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Immunity. Author manuscript; available in PMC 2014 February 21.

Published in final edited form as:
Immunity. 2013 February 21; 38(2): 225–236. doi:10.1016/j.immuni.2012.10.020.

Mitochondria are required for antigen-specific T cell activation through reactive oxygen species signaling

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SUMMARY

It is widely appreciated that T cells increase glycolytic flux during activation, however the role of mitochondrial flux is unclear. Here we have shown that mitochondrial metabolism, in the absence of glucose metabolism, was sufficient to support interleukin-2 (IL-2) induction. Furthermore, we used mice with reduced mitochondrial reactive oxygen species (mROS) production in T cells (T-*Uqcrfs*$^{-/-}$ mice) to show that mitochondria are required for T cell activation to produce mROS for activation of nuclear factor of activated T cells (NFAT) and subsequent IL-2 induction. These mice could not induce antigen-specific expansion of T cells *in vivo*, however *Uqcrfs1*$^{-/-}$ T cells retained the ability to proliferate *in vivo* under lymphopenic conditions. This suggests that *Uqcrfs1*$^{-/-}$ T cells were not lacking bioenergetically, but rather lacked specific ROS-dependent signaling events needed for antigen-specific expansion. Thus, mitochondrial metabolism is a critical component of T cell activation through production of complex III ROS.

INTRODUCTION

T cells orchestrate the adaptive immune response and are critical for pathogen-specific defense and immunological memory. When quiescent naïve T cells are stimulated by antigen, they undergo an activation program that initiates rapid proliferation and primes them for differentiation to effector subtypes. Activation requires ligation of both the T cell receptor (TCR) and the co-stimulatory molecule CD28. The TCR and CD28 then initiate

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The authors declare no competing financial interests.

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integrated phosphorylation-based signaling cascades that result in activation of transcription factors, including nuclear factor of activated T cells (NFAT), activator protein-1 (AP-1), and nuclear factor of kappa light chain enhancer in B cells (NF-κB), to promote expression of genes that drive T cell activation such as interleukin 2 (IL-2) (Smith-Garvin et al., 2009).

A central part of the T cell activation program is a change in cellular metabolism. Proliferating cells have vastly different metabolic requirements than quiescent cells – while quiescent cells only need metabolism to support housekeeping functions and trafficking throughout the body, proliferating cells need to produce more ATP for enhanced activity, intermediates for biosynthesis, and signaling molecules to propagate anabolic metabolism.

The most dramatic change in T cell metabolism upon activation is a marked increase in glucose metabolism, which appears to be regulated by the PI3K and Akt pathway and the transcription factors Myc and ERRα (Frauwirth et al., 2002; Michalek et al., 2011; Wang et al., 2011). Activated T cells take up large amounts of glucose while simultaneously producing lactate. This has led to the widespread conceptual idea that activated T cells are primarily glycolytic despite ample oxygen supply, engaging in a process termed aerobic glycolysis (Jones and Thompson, 2007; Krauss et al., 2001; Pearce, 2010; Wang et al., 1976). However, while glycolysis may be important as a rapid source of ATP and as a conduit to the pentose phosphate pathway to generate NADPH and nucleotides, it is insufficient to provide the full complement of factors needed for cell proliferation. Interestingly, T cells also increase glutamine metabolism upon activation, and glutamine is required for T cell proliferation (Carr et al., 2010). Glutamine is primarily a mitochondrial substrate, as it can fuel the mitochondrial tricarboxylic acid (TCA) cycle through conversion to α-ketoglutarate in a process called glutaminolysis. Glutaminolysis is particularly important in proliferating cells in which TCA cycle intermediates are continually depleted for use in biosynthetic reactions (DeBerardinis et al., 2007). This suggests that mitochondrial metabolism may play an important role in T cell activation.

In addition to supporting biosynthesis, mitochondria are major sources of reactive oxygen species (ROS). Superoxide is generated at complexes I, II, and III of the mitochondrial electron transport chain (Turrens, 2003). Complexes I and II emit superoxide into the mitochondrial matrix where it is converted to hydrogen peroxide by superoxide dismutase 2 (SOD2). This hydrogen peroxide can then freely diffuse across the mitochondrial membranes into the intermembrane space and cytosol. Of note, complex III emits superoxide into both the matrix and the intermembrane space; intermembrane space superoxide can access the cytosol through VDAC channels without prior conversion to hydrogen peroxide (Han et al., 2003; Muller et al., 2004; Murphy, 2009). We and others have shown that mitochondrial ROS (mROS), particularly complex III ROS, can function as signaling intermediates (Byun et al., 2008; Schieke et al., 2008; Tormos et al., 2011; Weinberg et al., 2010). Interestingly, previous studies have shown that ROS are generated within 15 minutes of TCR cross-linking (Devadas et al., 2002) and treatment of mice with pan-antioxidants reduced T cell expansion (Laniewski and Grayson, 2004; Piganelli et al., 2002). Yet the source of these ROS and whether they are necessary for T cell activation has not previously been well defined.

Here we report a critical role for mitochondrial metabolism in T cell activation. Mitochondrial oxygen consumption increased during T cell activation, and fueling mitochondria was sufficient to support T cell activation. Mitochondrial ROS also increased during T cell activation, and this induction was regulated by calcium influx. Using mice with T cell-specific reduction of Rieske iron sulfur protein (RISP), a subunit of mitochondrial complex III, we show that mitochondrial metabolism, and specifically

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mitochondrial complex III ROS production, are essential for T cell activation both *in vitro* and *in vivo*.

RESULTS

Mitochondrial metabolism can support CD4⁺ T cell activation

In accordance with previous reports, we found that $CD4^+$ T cells increased their extracellular acidification rate (ECAR), a measure of lactic acid secretion indicative of rate of glycolysis, 4-fold following 24hr of CD3 and CD28 stimulation (Fig 1a). This phenomenon has historically eclipsed a role for mitochondria in T cell activation, yet we found that mitochondrial oxygen consumption (mitoOCR) also increased following stimulation (Fig 1b). To determine whether increased glycolysis or glucose metabolism is functionally important for T cell activation, we cultured $CD4^+$ T cells in media made with dialyzed serum with 10mM glucose added or omitted. In the absence of glucose, nearly all unstimulated and CD3 and CD28-stimulated cells were dead after 24 hours (Fig 1c). Interestingly, cell viability could be recovered by the addition of sodium pyruvate (Fig 1c), which bypasses upstream glucose metabolism and directly fuels the mitochondrial TCA cycle (Supplementary Fig 1a). $CD4^+$ T cells in pyruvate became dependent on mitochondrial metabolism, as they were sensitized to cell death by treatment with the mitochondrial complex I and complex III inhibitors, rotenone and antimycin (Supplementary Fig 1b). Furthermore, pyruvate did not rescue glucose deprivation-induced cell death by supporting glucose metabolism through gluconeogenesis, as the phosphoenolpyruvate carboxykinase inhibitor, 3-mercaptopropionic acid (3-MPA), had no effect on cell death (Supplementary Fig 1c). Thus, mitochondrial metabolism was sufficient to support $CD4^+$ T cell viability. Of note, the presence of glutamine was insufficient to maintain T cell viability in the absence of glucose (Fig 1c), however the downstream metabolite of glutaminolysis, α-ketoglutarate (cell permeable form is dimethylketoglutarate, DMK), partially rescued cell viability (Supplementary Fig 1d). This suggests that glutamine did not support viability in the absence of glucose because it failed to be converted to the TCA cycle intermediate, α-ketoglutarate. Wellen et al. similarly describe this effect and suggest that glucose is required for glycosylation events necessary for glutamine uptake (Wellen et al., 2010).

Importantly, mitochondrial metabolism was also sufficient to support $CD4^+$ T cell activation, as cells cultured with pyruvate or DMK in the absence of glucose upregulated surface expression of the activation markers CD69 and CD25 (Fig 1d and Supplementary Fig 1e–f) and induced interleukin-2 (IL-2) mRNA (Fig 1e and Supplementary Fig 1g) following CD3 and CD28 stimulation. Glutamine was required for full induction of IL-2 in glucose media, but was not necessary for full induction of IL-2 in pyruvate media (Fig 1e). This is likely because in the absence of glucose, pyruvate will not be converted to lactate due to lack of cytosolic NADH generated by glycolysis and will instead be fully available for use in the TCA cycle. In the presence of glucose, NADH is generated by glycolysis allowing for lactate dehydrogenase to convert pyruvate to lactate and NAD^+, reducing pyruvate availability to the TCA cycle and necessitating additional carbon influx through glutaminolysis (Supplementary Fig 1a). The requirement of glutamine for full induction of IL-2 in glucose media may support the idea that mitochondrial metabolism is also required for T cell activation. Nevertheless, these data clearly suggest that glucose metabolism is only required for T cell activation insomuch as it can provide pyruvate to fuel the mitochondria. As previously reported, both glucose and glutamine were necessary for $CD4^+$ T cell growth and proliferation following CD3 and CD28 stimulation (Fig 1f). This was expected given that glucose and glutamine are required for biosynthesis through the pentose phosphate, hexosamine, and glutaminolysis pathways (DeBerardinis et al., 2008; Lunt and Vander Heiden, 2011; Wellen et al., 2010). The unexpected aspect of these results is that

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mitochondrial metabolism alone is sufficient to support T cell viability and signaling for activation prior to cell growth and proliferation.

CD3-dependent calcium influx induces mROS, which are required for CD4$^+$ T cell activation

A consequence of mitochondrial metabolism is production of mitochondrial reactive oxygen species (mROS). Using CD4$^+$ T cells isolated from transgenic mice expressing a mitochondrial-targeted redox-sensitive GFP (mito-roGFP) (Guzman et al., 2010), we found that mROS spiked at 10 minutes following stimulation and were maintained at a higher level than baseline through 120 minutes (Fig 2a). To determine whether these ROS are functionally important for T cell activation, we treated CD4$^+$ T cells with the mitochondrial-targeted antioxidant Mito-Vitamin E (MVE). MVE is targeted to the mitochondria by covalent coupling to a triphenylphosphonium cation (TPP), which served as a control compound (Dhanasekaran et al., 2005). MVE attenuated IL-2 induction (Fig 2b) without affecting cell viability (Supplementary Fig 2), indicating that mROS are necessary for T cell activation.

Interestingly, mROS induction was mediated by CD3 stimulation, not CD28 stimulation (Fig 2c). Since CD3 activation leads to rapid influx of calcium, we predicted this calcium influx could regulate mROS induction, which also occurs early. Indeed, we found that influx of extracellular calcium was required for mROS induction, as chelation of extracellular calcium by EDTA or inhibition of the calcium release-activated calcium (CRAC) channel by BTP2 was sufficient to inhibit the mROS response (Fig 2d). Furthermore, influx of calcium into mitochondria was also required for mROS induction, as Ruthenium Red and Ru360 also inhibited the mROS response following CD3 and CD28 stimulation (Fig 2d). It is known that several TCA cycle enzymes are regulated by calcium (McCormack et al., 1990); thus calcium influx into mitochondria may increase TCA cycling, electron transport, and consequently mitochondrial membrane potential, which is associated with increased mROS production. In support of this, we found that reduction of mitochondrial membrane potential by FCCP diminished IL-2 induction, and this defect could be partially rescued by treatment with galactose oxidase (GaO) and 500uM galactose (Fig 2e). In the presence of galactose, GaO produces H_2O_2 in the culture media, which can freely diffuse into the cell (Wang et al., 1998). Thus, calcium influx through CRAC channels and subsequent calcium influx into mitochondria increase mROS production likely by increasing mitochondrial membrane potential, and this induction of mROS is required for T cell activation.

Mitochondrial complex III ROS are required for CD4$^+$ T cell activation

Previous reports indicate that the mitochondrial complex I inhibitor rotenone reduces CD4$^+$ IL-2 induction as well as CD8$^+$ T cell blasting, proliferation, and cytokine production (Kaminski et al., 2010; Yi et al., 2006), however inhibition of complex I coordinately reduces activity of the downstream complex III (Supplementary Fig 3). Previous work in our laboratory indicated that complex III is an important source of ROS for cell signaling (Bell et al., 2007; Tormos et al., 2011; Weinberg et al., 2010). Thus we hypothesized that complex III is the major site of mROS production in CD4$^+$ T cells. To test this, we sought to conditionally delete the *Uqcrfs1* gene in T cells in mice. *Uqcrfs1* is the gene that encodes Rieske Iron Sulfur Protein (RISP), a complex III subunit that is required for transfer of electrons downstream of complex III as well as complex III ROS production (Snyder et al., 1999). Mice that possess the *Uqcrfs1* gene flanked by loxP sites (*Uqcrfs1*$^{fl/fl}$) were crossed to mice that express *Cd4-cre*. Thymic cellularity (Fig 3a) and proportions and numbers of DN, DP, and SP cells (Fig 3b) were normal in *Uqcrfs1*$^{fl/fl}$; *Cd4-cre* mice, which permitted analysis of peripheral CD4$^+$ T cells. CD4$^+$ T cells isolated from *Uqcrfs1*$^{fl/fl}$; *Cd4-cre* mice had little detectable RISP protein by immunoblot (hereon called "*Uqcrfs1*$^{-/-}$ T cells" or "T-

Uqcrfs1$^{-/-}$ mice”), while non-T cell splenocytes possessed normal amounts (Fig 3c). T-*Uqcrfs1*$^{-/-}$ mice possessed similar numbers of total splenocytes, B cells, and macrophages as *Uqcrfs1*$^{fl/fl}$ littermate controls (hereon called “WT”), but a trend toward fewer CD4^{+} T cells and significantly fewer CD8^{+} T cells in the periphery (Fig 3d).

As expected, *Uqcrfs1*$^{-/-}$ CD4^{+} T cells produced significantly reduced amounts of activation-induced mROS as measured by MitoSOX Red (Fig 4a) and exhibited a diminished oxygen consumption rate compared to WT T cells (Supplementary Fig 4a). Surprisingly, these cells possessed a normal quantity of mitochondria, normal mitochondrial membrane potential, and a normal rate of glycolysis as determined by rate of extracellular acidification (Supplementary Fig 4b–d). Thus, we expect *Uqcrfs1*$^{-/-}$ T cells to specifically lack mitochondrial complex III ROS and mitochondrial production of ATP (oxidative phosphorylation), but not all mitochondrial function such as orchestration of cell death. In fact, there was no change in cell viability after 24h of cell culture between WT CD4^{+} T cells and *Uqcrfs1*$^{-/-}$ CD4^{+} T cells (Supplementary Fig 4e). However, *Uqcrfs1*$^{-/-}$ CD4^{+} T cells failed to induce IL-2 (Fig 4c) and had reduced expression of activation markers CD69 and CD25 (Fig 4b) when stimulated with anti-CD3 and anti-CD28. Importantly, IL-2 induction could be rescued by treatment with GaO and Gal (Fig 4c), which indicates that hydrogen peroxide derived from complex III is required for IL-2 induction. Further, IL-2 induction could also be rescued by stimulation with PMA and ionomycin (Fig 4d), which induced mROS production (likely at complexes I and II) to a comparable amount as stimulation of WT T cells with anti-CD3 and anti-CD28 (Fig 4e). IL-2 induction by PMA and ionomycin in *Uqcrfs1*$^{-/-}$ T cells was sensitive to MVE (Fig 4d), further indicating that PMA and ionomcyin stimulation could compensate for lack of complex III ROS by inducing ROS through complexes I and II. Thus complex III ROS are required for IL-2 induction and T cell activation.

IL-2 induction necessitates activation of the MAPK pathway, which has been reported to be redox-sensitive (Kamata et al., 2005). Yet we found that ERK1/2 phosphorylation was intact following CD3 and CD28 stimulation of *Uqcrfs1*$^{-/-}$ T cells (Supplementary Fig 4f). Multiple reports also suggest that NF-κB requires ROS for activation (Morgan and Liu, 2011), however phosphorylation of IκB appeared normal in the *Uqcrfs1*$^{-/-}$ T cells (Supplementary Fig 4g). Instead, we found that NFAT activation was defective in the *Uqcrfs1*$^{-/-}$ T cells – although *Uqcrfs1*$^{-/-}$ cells expressed similar quantities of cytosolic NFAT1, they failed to induce translocation of NFAT1 to the nucleus upon CD3 and CD28 stimulation (Fig 4f). To confirm this, we performed ChIP and found that NFAT1 failed to bind the IL-2 promoter upon CD3 and CD28 stimulation in *Uqcrfs1*$^{-/-}$ cells (Supplementary Fig 4h). As expected, PMA and ionomycin stimulation rescued NFAT1 nuclear translocation in *Uqcrfs1*$^{-/-}$ cells (Supplementary Fig 4i). Expression of the NFAT-dependent, activation-induced gene IL-3 was similarly diminished in *Uqcrfs1*$^{-/-}$ T cells as IL-2, while expression of the NFAT-independent, activation-induced gene Myc was unchanged (Supplementary Fig 4j–k). Therefore complex III ROS are required for NFAT activation.

NFAT is activated by dephosphorylation by calcineurin, which allows it to translocate to the nucleus and function as a transcription factor. Calcineurin is dependent on calcium and calmodulin; therefore we wanted to determine whether the CRAC channel was functional in the *Uqcrfs1*$^{-/-}$ cells. We found that intracellular calcium store release by IP3-dependent calcium channels was normal in the *Uqcrfs1*$^{-/-}$ cells (Fig 4g, arrow) indicating that assembly and activation of the upstream TCR complex is intact in these cells. CRAC channel activation was also intact (Fig 4g), which supports the data that calcium influx through CRAC channels is upstream of mROS induction in T cell activation. This suggests that calcium plays multiple roles in T cell activation, including activation of calcineurin

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through calmodulin, as well as stimulation of mROS. Together, these data indicate that mitochondrial complex III ROS are required for $CD4^+$ T cell activation through NFAT *in vitro*.

Mitochondrial complex III ROS are required for antigen-specific $CD4^+$ T cell expansion *in vivo*

Uqcrfs1$^{-/-}$ $CD4^+$ T cells did not proliferate *in vitro* upon CD3 and CD28 stimulation or PMA and ionomycin stimulation, even with the addition of recombinant IL-2 protein (Supplementary Fig 5). This was likely due to an inability to generate biosynthetic intermediates that require an intact TCA cycle for their synthesis, not due to lack of CD25 (IL-2 receptor) surface expression, as CD25 was reduced but not absent (Fig 4b). To determine whether *Uqcrfs1*$^{-/-}$ $CD4^+$ T cells could proliferate *in vivo*, we measured homeostatic expansion of purified *Uqcrfs1*$^{fl/fl}$ or *Uqcrfs1*$^{-/-}$ $CD4^+$ T cells in Rag1-deficient mice. Surprisingly, the *Uqcrfs1*$^{-/-}$ cells had no defect in homeostatic expansion (Fig 5a), suggesting that these cells meet their ATP and biosynthetic demands necessary for proliferation in the *in vivo* environment. However, when we co-injected *Uqcrfs1*$^{fl/fl}$ or *Uqcrfs1*$^{-/-}$ T cells with congenic WT T cells, we found that under competitive conditions, *Uqcrfs1*$^{-/-}$ T cells did not proliferate as well as *Uqcrfs1*$^{fl/fl}$ T cells (Fig 5b). Interestingly, T-*Uqcrfs1*$^{-/-}$ mice possessed more naïve $CD4^+CD62L^+CD44^-$ cells and fewer activated $CD4^+CD62L^-CD44^+$ cells than littermate controls (Fig 5c). This suggested that the *Uqcrfs1*$^{-/-}$ $CD4^+$ T cells may possess a defect in activation and antigen-specific, TCR-dependent proliferation that could explain the decrease in abundance. To test this possibility, we immunized mice with the LCMV peptide GP61 (amino acids 61–80 in the glycoprotein) in complete Freund's adjuvant (CFA) and used MHC class II I-A^b LCMV GP61 tetramers to measure expansion of GP61-specific $CD4^+$ T cells after 6 days (Wojciechowski et al., 2006). While GP61-specific $CD4^+$ T cells were observed in WT T cells, the T-*Uqcrfs1*$^{-/-}$ mice failed to induce expansion of GP61-specific $CD4^+$ T cells (Fig 5d). These data indicate that RISP is required for antigen-specific expansion of $CD4^+$ T cells *in vivo*. Because the *Uqcrfs1*$^{-/-}$ $CD4^+$ T cells are capable of undergoing lymphopenia-induced homeostatic expansion *in vivo*, their deficiency in antigen-specific expansion is likely due to insufficient complex III ROS generation required for T cell activation, not due to an inability to meet biosynthetic or bioenergetic demands.

Mitochondrial complex III ROS are required for antigen-specific $CD4^+$ T cell-dependent inflammation *in* vivo

To determine whether deficiency of RISP in T cells would affect progression of a $CD4^+$ T cell-dependent inflammatory disease, we assessed the susceptibility of the T-*Uqcrfs1*$^{-/-}$ mice to the ovalbumin model of allergic asthma. Briefly, T-*Uqcrfs1*$^{-/-}$ mice and littermate controls were sensitized and boosted with a mixture of OVA peptide in alum or PBS in alum, challenged 3 weeks later with aerosolized OVA inhalation, and sacrificed for analysis of lung inflammation (Bryce et al., 2006). Lung inflammation in this model is entirely dependent on $CD4^+$ T cell function (Afshar et al., 2008). As expected, we found that the T-*Uqcrfs1*$^{-/-}$ mice did not exhibit an eosinophilic infiltrate in the bronchoalveolar lavage (BAL) fluid (Fig 6a) nor signs of lung inflammation by histology (Fig 6b). Furthermore, the Th2-associated cytokines, interleukin-4 (IL-4), interleukin-5 (IL-5), and interleukin-13 (IL-13), were not increased in the lung tissue as is typically seen in this model (Fig 6c). We also noted that the T-*Uqcrfs1*$^{-/-}$ mice failed to induce production of OVA-specific IgE (Fig 6d), a B cell process that requires assistance from $CD4^+$ T cells for activation and class switching.

To ensure that the T-*Uqcrfs1*$^{-/-}$ mice are capable of inducing an allergic response when T cell participation is bypassed, we administered recombinant IL-4 protein intratracheally for

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3 days, and then assessed lung inflammation. We detected an eosinophilic infiltration into the BAL fluid (Fig 6e) as well as inflammation by histology (Supplementary Fig 6) in the T-*Uqcrfs1*$^{-/-}$ mice. Therefore the defect in induction of allergic asthma in the OVA model in these mice is due to dysfunctional CD4$^+$ T cells, not dysfunction of a downstream cell type. Together, these experiments suggest that RISP is required for expansion of antigen-specific CD4$^+$ T cells to promote lung inflammation.

Mitochondrial complex III ROS are required for antigen-specific CD8$^+$ T cell expansion *in vivo*

Given that *Cd4-cre* drives expression of Cre recombinase in both CD4$^+$ and CD8$^+$ T cells during the double positive phase of T cell development and we saw a decrease in numbers of CD8$^+$ T cells in the spleen (Fig 3d), we wanted to determine if CD8$^+$ T cells also require RISP for antigen-specific expansion and function. To do this, we infected T-*Uqcrfs1*$^{-/-}$ mice with a sublethal dose of *L. monocytogenes* expressing OVA (LM-OVA). Clearance of *L. monocytogenes* after infection is mediated by T cells, with CD8$^+$ T cells providing the most substantial contribution to protective immunity (Pamer, 2004). Following a primary infection, antigen-specific H2-M3-restricted T cells reach peak frequencies 5 to 6 days later, while MHC class Ia-restricted T cells reach peak frequencies 7 to 8 days following inoculation (Cho et al., 2011). Thus, we harvested splenic and hepatic leukocytes 7 days after infection and analyzed by flow cytometry. Like CD4$^+$ T cells, the *Uqcrfs1*$^{-/-}$ CD8$^+$ T cells failed to undergo antigen-specific expansion (Fig 7a). Furthermore, when splenic and hepatic leukocytes were restimulated *in vitro* with OVA peptide$_{257–264}$ (SIINFEKL) or heat-killed LM (HKLM), very few *Uqcrfs1*$^{-/-}$ CD8$^+$ T cells produced interferon-γ (IFN-κ) (Fig 7b).

To see if the *Uqcrfs1*$^{-/-}$ CD8$^+$ T cells could mount a memory response, we reinfected mice with LM-OVA 1 month after primary infection. We saw a robust induction of the OVA-specific CD8$^+$ T cells in the WT mice after 3 days, but few in the T-*Uqcrfs1*$^{-/-}$ mice (Fig 7c). While bacterial burden following primary infection can be regulated by the innate immune response, bacterial burden following secondary infection is more dependent on the memory T cell response. We noted that the bacterial burden in the spleen was greatly increased in the T-*Uqcrfs1*$^{-/-}$ mice, suggesting ineffective immune response and clearance of this secondary infection (Fig 7d). Thus, as for CD4$^+$ T cells, RISP is required for antigen-specific expansion of CD8$^+$ T cells.

DISCUSSION

Activated T cells have a very different metabolic profile from naïve T cells. Previous studies have highlighted the critical role of increased glucose metabolism in T cell activation. In contrast, this study highlights the critical role of increased mitochondrial metabolism in T cell activation. Our results indicate that glucose metabolism only becomes important during T cell proliferation. Prior to proliferation, mitochondrial metabolism is sufficient to support cell signaling for T cell activation. Importantly, our results show that mitochondrial ROS specifically derived from complex III were required for CD4$^+$ T cell activation *in vitro* and antigen-specific CD4$^+$ and CD8$^+$ T cell expansion *in vivo*. We found that *Uqcrfs1*$^{-/-}$ T cells, which lack oxidative phosphorylation and complex III ROS production, did not express IL-2 following CD3 and CD28 stimulation *in vitro*. Exogenous hydrogen peroxide produced by addition of GaO and Gal rescued IL-2 induction in *Uqcrfs1*$^{-/-}$ cells, and treatment of WT T cells with a mitochondrial-targeted antioxidant phenocopied *Uqcrfs1*$^{-/-}$ T cells, indicating that mROS, not oxidative phosphorylation, are necessary for T cell activation *in vitro*. Moreover, *Uqcrfs1*$^{-/-}$ T cells did not proliferate upon various antigen stimulation *in vivo*, but retained the ability to proliferate under lymphopenic conditions. Thus, these cells do not possess major defects in bioenergetic or biosynthetic pathways *in vivo*, and we can attribute

their inability to expand upon antigen stimulation *in vivo* to lack of mROS for T cell activation.

Historically, mROS were thought to be primarily cytotoxic by directly damaging DNA, lipids, and proteins (Finkel and Holbrook, 2000). Recent studies indicate that mROS are not categorically harmful, and low levels of ROS are important for healthy cell function (Sena and Chandel, 2012). This is becoming clear in innate immunity, as mROS appear to be essential for a wide range of innate immune function, including antiviral, antibacterial, and antiparasitic responses (West et al., 2011b). For example, West et al. recently reported that stimulation of cell surface toll-like receptors (TLRs) leads to an increase in mROS production that is needed for clearance of *Salmonella typimurium* (West et al., 2011a). TLR signaling through mROS appears to be important in human disease, as cells from TRAPS (tumor necrosis factor receptor-associated periodic symdrome) patients exhibit greater responsiveness to LPS due to increased mROS production (Bulua et al., 2011). Other studies have identified a critical role for mROS in RIG-1-like receptor (RLR) signaling (Tal et al., 2009) and NLRP3 inflammasome activation (Zhou et al., 2011). The present study suggests that adaptive immune cells follow suit with innate immune cells and also require mROS for activation and function.

Our results suggest that mROS induction following CD3 and CD28 stimulation of T cells is mediated by CD3-initiated calcium signaling. We found that both influx of calcium into the cytosol through CRAC channels and influx of calcium into the mitochondria were required for mROS induction. Several TCA cycle enzymes, including pyruvate dehydrogenase and α-ketoglutarate dehydrogenase, are regulated by calcium (McCormack et al., 1990), thus we predict calcium influx into mitochondria increases TCA cycling and electron transport chain (ETC) flux, consequently increasing mitochondrial membrane potential and mROS production. Indeed we show here that mitochondrial membrane potential is required for IL-2 induction through ROS. Previous studies have shown that mitochondria localize to the immunological synapse during T cell activation, where they appear to regulate local Ca^{2+} influx (Schwindling et al.). This localization would also allow for efficiency of short-lived ROS signals that are continually targeted for neutralization by antioxidant proteins, such as superoxide dismutases, catalases, glutathione, peroxiredoxins, among others.

We have identified the target of mROS to be downstream of the CRAC channel and upstream of NFAT in the calcium, calcineurin, and NFAT pathway in T cell activation. Despite precedents in the literature for ROS regulation of NF-κB and MAPK activity, we found these pathways to be unaffected by lack of complex III mROS in the present system. Given that we can rescue IL-2 induction in RISP-KO T cells within 24h by treatment with exogenous peroxide, we suspect that mROS are required for a post-translational protein modification, rather than alteration of protein expression. Future studies will determine the specific complex III mROS molecular target in T cell NFAT activation. It is possible that mROS alter dephosphorylation of NFAT by calcineurin or rephosphorylation of NFAT by kinases like GSK3β to regulate the quantity of nuclear, active NFAT.

An important implication of this study is that therapeutic strategies must consider the essential role of low levels of ROS in normal physiology. We predict that antioxidant therapy or supplements may prove to be detrimental during infection due to the necessity of mROS for T cell activation.

Furthermore, this study promotes the viewpoint that cell metabolism can regulate cell function. It is clear that T cells engage in a specified or programmed change in metabolism upon antigen stimulation and do not solely exhibit homeostatic changes in metabolite flux dictated by increased demands (Jones and Thompson, 2007). This programmed change in

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metabolism anticipates metabolic requirements for progression to a new cell fate. For maximal efficiency, we propose that a readout of metabolism, mROS, feeds back and impinges on signaling pathways to ensure cooperation of both cellular signaling and cellular metabolism for important cell fate decisions. Thus, cellular metabolism is not merely a structured series of reactions responsive only to energetic needs, but rather a malleable, adaptive program that is highly integrated with cell signaling. Our data indicates that mitochondria, classically known as 'the powerhouse of the cell,' are also signaling organelles that play a critical role in regulation of T cell activation.

METHODS

Animal experiments

Animal experiments were conducted in accordance with Northwestern University's institutional guidelines on the treatment of animals.

Oxygen consumption, extracellular acidification, and ROS measurement

Oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) were measured using the Extracellular Flux Analyzer (XF24, Seahorse Bioscience) according to the manufacturer's protocol. Mitochondrial OCR was determined by subtracting OCR following 2uM rotenone (Sigma) and 2uM antimycin (Sigma) treatment from basal OCR. Mitochondrial ROS production was measured either by mito-roGFP fluorescence or by MitoSOX Red (Invitrogen) fluorescence. For mito-roGFP experiments, $CD4^+$ T cells were isolated from spleens of Mito-roGFP transgenic mice (gift from Paul Schumacker). Mito-roGFP contains two surface-exposed cysteine residues that can form a disulfide bond when oxidized by hydrogen peroxide or superoxide. Disulfide bond formation increases the excitation spectrum peak near 400nm at the expense of the peak near 490nm. The ratios of fluorescence from excitation at 400 and 490nm indicate the extent of oxidation of the probe and thus the redox potential within the mitochondrial matrix. Cells were stimulated with anti-CD3 and anti-CD28 and ratiometric measurements of excitation were taken. The mito-roGFP probe was calibrated by treating cells with 1mM DTT for complete reduction and with 1mM t-butyl H_2O_2 for complete oxidation. Percent oxidized probe was determined by using the formula: % oxidized probe = X-DTT / H2O2-DTT. For MitoSOX Red experiments, 5uM MitoSOX Red in complete media was added to cells at 37°C 15 mins prior to harvesting. MFI was measured by flow cytometry.

Homeostatic Expansion in Rag1-deficient mice

$CD4^+$ T cells were purified, stained with CFSE, and 2×10^6 were injected retroorbitally IV into Rag1-deficient mice. Mice were sacrificed four days later and spleens and mesenteric lymph nodes were removed for analysis by flow cytometry. For competitive homeostatic expansion, $CD4^+$ T cells were purified from both Thy1.1 mice (Jackson Laboratory) and Thy1.2, *Uqcrfs1*$^{fl/fl}$ or *Uqcrfs1*$^{fl/fl}$; *Cd4-cre* mice, stained with CFSE, and co-injected (1:1 ratio; 2×10^6 cells each) retroorbitally IV into Rag1-deficient mice.

GP61-specific $CD4^+$ Expansion

Six- to 8-week-old mice were immunized with 150ug GP61 peptide in CFA or CFA alone subcutaneously. Mice were sacrificed 6 days later and spleens and inguinal lymph nodes were removed for analysis by flow cytometry (Wojciechowski et al., 2006). I-A^b-GP61 tetramer was a gift from David Hildeman.

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OVA Immunization, airway inflammation, and serum antibody levels

Four- to 8-week-old female mice were sensitized to OVA by intraperitoneal injection of 10 μg OVA (Grade VI; Sigma-Aldrich) in alum (3 mg) or alum alone at days 0 and 14. These mice were then challenged for 20 minutes with aerosolized 1% OVA by ultrasonic nebulization on days 21, 22, and 23 and were studied on day 24. Mice were killed and the lungs immediately flushed with 0.8 ml BAL fluid (10% FCS, 1 mM EDTA, ×1 PBS) via the trachea. Total cells were counted and remaining BAL fluid was cytospun onto slides and differential cell counts performed after staining with DiffQuik (Baxter). Tissue sections and histological staining were performed by Histo-Scientific Research Laboratories. Serum was collected on day 24 and OVA-specific IgE was determined by sandwich ELISA. OVA-specific IgE was determined by comparison with a standard curve constructed with the use of purified mouse OVA-specific IgE secreted by the hybridoma TOε.

Bacteria, listeria infections, and CFU assays

The recombinant LM strain rLM-ovalbumin (OVA) was grown in brain-heart infusion broth supplemented with 5 μg/mL erythromycin. For primary infections, mice were intraobitally infected with 2×10^4 cfu rLM-OVA. Recall infection with 4×10^4 CFU rLM-ova was performed 1 month after primary infection. At the indicated times after infection, mice were sacrificed and bacterial burden were determined (Dal Porto et al., 1993).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Drs. Balaraman Kalyanaraman and Joy Joseph for their kind contribution of MVE. This work was supported by NIH grants F30ES019815 (L.A.S.), T32-HL76139 (L.A.S.), 5P01HL071643 (N.S.C.), R01-AI076456 (P.B.), NS047599 (A.J.), R01-AI057753 (D.A.H.), R01-AI040310 (C.W.), RR025355 (P.T.S.), HL35440 (P.T.S.), AR050250 (H.P.), AR054796 (H.P.), AI092490 (H.P.), and HL108795 (H.P.) and funds provided by Northwestern University, Feinberg School of Medicine to Harris Perlman.

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HIGHLIGHTS

- Mitochondrial metabolism is sufficient to support T cell activation *in vitro*
- Mitochondrial ROS are induced by TCR-initiated calcium influx
- Mitochondrial complex III ROS are required for NFAT activation and IL-2 induction
- Induction of mROS is required for antigen-specific T cell responses *in vivo*

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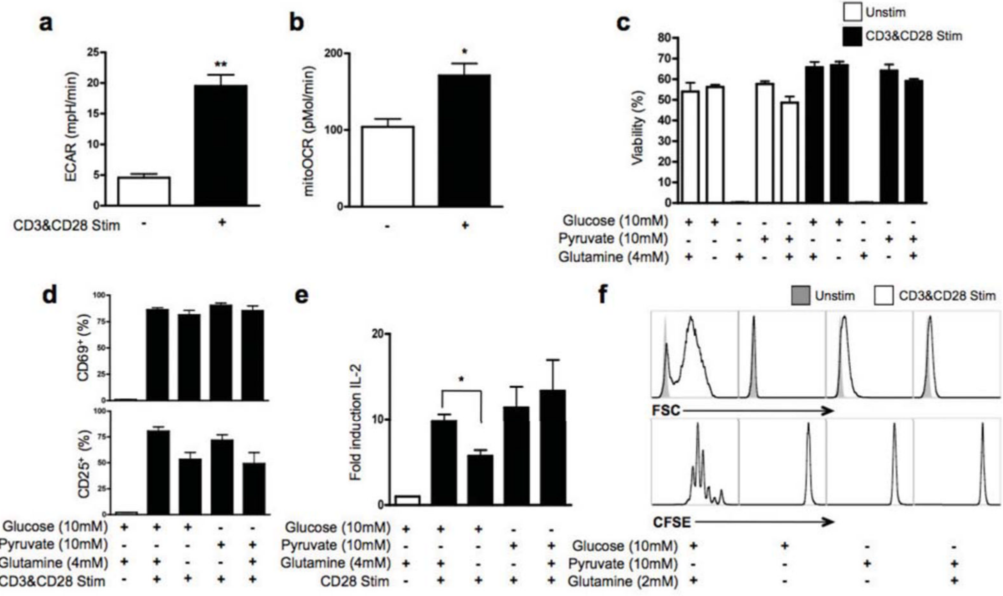

Figure 1. Mitochondrial metabolism is sufficient to support CD4$^+$ T cell activation

a, Extracellular acidification rate (ECAR) and **b,** mitochondrial oxygen consumption rate (mitoOCR) of freshly isolated CD4$^+$ T cells and 24h CD3 and CD28-stimulated CD4$^+$ T cells (n=3 ± SEM). **c**, Cell viability of CD4$^+$ T cells cultured in indicated media with and without CD3 and CD28 stimulation at 24h (n=3 ± SEM). **d**, Surface expression of CD69 and CD25 of CD4$^+$ T cells cultured in indicated media at 24h (n=3 ± SEM). **e**, Relative IL-2 mRNA expression of CD4$^+$ T cells cultured in indicated media at 24h, normalized to ML-19 expression (n=3 ± SEM). **f**, Cell growth (48h) and cell proliferation (96h) of CD4$^+$ T cells cultured in indicated media (n=3). *$p<0.05$, **$p<0.01$

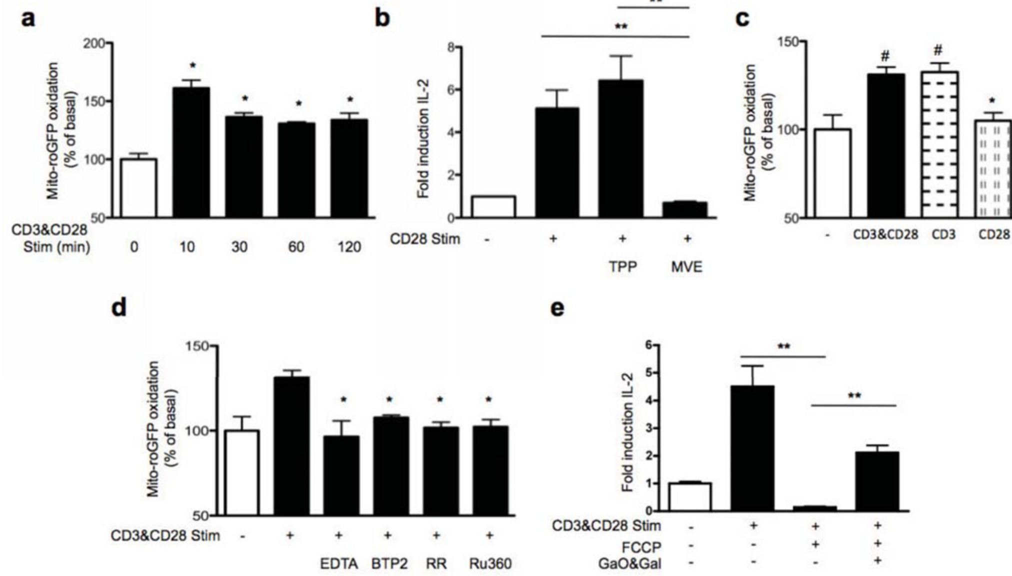

Figure 2. CD3-dependent calcium influx induces mROS, which are required for T cell activation

a, Mitochondrial ROS production measured by percent oxidized mito-roGFP probe of $CD4^+$ T cells isolated from Mito-roGFP transgenic mice following CD3 and CD28 stimulation (n=3 ± SEM). **b**, Relative IL-2 mRNA expression of $CD4^+$ T cells cultured with MVE and TPP (1.5uM each) at 8h, normalized to ML-19 expression (n=3 ± SEM). **c**, Mitochondrial ROS production measured by percent oxidized mito-roGFP probe of $CD4^+$ T cells isolated from Mito-roGFP transgenic mice following indicated stimulation (n=3 ± SEM). # indicates a significant difference from column 1, * indicates a significant difference from column 2. **d**, Mitochondrial ROS production measured by percent oxidized mito-roGFP probe of $CD4^+$ T cells isolated from Mito-roGFP transgenic mice following CD3 and CD28 stimulation with calcium inhibitors (EDTA 1mM, BTP2 200nM, Ruthenium Red (RR) 5uM, and Ru360 5uM) (n=3 ± SEM). **e**, Relative IL-2 mRNA expression of $CD4^+$ T cells cultured with FCCP (1uM) and GaO (0.045U/ml) & Gal (500uM) at 4h, normalized to ML-19 expression (n=3 ± SEM). */#p<0.05, **p<0.01

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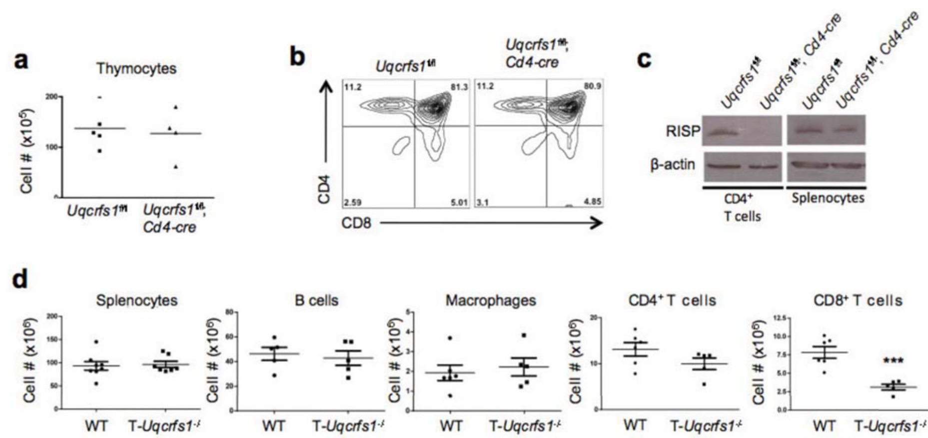

Figure 3. *Uqcrfs1*$^{fl/fl}$; *Cd4-cre* mice have reduced expression of RISP in T cells

a, Total number of cells isolated from mouse thymuses (n=5 ± SEM). **b,** Surface protein expression of CD4 and CD8 in mouse thymus (n=5 ± SEM). **c**, RISP protein expression in purified $CD4^+$ splenic T cells and total splenocytes from *Uqcrfs1*$^{fl/fl}$ and *Uqcrfs1*$^{fl/fl}$; *Cd4-cre* mice (n=4). **d**, Total number of splenocytes (following RBC lysis), B cells ($CD45^+B220^+$), macrophages ($CD45^+F4/80^+$), $CD4^+$ T cells ($CD45^+CD4^+$), and $CD8^+$ T cells ($CD45^+CD8^+$) in *Uqcrfs1*$^{fl/fl}$ and *Uqcrfs1*$^{fl/fl}$; *Cd4-cre* spleens (n=5–7 ± SEM). *p<0.05, **p<0.01, ***p<0.001

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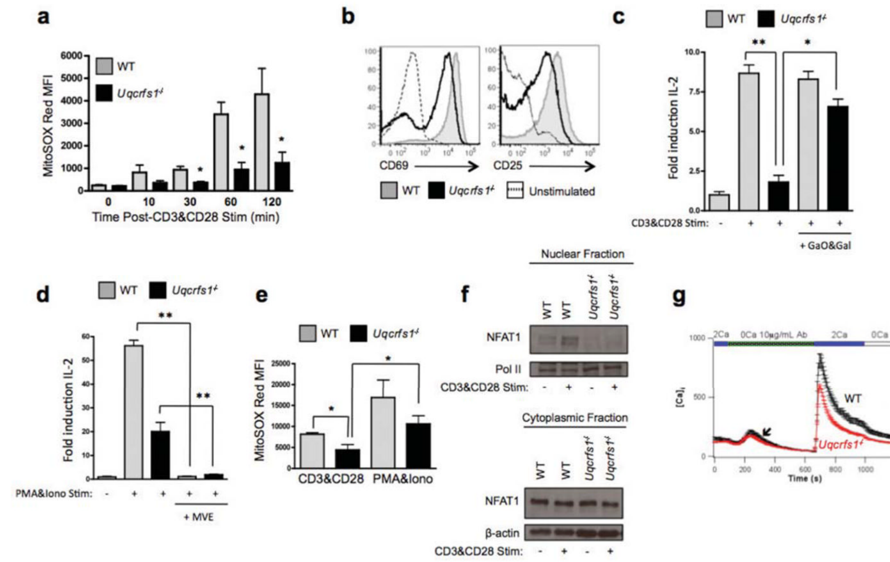

Figure 4. Mitochondrial complex III reactive oxygen species are required for antigen-specific CD4⁺ T cell expansion

a, Mitochondrial ROS production measured by MitoSOX Red fluorescence following CD3 and CD28 stimulation (n=3 ± SEM). **b**, Surface protein expression of CD69 and CD25 in $CD4^+$ T cells isolated from *Uqcrfs1*$^{fl/fl}$ and *Uqcrfs1*$^{fl/fl}$; *Cd4-cre* mice (n=3 ± SEM). **c**, Relative IL-2 mRNA expression of $CD4^+$ T cells isolated from *Uqcrfs1*$^{fl/fl}$ and *Uqcrfs1*$^{fl/fl}$; *Cd4-cre* mice stimulated with anti-CD3 and anti-CD28, with and without treatment with GaO (0.045U/ml) and Gal (500uM), normalized to ML-19 expression (n=2 ± SEM). **d**, Relative IL-2 mRNA expression of $CD4^+$ T cells isolated from *Uqcrfs1*$^{fl/fl}$ and *Uqcrfs1*$^{fl/fl}$; *Cd4-cre* mice stimulated with PMA/ionomycin, with and without treatment with MVE (1.5uM), normalized to ML-19 expression (n=3 ± SEM). **e**, Mitochondrial ROS production measured by MitoSOX Red fluorescence (n=4 ± SEM). **e**, Nuclear and cytosolic NFAT1 levels measured by isolation of nuclear and cytosolic lysates, then immunoblot (n=3). **g,** Intracellular calcium concentration measured by Fura2-AM. Cells were transferred from 2mM calcium Ringer solution to 0mM calcium solution with 10ug/ml anti-CD3 antibody to stimulate intracellular store release (arrow). Cells were then transferred to 2mM calcium to allow for store-operated calcium influx (CRAC channel influx) (n=3). *p<0.05, **p<0.01, ***p<0.001

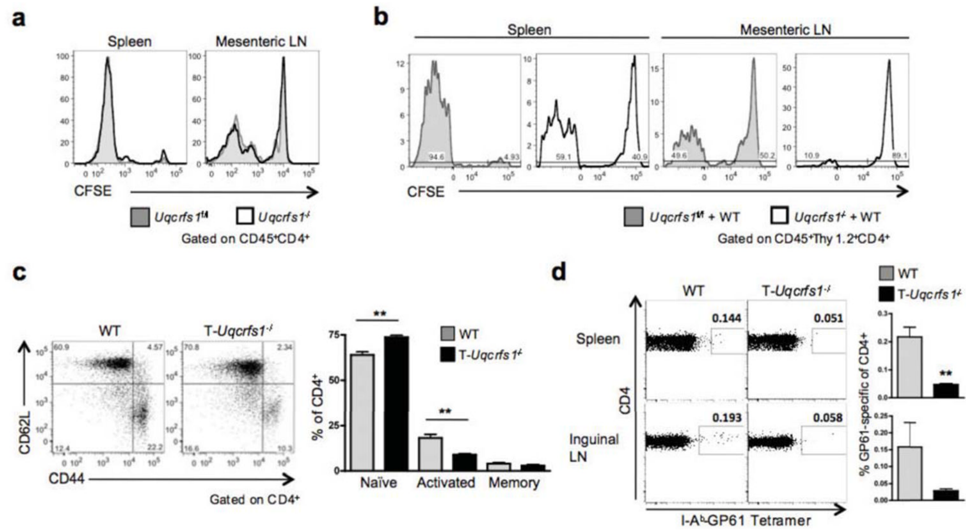

Figure 5. Mitochondrial complex III ROS are required for CD4+ antigen-specific CD4+ T cell expansion

a, Cell proliferation on day 4 of CD4+ T cells isolated from RISP$^{fl/fl}$ or RISP T KO mice and transferred into Rag1 KO mice (n=3). **b**, Cell proliferation on day 4 of CD4+ T cells isolated from RISP$^{fl/fl}$ or RISP T-KO mice and co-transferred with Thy1.1 CD4+ T cells into Rag1 KO mice (n=3). **c**, Surface protein expression of CD62L and CD44 in CD4+ populations from RISP$^{fl/fl}$ or RISP T KO spleens (n=5 ± SEM). **d**, Expansion of GP61-specific CD4+ T cells 6 days following IP injection of GP61 peptide in CFA into *Uqcrfs1*$^{fl/fl}$ or *Uqcrfs1*$^{fl/fl}$; *Cd4-cre* mice (n=4 ± SEM). *p<0.05, **p<0.01

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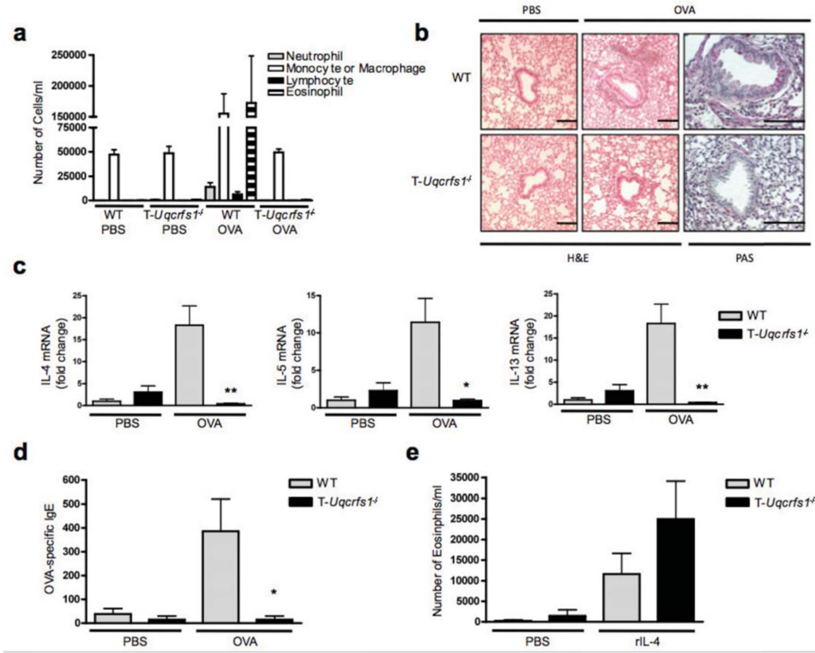

Figure 6. Mitochondrial complex III reactive oxygen species are required for antigen-specific CD4^{+} T cell-dependent inflammation *in vivo*

a–d, Ova-immunized (OVA) or sham-immunized (PBS) *Uqcrfs1*$^{fl/fl}$ and *Uqcrfs1*$^{fl/fl}$; *Cd4-cre* mice were exposed to OVA by inhalation and airway inflammation was assessed (n=4 ± SEM, representative of 2 experiments). **a**, Cellular composition of BAL fluid. **b**, Representative histological lung sections stained with H&E or PAS. Bars, 100um. **c**, Relative IL-4, IL-5, IL-13 mRNA expression from homogenized lungs, normalized to β-actin expression. **d**, Serum ova-specific IgE antibody level determined by ELISA. **e**, Five micrograms of recombinant mouse IL-4 or PBS was administered intranasally for 3 days and airway inflammation was assessed. Number of eosinophils in BAL fluid (n=5 ± SEM). *p<0.05, **p<0.01

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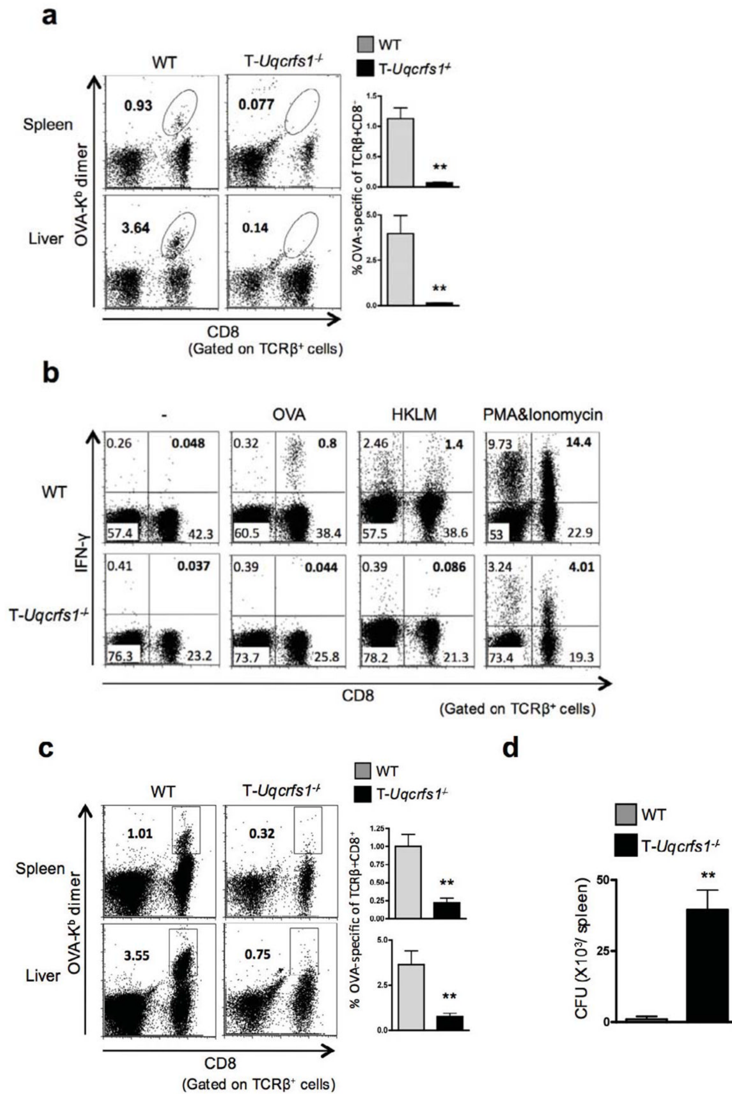

Figure 7. Mitochondrial complex III reactive oxygen species are required for antigen-specific $CD8^+$ T cell expansion

a, Frequency of OVA/K^b-specific $CD8^+$ T cells in LM-infected *Uqcrfs1*$^{fl/fl}$ and *Uqcrfs1*$^{fl/fl}$; *Cd4-cre* mice. RISP$^{fl/fl}$ and RISP T KO mice were infected with rLM-OVA. On day 7 post-infection, splenocytes and hepatic leukocytes were stained with anti-TCRb, anti-CD8, and OVA/K^b dimer and analyzed by flow cytometry (n=5 ± SEM). **b,** Frequency of OVA/K^b-specific, IFN-g–producing $CD8^+$ T cells in LM-infected *Uqcrfs1*$^{fl/fl}$ and *Uqcrfs1*$^{fl/fl}$; *Cd4-cre* mice. Splenocytes from indicated mice were harvested on day 7 after LM infection and stimulated with either OVA peptide or HKLM. Cells were stained for CD8 expression and intracellular IFN-g, and analyzed by flow cytometry (n=5 ± SEM). **c,** Frequency of OVA/K^b-specific $CD8^+$ T cells in *Uqcrfs1*$^{fl/fl}$ and *Uqcrfs1*$^{fl/fl}$; *Cd4-cre* mice following a second LM-infection. One month after primary LM infection (2×10^3 CFU), mice were re-challenged with 4×10^4 CFU of rLM-OVA. On day 3 after secondary infection, splenocytes and hepatic leukocytes were harvested and stained with anti-TCRb, anti-CD8, and OVA/K^b dimer and analyzed by flow cytometry (n=5 ± SEM). **d,** Bacterial burden in spleens of LM-

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infected *Uqcrfs1*$^{fl/fl}$ and *Uqcrfs1*$^{fl/fl}$; *Cd4-cre* mice on day 3 following secondary infection (n=5 ± SEM). *p<0.05, **p<0.01