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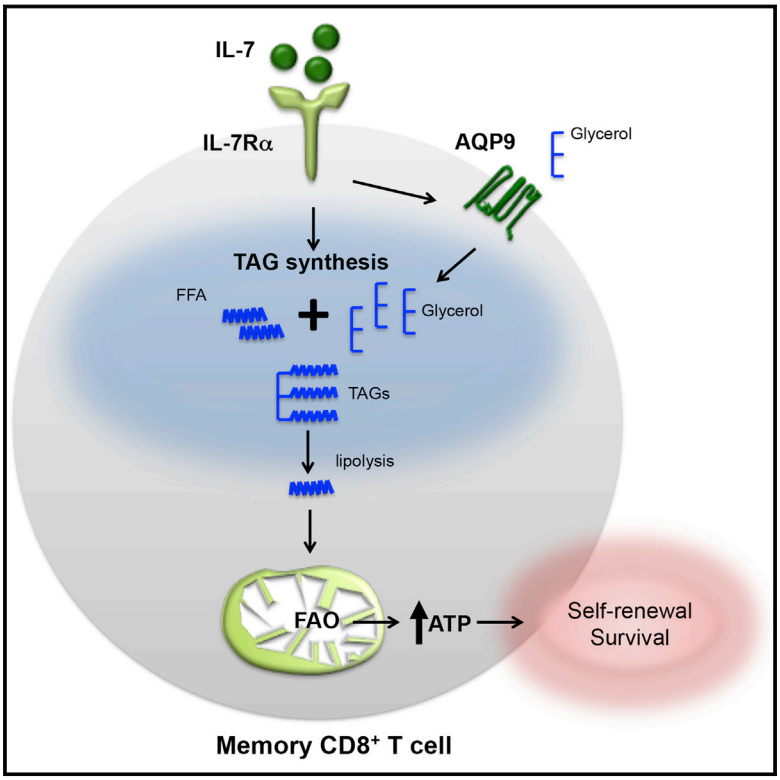
IL-7-Induced Glycerol Transport and TAG Synthesis Promotes Memory CD8$^+$ T Cell Longevity

Graphical Abstract



Highlights

- IL-7 induces glycerol channel AQP9 expression in CD8$^+$ T cells
- AQP9 is required for memory CD8$^+$ T cell survival and self-renewal
- AQP9 imports glycerol, promotes TAG synthesis, and sustains ATP levels in T cells
- IL-7 enhances TAG synthesis to promote memory CD8$^+$ T cell survival

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In Brief

Interleukin-7 induces expression of the glycerol channel aquaporin 9, allowing memory CD8$^+$ T cells to import glycerol, use it for triglyceride synthesis and storage, and sustain ATP levels required for long-term metabolic fitness and fast responses to reinfection.

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IL-7-Induced Glycerol Transport and TAG Synthesis Promotes Memory CD8$^+$ T Cell Longevity

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SUMMARY

Memory T cells are critical for long-term immunity against reinfection and require interleukin-7 (IL-7), but the mechanisms by which IL-7 controls memory T cell survival, particularly metabolic fitness, remain elusive. We discover that IL-7 induces expression of the glycerol channel aquaporin 9 (AQP9) in virus-specific memory CD8+ T cells, but not naive cells, and that AQP9 is vitally required for their long-term survival. AQP9 deficiency impairs glycerol import into memory CD8+ T cells for fatty acid esterification and triglyceride (TAG) synthesis and storage. These defects can be rescued by ectopic expression of TAG synthases, which restores lipid stores and memory T cell survival. Finally, we find that TAG synthesis is a central component of IL-7-mediated survival of human and mouse memory CD8+T cells. This study uncovers the metabolic mechanisms by which IL-7 tailors the metabolism of memory T cells to promote their longevity and fast response to rechallenge.

INTRODUCTION

Immunological memory is the foundation of protective vaccines, and therefore, understanding how memory lymphocytes form and persist after vaccination or infection is of great clinical importance. During acute viral infections, antigen-specific CD8$^+$ T cells undergo clonal expansion and differentiate into effector T cells that help fight off invading pathogens. After pathogen clearance, the majority of effector cells die and a small population survives as memory T cells, which can be further categorized into central memory T cells (T_{CM}), effector memory T cells (T_{EM}), and tissue resident memory T cells (T_{RM}) based on different migratory and functional properties (Beura and Masopust, 2014). Memory T cells can persist for decades and their longevity in many tissues is dependent on the cytokines IL-7 and IL-15, which promote cell survival and self-renewal (Becker et al., 2002; Kaech et al., 2003; Kennedy et al., 2000; Kieper et al., 2002; Kondrack et al., 2003; Lenz et al., 2004; Schluns et al., 2000). Voluminous evidence indicates that IL-7 plays an essential role in lymphopoiesis and peripheral T cell survival (Peschon et al., 1994; von Freeden-Jeffry et al., 1995), and our current understanding is that IL-7 promotes survival of naive and memory T cells as well as thymocytes through sustained expression of the anti-apoptotic factors Bcl-2 and Mcl1 (Opferman et al., 2003; Rathmell et al., 2001). However, other IL-7-dependent cellular processes are involved because Bcl-2 overexpression or deletion of Bim or Bax is insufficient to fully rescue T cell development in IL-7 receptor alpha (IL-7Rα)-deficient mice (Akashi et al., 1997; Khaled et al., 2002; Maraskovsky et al., 1997; Pellegrini et al., 2004). Indeed, IL-7 also controls amino acids uptake and glucose utilization in normal and leukemic T cells via its ability to enhance Glut1 trafficking and glycolysis through signal transducer and activator of transcription 5 (STAT5) and AKT activation (Barata et al., 2004; Pearson et al., 2012; Wofford et al., 2008). However, it is not known if IL-7 controls other processes essential for long-term survival of memory T cells nor how naive and memory T cells, which both rely on IL-7, avoid competition with one other for this limited resource.

Recent studies have suggested that a metabolic switch accompanies the differentiation of memory CD8$^+$ T cells from activated effector cells. After viral clearance, effector T cells that were once performing high rates of aerobic glycolysis, glutaminolysis, and anabolic metabolism rest down and become more reliant on fatty acid oxidation (FAO) and mitochondrial oxidative phosphorylation (OXPHOS) to generate energy (Fox et al., 2005; Pearce et al., 2009). In support of this model, knock down of lysosomal acid lipase (LAL), an enzyme that releases FAs from triacylglyceride (TAG)s in the lysosome, or carnitine palmitoyltransferase 1a (CPT1a), an enzyme required for mitochondrial FA transport, suppresses FAO and memory T cell survival following infection (van der Windt et al., 2012). Interestingly, at steady state, memory CD8$^+$ T cells do not display high rates of FA uptake, as opposed to activated T cells (O'Sullivan et al., 2014), and therefore, it is not known how these cells maintain an ample supply of FAs over long periods of time to sustain lipid burning. Most cell types, particularly adipocytes, store FAs in the form of TAGs by esterifying three FA chains to glycerol, which can then be broken down to supply FAs for FAO to meet energy demands (Lass et al., 2011).

To better understand the metabolic control of memory CD8$^+$ T cell longevity and homeostasis, we profiled the expression of genes involved in cellular metabolism as CD8$^+$ T cells

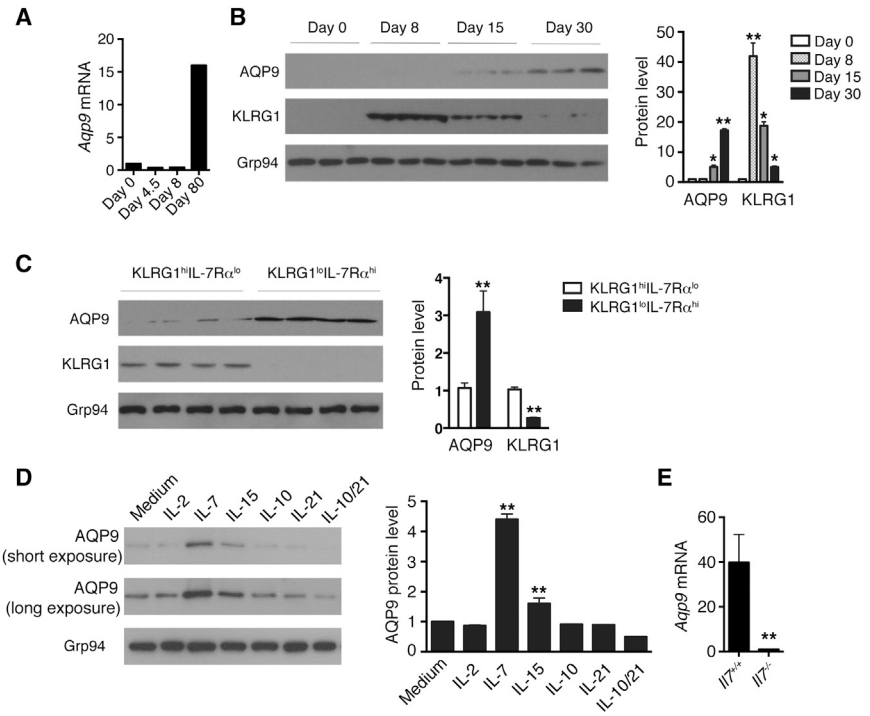

Figure 1. IL-7 Induces AQP9 Expression Selectively in Anti-viral Memory CD8$^+$ T Cells and Their Precursors

(A–C) Naive, effector, and memory P14 CD8$^+$ T cells were purified on the indicated dpi and the amount of *Aqp9* mRNA was measured using DNA microarrays and analyzed by GeneSpring software (A) or protein using western blotting (B and C).

(A) mRNA is normalized to naive samples.

(B and C) Each lane represents an individual biological sample; Grp94 and KLRG1 were used as loading and internal monitoring controls, respectively.

(C) KLRG1^{hi}IL-7R^{lo} and KLRG1^{lo}IL-7R^{hi} effector CD8 T cell subsets were isolated at 14 dpi. The bar graphs on the right show densitometry quantification of the immunoblot bands.

(D) P14 CD8$^+$ T cells were primed with $GP_{33–41}$ peptide for 3 days and then stimulated with various cytokines as indicated for 3 days before western blotting for AQP9. The bar graph on the right shows densitometry quantification of the immunoblot bands.

(E) In vitro primed P14 CD8$^+$ T cells were transferred to *Il7*$^{+/+}$ or *Il7*$^{-/-}$ mice. At 7 days later, the donor cells were purified and *Aqp9* mRNA levels were measured by qRT-PCR.

Data in (B)–(E) are representative of two independent experiments (n = 3–6 mice/group): *p < 0.05 and **p < 0.01 (see also Figure S1).

differentiate from naive→effector→memory stages. This identified that AQP9, a critical glycerol channel in mammals (Carbrey et al., 2003; Rojek et al., 2007), was selectively expressed in CD8$^+$ memory T cells compared with naive and effector T cells. Through biochemical and genetic analyses, we found that IL-7 induced AQP9 expression, glycerol importation, and TAG synthesis, which was necessary for memory CD8$^+$ T cell survival and homeostasis. Thus, this study reveals a previously unknown metabolic role for IL-7 in directing glycerol uptake and TAG storage to sustain memory CD8$^+$ T cells long-term survival, and identifies TAG synthesis as a critical biochemical process for therapeutic modulation of memory T cell survival and self-renewal.

RESULTS

IL-7 Induces AQP9 Expression in Memory CD8$^+$ T Cells

Aqp9 has a unique temporal gene expression pattern in virus-specific CD8$^+$ T cells, shared with only a handful of other genes, being expressed at very low levels in virus-specific naive and effector T cells and progressively increasing as memory T cells form following viral infection (Best et al., 2013) (Figure 1A). Consistent with the *Aqp9* mRNA expression pattern, AQP9 protein was more abundant in memory CD8$^+$ T cells (30 days post infection [dpi]) than in naive or effector CD8$^+$ T cells isolated 8 and 15 dpi (Figure 1B). Conversely, the expression of KLRG1, an inhibitory receptor expressed on the most terminally differentiated effector CD8$^+$ T cells, declined as these cells waned overtime (Joshi et al., 2007; Kaech et al., 2003). Further fractionation of the effector CD8$^+$ T cells into KLRG1^{hi} IL-7Rα^{lo} terminal effector and KLRG1^{lo} IL-7Rα^{hi} memory precursor effector cell subsets at 14 dpi revealed that AQP9 was selectively expressed in the IL-7Rα^{hi} effector cells that preferentially seed the memory T cell pool (Figure 1C) (Kaech et al., 2003; Schluns et al., 2000).

To determine if cytokines that regulate effector and memory CD8$^+$ T cell development and homeostasis induce AQP9 in CD8$^+$ T cells, we stimulated lymphocytic choriomeningitis virus (LCMV)-specific P14 TCR tg CD8$^+$ T cells, which recognize the LCMV epitope $GP_{33–41}$, in vitro with peptide for three days and then with IL-2, IL-7, IL-15, IL-10, and IL-21 and examined AQP9 expression using western blotting three days later. This showed that IL-7, and to a lesser extent IL-15, induced AQP9 expression in activated CD8$^+$ T cells (Figure 1D). To further test the requirement of IL-7 for AQP9 expression in an in vivo setting, we transferred in vitro primed P14 CD8$^+$ T cells to *Il7*$^{+/+}$ or *Il7*$^{-/-}$ mice and analyzed *Aqp9* expression in the cells 7 days later using quantitative (q)RT-PCR. This showed that *Aqp9* mRNA was dramatically decreased in cells isolated from *Il7*$^{-/-}$ host mice, indicating that IL-7 signaling was both necessary and sufficient to sustain *Aqp9* expression in antigen-experienced CD8$^+$ T cells (Figure 1E).

AQP9 Deficiency Impairs Memory CD8$^+$ T Cell Survival following Infection

The observation that AQP9 was selectively expressed in mature memory CD8$^+$ T cells following acute viral infection prompted us to examine its functional role in memory T cell generation. To this end, we generated 50:50 mixed *Aqp9*$^{+/+}$ and *Aqp9*$^{-/-}$ bone marrow chimeric mice. At eight weeks after reconstitution, the mice were infected with LCMV and the virus-specific T cells

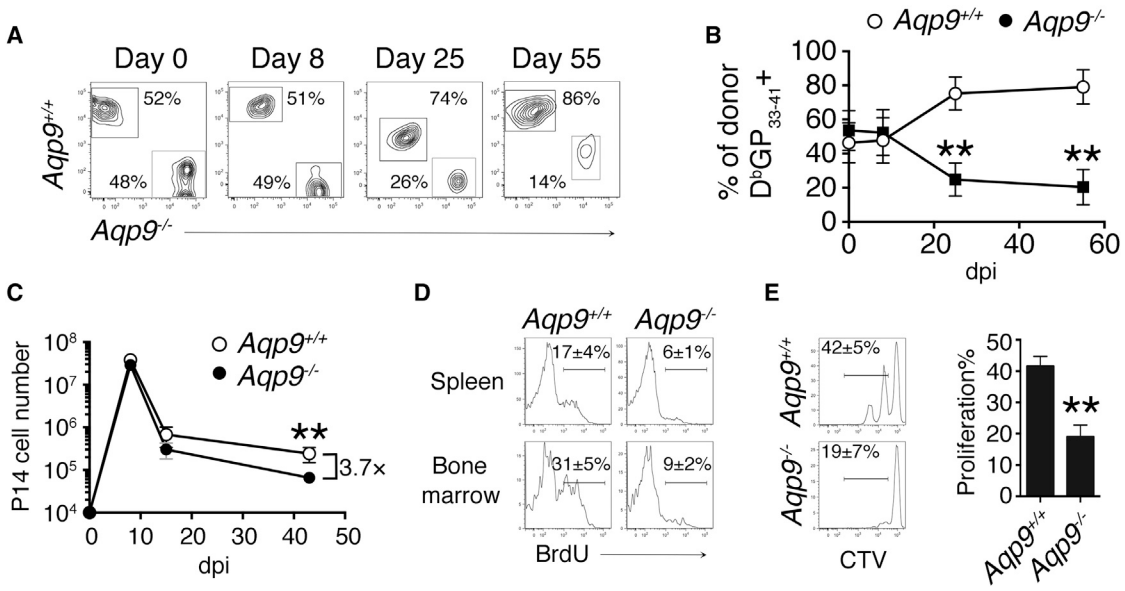

Figure 2. AQP9 Deficiency Impairs Formation of LCMV-Specific Memory CD8$^+$ T Cells

(A and B) Bone marrow chimeric mice containing a 1:1 ratio of *Aqp9*$^{+/+}$ (open circles, Ly5.2$^+$Thy1.1$^+$) and *Aqp9*$^{-/-}$ (black squares, Ly5.2$^+$Thy1.2$^+$) bone marrow cells were infected with LCMV-Armstrong and the frequency of the two populations within the D^{b}GP$_{33-41}$-specific CD8$^+$ T cells were analyzed longitudinally by flow cytometry.

(C) *Aqp9*$^{-/-}$ or littermate *Aqp9*$^{+/+}$ P14 CD8$^+$ T cells (10^4 cells) were adoptively transferred into B6 mice that were subsequently infected with LCMV-Armstrong. The numbers of donor P14 CD8$^+$ T cells were determined at the indicated dpi.

(D) P14 chimeric mice described in (C) were given BrdU drinking water (1 mg/ml) from 30–51 dpi to measure the rates of homeostatic proliferation in the memory CD8$^+$ T cells. Amounts of nuclear BrdU were measured by flow cytometry.

(E) *Aqp9*$^{-/-}$ or littermate *Aqp9*$^{+/+}$ P14 CD8$^+$ T cells were purified at 25 dpi, labeled with CTV, adoptively transferred to naive B6 mice, and analyzed by flow cytometry for CTV dilution. The bar graph shows the percentages of divided cells.

Data are representative (A, C–E) or cumulative (B) of three and four (C) independent experiments (n = 5–15 mice/group): **p < 0.01 (see also Figure S2).

were analyzed longitudinally. This showed that LCMV-specific *Aqp9*$^{-/-}$ effector CD8$^+$ T cell expansion was not affected compared with *Aqp9*$^{+/+}$ cells at 8 dpi. However, the *Aqp9*$^{-/-}$ CD8$^+$ T cells revealed a profound defect in their survival thereafter, and the frequency of *Aqp9*$^{-/-}$ memory CD8$^+$ T cells steadily declined over time (Figures 2A and 2B). This result demonstrated a critical role for AQP9 in memory CD8$^+$ T cell formation and maintenance. To more rigorously examine the CD8$^+$ T cell-intrinsic requirement of *Aqp9* in memory CD8$^+$ T cell formation, we created P14 mice lacking *Aqp9* and transferred small numbers of naive *Aqp9*$^{-/-}$ or *Aqp9*$^{+/+}$ P14 CD8$^+$ T cells into *Aqp9*$^{+/+}$ littermates that were subsequently infected with LCMV. The numbers of donor *Aqp9*$^{-/-}$ or *Aqp9*$^{+/+}$ P14 CD8$^+$ T cells were assessed at 8, 15, and 43 dpi (Figure 2C). Similar to the bone marrow chimeras, the P14 CD8$^+$ T cells lacking *Aqp9* expanded similarly to their wild-type counterparts, but were poorly maintained during the effector→memory transition and generated a pool of memory CD8$^+$ T cells that was ~4-fold smaller than the wild-type (WT) cells (Figure 2C). Furthermore, BrdU-labeling experiments from 30–51 dpi and cell tracer violet (CTV)-labeling experiments revealed that *Aqp9*$^{-/-}$ memory CD8$^+$ T cells displayed a profound defect in homeostatic proliferation in the spleen and bone marrow (Figures 2D and 2E). Closer interrogation of the quality of the effector and memory CD8$^+$ T cells revealed that the *Aqp9* was required for optimal differentiation of memory CD8$^+$ T cells (Figure S1). That is, *Aqp9*$^{-/-}$ memory CD8$^+$ T cells contained fewer IL-7Rα^{hi} CD27^{hi} and CD62L^{hi} cells than the *Aqp9*$^{+/+}$ cells. Thus, there was a block in the development of T_{CM} cells. These studies identified a new protein AQP9 that is critical for memory T cell development and survival after viral infection.

AQP9 Deficiency Impairs Glycerol Uptake and TAG Synthesis in CD8$^+$ T Cells

AQP9 transports water, glycerol, and urea. To investigate which solute was involved in AQP9-mediated memory CD8$^+$ T cells survival, we attempted to rescue AQP9-deficient CD8$^+$ T cells by retroviral (RV) overexpression of *Aqp3* (also permeable to water, glycerol, and urea) or *Aqp1* (permeable to water). This showed that *Aqp3* overexpression could partially rescue *Aqp9*$^{-/-}$ memory CD8$^+$ T cell formation, but *Aqp1* could not. This result suggested that glycerol or urea, as opposed to water, were the critical AQP9-dependent solutes for memory T cell formation (Figure S2A). Moreover, as prior studies found aberrantly high levels of glycerol in the serum of *Aqp9*$^{-/-}$ mice because of impaired glycerol uptake by the liver (Rojek et al., 2007), we hypothesized that defective glycerol import in *Aqp9*$^{-/-}$ T cells may contribute to their poor memory T cell survival. In support of this idea, we observed that the *Aqp9*$^{-/-}$ P14 CD8$^+$ T cells contained ~50% less intracellular glycerol than their *Aqp9*$^{+/+}$ counterparts following in vitro activation (Figure 3A).

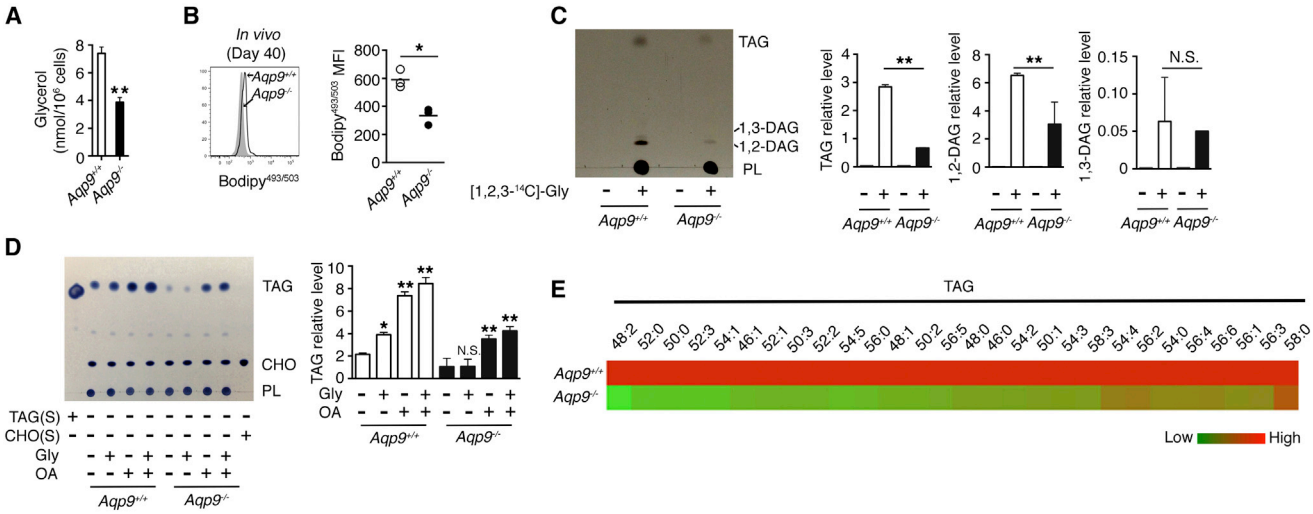

Figure 3. AQP9 Deficiency Impairs TAG Synthesis and Storage in CD8$^+$ T Cells

(A) *Aqp9*$^{-/-}$ or *Aqp9*$^{+/+}$ P14 CD8$^+$ T cells were cultured in vitro with $GP_{33–41}$ peptide for 3 days and then in IL-7 for two days. The amount of free glycerol was measured in total cell lysates by a coupled enzymatic reaction system.

(B) *Aqp9*$^{-/-}$ or *Aqp9*$^{+/+}$ P14 memory CD8 T cells from 40 dpi were stained with the neutral lipid indicator Bodipy$^{493/503}$ and analyzed by flow cytometry.

(C) P14 CD8$^+$ T cells described in (A) were pulsed with 0.1 μcurie per milliliter (Ci/ml) [1,2,3-^{14}C]-Glycerol for 4 hr, then lipids were extracted and resolved by TLC. The bar graphs on the right show densitometry quantification of TAG and DAG autoradiography bands after a 2-week exposure.

(D) P14 CD8$^+$ T cells described in (A) were cultured in the presence or absence of glycerol (Gly) or OA for 2 days before lipid extraction and TLC assay. Standards for TAG and CHO were loaded on the left- and right-most lanes. The bar graph on the right shows densitometry quantification of TAG band.

(E) Lipids were extracted from *Aqp9*$^{+/+}$ and *Aqp9*$^{-/-}$ P14 CD8$^+$ T cells described in (A) for LC-MS analysis of TAG isobaric species.

Data are representative of two (B and E) and three (A, C, and D) independent experiments (n = 3–7 mice/group): *$p < 0.05$, **$p < 0.01$, and not significant (n.s.) (see also Figure S3).

Glycerol is the molecular backbone of TAGs and most phospholipids (PLs). To determine if AQP9 deficiency affected glycerolipid homeostasis in CD8$^+$ T cells, we first evaluated the total cellular neutral lipid content (e.g., TAGs) in the *Aqp9*$^{-/-}$ LCMV-specific memory CD8$^+$ T cells (40 dpi) using Bodipy$^{493/503}$ labeling and observed that it was approximately one-half that of the *Aqp9*$^{+/+}$ control cells (Figure 3B). The reduction of Bodipy$^{493/503}$ mean fluorescence intensity (MFI) could be due to either decreased TAG synthesis or increased lipolysis or both. To more closely monitor the incorporation of glycerol into TAGs (i.e., synthesis), *Aqp9*$^{+/+}$ or *Aqp9*$^{-/-}$ CD8$^+$ T cells were pulsed with radioactive ^{14}C glycerol (for 4 hr) before lipid extraction and thin layer chromatography (TLC). Glycerol incorporation into diacylglycerol (DAG) and TAG was detected in *Aqp9*$^{+/+}$ cells, but virtually none was detected in *Aqp9*$^{-/-}$ cells (Figure 3C). We then compared the ability of *Aqp9*$^{-/-}$ and *Aqp9*$^{+/+}$ CD8$^+$ T cells to synthesize TAGs by culturing the activated CD8$^+$ T cells in glycerol and the free FA oleic acid (OA) for 48 hr. This treatment boosted TAG synthesis in *Aqp9*$^{+/+}$ CD8$^+$ T cells, but the *Aqp9*$^{-/-}$ cells were considerably less efficient (Figure 3D). Separation of glycerol and OA in the cultures demonstrated that exogenous OA could promote TAG synthesis in both *Aqp9*$^{+/+}$ and *Aqp9*$^{-/-}$ T cells, but glycerol could only enhance TAG synthesis in the *Aqp9*$^{+/+}$ CD8$^+$ T cells. This result provided greater evidence that AQP9 was necessary for glycerol import and TAG synthesis in CD8$^+$ T cells. To further characterize the various glycerolipid species in the CD8$^+$ T cells, we performed lipidomic analysis using liquid chromatography-mass spectrometry (LC-MS). This validated the TLC results by demonstrating a marked decrease in all TAG isobaric species in the *Aqp9*$^{-/-}$ CD8$^+$ T cells compared with *Aqp9*$^{+/+}$ cells (Figure 3E). Interestingly, the amounts of intracellular PLs or cholesterol (CHO) were only marginally affected by AQP9 deficiency, indicating a more specific defect in TAG biogenesis (Figures 3D and S2B). Altogether, these results show that AQP9 is necessary to maintain normal levels of glycerol and TAGs in antigen-specific CD8$^+$ T cells, and when coupled to the data shown in Figure 2, reveal a regulatory mode of TAG synthesis in memory CD8$^+$ T cell survival.

AQP9 Deficiency Reduces ATP Levels and Alters Metabolic States in CD8$^+$ T Cells

Next, we determined if the reduced amounts of TAGs in the CD8$^+$ T cells affected their bioenergetic states. To this end, we compared the rates of glycolysis and mitochondrial respiration using the Seahorse Extracellular Flux Analyzer. This showed that in vitro activated P14 *Aqp9*$^{-/-}$ CD8$^+$ T cells had substantially higher extracellular acidification rates (ECAR) (i.e., glycolytic rates) and modestly higher oxygen consumption rates (OCR) (i.e., mitochondrial respiration) than the *Aqp9*$^{+/+}$ cells (Figures 4A and S3). The increased ECAR:OCR ratios in *Aqp9*$^{-/-}$ CD8$^+$ T cells indicate a shift toward preferential use of glycolysis over OXPHOS (Figures 4A, right graph, and S3E). Furthermore, *Aqp9* deficiency affected the mitochondrial spare respiratory capacity (SRC) of LCMV-specific CD8 T cells, which is a measurement of the maximal rate of respiration after mitochondrial membrane

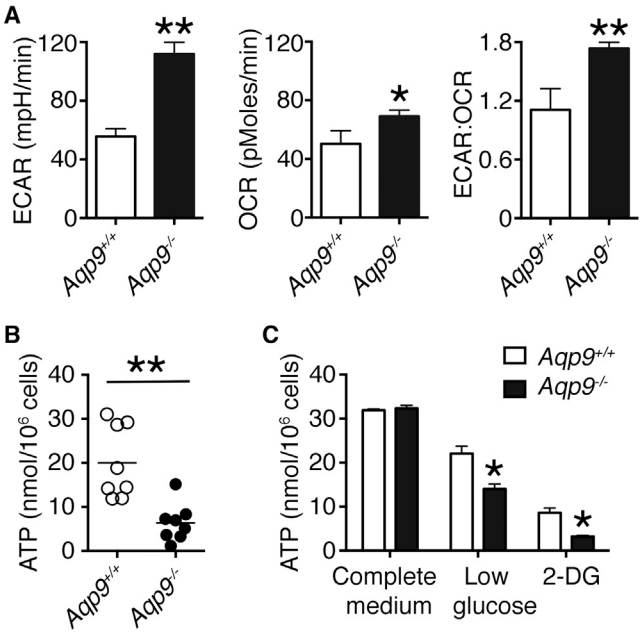

Figure 4. AQP9 Deficiency Reduces ATP Levels and Increases Glycolytic Rates in CD8$^+$ T Cells

(A) *Aqp9*$^{-/-}$ or *Aqp9*$^{+/+}$ P14 CD8$^+$ T cells were cultured in vitro with $GP_{33–41}$ peptide for 3 days and then in IL-7 for two days. Rates of ECAR and OCR were then measured using the Seahorse Extracellular Flux Analyzer. The bar graphs show the basal levels of ECAR, OCR, and the ratio between ECAR and OCR.

(B) The amount of intracellular ATP was measured in *Aqp9*$^{-/-}$ or *Aqp9*$^{+/+}$ P14 memory CD8 T cells from 40 dpi by a bioluminescence assay as described in Experimental Procedures.

(C) P14 CD8$^+$ T cells described in (A) were cultured in complete medium (11 millimolar [mM] glucose), low glucose medium (2.2 mM glucose), or complete medium plus the glycolysis inhibitor 2-DG and ATP levels were measured 12 hr later.

Data are cumulative from three independent experiments (n = 8 mice/group) (B) or representative of three independent experiments (n = 3 mice/group) (A and C): *p < 0.05 and **p < 0.01.

uncoupling (Figures S3A and S3B). The mitochondrial SRC in memory T cells has been suggested to be affected by FA availability (O'Sullivan et al., 2014), and indeed, *Aqp9*$^{-/-}$ LCMV-immune CD8$^+$ T cells had lower amounts of intracellular FFA (Figure S3F). Given that TAGs are an important biofuel that supply FAs, through lipolysis, for mitochondrial FAO, it is likely that TAG insufficiency in *Aqp9*$^{-/-}$ memory CD8$^+$ T cells prevents these cells from sustaining high rates of FAO necessary for memory T cell survival.

To determine if the metabolic alterations affected total ATP levels, we measured the amount of intracellular ATP between the two groups of memory P14 CD8$^+$ T cells isolated 40 dpi and found indeed, that the *Aqp9*$^{-/-}$ CD8$^+$ T cells had markedly lower ATP levels compared with the *Aqp9*$^{+/+}$ cells (Figure 4B). This finding suggested that despite the increase in glycolysis, *Aqp9*$^{-/-}$ CD8$^+$ T cells were unable to sustain normal ATP levels. To evaluate this further, in vitro activated *Aqp9*$^{-/-}$ and *Aqp9*$^{+/+}$ P14 CD8$^+$ cells were cultured in medium containing low glucose concentrations or 2-deoxy-D-glucose (2-DG), an inhibitor of the first step of glycolysis. This demonstrated that the *Aqp9*$^{-/-}$ CD8$^+$ T cells were more sensitive than the *Aqp9*$^{+/+}$ cells to glucose deprivation based on lowered amounts of ATP (Figure 4C). Together, these findings suggested that AQP9-deficient memory T cells were more reliant on glycolysis, likely because of reduced lipid stores, but nonetheless, were unable to generate sufficient amounts of ATP for long-term survival.

Increased TAG Synthesis Rescues *Aqp9*$^{-/-}$ Memory CD8$^+$ T Cell Survival

The above data demonstrated that AQP9 is required for glycerol import to support TAG synthesis and survival in memory CD8$^+$ T cells. To further investigate the regulation of TAG synthesis in CD8$^+$ T cells during viral infection, we examined the mRNA expression patterns of enzymes involved in TAG synthesis (Figure 5A) in *Aqp9*$^{+/+}$ or *Aqp9*$^{-/-}$ LCMV-specific CD8$^+$ T cells as they differentiated from naive→effector→memory CD8$^+$ T cells (Rodríguez et al., 2011; Shi and Burn, 2004). This showed that a few of the enzymes were upregulated in the *Aqp9*$^{+/+}$ virus-specific CD8$^+$ T cells at early (4.5 dpi) or late (8 dpi) effector time points relative to naive CD8$^+$ T cells, but stunningly, all of the enzymes assessed in this process were coordinately upregulated in memory T cells (40 dpi) (Figure 5B). In contrast, several of these enzymes, such *glycerol kinase* (*Gyk*), *glycerol-3-phosphate acyltransferase mitochondrial* (*Gpat1*), *monoacylglycerol O-acyltransferase 1* (*Mogat1*), and *DAG O-acyltransferase 1* (*Dgat1*), were not elevated in *Aqp9*$^{-/-}$ memory P14 CD8$^+$ T cells to the same extent as the WT cells (Figure 5B).

To determine if the reduction of TAG synthesis-related genes in *Aqp9*$^{-/-}$ cells contributed to their defects in memory T cell formation, we transduced *Aqp9*$^{-/-}$ P14 CD8$^+$ T cells with RVs overexpressing *Gyk*, *Dgat1*, *Gpat1*, and *Mogat1* or an empty vector (EV) control and analyzed the number of memory CD8 T cells and Bodipy staining at 40 dpi (Figure 5C). In addition, *Aqp9*$^{+/+}$ P14 cells expressing an EV RV were included as a comparison. This showed that overexpression of these genes in the *Aqp9*$^{-/-}$ P14 T cells restored intracellular neutral lipid content and memory CD8 T cell numbers to levels similar to that observed in A*qp9*$^{+/+}$ T cells (Figure 5C). A longitudinal analysis of these experiments (Figure 5D) revealed that overexpression of the TAG synthesis-related genes had a relatively bigger effect on the frequency of *Aqp9*$^{-/-}$ memory T cells (40 dpi) as opposed to effector cells (8 dpi). Likewise, a similar effect was observed when the genes were overexpressed in *Aqp9*$^{+/+}$ P14 CD8$^+$ T cells (Figure 5D). This showed that boosting TAG synthesis had a more prominent effect on the development of memory than on effector CD8 T cells. Notably, *Dgat1* overexpression had a greater impact on *Aqp9*$^{-/-}$ P14 memory CD8$^+$ T cell survival than the other enzymes or than that in *Aqp9*$^{+/+}$ cells, suggesting decreased DGAT1 activity is an underlying cause of the *Aqp9*$^{-/-}$ memory CD8 T defect. These results convincingly demonstrate that impaired TAG synthesis and storage is an underlying cause of defective memory CD8$^+$ T cell formation in *Aqp9*$^{-/-}$ cells, thereby illuminating the vital role of TAG synthesis in CD8$^+$ memory T cell formation and homeostasis.

IL-7 Enhances TAG Synthesis to Promote CD8$^+$ Memory T Cell Survival

To better understand how TAG synthesis is regulated in memory CD8$^+$ T cells, we returned to IL-7 because our initial data

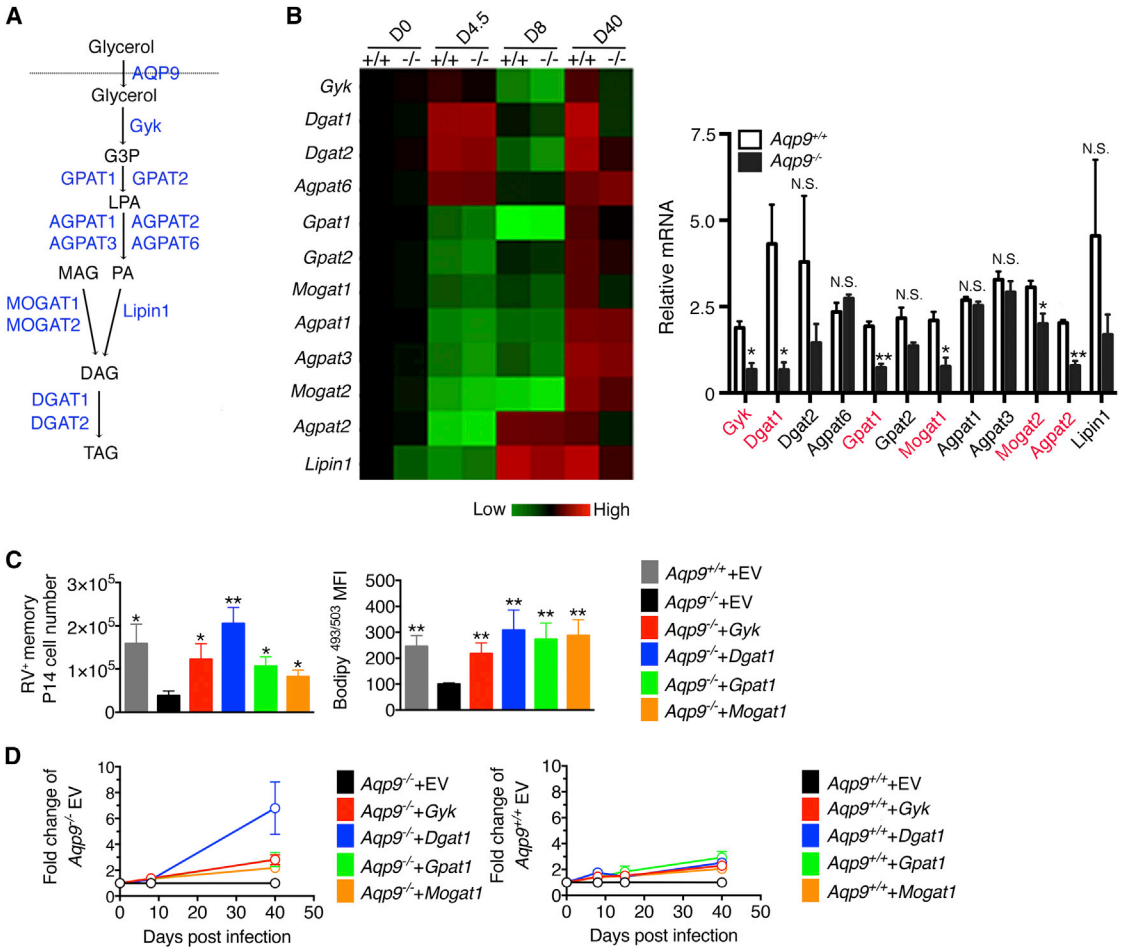

Figure 5. Overexpression of Glycerol Kinase and TAG Synthases Rescues Survival of *Aqp9*$^{-/-}$ Memory CD8$^+$ T Cells

(A) Outline of TAG synthesis pathway with AQP9 and TAG synthases highlighted in blue.

(B) Heat map shows the mean mRNA expression level of the indicated genes in virus-specific *Aqp9*$^{-/-}$ or *Aqp9*$^{+/+}$ P14 CD8$^+$ T cells purified at 0, 4.5, 8, and 40 dpi using qRT-PCR (values are normalized to naive CD8 T cells [day 0]). The bar graph shows the amount of mRNA relative to *L9* mRNA in memory CD8 T cells (40 dpi) and the statistically significant differentially expressed genes are highlighted in red.

(C) *Aqp9*$^{-/-}$ P14 CD8$^+$ T cells were transduced with RVs overexpressing *Gyk, Dgat1, Gpat*1, *Mogat1*, or control EV and adoptively transferred into B6 mice that were subsequently infected with LCMV-Armstrong. *Aqp9*$^{+/+}$ P14 CD8 T cells transduced with EV were included for comparison (dark gray). The bar graphs show the numbers of donor P14 CD8$^+$ T cells and MFI of Bodipy$^{493/503}$ staining at 40 dpi.

(D) Longitudinal analysis of the RV-transduced *Aqp9*$^{-/-}$ and *Aqp9*$^{+/+}$ P14 CD8$^+$ T cells, as described in (C). At each time point, the frequency of RV$^+$ cells was normalized to EV control cells and plotted in the line graph.

Data are cumulative of three (B–D) experiments (n = 6–10 mice/group): *p < 0.05, **p < 0.01, and not significant (n.s.).

indicated that IL-7 was sufficient and necessary for AQP9 expression in antigen-specific CD8$^+$ T cells. Given that IL-7Rα-signaling is critical for memory CD8$^+$ T cell survival (Goldrath et al., 2002; Kaech et al., 2003; Kieper et al., 2002; Prlic et al., 2002; Schluns et al., 2000; Tan et al., 2002), we hypothesized that IL-7 may directly regulate TAG synthesis in these cells. First, we examined if IL-7 signaling was necessary to sustain TAG levels in memory CD8$^+$ T cells by transferring such cells into *Il7*$^{+/+}$ or *Il7*$^{-/-}$ animals for 5 days. These experiments revealed that the donor memory CD8$^+$ T cells isolated from IL-7-deficient hosts had lower amounts of Bodipy$^{493/503}$ staining relative to the IL-7-sufficient hosts, which indicated that IL-7 was required to sustain neutral lipid levels in memory CD8$^+$ T cells (Figure 6A). Second, stimulating naive or LCMV-specific memory CD8$^+$ T cells in vitro with recombinant IL-7 (Figures 6B and 6C) or in vivo with IL-7/anti-IL-7 (M25) mAb complexes (Figure 6D) demonstrated that IL-7 treatment induced lipogenesis and TAG synthesis in memory CD8$^+$ T cells profoundly more than in naive CD8$^+$ T cells. Additionally, in vitro stimulation with IL-7 induced the expression of several TAG synthesis-related genes in activated, but not naive, CD8 T cells (Figure S4). Together, these findings indicated that, on a per cell basis, memory CD8$^+$ T cells had a greater capacity to synthesize neutral lipids (i.e., TAGs) than naive CD8$^+$ T cells and suggested that IL-7 induces distinct metabolic programs between naive and memory T cells. Because IL-7 is a critical memory T cell survival factor,

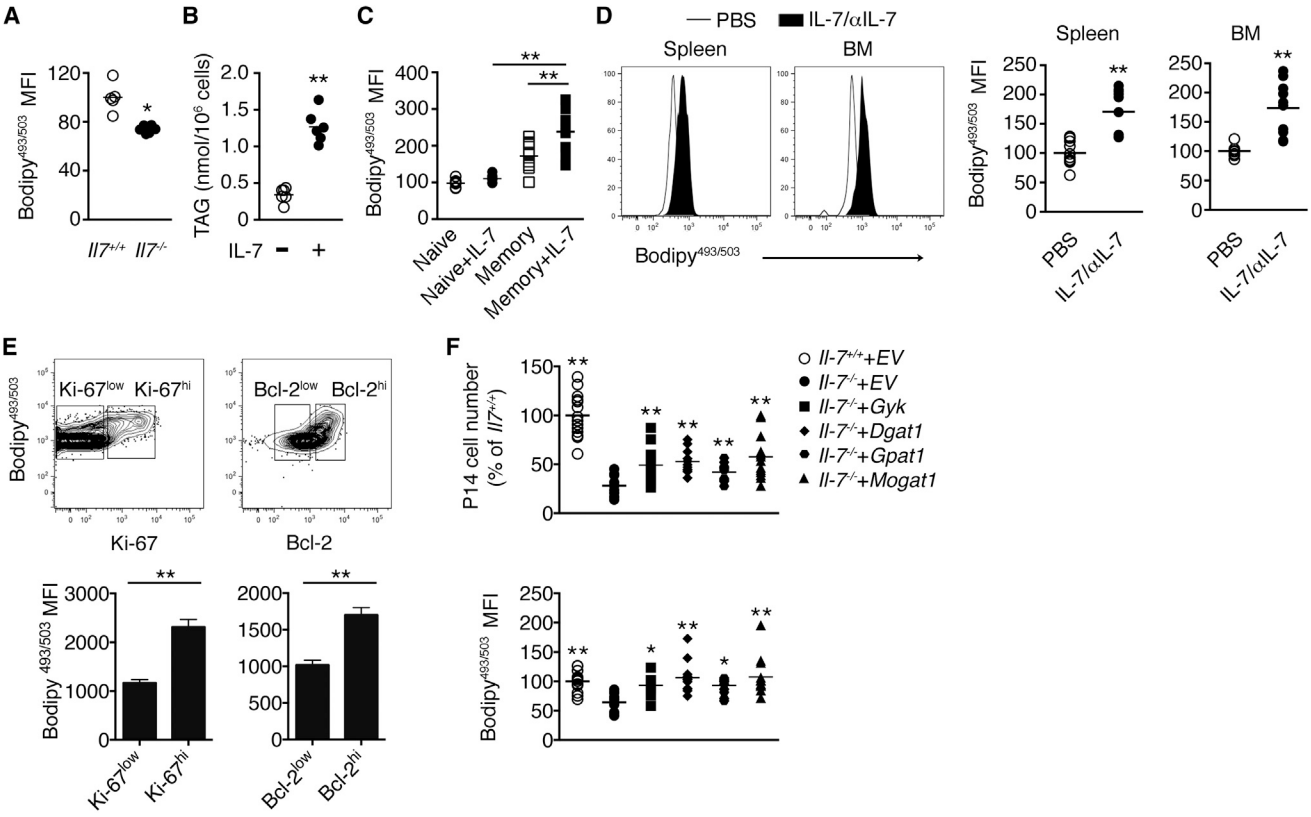

Figure 6. IL-7-Driven Glycerol Metabolism and TAG Synthesis Are Critical for Memory CD8$^+$ T Cell Survival

(A and B) Purified memory P14 CD8$^+$ T cells from 50–70 dpi were (A) transferred to *Il7*$^{+/+}$ and *Il7*$^{-/-}$ mice and 5 days later the donor cells were stained with Bodipy$^{493/503}$ and analyzed by flow cytometry or (B) stimulated with IL-7 for 12 hr and then TAG levels were measured as described in Experimental Procedures.

(C) Purified naive or memory P14 CD8$^+$ T cells (from 50–70 dpi) were stimulated with IL-7 for 12 hr and then TAG levels were measured using Bodipy$^{493/503}$ staining and flow cytometry.

(D) P14 chimeric mice containing memory P14 CD8$^+$ T cells from 40–60 dpi were injected with IL-7/anti–IL-7 (M25) complex. At 3 days later, TAG levels in donor P14 cells in the spleen and bone marrow were measured using Bodipy$^{493/503}$ staining and flow cytometry.

(E) Memory P14 CD8$^+$ T cells purified at 50–150 dpi were stained with Bodipy$^{493/503}$, Ki-67, and Bcl-2 and analyzed by flow cytometry. The bar graphs show Bodipy$^{493/503}$ MFI in the indicated cell populations.

(F) Memory P14 CD8$^+$ T cells (from 30–40 dpi) overexpressing *Gyk, Dgat1, Gpat1*, *Mogat1*, or EV were adoptively transferred into *Il7*$^{-/-}$ mice. For comparison, the EV-transduced cells were also transferred into *Il7*$^{+/+}$ mice (open circles). At 2–4 weeks later, donor RV$^+$ P14 CD8$^+$ T cells were enumerated and stained with Bodipy$^{493/503}$. Values were normalized to those of EV-transduced cells in *Il7*$^{+/+}$ mice (open circles).

Data shown are cumulative of two (D), three (A–C), and four (F) independent experiments or representative of five independent experiments (E) (n = 5–20 mice/group): *p < 0.05 and **p < 0.01 (see also Figure S4).

we asked if TAG synthesis was associated with memory T cell survival and homeostatic turnover. Interestingly, dividing (Ki-67$^+$) memory T cells had higher Bodipy$^{493/503}$ staining than the resting Ki-67$^-$ counterparts, suggesting a close connection between lipogenesis and CD8$^+$ memory T cell self-renewal (Figure 6E). Furthermore, the memory CD8$^+$ T cells that contained the greatest amount of neutral lipids also had the highest Bcl-2 expression, possibly highlighting a link between TAG levels and memory T cell survival.

Given the strong association between TAG synthesis and memory CD8$^+$ T cell homeostatic proliferation and survival, we wondered if increasing TAG synthesis could rescue memory T cell survival in *Il7*$^{-/-}$ hosts. To this end, *Aqp9*$^{+/+}$ P14 memory CD8$^+$ T cells overexpressing *Gyk* or TAG synthases (as in Figure 5D) were transferred into *Il7*$^{-/-}$ mice and analyzed 2–4 weeks later. This showed that overexpression of these enzymes in memory CD8$^+$ T cells could significantly boost neutral lipid levels and partially rescue cell survival in IL-7-deficient hosts (Figure 6F). These results indicated that TAG synthesis, a process not previously known to be controlled by IL-7, largely contributes to IL-7-mediated memory T cell survival.

IL-7 Drives TAG Synthesis and Promotes Human CD8$^+$ T Cell Survival

Finally, we asked if IL-7 signaling could similarly induce *Aqp9* expression and TAG synthesis in human memory T cells. As a first step, we characterized *Aqp9* gene expression in different CD8$^+$ T cell subsets, including CCR7$^+$CD45RA$^+$ naive cells, CCR7$^-$CD45RA$^+$ T_{EMRA} cells, CCR7$^-$CD45RA$^-$ T_{EM} cells, and CCR7$^+$CD45RA$^-$ T_{CM} cells and found that *Aqp9* mRNA was

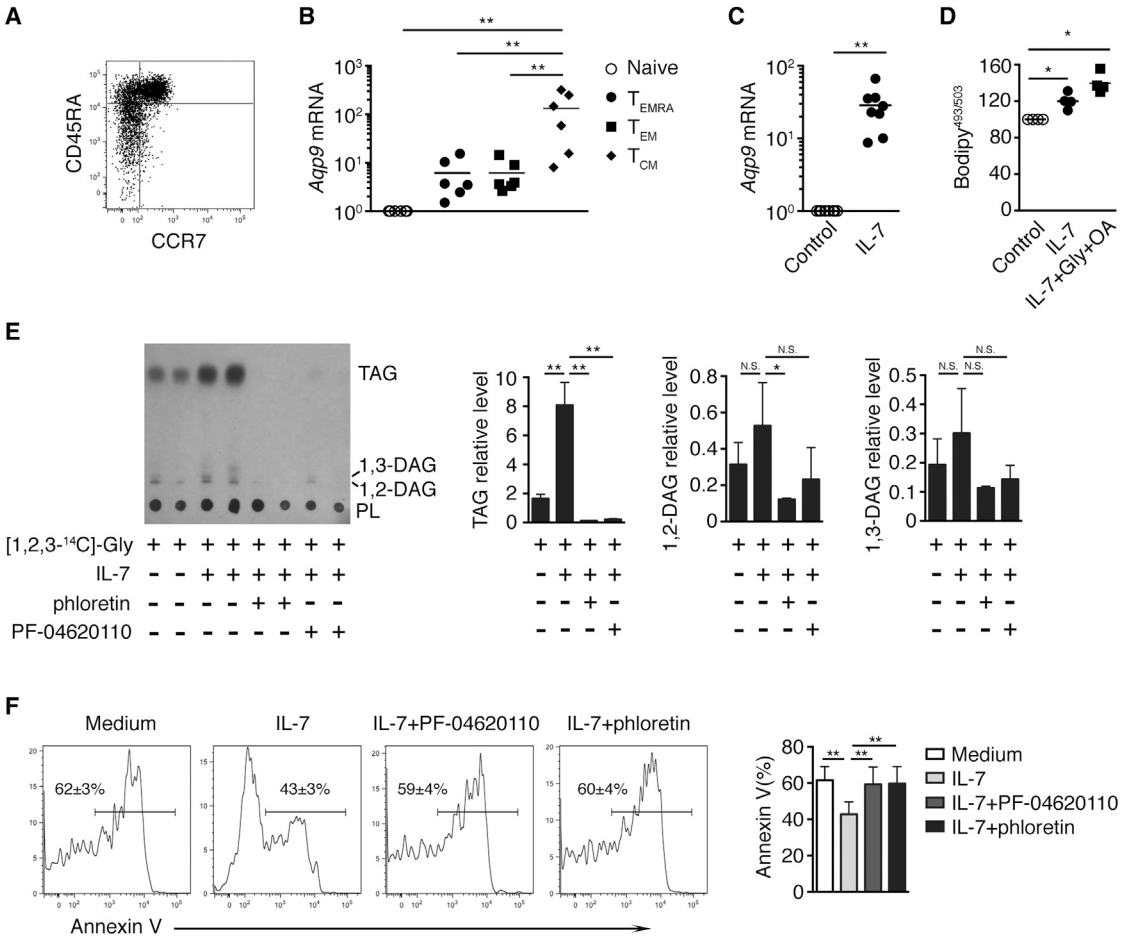

Figure 7. IL-7 Drives TAG Synthesis and Promotes Human Memory CD8$^+$ T Cell Survival

(A and B) CCR7$^+$ CD45RA$^+$ CD8$^+$ naive T cells, CCR7$^-$ CD45RA$^+$ CD8$^+$ T_{EMRA}, CCR7$^-$ CD45RA$^-$ CD8$^+$ T_{EM} cells, and CCR7$^+$ CD45RA$^-$ CD8$^+$ T_{CM} cells were FACS purified and *Aqp9* mRNA expression was measured by qRT-PCR (values shown are normalized to naive T cells).

(C) Scatter plot shows *Aqp9* mRNA expression in CD45RA$^-$ CD8$^+$ T cells stimulated with IL-7 for 24 hr, measured by qRT-PCR, and normalized to the untreated control cells.

(D) Freshly isolated human CD45RA$^-$ CD8$^+$ T cells were stimulated with or without IL-7 or IL-7 plus glycerol and OA for 24 hr before Bodipy$^{493/503}$ staining. Bodipy$^{493/503}$ MFI was normalized to the untreated control cells.

(E) Freshly isolated human CD45RA$^-$ CD8$^+$ T cells from two individuals were pulsed with 0.1 μCi/ml [1,2,3-^{14}C]-Glycerol in the absence or presence of IL-7 or the indicated drugs for 4 hr before lipid extraction and TLC assay. The bar graphs on the right show densitometry quantification of TAG and PL autoradiography bands after a 10-week exposure.

(F) Histograms show the percentage of Annexin V$^+$ CD45RA$^-$ CD8$^+$ T cells after a 12 hr treatment as indicated. The bar graph on the right shows the cumulative data of six samples.

Data shown are a cumulative of three (B–D) independent experiments or representative of three (E and F) independent experiments (n = 5–8 subjects/group): *p < 0.05 and **p < 0.01.

expressed to a higher extent in T_{EMRA}, T_{EM}, and T_{CM} cells compared to naive cells, with the T_{CM} cells expressing the most (Figures 7A and 7B). Importantly, IL-7 treatment induced *Aqp9* expression in CD45RA$^-$ memory T cells based on qRT-PCR (Figure 7C) and TAG synthesis in human CD8$^+$ T cells based on increased Bodipy$^{493/503}$ labeling (Figure 7D). These results suggested the ability of IL-7 to induce AQP9 and promote TAG synthesis was conserved in both human and murine memory T cells.

Next, we cultured human T cells with ^{14}C-labeled glycerol in the presence or absence of IL-7 to directly measure glycerol uptake and TAG synthesis by TLC (Figure 7E). Phloretin, an inhibitor of AQPs (Abrami et al., 1996), or PF-04620110, an inhibitor of DGAT1 (Dow et al., 2011) were used as specificity controls. These results showed that IL-7 increased TAG synthesis in human T cells in an AQP9- and DGAT1-dependent manner. Finally, we asked if TAG synthesis contributed to IL-7-mediated CD8$^+$ T cell survival in human T cells in vitro. As shown in Figure 7F, the addition of IL-7 to in vitro cultures of CD45RA$^-$ memory CD8$^+$ T cells augmented T cell survival considerably compared with cultures maintained without IL-7. Moreover, the addition of phloretin or PF-04620110 reversed IL-7-mediated survival

effects, suggesting that IL-7 also promoted survival of human memory T cells in a manner dependent on AQP9 and TAG synthesis.

DISCUSSION

The IL-7/IL-7R signaling axis is a well-established pathway necessary for memory T cell formation and homeostasis (Kaech et al., 2003; Schluns et al., 2000). The pro-survival effect of IL-7 on lymphocytes has mainly been attributed to the induction of anti-apoptotic factors and enhancement of glut1 expression and glucose metabolism (Barata et al., 2004; Opferman et al., 2003; Rathmell et al., 2001; Schluns et al., 2000; von Freeden-Jeffry et al., 1997; Wofford et al., 2008), but little else is known about how IL-7 controls memory T cell longevity and homeostasis. Our findings illuminate new mechanisms by which IL-7 promotes immunological memory after viral infection through tailoring memory $CD8^+$ T cell metabolism and survival via glycerol import (via AQP9) and TAG synthesis and storage.

The importance of FAO in maintaining pathogen-specific memory $CD8^+$ T cells after infection has been emphasized by recent studies (van der Windt et al., 2012), however, little is known about how and where memory T cells obtain lipids to sustain FAO long-term. A recent report demonstrated that memory $CD8^+$ T cells do not take up free FAs as well as effector $CD8^+$ T cells, and the lipids that fuel memory T cell FAO are generated intrinsically through LAL-mediated lipolysis of TAGs (O'Sullivan et al., 2014). This raises the relevant question of what controls TAG availability in memory T cells. Our data shed insight on this question by identifying that IL-7 sustains TAG stores in memory $CD8^+$ T cells through the specific induction of glycerol uptake and lipogenesis by AQP9. The ability to store TAGs efficiently may confer upon memory $CD8^+$ T cells a greater ability to survive in stressed or nutrient poor niches. For example, *Aqp9*$^{-/-}$ cells produced as much ATP as control cells in the presence of high glucose concentrations, but this was not the case when glucose was limiting. We also found that the *Aqp9*$^{-/-}$ memory CD8 T cells have decreased mRNA expression of several TAG synthases that could further contribute to TAG insufficiency, and indeed, overexpression of *Gyk, Gpat1, Dgat1*, or *Mogat1* could restore TAG storage and survival in *Aqp9*-deficient memory T cells. However, the *Aqp9*$^{-/-}$ $CD8^+$ T cells still contain roughly one-half the normal amount of glycerol, and perhaps, the overexpression of these enzymes boosts TAG synthesis by increasing their competition for the limited glycerol pool. It was interesting that PLs were not as sensitive as TAGs to the decreased glycerol availability in *Aqp9*$^{-/-}$ CD8 T cells. This may be due to differences in the rates of synthesis or half-lives between PLs and TAGs, or possibly, recycling of cytidine diphosphate glucose (CDP)-DAG enables PL synthesis to occur under limited glycerol conditions (Liu et al., 2014).

In addition to IL-7, IL-15 is another critical cytokine that regulates memory T cell homeostasis and self-renewal (Becker et al., 2002; Goldrath et al., 2002; Kennedy et al., 2000; Lodolce et al., 1998; Prlic et al., 2002; Tan et al., 2002). IL-15 can also affect $CD8^+$ T cell metabolism and in vitro it has been shown to stimulate CPT1a expression and FAO (van der Windt et al., 2012). Furthermore, IL-15 is known to accelerate lipolysis in adipocytes in rodents (Barra et al., 2010), and its plasma level is negatively associated with total fat mass (Nielsen et al., 2008). This suggests a possible "store-and-burn" model whereby IL-7 and IL-15 work in concert to trigger both TAG synthesis and lipolysis simultaneously in memory $CD8^+$ T cells to sustain lipid supplies and FAO. However, further in vivo studies are needed to more precisely define the relationship between IL-7 and IL-15 signaling on TAG metabolism in memory $CD8^+$ T cells and the metabolic regulation of memory T cell survival and self-renewal.

Both naive and memory T cells express the IL-7R and rely on it for survival (Kaech et al., 2003; Schluns et al., 2000; Tan et al., 2002; von Freeden-Jeffry et al., 1995). Therefore, another important question is how naive and memory T cells avoid competition for IL-7. Our data indicate that one answer to this question is that naive and memory T cells have adopted different metabolic responses downstream of IL-7R that help diversify the kinds of nutrients utilized by the two types of T cells. IL-7 promotes glucose utilization in T cells (Barata et al., 2004; Wofford et al., 2008), but its added ability to preferentially induce AQP9 and TAG synthesis in memory $CD8^+$ T cells endows memory cells with another capability to utilize glycerol and lipids more effectively than naive $CD8^+$ T cells. The biochemical basis for the differential responses of memory and naive T cells to IL-7 is not clear, but this provides a mechanism by which naive and memory T cells may avoid nutrient competition, thereby maximizing both T cell numbers and diversity in the periphery. However, it is important to emphasize that the differential dependence of naive and memory T cells on other critical survival factors such as self-major histocompatibility complex (MHC)/peptide and IL-15, respectively (Ge et al., 2004; Murali-Krishna et al., 1999; Swain et al., 1999; Tan et al., 2002; Tanchot et al., 1997), are equally important mechanisms that prevent resource competition between naive and memory T cells and regulate their homeostasis.

Another interesting observation in this study is the association between TAG synthesis and memory T cell homeostatic turnover. Proliferating cells have higher lipid content compared with their non-proliferating counterparts. Our work did not distinguish whether lipid storage fuels cell proliferation or homeostatic proliferation drives TAG synthesis, however, it is possible that the synthesis of TAGs that occurs as memory T cells divide is important for refilling their "gas tank" to sustain FAO and ATP generation during quiescent, non-cycling stages, or under growth factor- or nutrient-poor conditions. In this model, niches rich in IL-7 (and IL-15) could be considered as memory T cell gas stations for bioenergetic refueling. How this concept relates to the maintenance and longevity of other types of adult tissue stems cells will be of great interest. This study highlights that regulation of glycerol stores is critical for memory T cell development, but it also illuminates a previously unrecognized feature that T cells utilize different mechanisms to regulate their glycerol stores at different stages of immune responses. For instance, neither AQP9 nor IL-7R signaling is required during effector $CD8^+$ T cell clonal expansion (Schluns et al., 2000), which is associated with a burst of lipogenesis (O'Sullivan et al., 2014 and data not shown). Therefore, the glycerol must be obtained from alternative (AQP9-independent) pathways in effector T cells, possibly through increased abundance of glycolytic intermediates (e.g., dihydroxyacetone phosphate [DHAP]) that

can be converted to glycerol. However, as the effector T cells transition to memory cells and the rates of aerobic glycolysis decline, our work demonstrates that the T cells adopt a new method to obtain sufficient amounts of glycerol. Since naive T cells do not express AQP9, it will be interesting to learn how they regulate glycerol stores.

In summary, this work identifies a previously uncharacterized role of IL-7 in regulating lipid metabolism and maintaining cellular bioenergetics in memory $CD8^+$ T cells, which have important implications for development of vaccines and immunotherapies, especially those involving IL-7. Previous studies have demonstrated that recombinant IL-7 is a promising vaccine adjuvant or therapeutic option for treatment of chronic infection or cancer. For example, in mice, IL-7 treatment could boost antiviral immunity following vaccination or lead to better control of chronic LCMV infection (Nanjappa et al., 2008; Pellegrini et al., 2011). In humans, it could increase circulating $CD4^+$ and $CD8^+$ T cell numbers and homeostatic proliferation in patients infected with HIV, HBV, HCV, or those that have melanoma, renal cell carcinoma, and other cancers (Rosenberg et al., 2006; Sereti et al., 2009; Sportes et al., 2010). Given our discoveries on IL-7 regulating TAG pools in T cells, it will be important to consider how this contributes to the benefits of IL-7-based therapies and whether IL-7 has similar effects on non-immune cells. Additionally, our work uncovers possibilities for boosting or inhibiting T cell memory through drugs that manipulate rates of TAG synthesis.

EXPERIMENTAL PROCEDURES

Mice and Infections

Aqp9$^{-/-}$ mice were kindly provided by Drs. Aleksandra Rojek and Soren Nielsen in Aarhus University (Aarhus, Denmark) via Dr. Peter Agre in Johns Hopkins University (Rojek et al., 2007). *Il7*$^{-/-}$ mice were provided by Schering-Plough Biopharma (Palo Alto, CA). Mice were infected with LCMV-Armstrong (intraperitoneally [i.p.] with 2×10^5 plaque-forming unit [pfu]). All the studies have been approved by Yale University Institutional Animal Care and Use Committee.

TLC

Lipids were loaded onto TLC plates and resolved in heptane/isopropyl ether/acetic acid (60:40:4) solution. The TLC plates were developed in cerium molydbate solution and lipid classifications were made using known lipid standards. For ^{14}C-glycerol labeling studies, the signals were detected by autoradiography.

LC-MS

Aqp9$^{-/-}$ or littermate *Aqp9*$^{+/+}$ P14 $CD8^+$ cells were activated with GP_{33-41} peptide for 3 days and then stimulated with IL-7 for another 2 days. At least 1×10^7 cells were used for lipid extraction by methanol and CH_2Cl_2. After drying down in an argon evaporator, lipids were dissolved and loaded for LC-MS assay by LIPID MAPS Lipidomics Core at the University of California, San Diego, CA.

Metabolic Assays

In vitro activated *Aqp9*$^{-/-}$ or littermate *Aqp9*$^{+/+}$ P14 $CD8^+$ cells were stimulated with IL-7 for 2 days. After that, cells were washed and plated in XF media containing glucose, L-glutamine, and sodium pyruvate. OCR and ECAR were measured using the XF-96 Extracellular Flux Analyzer (Seahorse Bioscience).

Measurement of ATP, Glycerol, and TAG

Details of these procedures are found in Supplemental Information.

Statistical Analysis

Where indicated, p values were determined by a two-tailed Student's t test. $p < 0.05$ was considered statistically significant. All the data were presented as mean ± SEM (error bar). In scatter plots, a short black line represented the mean.

For more information, see Extended Experimental Procedures.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Extended Experimental Procedures and four figures and can be found with this article online at http://dx.doi.org/10.1016/j.cell.2015.03.021.

AUTHOR CONTRIBUTIONS

G.C., M.M.S., S.M.G., P.-C.H., R.A.A., J.W., and S.M.K. designed the experiments. G.C., M.M.S., and S.M.G. performed the experiments. G.C. and S.M.K. wrote the manuscript.

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