vs. Pmel
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