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Memory CD8$^+$ T Cells Use Cell-Intrinsic Lipolysis to Support the Metabolic Programming Necessary for Development

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SUMMARY

Generation of CD8$^+$ memory T cells requires metabolic reprogramming that is characterized by enhanced mitochondrial fatty-acid oxidation (FAO). However, where the fatty acids (FA) that fuel this process come from remains unclear. While CD8$^+$ memory T cells engage FAO to a greater extent, we found that they acquired substantially fewer long-chain FA from their external environment than CD8$^+$ effector T (Teff) cells. Rather than using extracellular FA directly, memory T cells used extracellular glucose to support FAO and oxidative phosphorylation (OXPHOS), suggesting that lipids must be synthesized to generate the substrates needed for FAO. We have demonstrated that memory T cells rely on cell intrinsic expression of the lysosomal hydrolase LAL (lysosomal acid lipase) to mobilize FA for FAO and memory T cell development. Our observations link LAL to metabolic reprogramming in lymphocytes and show that cell intrinsic lipolysis is deterministic for memory T cell fate.

INTRODUCTION

Upon infection, activated CD8$^+$ T cells undergo a distinct pattern of differentiation characterized by the proliferation of antigen (Ag)-specific effector T (Teff) cells, followed by contraction of these cells and development of long-lived memory T cells (Cui and Kaech, 2010; Harty and Badovinac, 2008). During this process, T cells metabolically reprogram to provide for the divergent energetic and functional needs of these distinct cell types. Teff cells, which require precursors for biomass accumulation and effector functions, dramatically increase aerobic glycolysis (Caro-Maldonado et al., 2012), whereas memory T cells use oxidative phosphorylation (OXPHOS) to meet metabolic demands (van der Windt and Pearce, 2012). Although Teff cells can engage OXPHOS (Chang et al., 2013; Wang et al., 2011), which is necessary for their Ag driven proliferation (Sena et al., 2013), memory T cells rely on this metabolic pathway, and in particular, the use of fatty acids (FA) to fuel this process (Pearce et al., 2013). We previously demonstrated that fatty-acid oxidation (FAO) provides a metabolic advantage for the survival of memory T cells and for their rapid recall after reinfection (van der Windt et al., 2012; van der Windt et al., 2013). However, how memory T cells access FA to fuel this process remains unclear.

There is a strong association between burning fat and living longer (Hansen et al., 2013; Wang et al., 2008). Memory T cells are long-lived and previous studies demonstrating that they engage FAO to support survival have helped establish the link between lipid metabolism and cellular longevity in the immune system (Pearce, 2010; van der Windt et al., 2012). Given that long-lived lymphocytes are a goal of vaccination, there is interest in understanding the pathways that regulate their longevity.

Lipolysis is the hydrolysis of stored lipids to liberate FA that can then be used as energy substrates, essential precursors for membrane synthesis, or signaling mediators (Farese and Walther, 2009; Lass et al., 2011; Zechner et al., 2012). Consistent with the importance of lipolysis in energy homeostasis, it is thought to occur in all cell types, but is most abundant in adipose tissue, where the release of stored fats into the vasculature supplies energy substrates to other cells (Lass et al., 2011; Zechner et al., 2012). Several enzymes and regulatory factors, such as adipose triglyceride lipase (ATGL) and hormone-sensitive lipase (HSL), regulate the release of lipids from lipid droplets in response to changes in the nutritional state (Brasaemle, 2007; Farese and Walther, 2009). Other lipases, such as lysosomal acid lipase (LAL) can also contribute to lipolytic processes (Sheriff et al., 1995). Tissues around the body that use FAO, such as cardiac and skeletal muscle, liver, and kidney, can acquire FA from the blood and oxidize them in mitochondria to fuel energy production (Kodde et al., 2007; Reddy and Rao, 2006; Weinberg, 2011; Zhang et al., 2010). While lipolysis in adipocytes has been extensively studied, how other cells store, access, or mobilize FA is less well understood (Zechner et al., 2012).

We show that although CD8$^+$ memory T cells depend on FAO (van der Windt et al., 2012), they do not acquire appreciable amounts of extracellular free FA to fuel this process, and in contrast to Teff cells, do not readily store exogenous long-chain

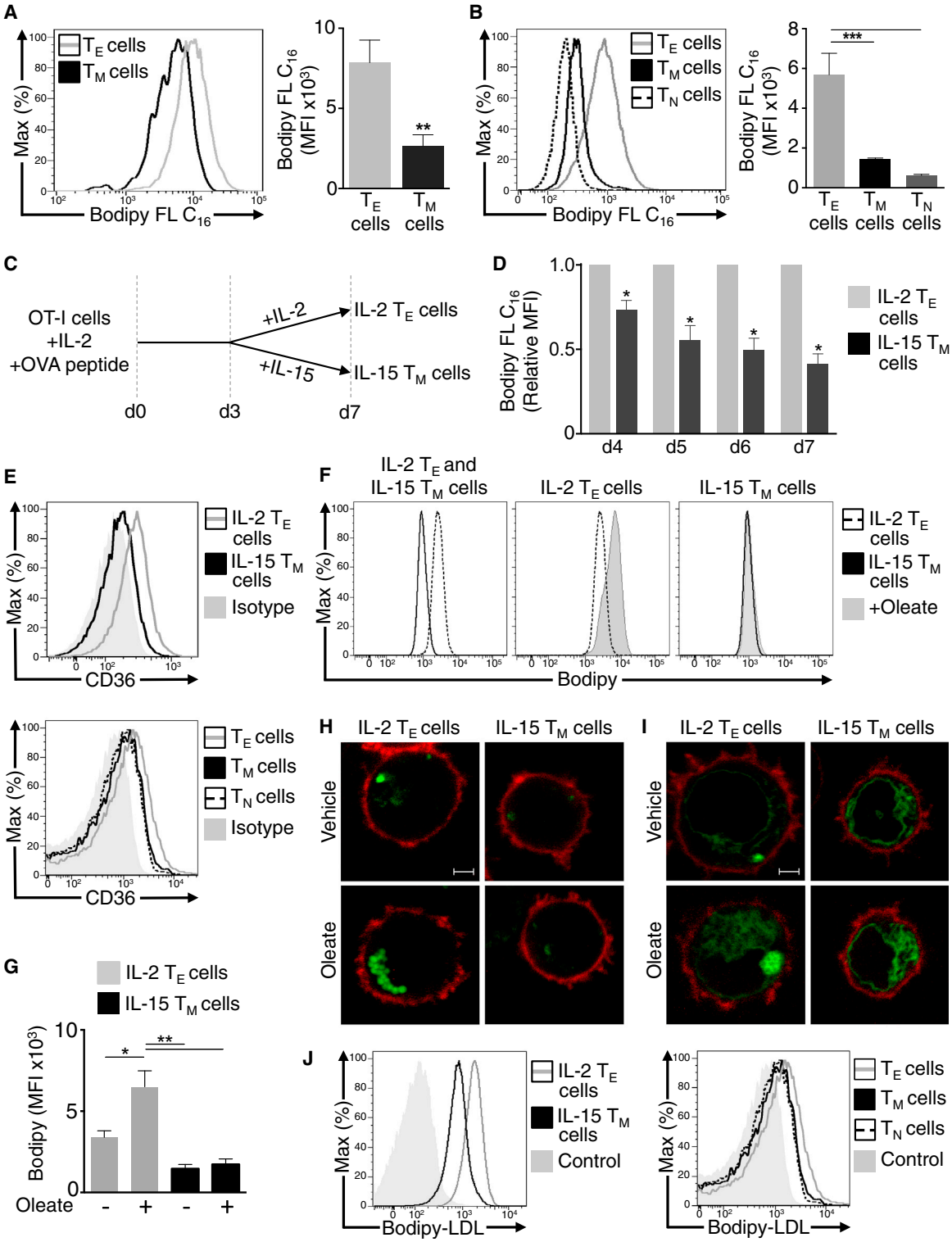

Figure 1. Unlike CD8+ Teff Cells, CD8+ Memory T Cells Do Not Acquire Substantial Amounts of Extracellular Fatty Acids

OT-I cells were injected i.v. into congenic recipients. Mice were infected with LmOVA to generate Teff (T_E; 7 days) and memory T (T_M; ≥21 days) cells, and peripheral blood was collected 1 hr after i.v. Bodipy FL C_{16} injection and uptake quantified by FACS.

(A) Representative plots (histogram) and average MFI (bar graph) of Bodipy FL C_{16} in Teff and memory T K^bOVA-specific $CD8^+$ $OT\text{-}I^+$ cells from two experiments (n = 7-8 mice/group). Error bars show mean ± SEM, **p < 0.01.

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FA in lipid droplets. Instead, memory T cells use extracellular glucose to support FAO and OXPHOS, indicating that these cells synthesize FA for mitochondrial FAO. Consistent with the reliance of memory T cells on FAO, LAL, an enzyme that hydrolyzes cholesterol esters (CE) and triacylglycerol (TAG) to generate free FA and cholesterol in the lysosomes of cells (Sheriff et al., 1995), is expressed in $CD8^+$ memory T cells and supports the metabolic reprogramming necessary for their development.

RESULTS

Unlike Teff Cells, Memory T Cells Do Not Acquire Substantial Amounts of Extracellular FA

Because memory T cells use long-chain FA to fuel FAO (van der Windt et al., 2012), we investigated whether these cells, like other cells that use FAO, acquire free FA from their external environment (Kiens, 2006; Koonen et al., 2005). To this end, we isolated $CD8^+$ T cells from OT-I transgenic mice and transferred them into congenic recipients and then infected the mice with *Listeria monocytogenes* expressing ovalbumin (OVA) (LmOVA) to induce an OVA-specific $CD8^+$ T cell response. We then injected, fluorescently labeled palmitate (Bodipy FL C_{16}), a long-chain FA, into the mice 7 days (Teff phase) or 21 days (memory T phase) after LmOVA infection. One hour after Bodipy FL C_{16} injection, OVA-specific donor T cells were analyzed for FA uptake. OVA-specific $CD8^+$ memory T cells acquired significantly less palmitate compared to OVA-specific $CD8^+$ Teff cells (Figure 1A). There was no difference in Bodipy FL C_{16} uptake between KLRG-1^{hi} and KLRG-1^{lo} Ag-specific Teff cells (Sarkar et al., 2008), indicating that increased FA uptake is not specific to short-lived Teff cells, but to Teff cells as a whole (data not shown). Since Bodipy FL C_{16} was injected at distinct time points to assess differences in FA acquisition between Teff and memory T cells, we also compared cells within an individual animal. We injected Bodipy FL C_{16} 7 days postinfection and 1 hr later assessed its incorporation into $CD8^+$ $CD44^{hi}CD62L^{lo}$ Teff cells, and into $CD8^+$ T cells expressing naive (Tn; $CD44^{lo}CD62L^{hi}$) or memory T ($CD44^{hi}CD62L^{hi}$) markers (Figure 1B, histogram only). We observed the same trend in these polyclonal T cells as compared to day 7 Teff cells and to memory T cells and to T cells expressing Tn markers day 21 postinfection, because memory T cells took up less palmitate than Teff cells (Figure 1B, bar graph). Although acquisition by memory T cells appeared higher than Tn cells, the difference was not statistically significant. Consistent with Teff cells acquiring more Bodipy FL C_{16} than memory T cells, secondary $CD8^+$ Teff cells (2° Teff) from mice challenged with LmOVA 4 days prior exhibited increased Bodipy FL C_{16} uptake relative to memory T cells (see Figure S1A available online). Polyclonal Teff cells from these mice, which were comparable in size to memory T cells, also acquired more Bodipy FL C_{16} (Figure S1A), indicating that differences in FA acquisition are not only due to cell size differences.

To further investigate FA acquisition we stimulated $CD8^+$ OT-I cells with OVA peptide and interleukin-2 (IL-2) for 3 days, then differentially cultured the cells in IL-2 or IL-15 for 4 more days (Figure 1C) to approximate in vitro the conditions that program $CD8^+$ Teff and memory T cells, respectively, after infection (Carrio et al., 2004; van der Windt et al., 2012). Like our in vivo findings, IL-15 differentiated memory-like T (IL-15 memory T) cells acquired much less Bodipy FL C_{16} than IL-2 driven effector-like (IL-2 Teff) cells (Figure 1D). As IL-2 Teff cells differentiated into IL-15 memory T cells, their ability to acquire Bodipy FL C_{16} decreased. Following overnight incubation with Bodipy FL C_{16}, the difference in uptake between IL-2 Teff and IL-15 memory T cells remained (Figure S1B). Surface expression of CD36, a receptor that binds long-chain FA and low-density lipoprotein (LDL) (Silverstein and Febbraio, 2009), was lower on IL-15 memory T cells and on in vivo generated memory T cells compared to their Teff cell counterparts (Figure 1E), consistent with their reduced capacity to acquire FA.

Given the difference in FA uptake between these cells, we assessed their total neutral lipid (indicates stored fat). IL-2 Teff cells stained with unconjugated Bodipy had more neutral lipid than IL-15 memory T cells (Figure 1F), and when incubated with the long-chain FA oleate, only IL-2 Teff cells exhibited an increase in Bodipy staining (Figures 1F and 1G). Furthermore, IL-2 Teff cells cultured with oleate formed lipid droplets (Figure 1H). These structures were not evident in IL-15 memory T cells. Supporting these observations, Tip47, a lipid droplet protein (Bulankina et al., 2009), was expressed more in IL-2 Teff than IL-15 memory T cells (Figure S1C). To confirm that these structures were lipid droplets, we transduced T cells with a retrovirus containing a lipid droplet-targeting construct (LD-GFP), where GFP was fused to the N-terminal hydrophobic region of METTL7B (ALDI), a protein that localizes to lipid droplets (Zehmer et al., 2008), and differentially cultured them in IL-2 or IL-15. LD-GFP localized to lipid droplets in IL-2 Teff cells, which became abundant in oleate-supplemented IL-2 Teff cells (Figure 1I). In contrast, the IL-15 memory T cells exhibited diffuse fluorescence, even when supplemented with oleate. We interpret the diffuse fluorescence to indicate retroviral expression of METTL7B-GFP in the cytoplasm. These data show that unlike

(B) Representative plots (histogram) of Bodipy FL C_{16} MFI in polyclonal Teff ($CD44^{hi}$ $CD62L^{lo}$), memory T ($CD44^{hi}$ $CD62L^{hi}$), and Tn (T_N; $CD44^{lo}$ $CD62L^{hi}$) $CD8^+$ T cells 7 days postinfection and average MFI (bar graph) of polyclonal $CD8^+$ T cells from two experiments (n = 9 mice/group). Error bars show mean ± SEM. ***$p < 0.001$.
(C) OT-I cells were activated with OVA peptide and IL-2 for 3 days and subsequently cultured in IL-15 or IL-2 for 4 more days to generate IL-15 memory T and IL-2 Teff cells, respectively.
(D) OT-I cells were incubated with Bodipy FL C_{16} on days 4–7 of culture, and uptake was measured. MFIs were normalized to the daily MFI of IL-2 Teff cells. Data from three experiments is shown as mean ± SEM, *$p < 0.05$ by one-sample t test.
(E) CD36 expression on day 7 IL-2 Teff and IL-15 memory T cells, or polyclonal T cells. Data represent three experiments.
(F–H) Day 6 IL-2 Teff or IL-15 memory T cells were cultured overnight ± oleate then stained with Bodipy. (F) Representative plots showing Bodipy MFI and (G) average Bodipy MFI from three experiments show mean ± SEM, *$p < 0.05$, **$p < 0.01$. (H) Images showing Bodipy (green) and CD8 (red) represent four experiments. Scale bars represent 2 μM.
(I) OT-I cells expressing a lipid droplet-targeting construct (LD-GFP) were cultured overnight ± oleate. Images show LD-GFP (green) and CD8 (red) and represent two experiments.
(J) Cells were incubated with Bodipy-LDL or left unstained (control), data represent two experiments.

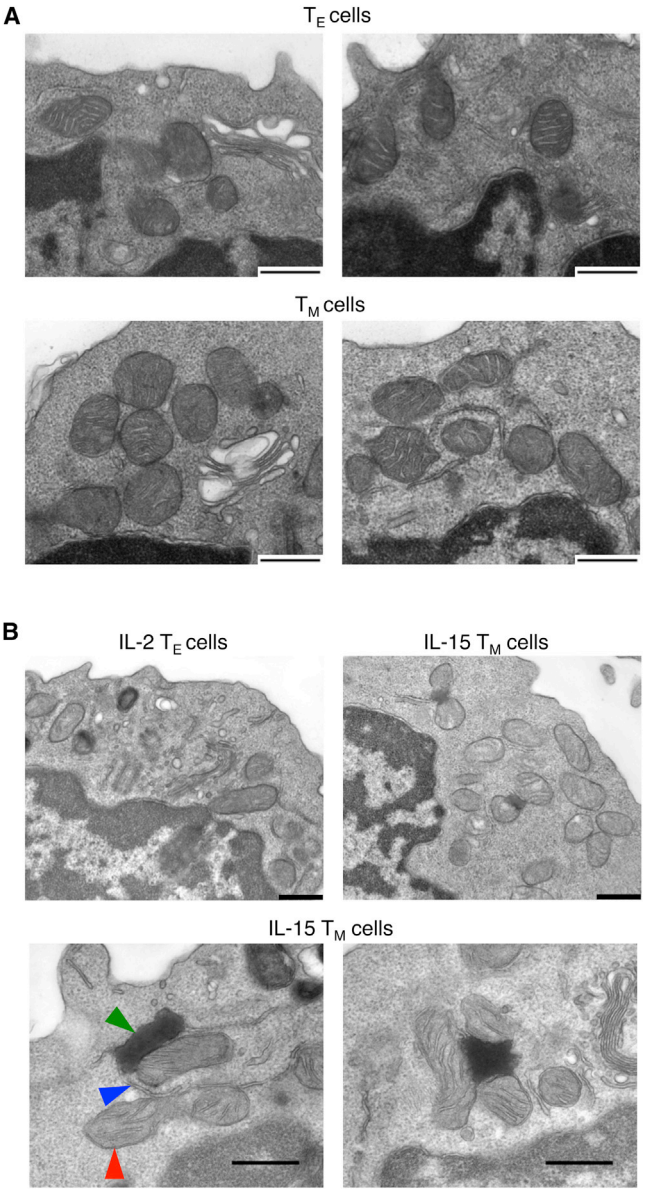

Figure 2. Ultrastructure Analysis Reveals Densely Packed Mitochondria in Close Proximity to ER in Memory T Cells

EM images of mitochondria associated with ER from (A) Teff and memory T cells and (B) IL-2 Teff and IL-15 memory T cells; mitochondrion, red arrow; ER, blue arrow; electron dense structure, green arrow. Data represent ≥2 experiments.

IL-15 memory T cells, IL-2 Teff cells acquire long-chain FA and store them as neutral lipid (e.g., TAG and CE) in lipid droplets.

We questioned whether memory T cells are able to acquire other forms of lipid produced in vivo, such as those that provide cholesterol. We added Bodipy-conjugated LDL in vitro and found that IL-15 memory T cells acquired LDL, although to a lesser extent than IL-2 Teff cells (Figure 1J). In addition, Tn, as well as Teff and memory T cells, acquired LDL to a somewhat similar extent in vivo (Figure 1J), a pattern distinct from what we observed for Bodipy FL C_{16}. These data, and those shown in Figures 1A and 1B, indicate that different T cells exhibit distinct preferences for lipid substrates, suggesting that acquisition of lipids that provide cholesterol for membranes and other processes might be different from those that fuel energy metabolism, fitting with published data (Bensinger et al., 2008; Kidani et al., 2013).

Glucose Fuels Mitochondrial FAO and OXPHOS in IL-15 Memory T Cells

We did not visualize abundant lipid droplets by electron microscopy (EM) in either Teff or memory T cells. We did, however, observe accumulations of mitochondria in close association with endoplasmic reticulum (ER) in memory T cells, but not Teff cells (Figures 2A and 2B). Furthermore, we observed electron-dense structures localized to mitochondria and ER in IL-15 memory T cells (Figure 2B), which were always proximal to mitochondria and did not appear in other areas of the cytoplasm.

Lipids imported into the mitochondria can be synthesized in the ER, requiring an interaction between these two compartments (Flis and Daum, 2013), and glycerol-3-phosphate acyltransferases, critical enzymes for glycerolipid synthesis and ultimately TAG production, can associate with ER and mitochondria (Wendel et al., 2009). Given that memory T cells engage FAO to fuel OXPHOS (van der Windt et al., 2012), but do not acquire FA to a great extent, nor have appreciable lipid droplets, we wondered whether the localization of ER and mitochondria in memory T cells suggested that lipids synthesized in the ER are substrates for FAO. The electron-dense structures in IL-15 memory T cells might indicate an abundance of synthesized lipid from the ER in nutrient replete culture conditions, whereas in memory T cells generated after infection, lipids might be metabolized as they are made, thereby accounting for the absence of these structures in memory T cells.

To assess lipid synthesis, we cultured IL-15 memory T and IL-2 Teff cells with C75, an inhibitor of fatty-acid synthase (FASN) (Hansen et al., 2013). Consistent with the idea that IL-15 memory T cells synthesize FA for survival, IL-15 memory T cell survival decreased when cultured with C75. At the same concentration of C75, IL-2 Teff cell survival was unaffected, although proliferation was attenuated (Figures 3A and 3B), supporting that IL-2 Teff cells synthesize lipid for biomass (Wang et al., 2011). However, higher concentrations of C75 reduced IL-2 Teff cell survival (Figure S2A), consistent with a recent finding that proliferating T cells defective in FA synthesis (FAS) have decreased survival (Lee et al., 2014). We also attempted to genetically block FAS by silencing ATP-citrate lyase (ACL), an enzyme that generates cytosolic acetyl-CoA, a substrate needed in FAS (Hatzivassiliou et al., 2005). IL-2 Teff cells transduced with a retrovirus expressing shRNA against ACL (hpACL) had no defect in survival, but had attenuated proliferation compared to control shRNA IL-2 Teff cells (Figures S2B–S2D). Unexpectedly, survival of hpACL IL-15 memory T cells was not impaired (Figure S2B). These data are in contrast to the decreased survival of IL-15 memory T cells cultured with C75. This disparity could be due to two possibilities, both of which center on the idea that metabolic and biosynthetic demands are low in memory T compared to Teff cells. Incomplete silencing of ACL (Figure S2D) might result in enough acetyl-CoA produced to support FAS needed for IL-15 memory T cell survival, but not

for the extensive proliferation of IL-2 Teff cells. Alternatively, IL-15 memory T cells might be able to switch from using ACL derived acetyl-CoA to that produced from acetate via acyl-CoA synthetase, an effect previously observed after ACL deletion (Hatzivassiliou et al., 2005; Zaidi et al., 2012). As C75 inhibits FASN directly, these potential confounding factors are removed. Therefore, the results using C75 indicate that despite IL-15 memory T cells having a more quiescent phenotype, these cells are acutely sensitive to inhibition of FAS. Because IL-15 memory T cells have a reduced demand for lipid for anabolic processes, as well as reduced extracellular FA uptake, these cells likely use synthesized lipids for catabolic processes such as FAO.

Most cells can synthesize lipid from glucose carbon that enters the TCA cycle, which is exported from the mitochondria as citrate (Vander Heiden et al., 2009) and is used for FAS (Wakil et al., 1983). To explore whether memory T cells synthesize lipids from glucose, we cultured IL-2 Teff and IL-15 memory T cells with ^{13}C-labeled glucose and analyzed ^{13}C-glucose incorporation into the FA chains of PG and PE lipid via mass spectrometry (MS). Both cell types synthesized lipids from glucose; although the overall rate of glucose incorporation into lipid was lower in IL-15 memory T cells, a similar relative incorporation of ^{13}C-glucose into these lipids occurred in IL-2 Teff and IL-15 memory T cells (Figure S2E). Consistent with active lipid synthesis, FASN, and acetyl-CoA carboxylase 1 and 2 mRNA, were similarly expressed in both cell types (data not shown).

To explore whether IL-15 memory T cells use glucose to produce FA for OXPHOS, we assessed O_2 consumption rates (OCR, an indicator of OXPHOS) in cells cultured in normal media (11 mM glucose) or media with low (2 mM) glucose (LG) (Figure 3C). The substantial spare respiratory capacity (SRC) prominent in memory T cells (van der Windt et al., 2012) (OCR after FCCP/basal OCR) was reduced in LG, indicating that in these cells, glucose contributes to OXPHOS and maximal respiratory capacity (Figure 3C). The precipitous reduction in SRC of cells in LG is consistent with the view that glucose supports FAO, as the majority of the SRC in glucose replete-media is derived from long-chain FAO, indicated by the substantial reduction in this parameter in the presence of etomoxir, a specific inhibitor of carnitine palmitoyl transferase 1a (CPT1a), which transports long-chain FA into the mitochondria and is a rate limiting step of FAO (Figure 3C) (van der Windt et al., 2012). IL-15 memory T cells in LG also had decreased basal OXPHOS, indicating that glucose supports OXPHOS in the steady state. A similar trend was detected when cells cultured in glucose-replete media were subsequently transferred to LG right before OCR was measured (data not shown), indicating that the decreased OXPHOS in LG was not due to alterations in mitochondrial mass that could occur in prolonged culture. In cancer cells, glutamine provides an alternate carbon source for lipid synthesis in LG (Le et al., 2012); however, we found that excess glutamine did not rescue the defects in IL-15 memory T cell metabolism (Figure S2F). In contrast to IL-15 memory T cells in LG, OXPHOS in IL-2 Teff cells was not impaired (Figure 3C). Consistent with published observations (Buzzai et al., 2005), T cells cultured in IL-2 compensated for reduced glycolysis in LG by increasing FAO supported OXPHOS, as indicated by the reduction in OCR following etomoxir in IL-2 Teff cells in LG.

To support that memory T cells use glucose for FAO and OXPHOS, we assessed glucose uptake in T cells by using the fluorescent glucose analog 2-NBDG. On day 7 after infection we compared Teff cells to Tn and memory T cells from mice infected 1 month previously. We found that Tn and memory T cells acquired more 2-NBDG than Teff cells (Figure 3D, bar graph). This trend was also apparent in polyclonal T cells expressing Tn, Teff, and memory T markers, when analyzed from a single mouse at day 7 postinfection (Figure 3D, histogram). The increased 2-NBDG uptake in these quiescent cells is consistent with their use of glucose for survival. Importantly, although the data showing that memory T and Tn cells acquire more 2-NBDG than Teff are surprising, they do not argue against a role for glucose and glycolysis in Teff cell proliferation or effector function (Chang et al., 2013; Fox et al., 2005; Jacobs et al., 2008; Wang et al., 2011). We find that early Teff cells, present day 5 after Lm infection, when clonal expansion has yet to peak, have higher ECAR than day 7 Teff cells (data not shown). We speculate that the later day 7 Teff cells acquire less glucose than early Teff cells, because late Teff cells are on the verge of contraction, whereas early Teff cells, which are highly glycolytic, are more similar to in vitro generated IL-2 Teff cells. This would be in keeping with the fact that IL-2 Teff cells acquire more 2-NBDG than IL-15 memory T cells in vitro (Figure S2G). Also, these data are not inconsistent with a recent paper showing that restraining terminal differentiation of Teff cells in vitro by treating with the glycolysis inhibitor 2-DG forms better memory T cells in vivo (Sukumar et al., 2013), because 2-DG was administered in the Teff phase, but not during memory T cell development.

Because intracellular free FA can be toxic, most cells store excess FA in less reactive forms, such as TAG, and then release FA from these stores as needed (Thiam et al., 2013). To explore whether synthesized TAG contributes to FAO in IL-15 memory T cells, we inhibited diacylglycerol acyltransferases (DGAT) 1 and 2, critical enzymes for TAG synthesis, with the inhibitor Amidepsine A (AmA) (Tomoda et al., 1995). IL-15 memory T cells treated with AmA exhibited reduced SRC and were insensitive to etomoxir (Figure 3E), indicating that these cells were no longer using long-chain FA to fuel OXPHOS. This effect was specific to IL-15 memory T cells, because AmA treated IL-2 Teff cells did not exhibit OXPHOS defects (data not shown).

To confirm that IL-15 memory T cells synthesize lipids for FAO, we cultured IL-15 memory T or IL-2 Teff cells in lipid-depleted (LD) media. We observed no defect in basal OXPHOS, SRC, or long-chain FAO in IL-15 memory T cells cultured in LD media (Figure 3F). We did find reduced OXPHOS in IL-2 Teff cells (Figure 3F), which was accompanied by decreased glycolysis (data not shown), likely due to reduced availability of extracellular lipids impairing anabolic processes and leading to an overall reduction in metabolic activity in these cells. Collectively our data indicate that IL-15 memory T cells synthesize lipids and are not dependent on extracellular FA to fuel OXPHOS.

The Lipid Signature of Memory T Cells Suggests Active Lipolysis

Most cells can store lipids as TAG and CE then subsequently release FA from these stores when needed (Thiam et al., 2013). Because we did not detect lipid droplets in memory T cells, we

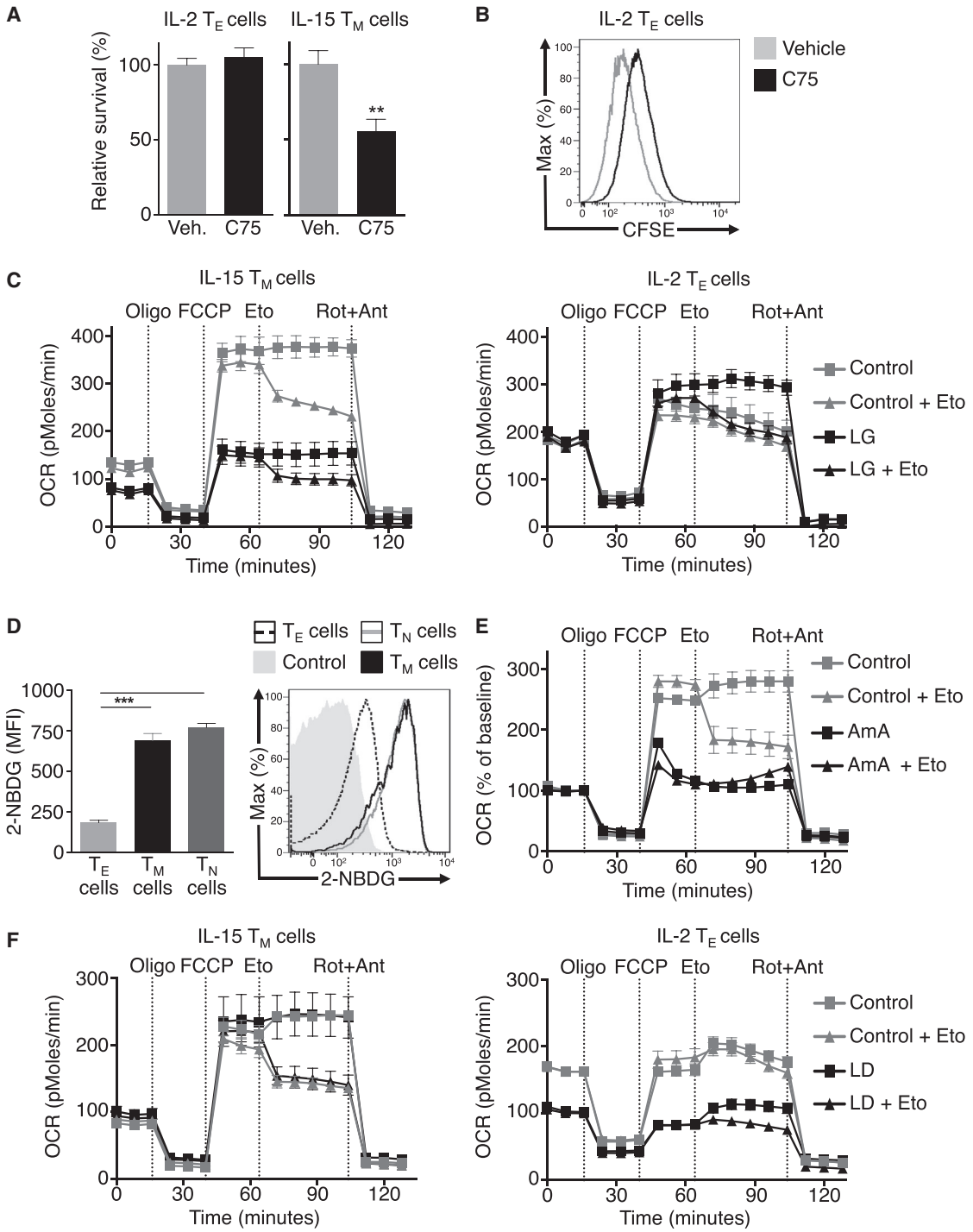

Figure 3. Glucose Fuels Mitochondrial FAO and OXPHOS in IL-15 Memory T Cells

(A) IL-2 Teff and IL-15 memory T cell survival (7-AAD exclusion) ± C75, shown as survival relative to vehicle (Veh.) treated cells. Data from five experiments show mean ± SEM, $^{**}p < 0.01$.

(B) IL-2 Teff proliferation in the presence of C75 or vehicle. Data represent three experiments.

(C) OCR of IL-2 Teff or IL-15 memory T cells cultured in TCM (Control) and low glucose TCM (LG) was measured under basal conditions and in response to oligomycin (Oligo), FCCP, etomoxir (Eto), and rotenone + antimycin (Rot+Ant). Data represent two experiments shown as mean ± SEM.

(D) Representative plot (histogram) and average MFI (bar graph) of 2-NBDG uptake in polyclonal Teff and memory T cells from one experiment (n = 4 mice/group). Data in error bar show mean ± SEM, $^{***}p < 0.001$.

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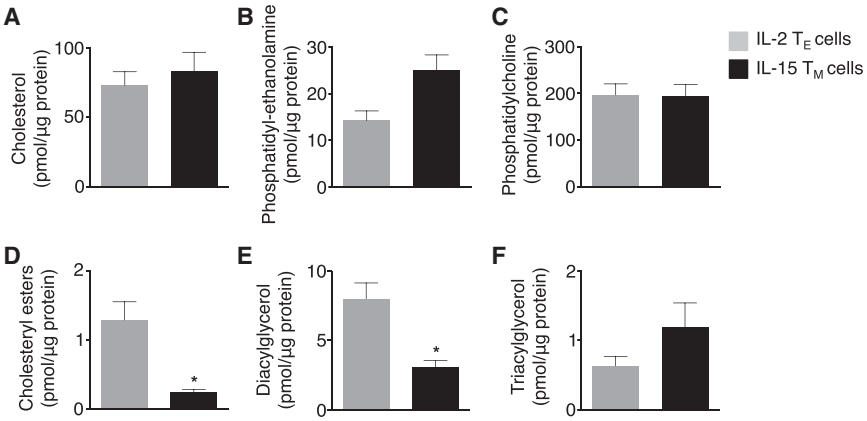

Figure 4. The Lipid Signature of Memory T Cells Suggests Active Lipolysis

(A–F) Lipids were quantified from day 7 cultured IL-2 Teff and IL-15 memory T cells. Data represent three experiments shown as mean ± SEM, *$p < 0.05$.

reasoned that they might oxidize fat shortly after its synthesis, and any synthesized TAG could be quickly lipolyzed, for fuel. This would agree with our data demonstrating that inhibiting DGAT impairs FAO in IL-15 memory T cells. We next questioned whether we could detect altered concentrations of lipid species that are broken down during lipolysis in memory T cells. We assessed the abundance of TAG, diacylglycerol (DAG), and CE, which are targets of lipolysis. Relative abundance of cholesterol, phosphatidylcholine (PC), and phosphatidylethanolamine (PE) were similar between IL-15 memory T and IL-2 Teff cells (Figures 4A–4C). However, consistent with active lipolysis, we found that CE and DAG were significantly decreased in IL-15 memory T cells as compared to IL-2 Teff cells (Figures 4D and 4E). In contrast, TAG abundance was similar between these cells (Figure 4F), perhaps suggesting that TAG is actively synthesized in IL-15 memory T cells and was evident as the electron-dense deposits between mitochondria and ER (Figure 2B). A trend toward decreased neutral lipids was observed in memory T compared to Teff cells after LmOVA infection, with significantly reduced CE (Figure S3). Overall, the alterations in neutral lipids in memory T cells support the idea that these species are hydrolyzed to provide FA for FAO.

Lysosomes Associate with Neutral Lipids in Memory T Cells

While glucose-derived ^{13}C incorporation into lipids indicated that lipid synthesis occurs in IL-2 Teff and IL-15 memory T cells, the differences in TAG, CE, and DAG suggested that lipolysis actively occurs in memory T cells, which agrees with the fact that they engage FAO (van der Windt et al., 2012). Because we did not find evidence of adipocyte-like lipid droplets in memory T cells (Figures 2A and 2B), we considered that their lipolytic machinery might differ from those characterized in adipocytes, such as that mediated by ATGL and HSL (Zechner et al., 2012). LAL is a lipase capable of hydrolyzing TAG, DAG, and CE to generate free FA and cholesterol in lysosomes (Sheriff et al., 1995). We assessed its expression and found that LAL protein and/or mRNA were increased in IL-15 memory T and ex vivo memory T cells when compared to Teff cells, as were memory T cells when compared to 2° Teff cells (Figures 5A–5C; Figure S4).

We reasoned that if LAL regulates lipolysis in memory T cells, colocalization of neutral lipids and lysosomes might occur shortly after exposure to IL-15, when cells are differentiating toward a memory T cell phenotype. IL-15 promotes FAO and the memory T cell phenotype in these cells (Carrio et al., 2004; van der Windt et al., 2012). We stained day 4-activated IL-2 Teff cells, and day 4 IL-15 early memory T cells (i.e., only 1 day of exposure to IL-15), with Bodipy and LysoTracker, to visualize neutral lipids and lysosomes, respectively. Confocal microscopy revealed that lysosomes associated with neutral lipid in day 4 IL-15 memory T cells, but not in day 4 IL-2 Teff cells (Figure 5D). In line with the idea that lysosome association with lipid would lead to lipolysis and subsequent fueling of FAO, the neutral lipid that was evident 1 day after exposure to IL-15 was nearly undetectable after 3 days in culture with IL-15 (Figure 5E). This finding is corroborated by data in Figure S5, showing that LAL mRNA expression increased 1 day following IL-15 exposure, and significant reductions in DAG and CE were detected within 3 days. These data support the hypothesis that memory T cells hydrolyze intracellular neutral lipid.

Lysosomal Lipolysis Metabolically Programs T Cells toward the Memory T Cell Phenotype

We wondered whether LAL plays an important cell intrinsic role in memory T cells. Mice deficient in LAL have defects in thymocyte development and in maturation of peripheral T cells (Qu et al., 2009). Given the defects in T cell development in LAL-deficient mice, this model was not conducive to studying LAL in memory T cells. We took a different genetic approach and transduced activated T cells with a retrovirus expressing either shRNA against LAL (hpLAL) or a control shRNA. This allowed us to suppress the expression of LAL (Figures S6A and S6B) after the T cells were activated. Neutral lipid was increased in hpLAL IL-15 memory T cells compared to controls (Figure 6A), and no increase was observed in hpLAL expressing IL-2 Teff cells. These results suggest that LAL actively hydrolyzes lipid in IL-15 memory T cells, and silencing LAL blocks lipolysis, resulting in increased neutral lipid. To confirm that hpLAL specifically targets lipolysis, we measured TAG and CE in control

(E) OCR represented as percentage of baseline for IL-15 memory T cells cultured with AmA or vehicle (Control) measured in response to indicated drugs. Data represent two experiments shown as mean ± SEM.

(F) OCR of IL-2 Teff or IL-15 memory T cells cultured in TCM (control) and lipid depleted TCM (LD), measured under basal conditions and in response to indicated drugs. Data represent two experiments shown as mean ± SEM.

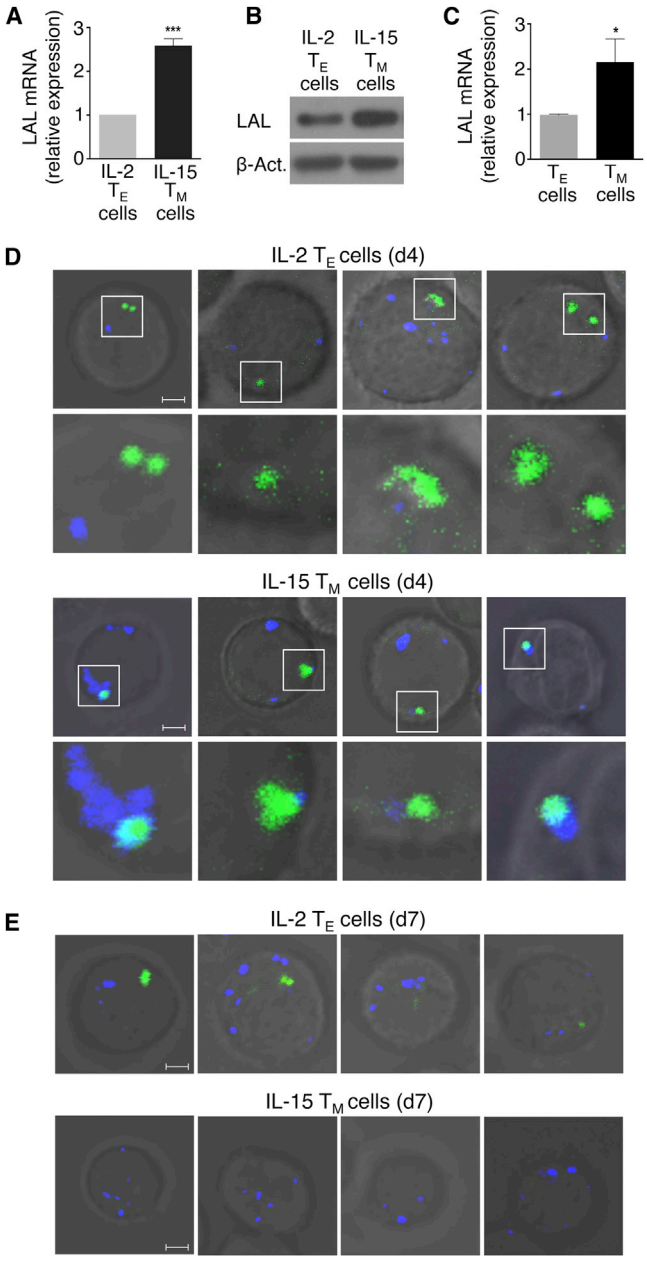

Figure 5. Lysosomes Associate with Intracellular Neutral Lipids during the Memory T Cell Transition In Vitro

(A) Relative mRNA expression of LAL in day 7 IL-2 Teff and IL-15 memory T cells. Data from three experiments shown as mean ± SEM, ***$p < 0.001$.

(B) LAL protein expression compared to β-Actin. Data represent two experiments.

(C) Relative mRNA expression of LAL in Teff and memory T cells. Data from three experiments shown as mean ± SEM, *$p < 0.05$. IL-2 Teff and IL-15 memory T cells were stained with LysoTracker (blue) and Bodipy (green) on day 4 (D) or day 7 (E) of culture. Scale bar represents 2 μM. Data represent three experiments.

and hpLAL IL-15 memory T cells and found an increase in these species, indicating that lipolysis was blocked (Figure 6B).

Because IL-15 promotes FAO in T cells (van der Windt et al., 2012), we investigated whether LAL-mediated lipolysis would provide FA for FAO in T cells after IL-15 exposure. As we observed lysosomal association with neutral lipid soon after IL-15 exposure, we expected that any effects on metabolism should be detectable at this time. Therefore, 3 days after activation we exposed control or hpLAL transduced IL-2 Teff cells to IL-15 and immediately analyzed OCR in real time. When compared to hpLAL cells, control cells in IL-15 showed a progressive and substantial increase in OCR (Figures 6C and 6D). The rapid but blunted increase in IL-15 induced OXPHOS in the hpLAL T cells suggested that the residual amount of LAL expressed (Figure S6A) is sufficient to metabolically reprogram the cells toward lipolysis and FAO under memory T cell-inducing conditions (i.e., IL-15 in the absence of IL-2) and that the induction of LAL expression by IL-15 is downstream of this process. When cells were exposed to etomoxir, OCR dropped precipitously, indicating that the increase in OXPHOS following IL-15 exposure was due to FAO. However, the magnitude of this decrease in OCR was substantially reduced in hpLAL cells, indicating that FAO-fueled OXPHOS is facilitated by LAL-mediated lipolysis (Figures 6C and 6D).

As the initial increase in OCR appeared rapid in hpLAL cells and at a slightly different kinetic from the control, it is also possible that a different substrate is fueling OXPHOS shortly after exposure to IL-15 in hpLAL cells, and that FAO only occurs after a few hours, when respiration becomes etomoxir sensitive. Because hpLAL cells remain more glycolytic, as indicated by the increased extracellular acidification rate (ECAR) (Figure 6D), the initial burst in OCR could be fueled by glucose oxidation in the mitochondria. Further experiments will be needed to determine whether this is the case. Regardless, the increase in glycolysis also suggested that LAL inhibition impaired the ability of T cells to metabolically transition toward a memory T cell phenotype. Consistent with this idea, hpLAL IL-15 memory T cells expressed increased CD25 and reduced CD62L compared to control cells, indicating that hpLAL IL-15 memory T cells retained a more activated phenotype (Figure 6E).

There is a strong link between FAO and $CD8^+$ T cell longevity (van der Windt et al., 2012). If LAL is important for FAO, reduced LAL expression should limit survival of these cells when cultured in IL-15. We found that while hpLAL IL-2 Teff cells exhibited no survival disadvantage compared to controls, hpLAL IL-15 memory T cells exhibited a significant survival defect (Figure 6F). A similar decrease in survival was also observed with IL-15 memory T cells, but not IL-2 Teff cells, when cultured with chloroquine, which prevents LAL activity by inhibiting lysosome acidification (Figure S6C) (Bowden et al., 2011). These results indicate that LAL supports FAO in T cells and that lysosomal lipolysis contributes to IL-15-mediated T cell survival in vitro. We considered a role for ATGL in lipolysis in memory T cells, despite the fact we could not detect classical lipid droplets in these cells. We cultured activated $CD8^+$ T cells from ATGL-deficient mice (*Pnpla2*$^{-/-}$) in IL-15 and found no differences in the expression of T cell activation markers, neutral lipid content, or survival when compared to wild-type $CD8^+$ T cells (Figures S7A and S7B). Likewise, we found no defect in memory T cell development by *Pnpla2*$^{-/-}$ OT-I cells following LmOVA infection (Figure S7C). These results support the hypothesis that unlike ATGL, LAL has a predominant role in memory T cell development.

T Cell-Intrinsic Lysosomal Lipolysis Supports Memory T Cell Development after Infection

To explore whether LAL, and thus T cell intrinsic lipolysis, supports Ag-specific CD8+ T cell survival in vivo after activation signals are withdrawn, we transferred control or hpLAL transduced congenic IL-2 Teff cells into C57BL/6 mice. The number of hpLAL GFP+ donor cells recovered from the spleen and lymph nodes (LN) 2 days later was dramatically lower than control cells, indicating that LAL regulates the survival of activated CD8+ T cells in vivo after the withdrawal of activation signals (Figures 7A and 7B). We view this environment to be similar to what occurs after infection, when pathogen is cleared, activation signals wane, and Ag-specific Teff cells contract. To establish that LAL is important for memory T cell development, we tracked OVA-specific responses of control or hpLAL transduced OT-I cells following immunization with LmOVA. To accomplish this, we infected a congenically marked OT-I donor mouse and C57BL/6 recipient mice with LmOVA. One day later, splenocytes from the donor mouse were isolated, transduced, and then transferred into the recipient mice. During the peak of the Teff cell response (7 days postinfection), the percentage of control and hpLAL cells (detectable by GFP expression) in the blood were similar, despite efficient silencing of LAL (Figure 7C; Figure S6B). However, after contraction of the Ag-specific populations (26 days postinfection), the frequency of hpLAL GFP+ cells was significantly less than control GFP+ cells, indicating that hpLAL cells fail to form a robust CD8+ memory T cell population (Figure 7C). Illustrating the defect in memory T cell development, when the mice were challenged with LmOVA to induce a 2° Teff cell response, the number of hpLAL GFP+ cells recovered from the spleen was also significantly less than control GFP+ cells (Figure 7D). Collectively, these results show that LAL regulates CD8+ memory T cell development.

DISCUSSION

While our results show that cell intrinsic lipolysis supplies FA for FAO in memory T cells, precisely which substrates T cells can use to produce TAG for lipolysis, especially in vivo, is still under investigation. At this time, we infer from our data that glucose is used to synthesize TAG in these cells. We base this on our observations that memory T cells do not acquire substantial amounts of free FA and that glucose supports FAO and OXPHOS in these cells in vitro. Regardless of which substrates these cells use to produce TAG, our data show that the coupling of TAG synthesis and subsequent FAO is how memory T cells meet metabolic demands.

Futile metabolic cycling of FAS and FAO has been documented in muscle and adipose tissue (Dulloo et al., 2004; Guan et al., 2002; Yu et al., 2002) and in cells overexpressing DGAT (Liu et al., 2009). How this futile cycling occurs is an intriguing question, as FAO and FAS pathways can negatively regulate each other. For example, malonyl-CoA, a substrate for FAS, is an allosteric inhibitor of CPT1a (McGarry et al., 1977). It remains to be determined whether FAS and FAO occur simultaneously or whether cells oscillate between periods of FAS and FAO. If the former occurs, then a mechanism for preventing malonyl-CoA inhibition of CPT1a would be needed. Perhaps cells can partition FAS and FAO into separate areas, or rapidly utilize malonyl-CoA for FAS, while inducing activity of CPT1a. Memory T cells express more CPT1a than Teff cells (van der Windt et al., 2012), suggesting that higher concentrations of malonyl-CoA would be needed for inhibition. However, oscillating cycles could also occur if malonyl-CoA concentrations fluctuate, with higher amounts resulting in CPT1a inhibition and increased FAS, and with lower amounts, due to its consumption through FAS, CPT1a inhibition would be relieved and FAO permitted. Although the coupling of FAS and FAO is not energetically efficient in terms of ATP production, and based on this notion is seemingly paradoxical, it is possible that this process allows memory T cells to preserve glycolytic and lipogenic machinery, while maintaining mitochondrial health over long periods of quiescence, rendering these cells "primed and ready" to tap into their substantial metabolic reserve required for rapid reactivation following Ag recognition.

Relatively little is known about lipolysis or lipolytic mechanisms in nonadipose tissues (Zechner et al., 2012). Our results showing that Teff cells have the capacity to acquire external FA and store excess in lipid droplets—whereas memory T cells, which rely on FAO for survival, do not—were unexpected. Most cell types are thought to be able to acquire and store excess lipids (Thiam et al., 2013). It has been shown that regulatory T (Treg) cells in adipose tissue express CD36 and have lipid droplets (Cipolletta et al., 2012), and that proliferating T cells during graft versus host disease increase in FA uptake (Byersdorfer et al., 2013). Although our data suggest that memory T cells do not acquire excess exogenous FA to fuel OXPHOS, more work is needed to determine why these T cells have this unique phenotype. Perhaps memory T cells, or even other long-lived immune cells, do not store lipid in lipid droplets as a protective mechanism against viruses, which often use these organelles as sites of replication and persistence (Camus et al., 2013; Miyanari et al., 2007).

We initially hypothesized that because memory T cells use FAO, these cells would acquire free FA as fuel, much like in vitro Treg cells and other cells that engage FAO (Dhalla et al., 1992; Kodde et al., 2007; Michalek et al., 2011; Zhang et al., 2010). This idea would have fit with the fact that CD8+ memory T cells reside in lipid-rich niches, such as bone marrow and LN. However, our findings that Teff, but not memory T cells, acquire substantial amounts of FA raises the possibility that fat around LN supplies substrate for rapidly dividing cells, such as would be required during an immune response. Unlike other cells in the body, lymphocytes divide extremely rapidly following activation, and there could be an advantage to having substrates "on hand," rather than depending on circulating lipids derived from central stores. Although FA derived from lipolysis can fuel OXPHOS, lipolysis is also required for the production of lipid signaling molecules, such as lipid ligands that activate the peroxisome proliferator-activated receptor (PPAR) pathway (Haemmerle et al., 2011). Future studies are needed to illuminate the precise destinies for all mobilized lipids in T cells.

The fact that we found no evidence that memory T cells form classical lipid droplets agrees with the lack of a role for ATGL in their survival. Our data suggest that memory T cells can synthesize FA by using glucose-derived carbon, and we speculate that these FA are converted to TAG in the ER, which in turn directly undergo LAL-mediated lipolysis to generate FA for

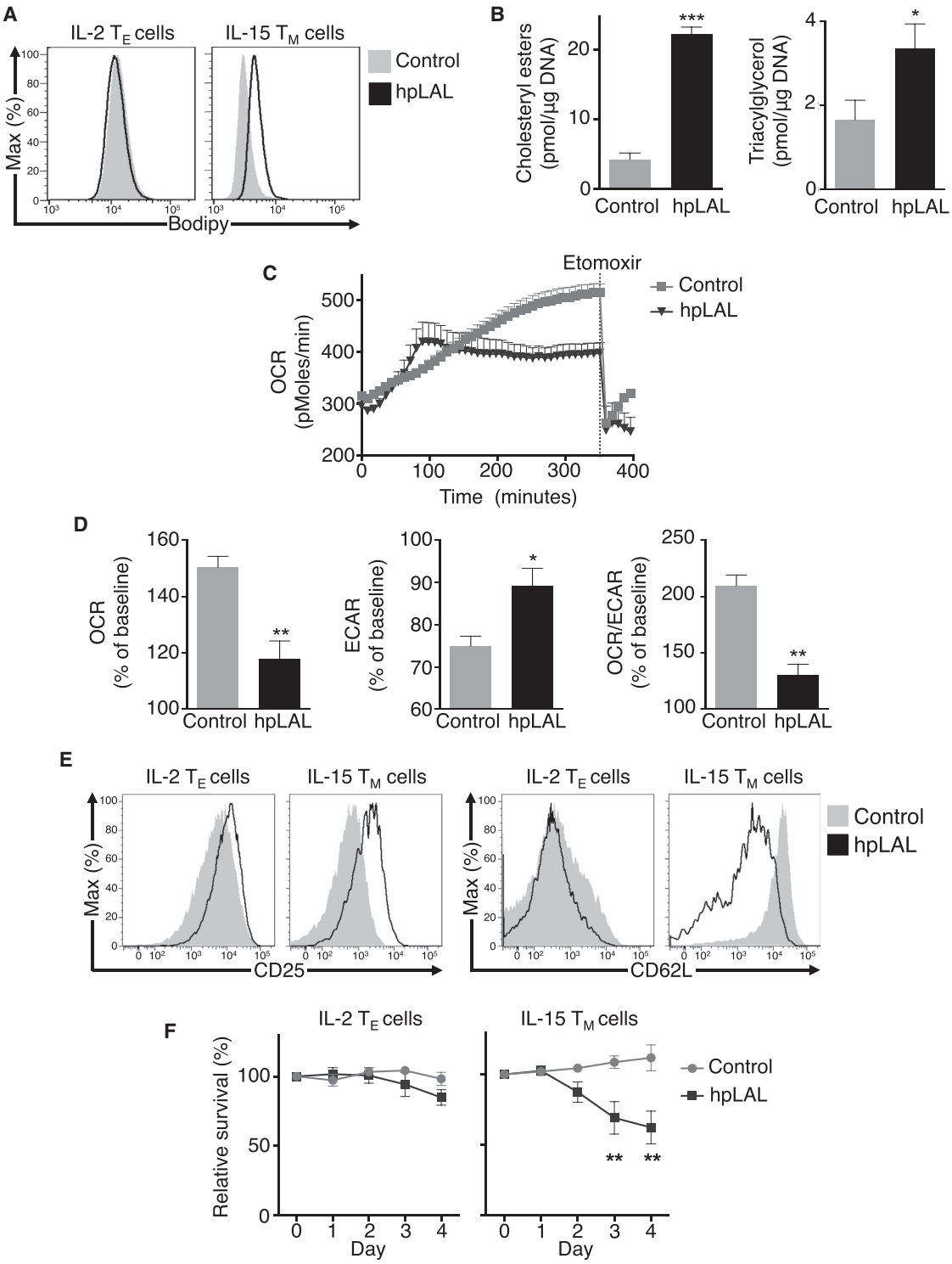

Figure 6. Lysosomal Lipolysis Metabolically Programs CD8+ T Cells toward the Memory T Cell Phenotype

IL-2 Teff or IL-15 memory T cells were transduced with retroviral vectors containing shRNA against luciferase (Control) or against LAL (hpLAL).

(A) Cells were stained for neutral lipids with Bodipy. Data represent three experiments.

(B) IL-15 memory T cells were analyzed for lipid content by MS. Data from three experiments show mean ± SEM, *$p < 0.05$, ***$p < 0.001$.

(C–E) On day 3 of culture, IL-2 Teff cells were washed and resuspended in media with IL-15. OCR and ECAR were measured. (C) Representative plot showing OCR. (D) Relative change in OCR, ECAR, and OCR/ECAR 5 hr after baseline, represented as percent change from baseline. Data from five

(legend continued on next page)

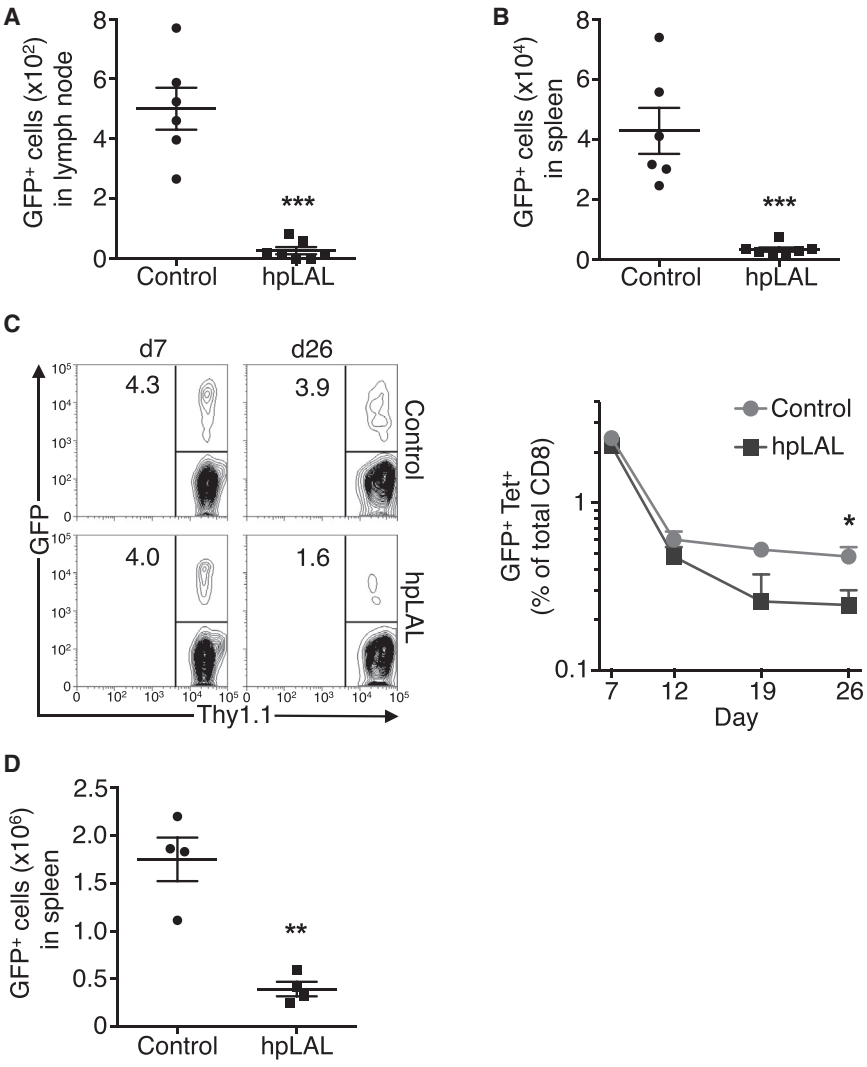

Figure 7. T Cell Intrinsic Lysosomal Lipolysis Supports CD8+ Memory T Cell Development after Infection

(A and B) Congenic IL-2 Teff cells were transduced with control or hpLAL and sorted on GFP. On day 6 of culture, 2 × 10^6 cells/mouse were injected i.v. and harvested from spleen or LN 2 days later. Data represent three experiments, shown as mean ± SEM, ***p < 0.001.

(C–E) Congenic OT-I control or hpLAL transduced cells were transferred into mice infected 1 day prior with LmOVA. (C and D) Blood was analyzed for CD8, K^bOVA, Thy1.1, and GFP. (C) Data shown as shown as mean ± SEM percentage of total $CD8^+$ (line graph); representative plots show percent GFP^+ of Thy 1.1^+ donor cells. Data represent two experiments, *p < 0.05. (D) Mice were challenged with LmOVA and 5 days later $CD8^+$ K^bOVA GFP^+ cells from spleen were quantified. Data shown as mean ± SEM and represent two experiments, **p < 0.01.

FAO. Because we do not detect the accumulation of lipid droplets in memory T cells, this suggests that lipolysis of TAG would occur shortly after synthesis. Consistent with this model, EM revealed close localization of ER and mitochondria in memory T cells. More work is needed to confirm this mechanism and to understand how lysosomes are involved in this process. Of note, in parallel studies, we have found that cell intrinsic expression of LAL is essential for IL-4-driven macrophage alternative activation (S.C.-C.H., data not shown), suggesting that LAL has broadly assumed a role in coordinating cytokine (IL-4 or IL-15)-induced increases in FAO.

It was shown that autophagy can regulate lipid metabolism, where lipid droplets and autophagic components associate during nutrient deprivation, leading to release of TAG (Singh et al., 2009). A recent study has also connected lysosomal lipolysis and autophagy to nutrient availability, fat storage, and aging in *C. elegans* (O'Rourke et al., 2013) and showed that lysosomal lipolysis and autophagy are transcriptionally linked to nutrients in mammals. Future studies delineating autophagy and lipolysis in memory T cell development and how nutritional status orchestrates these pathways to control cellular lifespan are needed. It has also been shown that the transcription factor forkhead homeobox type protein O1 (FoxO1) is induced by nutrient restriction in adipocytes and exerts transcriptional control of lipid catabolism via the induction of LAL (Lettieri Barbato et al., 2013). Given several papers showing the importance of FoxO1 in the development of memory T cells (Rao et al., 2012; Tejera et al., 2013), it is possible that this factor promotes memory T cell development by promoting lipolysis. Our preliminary results indicate that inhibiting the activity of FoxO1 in IL-15 memory T cells blocks LAL mRNA expression. It has also been shown that metformin, a drug used to treat type 2 diabetes, elicited FoxO1-dependent LAL induction and lipophagy in adipocytes (Lettieri Barbato et al., 2013). These results complement our previous findings that metformin promotes FAO in memory T cells and enhances their development in vivo (Pearce et al., 2009). Understanding how lipid metabolic programs are enacted in T cells and how these programs can be manipulated to increase cell longevity will be a subject of future study.

experiments show mean ± SEM, *p < 0.05 or **p < 0.01. (E) Expression of CD25 and CD62L on day 7 IL-2 Teff or IL-15 memory T cells. Data represent five experiments.

(F) Day 3 IL-2 Teff cells were cultured in IL-2 or IL-15 and analyzed daily (days 0–4) for survival (7AAD exclusion). Data represented as percent survival relative to nontransduced cells. Data from three experiments are shown as mean ± SEM, **p < 0.01 by two-way ANOVA.

EXPERIMENTAL PROCEDURES

See the Supplemental Information for details.

Mice and Immunizations

C57BL/6, C57BL/6-Tg (*TcraTcrb*)1100Mjb/J (OT-I), B6.129P2-*Pnpla2*tm^{1Rze}/J (*Pnpla2*$^{-/-}$), and OT-I CD90.1 mice were maintained at Washington University School of Medicine under protocols approved by the Institutional Animal Care and Use Committee. *L. monocytogenes* deficient for actA and expressing OVA (LmOVA) was used for infections.

Cell Culture

T cell media (TCM) contained RPMI 1640 with 10% FCS, 2 mM L-glutamine, 100 U/ml Pen Strep, and 55 μM 2-Me. LG TCM contained 2 mM glucose, and excess glutamine was added (8 mM) as indicated. LD TCM contained FCS depleted of lipids with PHM-L Liposorb. For drug cultures, 10 μM chloroquine, 20 or 40 μM C75, 20 μM AmA, or respective vehicles (H_2O or DMSO) were added on day 3 of culture. For oleate cultures, cells were incubated overnight in TCM ± 100 μM oleate conjugated to bovine serum albumin.

Flow Cytometry and Microscopy

OVA-specific CD8$^+$ T cells were quantified with H2-K^{b}OVA$_{257–264}$ (K^{b}OVA) MHC-peptide tetramers. To identify neutral lipids, we stained cells with 500 ng/ml Bodipy 493/503. For lipid uptake, in vitro or ex vivo cells were incubated with either 1 μM Bodipy FL C_{16} or 10 μg/ml Bodipy FL LDL in TCM. For Bodipy FL C_{16} uptake, in vivo mice were injected intravenously (i.v.) with 50 μg/mouse diluted in DPBS. For 2-NBDG uptake in vivo, mice were injected i.v. with 100 μg/mouse diluted in DPBS or in vitro: cells were incubated in media containing 50 μg/ml 2-NBDG. For transmission EM, cells were fixed in 2% paraformaldehyde 2.5% glutaraldehyde in 100 mM sodium cocodylate containing 0.05% malachite green.

Retroviral Transduction

Activated OT-I splenocytes were transduced with human CD8 or GFP expressing control (virus expressing shRNA against luciferase), hpLAL (virus expressing shRNA aganst LAL), or LD-GFP (virus expressing the sequence of the 38 AA N-terminal of METTL7B fused to eGFP).

Adoptive Transfers

For in vivo memory T cell experiments, donor and recipient mice were infected i.v. with 5 × 10^6 cfu of LmOVA. The following day, splenocytes from donor mice were transduced with control or hpLAL. Following transduction, cells were injected i.v. (5 × 10^5 CD8$^+$ cells/mouse) into recipient mice. For in vivo survival experiments, OT-I cells transduced with control or hpLAL and sorted on GFP expression. On day 6 of culture, 2 × 10^6 CD8$^+$ cells/mouse were injected i.v into naive C57BL/6 mice.

Metabolism Assays

Cells were plated in XF media ± 10% FCS added as indicated. For LG conditions, 2 mM glucose XF media was prepared ± L-glutamine (8 mM) as indicated. For LD conditions, XF media was prepared without FCS. OCR and ECAR were measured under basal conditions and following the addition of IL-15 (10 ng/ml) or the following drugs: 1 μM oligomycin, 1.5 μM FCCP, 200 μM etomoxir, and 100 nM rotenone + 1 μM antimycin A with an Extracellular Flux Analyzer.

Lipidomics and Glucose Tracing

For glucose tracing, cells were cultured into IL-2 Teff and IL-15 memory T. On day 6, cells were cultured for 24 hr in TCM containing only D-[U-^{13}C] labeled glucose and analyzed by MS.

SUPPLEMENTAL INFORMATION

Supplemental Information includes seven figures and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.immuni.2014.06.005.

AUTHOR CONTRIBUTIONS

D.O., G.J.W.v.d.W., S.C.-C.H., C-H.C., M.D.B., W.Y.L., L.M.D., M.J.B., E.L.P designed research. D.O., G.J.W.v.d.W., J.D.C., C-H.C., J.Q., F-F.H., and E.L.P. analyzed data. D.O., G.J.W.v.d.W., J.D.C., M.D.B., A.M.S., L.M.D., performed experiments. D.O., G.J.W.v.d.W., M.D.B., J.Q., M.J.B., F-F.H., E.J.P., and E.L.P. contributed to manuscript preparation. D.O. and E.L.P. wrote the manuscript.

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