Modulation of Endoplasmic Reticulum Stress Controls CD4$^+$ T-cell Activation and Antitumor Function

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Abstract

The endoplasmic reticulum (ER) is an energy-sensing organelle with intimate ties to programming cell activation and metabolic fate. T-cell receptor (TCR) activation represents a form of acute cell stress and induces mobilization of ER Ca$^{2+}$ stores. The role of the ER in programming T-cell activation and metabolic fate remains largely undefined. Gp96 is an ER protein with functions as a molecular chaperone and Ca$^{2+}$ buffering protein. We hypothesized that the ER stress response may be important for CD4$^+$ T-cell activation and that gp96 may be integral to this process. To test our hypothesis, we utilized genetic deletion of the gp96 gene Hsp90ab1 in a CD4$^+$ T cell–specific manner. We show that gp96-deficient CD4$^+$ T cells cannot undergo activation-induced glycolysis due to defective Ca$^{2+}$ mobilization upon TCR engagement. We found that activating naïve CD4$^+$ T cells while inhibiting ER Ca$^{2+}$ exchange, through pharmacological blockade of the ER Ca$^{2+}$ channel inositol trisphosphate receptor (IP$_3$R), led to a reduction in cytosolic Ca$^{2+}$ content and a pool of CD62L$^{hi}$/CD44$^{lo}$ CD44$^{hi}$/CD62L$^{lo}$ T cells compared with wild-type (WT) matched controls. In vivo IP$_3$R-inhibited CD4$^+$ T cells exhibited elevated tumor control above WT T cells. Together, these data show that ER-modulated cytosolic Ca$^{2+}$ plays a role in defining CD4$^+$ T-cell phenotype and function. Factors associated with the ER stress response are suitable targets for T cell–based immunotherapies.

Introduction

Upon antigen recognition in the proper immunogenic context, T cells undergo multiple rounds of proliferation (1). To fulfill the bioenergetic demands of activation and expansion naïve T cells undergo a metabolic shift from oxidative phosphorylation (OXPHOS) toward aerobic glycolysis (2). During the activation process, driven by T-cell receptor (TCR) engagement, a rapid rise in cytosolic Ca$^{2+}$ occurs that intimately controls subsequent programming of T-cell differentiation and effector function (3–5). The endoplasmic reticulum (ER) maintains cell Ca$^{2+}$ stores, and release of Ca$^{2+}$ into the cytosol promotes T-cell activation (6–8). In activated T cells, metabolites from the glycolytic pathway work to inhibit Ca$^{2+}$ re-uptake by the ER to potentiate the effector T-cell metabolic cycle (9). The role of initial Ca$^{2+}$ rise and the channels that drive this process has not been well studied in the context of the T-cell metabolic programs.

Materials and Methods

Mice

T cell–specific deletion of gp96 on a C57BL/6 background was accomplished by crossing Hsp90ab1$^{lox/lox}$ (16) mice with Cd4-Cre (Tg(Cd4-Cre)1Cwi/Bhfj) transgenic mice (The Jackson Laboratory) C57BL/6, Rag1$^{-/-}$ (B6.129S7-Rag1$^{+/+}$/J), OT-1 (C57BL/6-Tg(TcraTcrb)1100Mjb/J), TRP-1 (B6.Cg-Rag1$^{+/+}$/J Tg(Cd4-Cre)1Cwi/Bhfj)
or CD8+ T cells (Milenyi) were counted and loaded with Fluo-4 (0.5 μmol/L) (Molecular Probes) in T cell media for 30 minutes at 37°C. Cells were labeled with CD4-APC and spun onto poly-lysine coated plates (Tissue-Tec) and fresh media was added. Imaging was performed using the Olympus FV10 for 148 frames at 11s intervals directly after addition of CD3/28 beads (Dynabeads). Fiji Image J software was used to quantify Fluo-4 fluorescence over time on a single cell basis.

**Metabolic assays**

Oxygen consumption rates (OCR) and extracellular acidification rates (ECAR) were measured in non-buffered RS media supplemented with HEPES under basal conditions and in response to 1 μmol/L oligomycin, 1.5 μmol/L FCCP, and 100 nmol/L rotenone + 1 μmol/L Antimycin A (OAR) or 10 μmol/L glucose, 1 μmol/L oligomycin, and 10 μmol/L 2-deoxyglucose (ECAR) with XF-96 Extracellular Flux Analyzer (Seahorse Biosciences). Cell Traq was used for T-cell adherence. For in vivo 2-NBDG (Cayman Chemical) uptake, 100 μg/mouse was injected via tail vein and mice were bled 15 minutes post injection. Ex vivo 2-NBDG uptake was assessed 18 hours post T-cell activation after glucose-free medium treatment. Lactate in cell culture media was measured with Lactic Acid kit (Sigma).

**Adoptive cellular therapy (ACT)**

B16F10 cells were obtained from ATCC and tested negative for mycoplasma in March 2015. Cells were passaged three times prior to in vivo inoculation. All media used was supplemented with plasmocin prophylactic (Invivogen). Cells were not re-authenticated during the course of these experiments. Tumors were established on the right flank of C57Bl/6 male mice for 7 days. One day prior to ACT mice were irradiated (5Gy). Ex vivo-expanded TRP-1 TCR-transgenic T cells (2 x 10^6) treated for 7 days with vehicle or 2-Aminoethoxydiphenyl borate (2-APB) (5 μM) (Tocris) were injected into the tail vein and tumor growth was assessed every other day until the endpoint tumor size (≥ 400 mm²) was reached. For tumor-infiltrating lymphocyte (TIL) analysis, a tumor dissociation kit (Milenyi) was used.

**Results**

Due to the fact that ER stress chaperone gp96-folded proteins, such as the integrins, are involved in various T-cell functions, we asked whether the chaperone itself was modulated in response to T-cell activation. We purified CD4+ or CD8+ T cells from wild-type (WT) mouse spleens and assessed gp96 gene expression after 6 and 18 hours of activation. Both CD4+ and CD8+ T cells upregulated gp96 gene expression 6 and 18 hours post activation, indicating an acute ER stress response (Fig. 1A and B). To determine whether TCR activation induces the generalized ER stress response-associated with increased gene expression of chaperone proteins, we measured gp78 expression in the aforementioned conditions in both CD4+ and CD8+ T cells. In line with a general and acute ER stress response, gp78 gene expression was also increased at 6 and 18 hours post T-cell activation (Supplementary Fig. S1). To confirm the gene product of gp96, we measured gp96 protein expression via intracellular staining and flow cytometry at 18 hours post T-cell activation in both CD4+ and CD8+ T cells. At this time point gp96, as measured by mean fluorescent intensity (MFI), was significantly
upregulated in both CD4+ and CD8+ T-cell subsets above unstimulated naïve controls (Fig. 1C).

Due to the upregulation of the gp96 gene and protein in both CD4+ and CD8+ T cells upon CD3/28 activation (Fig. 1A–C), we asked whether this response was specific to TCR activation induced by cognate MHC class I–peptide complexes. We assessed the CD4+ T cell response using T cells from TCR transgenic mice with a TCR specific for tyrosinase-related protein-1 (TRP-1). CD4+ T cells were activated for 18 hours in the presence of cognate peptide, and the induction of intracellular gp96 was measured. TCR transgenic CD4+ T cells had significantly increased gp96 protein expression in response to cognate peptide, compared with naïve CD4+ T-cell controls (Fig. 1D; ref. 18). We next measured induction of gp96 in OT-1 TCR transgenic CD8+ T cells specific for chicken ovalbumin peptide (19). Similar to CD4+ T cells, activation of CD8+ T cells with cognate antigen induced gp96 protein expression (Fig. 1E). Given the critical role of the acute ER stress response in cellular activation and differentiation, our results agree with published data (20). However, our data demonstrate that TCR ligation is a form of acute stress that can induce ER chaperones in both primary CD4+ and CD8+ T cells.

T cell–specific gp96 mutants have altered CD4+ T-cell subsets

We have previously demonstrated that the function of regulatory T cells is greatly impaired by deletion of gp96, due to loss of GARP, which is a client protein of gp96 (17). We now investigated whether gp96 deficiency in the effector cell subset may impact T-cell activation and phenotype. We generated CD4− T cell–specific gene deletion mutants for gp96 via CD4cre x Hsp90b1flo/flox (96KO) genetic cross and confirmed gene deletion and loss of protein expression in naïve CD4+ bead–isolated fractions from both spleen and thymus (Supplementary Fig. S2A and S2B). Given reduced gp96 protein expression in CD4 single-positive (SP) thymocytes, we measured the expression of thymic differentiation markers CD25, CD44, and CD127 among WT or 96KO mice and found no differences (data not shown). To assess alterations in SP and double-positive (DP) thymocyte subsets, we measured CD4+/CD8+ T cells among thymocytes and found no differences in the size of the DP populations between WT and 96KO groups (Fig. 2A–C). Given that we aimed to assess the role of gp96 in T-cell activation, we examined TCR Vβ chain usage via flow cytometry in WT and 96KO mice. In both spleen and thymus, 14 commonly expressed Vβ chains were expressed to a similar extent in both WT and 96KO mice (Supplementary Fig. S2C).

CD4cre, expressed in the double-positive stage of thymocyte maturation, induced losp- mediated gp96 gene deletion in >90% of CD4+ and CD8+ T cells. We undertook phenotypic analysis of CD4+ and CD8+ thymocytes, splenocytes, and lymph node–associated populations between WT and 96KO mice (21). In agreement with our protein and gene deletion data (Supplementary Fig. S2A and B) we found that splenic populations were significantly reduced in gp96 expression in both CD4+ and CD8+ T-cell subsets (Fig. 2A). We noticed that splenic populations of CD4+ T cells significantly increased in 96KO mice compared with WT controls. CD8+ T-cell percentages in 96KO mice appeared slightly reduced (Fig. 2B and C; Supplementary Table S1). Our lab has shown that gp96 chaperones multiple integrins, many of which are essential for T-cell homing to lymph nodes. Thus, a direct functional consequence of T cell–specific gene deletion of gp96 was reduced percentages of CD4+ and CD8+ T cells that had trafficked to mesenteric lymph nodes in 96KO mice compared with WT controls (Fig. 2B and C; Supplementary Table S1; ref. 13).

Analysis of absolute T-cell numbers in both CD4+ and CD8+ T-cell subsets showed that absolute CD8+ T-cell numbers between WT and 96KO mice were not significantly different. However, the absolute numbers of CD4+ T-cell populations were affected at absolute levels between WT and 96KO mice (Supplementary Fig. S3A; Supplementary Table S1). Assessment of subpopulations of...
Figure 2.
Gp96 T-cell–specific gene deletion induces CD4+ subset changes. A, Representative histograms of gp96 expression in CD4+ or CD8+ single-positive thymocytes, splenocytes, or mesenteric lymph node (MLN) T cell preparations from CD4creHsp90b1fl/+;C3/C3creHsp90b1+/+ypical WT (WT) or CD4creHsp90b1fl/+;C3/C3creHsp90b1+/+otypical (96KO) mice. B, Representative FACS plots for CD4+ T cells from WT or 96KO mice from thymus, spleen, or MLNs and (C) quantification of populations. D, Representative FACS plots for CD4+ T cells from WT or 96KO splenocytes and (D) percentage or (E) absolute number quantification of splenic subpopulations. Data are mean ± SD of 5–7 mice per group. *, P < 0.05; **, P < 0.01; ***, P < 0.0001, two-tailed Student t test.

Loss of gp96 results in CD4+ T-cell activation defects

In order to rectify the finding that although 96KO mice possessed greater numbers of both naïve and highly activated CD44+ T cells, we used the gp96-folded molecule CD18 as a marker of gp96 deletion in vivo among CD4+ T-cell subsets. Our data show that the highly activated CD4+ T-cell subset in 96KO mice was an outgrowth of a remnant WT population (Supplementary Fig. S3B). To further substantiate this finding and investigate the role of gp96 in CD4+ T-cell activation, we CFSE-labeled CD4+ T cells isolated from spleen of WT or 96KO mice (depleted of CD4+CD25+ cells), and transferred them into sublethally irradiated Rag1−/− mice. After 14 days, we assessed splenic populations of recipient Rag1−/− mice for CFSE/CD62L+ populations, whereas transfers from WT mice showed complete activation, as shown by robust cell division and loss of CFSE+ transferred CD4+ T cells (Supplementary Fig. S4). To address the role of a cell intrinsic activation defect in 96KO CD4+ T cells not due to loss of Treg function, we undertook mixed bone marrow chimera analysis. As Fig. 3A indicates, T cell-depleted bone marrow preparations from WT and 96KO mice were transferred to lethally irradiated Rag1−/− mice and reconstitution was allowed for up to 2 months post transfer. After 1 month of bone marrow reconstitution, the vast majority of 96KO CD4+ T cells (CD45.2) were CD18+. However, 2 months after reconstitution a remnant WT population grew out and persisted within 96KO reconstituted populations (Fig. 3B and C; ref. 22). Direct evidence for an activation defect among 96KO populations was demonstrated by gating on CD18+ populations among the CD44+ 96KO group compared to WT matched controls. We assessed CD4+ T-cell subsets within this group and found that CD4+ T cells devoid of gp96 could not transition to the CD62L+/CD44+ or CD44+ subsets (Fig. 3D and E). A BrdUrd pulse-chase 24 hours before sacrifice demonstrated that homeostatic proliferation among naïve CD4+ T cells was not significantly affected between WT and 96KO groups (Fig. 3F). Our data suggest that CD4+ T cells devoid of ER stress protein gp96 were severely impaired in the ability to transition from a naïve state and that this is a cell intrinsic defect.

Ca2+ flow and mitochondrial membrane potential in CD4+ 96KO T cells

ER stress is tightly tied to fluctuations in cell Ca2+ concentrations. ER-associated and chaperone proteins have secondary capacities as Ca2+ buffering proteins (15, 23). For example, T cell–specific deletion of calreticulin greatly impairs cell Ca2+ oscillations resultant in a pool of less activated T cells and autoimmune conditions in vivo (24). We undertook ex vivo Ca2+ imaging of single cells from WT or 96KO-naïve CD4+ T-cell preparations. Dead-isolated naïve WT or 96KO CD4+ T cells were labeled with cytosolic Ca2+ imaging dye Fluo-4. This dye fluoresces upon cytosolic free Ca2+ elevation. We prelabeled CD4+ T cells with Fluo-4 and live-imaged fluorescent signal upon CD3/28 bead addition. Fluorescence in WT CD4+ T cells increased over the first 30 minutes of TCR engagement, whereas 96KO CD4+ T cells did not mobilize cytosolic free Ca2+ similar to WT patterns (Fig. 4A). Direct quantification of mobilization was obtained by measurement of peak Ca2+ intensity with the first 30 minutes of TCR ligation between WT and 96KO groups (Fig. 4B). 96KO CD4+ T cells did not mobilize cytosolic free Ca2+ to
the extent that WT CD4+ T cells did, lending potential mechanistic support to explain the activation defect in 96KO CD4+ T cells. However, more work is needed to attribute this effect directly to the Ca2+ buffering capacity, and not the chaperone function, of gp96.

Given that 96KO CD4+ T cells maintained a naïve state in vivo and that these cells showed impaired initial Ca2+ mobilization in response to TCR activation (Fig. 3 and 4A), we asked whether activation-induced long-term cytosolic Ca2+ concentrations were impaired in these cells. We found that though naïve cytotoxic Ca2+ concentrations between WT and 96KO cells did not differ, 18 hours post-activation 96KO CD4+ T cells showed significantly diminished cytosolic free Ca2+ (Fig. 4C). Thus, naïve CD4+ T cells maintained low cytosolic Ca2+ concentrations, which supported the notion of Ca2+ as a driver of T-cell activation.

Release of ER Ca2+ stores leads to elevated mitochondrial Ca2+ modulated through the IP3R (25). Though not yet described in T cells, mitochondrial activation through cell Ca2+ is a key regulator of cell bioenergetics (26, 27). We asked whether inhibition of mitochondrial Ca2+ signal could be detected in activated 96KO CD4+ T cells. We used Rhodamine-2 (Rhod-2) to assess mitochondrial Ca2+ content 0 and 18 hours post T-cell activation. We found that, although no basal differences in mitochondrial Ca2+ could be detected, activated 96KO cells showed impaired mitochondrial Ca2+ content compared with WT T cells (Fig. 4D). These data suggested a link between ER-modulated Ca2+ signals and mitochondrial activation. Ca2+ overload within the mitochondria can lead to induction of apoptotic signaling cascade, whereas impaired mitochondrial Ca2+ uptake can compromise mitochondrial metabolism (28). We used the mitochondrial membrane potential dye tetramethylrhodamine methyl ester (TMRM) to measure mitochondrial activity. We reasoned that initial loss of Ca2+ uptake in 96KO cells would lead to inactive mitochondria as assessed by TMRM and demonstrate that mitochondrial metabolism was compromised in these cells. Indeed, 18 hours after activation, WT cells increased TMRM fluorescence compared with

Figure 3.

WT CD4+ T cells outcompete 96KO CD4+ T cells, indicative of a cell intrinsic defect. A, Experimental design of 1:1 WT (CD45.1) or 96KO (CD45.2) T cell–depleted mixed bone marrow chimeras (BMC) transferred to irradiated (10 Gy) Rag–/– mice and harvested 1 and 2 months after transfer. B, Representative flow cytometry plots of splenocytes from 1 and 2 months after bone marrow reconstitution, gated on CD4+ T cells and probing for CD45.2+/CD18– as a measure of gp96 deletion. C, Quantification of CD4+ T cells probed for CD45.1/CD45.2/CD18 1 and 2 months after BMC reconstitution. D, Representative flow cytometry plots of splenocytes from 2 months after BMC reconstitution, gated on CD4+ T cells and further gated from (pink gates) CD45.1/CD18– (WT) or CD45.2/CD18– (96KO) populations assessed for CD62L/CD44 expression and (E) quantification of subset populations. F, Representative flow cytometry plots of BrdUrd uptake gated from CD4+ splenic T cells from 24-hour pulsed 1-month BMC mice and (G) quantification of BrdUrd uptake in CD4/CD62L+ T cells gated from WT (CD45.1/CD18+) or 96KO (CD45.2/CD18–) populations. Data are mean ± SD of 4 mice per group. *, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001, two-tailed Student t test. Two independent repeats were performed for these experiments.
Modulation of ER Ca$^{2+}$ in CD4$^+$ T Cells

Figure 4.
Gp96 is necessary for naive CD4$^+$ T-cell mobilization of intracellular Ca$^{2+}$. A, Real-time single-cell imaging in WT or 96KO CD4$^+$/CD62L$^+$ T cells. Naive T cells were isolated and labeled with Fluo4 (FITC) and CD4 (APC) and adhered to glass slide incubating chambers. At time 0, CD3/28 beads were added to media. Data are plotted as average fold increase in Fluo-4 for 12-16 cells per group over 11 second intervals for ~30 minutes. Analysis was performed with ImageJ software. B, Peak fluorescent increase in Fluo-4 was recorded. Data are mean ± SEM of 12 cells per group. ***, P < 0.001, two-tailed Student t test. Similar data were obtained for 4 mice per group in 4 separate experiments. Representative histograms and quantification from 0 or 18-hour CD3/28 activated splenocytes from WT or 96KO mice gated on CD4$^+$/CD62L$^+$ (WT) or CD4$^+$/CD62L$^+$ (96KO) cells and probed for (C) cytosolic Ca$^{2+}$ with Fluo-4 (D) mitochondrial Ca$^{2+}$ with Rhod-2 (E) or mitochondrial membrane potential with TMRM. Data are means from 3 mice, three independent experimental repeats were performed. *, P < 0.05, two-tailed Student t test.

0 hour controls, but 96KO cells were unable to significantly regulate TMRM levels to that of WT controls (Fig. 4E). Together, these data present evidence for the role of cell Ca$^{2+}$ to modulate mitochondrial metabolism in T cells.

Gp96 CD4$^+$ T-cell mutants are impaired in glucose consumption

The role of ER stress chaperones and sensors in T-cell biology and associated metabolomics has not been studied. Upon CD4$^+$ T-cell activation, glucose uptake and entry into glycolysis is modulated by Glut-1 cell surface expression (29). Further, it is known that cytosolic Ca$^{2+}$ can perpetuate glycolysis as glycolytic product phosphoenolpyruvate (PEP) directly inhibits ER-re-establishment of Ca$^{2+}$ homeostasis (9). However, the role of initial mobilization of Ca$^{2+}$ in promotion of glycolysis has not been addressed. We utilized 96KO CD4$^+$ T cells as a tool to study the induction of glycolysis within the first 18 hours of T-cell activation in the context of impaired Ca$^{2+}$ mobilization.

We asked whether there were in vivo differences between WT CD4$^+$ and 96KO CD4$^+$ T-cells in ability to consume glucose. We injected fluorescent glucose analog 2-[N-(7-nitrobenz-2-oxa-1,3-dioxol-4-yl)aminol]-2-deoxyglucose (2-NBDG) into WT or 96KO mice and measured in vivo glucose uptake in CD4$^+$ populations in peripheral blood (30). 96KO CD4$^+$ T-cells were severely impaired in their ability to uptake glucose compared with WT-matched controls (Fig. 5A). These results were not unexpected given that cytosolic Ca$^{2+}$ concentrations may be slightly affected in vivo in the context of homeostatic proliferation in 96KO T cells and highlight the importance of basal glucose uptake impacted by gp96 in vivo for cell survival.

In the context of 18-hour TCR activation, we saw impaired glucose uptake measured by in vitro 2-NBDG in CD4$^+$ T-cells (Fig. 5B). In accordance with diminished processing of glucose, we found that end-stage glycolytic product lactate was significantly reduced in 96KO CD4$^+$ T-cells (Fig. 5C). 96KO cells activated for 18 hours were also unresponsive to glucose addition, as measured by extracellular acidification rate (ECAR) in comparison with WT control rates (Fig. 5D). Thus, both gp96 and Ca$^{2+}$ mobilization may be required for CD4$^+$ T-cell activation and subsequent induction of glycolysis.

Cytosolic Ca$^{2+}$ defines CD4$^+$ T-cell activation and differentiation

Naive CD8$^+$ T cells activated in the presence of glycolysis inhibitor 2-deoxyglucose (2-DG) primarily form T memory cells in vitro as marked by preferential dependence on oxidative metabolism (31). Given the emerging role of metabolomics in programming T-cell lineages coupled to our finding that Ca$^{2+}$ inhibited CD4$^+$ T cells showed severe defects in glucose uptake, we asked whether cytosolic Ca$^{2+}$ alone could define murine CD4$^+$ T-cell activation and differentiation. We harvested splenocytes from WT mice and used Fluo-4 dye to assess cytosolic free Ca$^{2+}$ content in CD4$^+$ T-cells directly ex vivo. Cytosolic Ca$^{2+}$ concentrations could differentiate naive (CD62L$^+$/CD44$^+$), central memory (CD62L$^-$/CD44$^+$), and effector (CD62L$^-$/CD44$^+$) subsets among CD4$^+$ T-cell populations in mice (Fig. 6A and B).
In vitro analysis of CD8+ splenocytes from mice showed that cytoplasmic Ca2+ content was only able to differentiate CD44+ effector subsets from naïve and central memory groups (Supplementary Fig. S5A). However, in the context of in vitro cytokine driven differentiation of CD8+ T cells toward effector (IL2) or memory (IL15) lineages, cytosolic Ca2+ was able to discriminate subsets (Supplementary Fig. S5B). Thus, cytosolic Ca2+ is a means to discriminate T-cell lineages and suggests that mechanisms of Ca2+ control may be used to modulate lineage outcomes.

We next asked whether a similar differentiation based on cytosolic free Ca2+ content could be made among human CD4+ populations. We isolated peripheral mononuclear cells (PBMC) from donors and utilized CD62L/CD45RO subset analysis to differentiate between naïve (CD62L+/CD45RO−), central memory (CD62L+/CD45RO+), effector memory (CD62L−/CD45RO−), and effector (CD62L−/CD45RO+) subsets. Indeed, we found that in human CD4+ T cells, cytosolic Ca2+ content could distinguish CD4+ lineage populations. Among four PBMC samples assessed, there were no significant differences between naïve and central memory cytosolic Ca2+ levels, whereas Ca2+ was able to distinguish both effector memory and effector populations from the naïve group (Supplementary Fig. S6).

Our demonstration that a low cytosolic free Ca2+ signal is a property able to differentiate fully activated effector T-cell populations, coupled to our finding that inhibition of Ca2+ mobilization impairs glucose dependence, led us to ask whether direct inhibition of ER-mitochondrial Ca2+ exchange could modulate CD4+ T-cell phenotype and metabolic dependence. The IP3R is responsible for release of ER Ca2+ stores to both the cytosol and mitochondria, and similar to 96KO CD4+ T-cell phenotype, T cell-specific deletion of IP3R inhibits T-cell activation (25, 32). However, the specific role of IP3R inhibition in the context of CD4+ T-cell activation has not been addressed. We used IP3R inhibitor 2-aminoethoxydiphenyl borate (2-APB) to inhibit IP3R at the time of T-cell activation in CD4+ TCR transgenic T cells. As expected, 18 hours after peptide-specific activation of TRP TCR transgenic CD4+ T cells, cytosolic Ca2+ was significantly diminished in the IP3R inhibited T-cell group (Fig. 6C). After 7 days, TCR transgenic T cells activated in the presence of 2-APB maintained a significantly increased CD62Lhigh T-cell pool with less fully activated CD44+ T cells, compared with WT-matched controls (Fig. 6D).

A hallmark feature of CD62Lhigh/CD44+ CD8+ T cells is elevated spare respiratory capacity (SRC) demonstrative of continued oxygen consumption in the context of activation (33). Given the striking CD62Lhigh/CD44int phenotype of IP3R knockdown CD4+ T cells, we measured oxygen consumption rates (OCR) and associated SRC in these cells 7 days after initial activation. We found IP3R CD4+ T cells possessed greater OCR and marked SRC compared with WT-matched controls (Fig. 6E).

Modulation of cell Ca2+ enhances therapeutic efficacy of tumor-specific T cells

A hallmark feature of T cells shifted toward a CD62Lhigh/CD44+ phenotype through metabolic modulation is elevated tumor control above glycolytic effector counterparts. Such manipulations are well documented in CD8+ T-cell populations and not yet flourished among CD4+ T-cell counterparts (31, 34). We asked whether IP3R CD4+ T cells possessed the in vivo property of augmented tumor control. We adoptively transferred CD4+ TCR transgenic T cells activated and expanded in the presence of vehicle or 2-APB into syngeneic B16F10 melanoma-bearing mice. We found that IP3R 2-APB–treated T cells significantly inhibited tumor growth and extended survival times above vehicle matched control T cells (Fig. 7A and B).
Possible reasons for superior tumor control by CD62L$^{\text{high}}$/CD44$^+$ populations are increased lymph node homing and tumor entry due to expression of CD62L and energy reserves endowed by heightened SRC (35, 33). Given that IP$_3$RI TRP CD4$^+$ T cells showed increased CD62L expression and elevated SRC compared with effector matched controls, we asked whether more V$\beta$14$^+$ CD4$^+$ T cells were found in IP$_3$RI T cell–treated mice. Spleens from mice adoptively transferred with IP$_3$RI T cells showed significantly increased V$\beta$14$^+$ percentages and absolute numbers compared with WT matched controls (Fig. 7C and E). Phenotypic analysis of the V$\beta$14 populations found in mice treated with control or treated or IP$_3$RI–treated T cells showed that IP$_3$RI populations maintained significantly increased numbers of CD62L$^{\text{high}}$/CD44$^+$ associated populations compared with vehicle treated T-cell group (Fig. 7D). As expected, IP$_3$RI T cell–treated mice had significantly increased total CD4$^+$ T-cell numbers accumulated in tumors as compared with vehicle treated T-cell controls (Fig. 7E). Together, these data show that cytosolic Ca$^{2+}$ content is a novel property of antitumor immunity that could predict efficacy of adoptively transferred T cells.

**Discussion**

This work depicts the initial exploration of a vivid role for the ER in T-cell biology in both control of mitochondrial function and lineage fate. The defects in 96KO CD4$^+$ T cells were not unexpected due to the essential role of this chaperone protein in general immune cell biology previously elucidated by our laboratory (36, 16). Still, the profound impairment presented in 96KO T cells is particularly intriguing given that we show that antigenic stimulation specifically upregulates the ER stress response. Glucose-regulated proteins were discovered due to their upregulation in the context of glucose deprivation (37). In the face of acute cell stress, ER chaperones are mobilized at both the gene and protein levels to promote protein-folding functions in order to restore cellular homeostasis (38). It stands to reason that, as we have demonstrated, TCR engagement imparts a form of acute cell stress, as measured by upregulation of gp96 and grp78 gene and protein expression. Entry into glycolysis, a time of vigorous new protein synthesis, likely demands increased ER chaperone activity to support de novo protein biosynthesis.

In the context of TCR engagement, the specific role of the ER to promote T-cell metabolism has, to this point, remained largely unknown. We demonstrated that gp96 deficient CD4$^+$ T cells have defective Ca$^{2+}$ mobilization upon TCR engagement and are unable to undergo activation-induced glycolysis. The T-cell defect in the absence of gp96 is likely multifactorial and cannot be attributed to dysregulation of Ca$^{2+}$ mobilization only. However, we specifically addressed the roles of ER Ca$^{2+}$ exchange in the context of TCR engagement in CD4$^+$ T-cell activation using a pharmacological inhibitor of IP$_3$Rs. We found that that inhibition of IP$_3$Rs in T
cells results in a critical activation defect, followed by an inability to initiate glycolysis, consistent with the inability to mobilize cytosolic Ca\textsuperscript{2+} content (32). If results from other cell types may be extrapolated, IP\textsubscript{3}R-mediated Ca\textsuperscript{2+} release is a key driver of mitochondrial programming and function. Deletion of IP\textsubscript{3}Rs from B-lymphocytes show diminished mitochondrial Ca\textsuperscript{2+} uptake resulting in impaired cellular bioenergetics (39).

Cytosolic Ca\textsuperscript{2+} content was able to directly discriminate both CD4\textsuperscript{+} and CD8\textsuperscript{+} T-cell lineage fates. We reasoned that direct graded modulation of ER-mitochondrial Ca\textsuperscript{2+} exchange might similarly tune T-cell outcomes. Our data show that direct modulation of a second messenger ion could shift CD4\textsuperscript{+} T-cell fates. This was interesting given that costimulatory signals associated with graded crosslinking of CD28 drive IP\textsubscript{3}R-associated cytosolic Ca\textsuperscript{2+} rise only at the strongest levels of engagement (40).

We found in vivo that CD4\textsuperscript{+} T cells activated in the presence of IP\textsubscript{3}RI had augmented antitumor responses. Therapeutic efficacy of CD4\textsuperscript{+} T cells in the context of ACT generally involves intense cytokine conditioning (18). However, the requirements for generating therapeutically effective CD4\textsuperscript{+} T cells could be tempered simply by Ca\textsuperscript{2+} channel modulation. Together, our data suggest a role for Ca\textsuperscript{2+} in programming CD4\textsuperscript{+} T-cell fates and shed light on ER-mitochondrial crosstalk in T cells that may regulate cellular bioenergetics, survival, and function in vivo.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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References


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Modulation of Endoplasmic Reticulum Stress Controls CD4+ T-cell Activation and Antitumor Function

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