CD8 memory T cells have a bioenergetic advantage that underlies their rapid recall ability

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A characteristic of memory T (T_M) cells is their ability to mount faster and stronger responses to reinfection than naïve T (T_N) cells do in response to an initial infection. However, the mechanisms that allow this rapid recall are not completely understood. We found that CD8 T_M cells have more mitochondrial mass than CD8 T_N cells and, that upon activation, the resulting secondary effector T (T_E) cells proliferate more quickly, produce more cytokines, and maintain greater ATP levels than primary effector T cells. We also found that after activation, T_M cells increase oxidative phosphorylation and aerobic glycolysis and sustain this increase to a greater extent than T_N cells, suggesting that greater mitochondrial mass in T_M cells not only promotes oxidative capacity, but also glycolytic capacity. We show that mitochondrial ATP is essential for the rapid induction of glycolysis in response to activation and the initiation of proliferation of both T_N and T_M cells. We also found that fatty acid oxidation is needed for T_M cells to rapidly respond upon restimulation. Finally, we show that dissociation of the glycolysis enzyme hexokinase from mitochondria impairs proliferation and blocks the rapid induction of glycolysis upon T-cell receptor stimulation in T_M cells. Our results demonstrate that greater mitochondrial mass endows T_M cells with a bioenergetic advantage that underlies their ability to rapidly recall in response to reinfection.

metabolism | lymphocytes

Naïve T (T_N) and memory T (T_M) cells are quiescent, but upon T-cell receptor (TCR)-mediated recognition of antigen (Ag) and costimulation, they become activated, undergo clonal expansion, and acquire effector functions. Although both T_N and T_M cells acquire effector functions, a noted characteristic of T_M cells is their ability to respond more rapidly than T_N cells to Ag (1–4). Several factors underlie the accelerated recall response of T_M cells. For example, Ag-specific T_M cells are present in greater numbers than T_N cells. In addition, several intrinsic aspects of T_M cells have been suggested to contribute to their ability to respond more efficiently, such as increased activity of proximal TCR signaling components, an "open" chromatin conformation of cytokine genes, and altered transcriptional profiles (1, 2, 5–9). However, whether bioenergetic differences contribute to this process is not clear.

Resting cells like T_N and T_M cells interchangeably use glucose, amino acids, and lipids to fuel the tricarboxylic acid (TCA) cycle and oxidative phosphorylation (OXPHOS) for ATP production (10–12). However, proliferating cells like activated T cells promote aerobic glycolysis, which supports cell growth, proliferation, and effector functions (13–16). Although T_N and T_M cells both have a similar metabolism, there are metabolic differences between these cells (17). T_M cells from *Listeria monocytogenes*-infected mice have more mitochondria than T_N cells, which is consistent with our finding that T_M cells but not T_N cells have considerable spare respiratory capacity (SRC) (17). Because SRC is important for cellular survival and function (17–19) and T_M cells are characterized by their ability to respond vigorously to Ag reencounter (20), we investigated whether bioenergetic differences between T_M and T_N cells contribute to the ability of T_M cells to rapidly recall in response to reinfection. We show here that T_M cells have more mitochondria and ATP than T_N cells and that, upon activation, secondary T_E cells proliferate faster, produce cytokines more quickly, and maintain more ATP than primary effector T (T_E) cells. In addition, T_M cells use both OXPHOS and glycolysis to a greater and more prolonged degree after activation than T_N cells, suggesting that more mitochondria in T_M cells not only promote OXPHOS, but also glycolysis. We show that both the rapid proliferation and induction of glycolysis in T_M cells depend on mitochondrial ATP. We also found that optimal mitochondrial function is predominantly fueled by fatty acid oxidation (FAO) in T_M cells and depends on the association of hexokinase (HK) with mitochondria. Thus, our data demonstrate that greater mitochondrial mass underlies the rapid recall ability of T_M cells.

Results

To explore whether bioenergetic differences between T_M and T_N cells account for the ability of T_M cells to rapidly recall upon activation, we generated "memory" T cells in vitro by activating OT-I T_N cells with OVA peptide and IL-2 for 3 d and then differentially cultured cells for 3–4 additional days in IL-15 (17, 21). To determine whether IL-15 T_M cells have more mitochondria than T_N cells, as we have shown for T_M cells generated after infection (17), we stained cells with Mitotracker green and found that IL-15 T_M cells had more mitochondrial mass (Fig. 1 *A* and *B*). Although IL-15 T_M cells are slightly bigger than T_N cells, greater size does not necessarily correlate with increased mitochondrial content. We have shown that in vitro-generated IL-2–induced T_E (IL-2 T_E) cells are much larger than IL-15 T_M cells and actually have less mitochondrial content (17). IL-15 T_M cells also have a significantly higher ratio of mitochondrial DNA/nuclear DNA (mtDNA/nDNA) than T_N cells (Fig. 1*C*). Together these data indicate that in vitro-generated IL-15 T_M cells, like T_M cells generated after infection (17), have greater mitochondrial mass than T_N cells and provide a model from which to study bioenergetic differences between these cells.

To verify that IL-15 T_M cells respond faster to stimulation than T_N cells, we measured proliferation of T_N and IL-15 T_M cells after activation with anti-CD3/28. Secondary T_E cells (derived from IL-15 T_M cells) proliferated faster than primary T_E cells (derived from T_N cells; Fig. 2*A*). We also found that secondary T_E cells produced more IFN-γ (Fig. 2*B*) and IL-2 (Fig. S1). Together, these data confirm that IL-15 T_M cells proliferate more quickly and produce more cytokines after activation than T_N cells.

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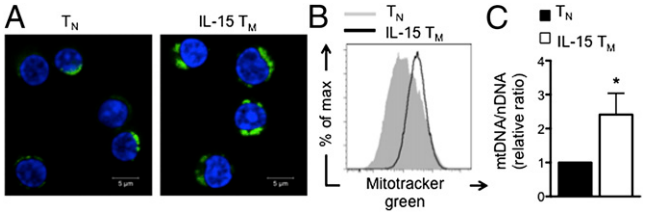

Fig. 1. IL-15 T_M cells have more mitochondrial mass than T_N cells. OT-I cells were activated with OVA peptide for 3 d in IL-2 and cultured in IL-15 for 3–4 d to generate IL-15 T_M cells, which were compared with OT-I T_N cells. (*A*) T_N and IL-15 T_M cells stained with Mitotracker green (green) and DRAQ5 (blue). (*B*) Mitotracker green staining was quantified by flow cytometry. Data are representative of three experiments. (*C*) mtDNA/nDNA ratio, mean ± SEM, from six experiments; $*P < 0.05$.

To determine whether differences in mitochondrial content between T_N and IL-15 T_M cells result in metabolic differences after activation, we stained cells with Mitotracker Deep Red, a dye that localizes to respiring mitochondria. Secondary T_E cells proliferated faster and had more respiring mitochondria than primary T_E cells (Fig. 3*A*). We also measured O_2 consumption rates (OCR), an indicator of OXPHOS, and extracellular acidification rates (ECAR), an indicator of aerobic glycolysis. Two days after restimulation, secondary T_E had higher OCR and ECAR than primary T_E cells (Fig. 3*B*), indicating increased OXPHOS and aerobic glycolysis, respectively, and correlating with the faster proliferation of these cells (Figs. 2*A* and 3*A*). We measured ATP and found that secondary T_E cells have more ATP than primary T_E cells after restimulation and that resting IL-15 T_M cells have more ATP than resting T_N cells (Fig. 3 *C* and *D*). Taken together, these data demonstrate that secondary T_E cells have more metabolic activity and ATP than primary T_E cells.

Given our findings that IL-15 T_M cells have enhanced mitochondrial content and more ATP than T_N cells, we next determined their metabolic phenotype in resting conditions. IL-15 T_M cells had higher OCR and ECAR than T_N cells (Fig. 4*A*), indicating that resting IL-15 T_M cells are metabolically more active. Relative utilization of glycolysis and OXPHOS, as indicated by OCR/ECAR ratio, is the same in both cells (Fig. 4*A*), suggesting that the IL-15 T_M cells revert to a quiescent metabolic state rather than preferentially using glycolysis over OXPHOS like activated T_E cells. Because T_M cells respond to reinfection more rapidly than T_N cells, we measured the bioenergetics of T cells immediately after activation. We activated T_N and IL-15 T_M cells with anti-CD3/28 and found that IL-15 T_M cells increased OCR and ECAR (Fig. 4*B*). As T_N cells minimally responded in this time frame, we used a stronger stimulus that would also bypass differences in TCR signaling (6). T_N and IL-15 T_M cells rapidly increased OCR after PMA/ionomycin (iono) (Fig. 4 *C* and *D*), which was proportionally similar at the peak, but more sustained in IL-15 T_M cells. Both T_N and IL-15 T_M cells increased ECAR after PMA/iono; however, this increase was higher at the peak and sustained longer in IL-15 T_M cells, indicating their greater capacity to promote and sustain glycolysis upon stimulation (Fig. 4 *C* and *D*). Furthermore, unlike T_N cells, mitochondrial activity in IL-15 T_M cells remained intact after PMA/iono, as indicated by increased OCR in response to the uncoupler carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone (FCCP), which reveals maximum respiratory capacity, in the presence of oligomycin (oligo) (blocks ATP synthase), and decreased OCR after etomoxir (FAO inhibitor), and rotenone plus antimycin A (R+A) [electron transport chain (ETC) inhibitors] (Fig. S2). Together these data indicate that IL-15 T_M cells are bioenergetically different from T_N cells and have a greater capacity to promote and sustain glycolysis and OXPHOS after activation.

We next questioned how having more mitochondrial mass could contribute to the rapid recall ability and the enhanced glycolytic capacity of IL-15 T_M cells. We activated T_N and IL-15 T_M cells with anti-CD3/28 and followed proliferation in the absence or presence of oligo. Similar to our findings in CD4 T_N cells (16), initiation of proliferation of both CD8 T_N and IL-15 T_M cells was impaired in the presence of low doses of oligo (5–10 nM) (Fig. 5*A* and Fig. S3). In contrast, even high doses of oligo (1 μM) did not stop proliferation of actively proliferating T cells (Fig. 5*B*), showing that the initiation of proliferation, rather than the process of active proliferation, requires mitochondrial ATP. The low doses of oligo that inhibited proliferation (Fig. 5*A*) also reduced OCR (Fig. 5*C*). Together these data indicate that although IL-15 T_M cells were previously activated, they have returned to a resting metabolic state and have the same requirements for activation as T_N cells. To determine whether mitochondrial ATP was required for the greater glycolytic capacity of IL-15 T_M cells, we added oligo together with PMA/iono. The increase in OCR after activation was diminished in IL-15 T_M cells when low doses of oligo were present (Fig. 5*D*), indicating that OXPHOS is induced after activation and produces mitochondrial ATP. The increase in ECAR in IL-15 T_M cells after activation was also inhibited by low doses of oligo (Fig. 5*D*), suggesting that mitochondrial ATP is needed for the induction of glycolysis and the rapid proliferation of IL-15 T_M cells after activation.

We next measured the bioenergetics of in vivo-generated T_M cells isolated from *L. monocytogenes* (expressing OVA, LmOva) infected mice. Anti-CD3/28 stimulation increased OCR and ECAR in T_M cells and, to a lesser extent, ECAR in polyclonal T_N cells (Fig. 6*A*). Two hours after activation, both cell types responded to oligo, FCCP, and R+A (Fig. 6*B*), indicating intact mitochondrial function. In addition, both T_N and T_M cells rapidly increased ECAR and OCR after PMA/iono, which was sustained in T_M but not T_N cells (Fig. 6 *C* and *D*). Like IL-15 T_M cells (Fig. S2), T_M cells increased OCR when exposed to oligo plus FCCP 2 h after activation and reduced OCR after R+A, whereas T_N cells did not (Fig. 6*C*). Together these data indicate that T_M cells sustain glycolysis and OXPHOS after activation and that their mitochondrial function remains intact after PMA/iono. T_N cells did not lose mitochondrial function when exposed to media (Fig. S4) or anti-CD3/28 (Fig. 6*B*), suggesting that this loss of mitochondrial function is not an intrinsic defect of T_N cells, but rather a result of PMA/iono. We also verified that resting T_M cells had more ATP than T_N cells, which was not due to greater cell size (Fig. 6*E*). Likewise, secondary T_E cells

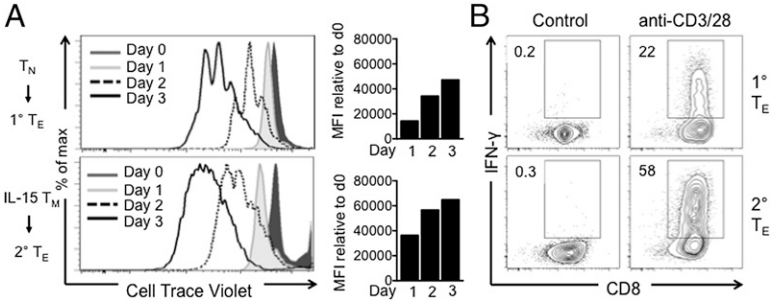

Fig. 2. Secondary T_E cells proliferate faster and make more IFN-γ than primary T_E cells. Naïve OT-I and IL-15 T_M cells were (re)stimulated with anti-CD3/28 for 3 d. (*A*) Proliferation by Cell Trace Violet dilution at indicated time points. Bar graphs represent difference in mean fluorescence intensity (MFI) at the indicated time point relative to $t = 0$. (*B*) IFN-γ production 2 d after (re)stimulation; representative of ≥2 experiments.

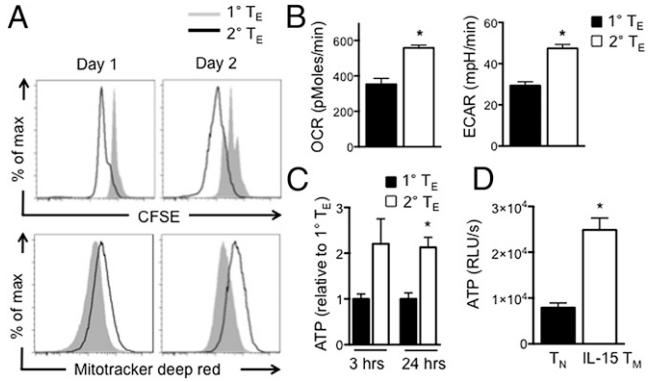

Fig. 3. Enhanced secondary T_E cell proliferation is marked by greater metabolic activity and ATP production. Naïve OT-I and IL-15 T_M cells were (re)stimulated with anti-CD3/28. (*A*) Proliferation and Mitotracker deep red staining 1 and 2 d after (re)stimulation. (*B*) OCR and ECAR in primary and secondary T_E cells 2 d after stimulation; mean ± SEM; $*P < 0.0001$. (*C* and *D*) ATP in primary and secondary T_E cells 3 and 24 h after (re)stimulation with anti-CD3/28 (*C*), and in naïve and IL-15 T_M cells (*D*). Data are shown as mean ± SEM; $*P < 0.005$ (*C*) and < 0.001 (*D*); representative of ≥2 experiments.

derived from T_M cells had more ATP (Fig. 6*F*) and proliferated faster than primary T_E cells, whereas the initiation of proliferation in both T_N and T_M cells was impaired by low doses of oligo (Fig. 6*G*). Together, these data indicate that T_M cells, like IL-15 T_M cells, have a greater glycolytic capacity and proliferate faster upon stimulation than T_N cells, which is facilitated by mitochondrial ATP.

We have shown that T_M cells preferentially use FAO for their energy (17). To determine whether FAO contributes to the mitochondrial ATP required for T_M cell activation, we activated T_M and IL-15 T_M cells in the presence of etomoxir and followed proliferation. We found that proliferation was attenuated, but not completely suppressed, when FAO was blocked (Fig. 7 *A* and *B* and Fig. S5), suggesting that FAO contributes to, but is not the only pathway fueling OXPHOS in these cells. T_N cell proliferation was also reduced when FAO was blocked (Fig. S6). To test whether FAO inhibition impairs the rapid induction of glycolysis after activation, we exposed IL-15 T_M cells to etomoxir immediately before activation with PMA/iono. Etomoxir inhibited the increase in OCR and blunted ECAR after activation (Fig. 7*C*), indicating that FAO fuels OXPHOS and contributes to the rapid activation of IL-15 T_M cells. T_M cells in the presence of etomoxir failed to increase OCR, but not ECAR, after PMA/iono (Fig. S7), and also failed to increase OCR after oligo plus FCCP, indicating that FAO contributes to the maximum respiratory capacity after activation. We also modulated the expression of carnitine palmitoyltransferase 1a (CPT1a), a protein that is the rate-limiting step to FAO, and the target of etomoxir (22, 23). We transduced T cells with retrovirus expressing shRNA against CPT1a (hpCPT1a) (17). Inhibiting CPT1a expression in IL-15 T_M cells slowed initial proliferation after anti-CD3/28 activation (Fig. 7*D*), although this effect was more subtle and variable than the consistently strong inhibition by etomoxir. To investigate whether pyruvate fuels this process, we used a retrovirus expressing shRNA against the mitochondrial pyruvate carrier (hpMPC1) (24). Although this shRNA resulted in lower MPC1 mRNA expression (Fig. S8), there was no difference in proliferation compared with the control cells (Fig. 7*D*). Consistent with our data showing that FAO contributes to the proliferation of IL-15 T_M cells after activation, we found that hpCPT1a IL-15 T_M cells had impaired induction of OCR and ECAR after PMA/iono stimulation, whereas hpMPC1 IL-15 T_M cells did not (Fig. 7 *E* and *F*). Flux analysis of ^{13}C-glucose comparing IL-2 T_E cells, which are highly glycolytic and use substantial amounts of glucose, to IL-15 T_M cells showed that the relative abundance of unlabeled carbon was greater than that of labeled carbon for TCA cycle intermediates in IL-15 T_M cells (Fig. S9). These data indicate that pyruvate flux into the TCA cycle is lower in IL-15 T_M cells, which may suggest that other substrates contribute to OXPHOS. Together these data indicate that FAO fuels the OXPHOS required for the rapid recall response of IL-15 T_M cells.

We next wanted to determine how mitochondrial ATP could contribute to the rapid increase in glycolysis in IL-15 T_M cells upon activation. HK mediates the ATP-dependent conversion of glucose into glucose-6-phosphate in the first step of glycolysis. Upon T-cell activation, HK is recruited to the mitochondria where ATP is abundant (25). We used clotrimazole (CLT), a drug that dissociates HK from the mitochondria (Fig. 8*A*) (26), or peptides that consist of the N-terminal sequence of HK and compete with HK for mitochondrial binding (27, 28), to explore whether mitochondrial-associated HK is critical for the rapid induction of glycolysis in (IL-15) T_M cells. We found that the ability of IL-15 T_M cells to rapidly increase OCR and ECAR after PMA/iono was impaired in the presence of CLT or HK peptides (Fig. 8*B* and Fig. S10). CLT did not destroy mitochondrial activity, because R+A decreased OCR after CLT (Fig. S11*A*). Likewise, 10 μM of either HK-1 or HK-2 peptides left OXPHOS intact, whereas the combination of both resulted in the inability to increase OCR after oligo + FCCP (Fig. S11*B*), indicating a loss of mitochondrial function. In addition, IL-15 T_M and T_N cell proliferation after anti-CD3/28 stimulation was impaired by CLT (Fig. 8*C* and Fig. S12) or HK-1 competing peptide (Fig. 8*D*). CLT also decreased proliferation (Fig. 8*E*) and impaired OCR and ECAR after activation (Fig. 8*F*) in T_M cells. Mitochondrial function remained intact in T_M cells treated with CLT as shown by the increase in OCR after oligo plus FCCP and decrease in OCR after R+A (Fig. 8*F*). These data indicate that mitochondria-associated HK is critical for the rapid induction of glycolysis and proliferation of (IL-15) T_M cells.

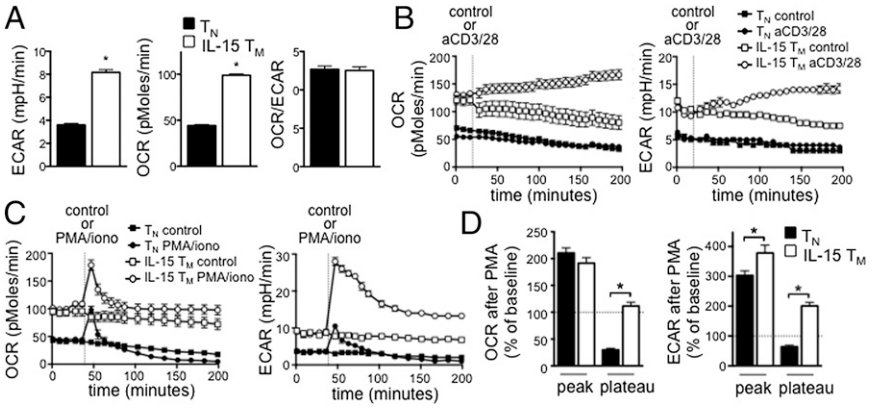

Fig. 4. IL-15 T_M cells have enhanced glycolytic capacity compared with T_N cells after activation. (*A*) Basal ECAR and OCR were measured in OT-I T_N and IL-15 T_M cells; $*P < 0.0001$. T_N and IL-15 T_M cells were stimulated with anti-CD3/28 beads (*B*) or with PMA/iono (*C*) and OCR and ECAR measured. Data in *A*–*C* are from the same experiment and are representative of ≥3 experiments. (*D*) Compiled and baselined data as shown in *C*, from two experiments; peak is first measurement after PMA/iono, plateau is at 120 min. $*P < 0.0001$ (*Left*), and < 0.05 and < 0.001 (*Right*); mean ± SEM.

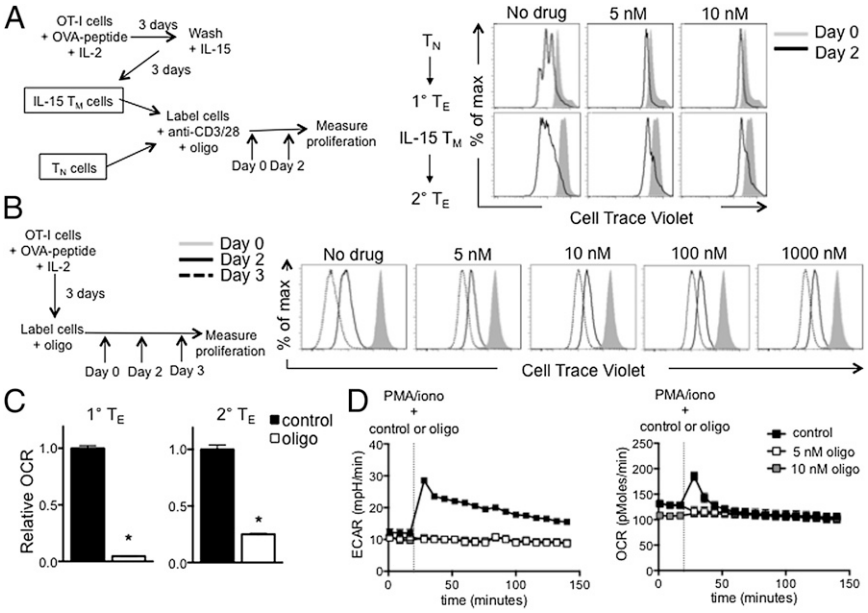

Fig. 5. Mitochondria-derived ATP is required for the initiation of proliferation and facilitates glycolysis in IL-15 T_M cells after PMA/iono. (*A*) OT-I T_N and IL-15 T_M cells were (re)stimulated with anti-CD3/28 with or without oligo (added at day 0), and proliferation is shown at days 0 and 2; representative of four experiments. (*B*) OT-I cells were activated with OVA peptide and IL-2 for 3 d, then labeled with Cell Trace Violet and oligo added (day 0). Proliferation was observed at days 0, 2, and 3; representative of two experiments. (*C*) Relative OCR in primary and secondary T_E cells 2 d after anti-CD3/28, ±5 nM oligo; mean ± SEM, representative of two experiments; $^*P < 0.0001$. (*D*) IL-15 T_M cells were restimulated with PMA/iono, with or without oligo, and OCR and ECAR were measured. Data are shown as mean ± SEM, representative of three experiments.

Discussion

We propose a model where substantial mitochondrial mass and ATP enable T_M cells to rapidly recall upon restimulation. The enhanced availability of mitochondrial ATP provides the energy that is required for the rapid engagement of glycolysis observed in T cells after activation. The HK-mediated conversion of glucose into glucose-6-phosphate requires ATP, and we show here that blocking the generation of mitochondrial ATP, and the dissociation of HK from the mitochondria, impair the rapid induction of glycolysis and hamper the fast proliferation of T_M cells after activation. The proliferation of T_N cells after activation was also impaired when mitochondrial ATP was unavailable, suggesting that both T_N and T_M cells have similar requirements for activation and that quantitative bioenergetic differences underlie the distinct abilities of these cells to respond to Ag. Qualitative bioenergetic differences may also exist between T_N and T_M cells. Although both T_N and T_M cells increased glycolysis after anti-CD3/28, and responded to uncoupling and ETC inhibitors, T_N cells lost metabolic activity after PMA/iono, suggesting that after this stimulation, T_N cells cannot efficiently fuel their mitochondria, whereas T_M cells can. Several factors could contribute to this disparity, such as differences in the strength of activation, the signaling pathways involved, or the acquisition or storage of substrates.

Although quantitative differences in Ag-specific T_N and T_M cells can contribute to the superior protection conferred by T_M cells upon reinfection, studies where equal cell numbers were transferred into mice have shown that these cells respond with distinct kinetics (1, 3, 5, 29, 30). T_M cells enter the cell cycle earlier and proliferate faster than T_N cells after activation (3–5). Because T_M cells are different from T_N cells in their ability to migrate to peripheral tissues (30–32), these experiments do not exclude the possibility that T_M cells respond more quickly because they encounter Ag before T_N cells. Consistent with previous

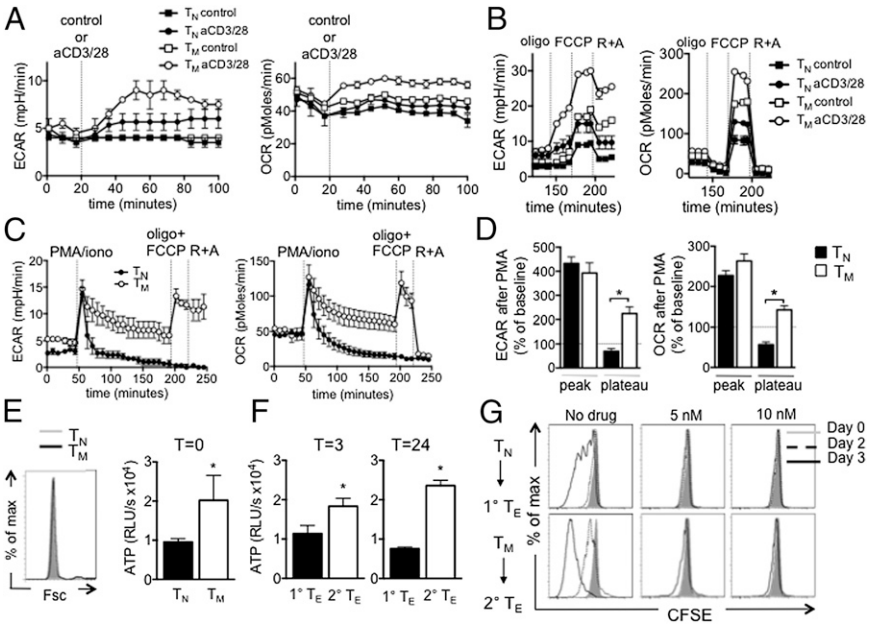

Fig. 6. T_M cells have enhanced glycolysis, more ATP, and proliferate faster than T_N cells after activation. CD8 T_N ($CD44^{lo}$ $CD62L^{hi}$) and T_M ($CD44^{hi}$ $CD62L^{hi}$) cells were isolated from naïve and LmOVA-infected mice. OCR and ECAR in T_N and T_M cells after stimulation with aCD3/28 coated beads (*A*), and after subsequent oligo (1 μM), FCCP (1.5 μM), and rotenone (100 nM) plus antimycin A (1 μM) injections (*B*) (data in A and B are from the same experiment). (*C*) T_N and T_M cells were PMA/iono stimulated, exposed to oligo + FCCP, and R+A, and OCR and ECAR measured. T_N is the same as Fig. S4, T_M is the same as Fig. 8*F* and Fig. S7. (*D*) Compiled and baselined data as shown in *C*, from two experiments; peak is first measurement after PMA/iono, plateau is at 120 min. $^*P < 0.0005$ (*Left*), and < 0.0001 (*Right*). Forward scatter and ATP of resting T_N and T_M cells (*E*), and ATP in primary and secondary T_E cells 3 and 24 h after aCD3/28 (*F*). (*G*) T_N and T_M cells stimulated with aCD3/28 with or without oligo (day 0) and proliferation is shown. T_N control is the same as in Figs. S6*B* and S12*B*. T_M control is the same as Fig. 7*A* and 8*E*; mean ± SEM, representative of two (*A*, *B*, and *E*), 3 (*C*), or one (*F* and *G*) experiment(s).

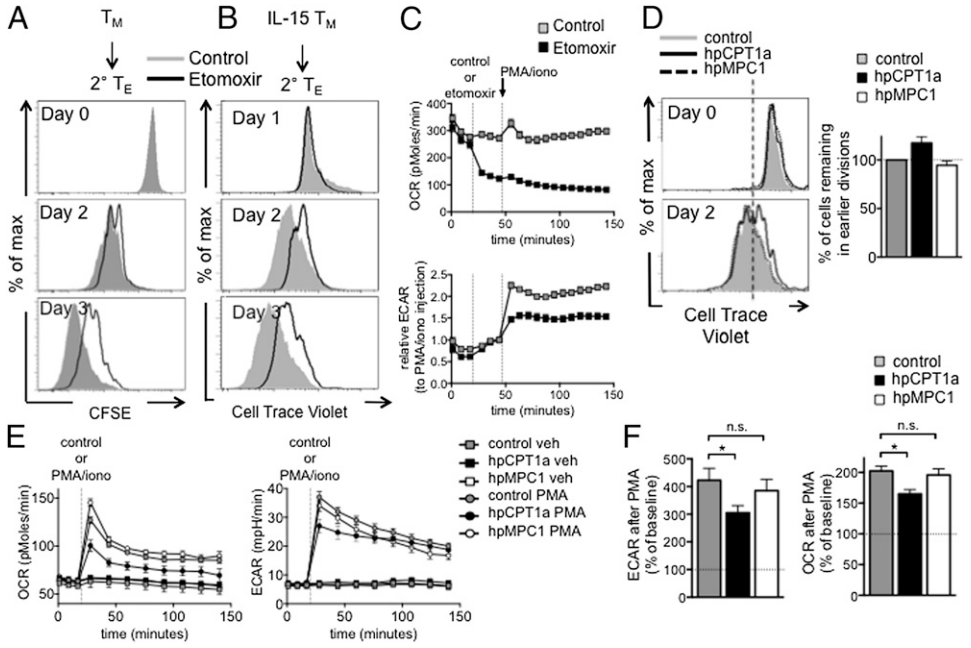

Fig. 7. FAO fuels the OXPHOS needed for rapid recall of T_M cells. (*A*) Proliferation of T_M cells from LmOVA-infected mice after anti-CD3/28 ± etomoxir (day 0); control is same as Fig. 6*G* and 8*E*. Data are from one experiment. (*B*) Proliferation of IL-15 T_M cells after anti-CD3/28 ± etomoxir; representative of four experiments. (*C*) OCR and ECAR of IL-15 T_M cells after PMA/iono ± etomoxir; mean ± SEM, representative of four experiments. (*D–F*) Proliferation of IL-15 T_M cells expressing control (shRNA against luciferase), hpCPT1a (shRNA against CPT1a), or hpMPC1 (shRNA against MPC1) retrovirus after anti-CD3/28 (*D*); graph shows percent of cells normalized to control cells in gate with fewer divisions (separated by line), generated from two experiments. (*E*) OCR and ECAR of control, hpCPT1a, and hpMPC1 IL-15 T_M cells after PMA/iono. (*F*) Compiled and baselined data as shown in *E*, from three experiments; peak is at first measurement after PMA/iono, plateau is at 120 min. n.s., not significant. $^*P < 0.05$; mean ± SEM (*E* and *F*) and representative of two (*D*) or four (*E*) experiments.

studies (3, 4, 33), we show here that T_M cells are intrinsically different as they proliferate faster and make more cytokine after activation in vitro. This more ready-to-respond state of T_M cells has been attributed to several intrinsic differences such as an "open" chromatin conformation of cytokine genes, altered transcription profiles, and enhanced activity of proximal TCR signaling components (1, 2). We now show here that rapid proliferation and engagement of glycolysis by T_M cells after activation requires mitochondrial ATP to enable optimal HK function. Although primary T_E cells have been shown to exhibit prolonged proliferation after infection compared with secondary T_E cells (5, 34), and ultimately T_N cells have a greater per-cell expansion than T_M cells (34), these observations do not contradict that T_M cells have a greater capacity to proliferate more rapidly immediately after stimulation.

CD8 T_M cells use FAO, which contributes to their substantial SRC that is important for survival and stable T_M development (17). We show here that FAO in T_M cells also provides the substrates, at least partly, for the OXPHOS that is required for the rapid induction of glycolysis and the fast proliferative response of these cells. When IL-15 T_M cells are transduced with the hpCPT1a retrovirus, the effect on the inhibition of OCR and proliferation is more subtle and variable than when fully differentiated IL-15 T_M cells are acutely exposed to etomoxir. Our published data show 50% reduction in CPT1a mRNA by using this construct (17), suggesting only partial FAO inhibition. Unlike direct exposure to etomoxir, the hpCPT1a retrovirus does not acutely impair FAO. When transduced cells are cultured for 5 d before proliferation and OCR are measured, it is likely that they adapt and begin to rely less on FAO. Given the importance of CPT1a in in vivo T_M cell development, and that IL-15 induces CPT1a expression in vitro (17), we expect that the hpCPT1a-transduced T cells that do survive until day 6 in the IL-15 cultures are selected on their ability to use other substrates, i.e.,

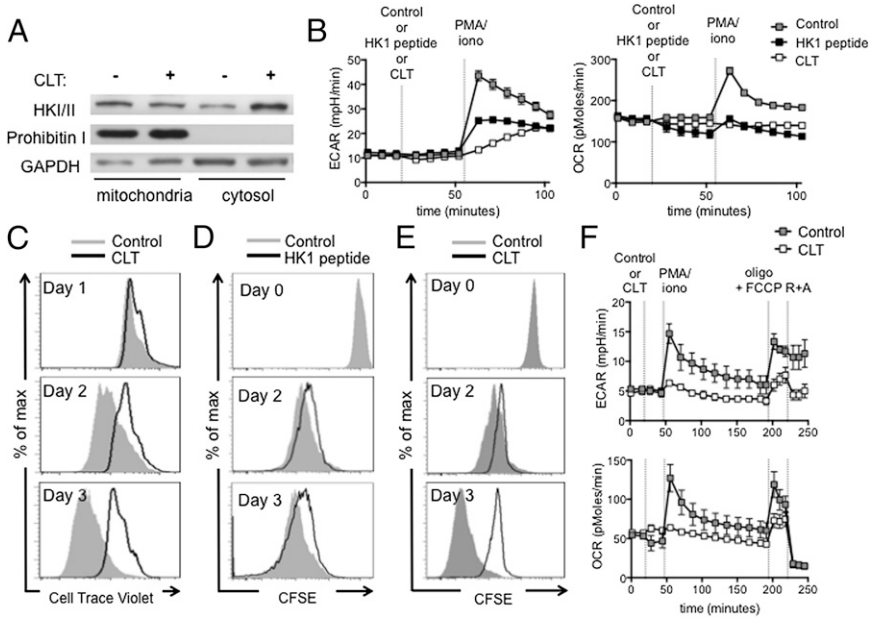

Fig. 8. Dissociation of HK from mitochondria impairs proliferation and the rapid engagement of glycolysis in (IL-15) T_M cells after activation. (*A*) Western blot analysis for HK I and II in IL-15 T_M cells incubated ± CLT for 2 h; GAPDH and prohibitin I are loading controls for cytosolic and mitochondrial fractions, respectively; representative of two experiments. (*B*) OCR and ECAR of IL-15 T_M cells stimulated with PMA/iono in the presence of control or HK-1 peptide (10 μM) or CLT (25 μM); mean ± SEM, representative of ≥2 experiments. IL-15 T_M cells were stimulated with anti-CD3/28 and proliferation ± CLT; representative of five experiments (*C*), or with control or HK-1 peptide (10 μM); representative of two experiments, is shown (*D*). (*E* and *F*) T_M cells from LmOVA-infected mice. Proliferation after anti-CD3/28 ± CLT; control is same as Fig. 6*G* and 7*A*, from one experiment (*E*); OCR and ECAR after PMA/iono ± CLT and exposed to oligo (1 μM) plus FCCP (1.5 μM), and rotenone (100 nM) plus antimycin A (1 μM); mean ± SEM and representative of two experiments; control is the same as Fig. 6*C* and Fig. S7 (*F*).

cells that rely most heavily on FAO while expressing hpCPT1a might not survive. This phenomenon could explain the more striking effect on proliferation and OCR when FAO is acutely inhibited by etomoxir. Our data also suggest that pyruvate flux via MPC does not substantially contribute to OXPHOS in T cells during the recall response. Although we have not ruled out the possibility that pyruvate still enters the mitochondria in hpMPC cells, it is likely that, in addition to long chain fatty acids, glutamine or medium and short chain fatty acids contribute to OXPHOS in T_M cells.

Our results indicate that mitochondrial association of HK is important for the rapid recall of T_M cells. Recruitment of HK to the mitochondria happens quickly in response to Akt activation (25), and the conversion of glucose into glucose-6-phosphate is a process that requires ATP. Because mitochondrial HK has been shown to exclusively use intramitochondrial ATP to phosphorylate glucose (35–37), we speculate that the direct availability of ATP supports the rapid activation of HK when associated with mitochondria. Higher expression of glycolysis enzymes in T_M cells might also account for their capacity to quickly increase glycolysis.

OT-I T_N cells did not increase OCR or ECAR after anti-CD3/28, whereas polyclonal T_N cells slightly increased ECAR. It is possible that this difference would not occur if given more time or a stronger stimulus, or perhaps polyclonal T_N cells exist in a slightly more activated state than transgenic T_N cells and that this more activated state contributes to their faster metabolic activity. We also found that etomoxir inhibited the increase in OCR, but not ECAR, in T_M cells after PMA/iono, differing from IL-15 T_M cells, which show a decrease in both ECAR and OCR. These data suggest that T_M cells are able to engage glycolysis briefly after PMA/iono in the presence of etomoxir, but importantly, this engagement is dissipated after oligo and FCCP. This difference may be due to the fact that IL-15 T_M cells are a homogenous population exposed to optimized culture conditions, whereas in vivo-generated T_M cells have varied substrate and growth factor availability and might not rely solely on FAO to initially engage glycolysis after activation, but need it to maintain mitochondrial and glycolytic activity when maximal respiratory capacity is required.

We show here that bioenergetic differences between CD8 T_N and T_M cells contribute to the differential responses of CD8 T_N and T_M cells to activation, i.e., greater mitochondrial mass endows T_M cells with the ability to rapidly recall. Agents that induce mitochondrial biogenesis have been of interest for treatment of numerous pathologies (38). Our findings indicate that drugs that induce mitochondrial biogenesis could hold promise as immunotherapeutics to improve vaccination strategies.

Materials and Methods

Full methods are available as *SI Materials and Methods*. Mice were purchased from Jackson Laboratory. OT-I cells were activated with OVA-peptide and IL-2 for 3 d and, subsequently, cultured in the presence of IL-2 or IL-15 for 3–4 d to generate IL-2 T_E and IL-15 T_M cells, respectively (17). OCR and ECAR were measured by using the XF-24 or XF-96 Extracellular Flux Analyzers (Seahorse Bioscience). Statistical comparisons for two groups were calculated by using unpaired two-tailed Student's *t* tests.

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