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Survival of Effector CD8+ T Cells during Influenza Infection Is Dependent on Autophagy

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The activation and expansion of effector CD8+ T cells are essential for controlling viral infections and tumor surveillance. During an immune response, T cells encounter extrinsic and intrinsic factors, including oxidative stress, nutrient availability, and inflammation, that can modulate their capacity to activate, proliferate, and survive. The dependency of T cells on autophagy for in vitro and in vivo activation, expansion, and memory remains unclear. Moreover, the specific signals and mechanisms that activate autophagy in T effector cells and their survival are not known. In this study, we generated a novel inducible autophagy knockout mouse to study T cell effector responses during the course of a virus infection. In response to influenza infection, Atg5−/− CD8+ T cells had a decreased capacity to reach the peak effector response and were unable to maintain cell viability during the effector phase. As a consequence of Atg5 deletion and the impairment in effector-to-memory cell survival, mice fail to mount a memory response following a secondary challenge. We found that Atg5−/− effector CD8+ T cells upregulated p53, a transcriptional state that was concomitant with widespread hypoxia in lymphoid tissues of infected mice. The onset of p53 activation was concurrent with higher levels of reactive oxygen species (ROS) that resulted in ROS-dependent apoptotic cell death, a fate that could be rescued by treating with the ROS scavenger N-acetylcysteine. Collectively, these results demonstrate that effector CD8+ T cells require autophagy to suppress cell death and maintain survival in response to a viral infection. The Journal of Immunology, 2015, 194: 4277–4286.

Within days of Ag exposure, naive T cells undergo rapid expansion and proliferate to reach the peak of the effector response. This process is of central importance for CD8+ T cells to form long-lived memory T cells capable of immune reactivation (1–7). Indeed, emerging studies show that the fate of effector cells and their differentiation into memory cells may be influenced by metabolic factors during the early stages of expansion (8–12). Autophagy is a survival process that digests intracellular contents to promote survival under cellular stress. This form of degradation involves sequestration of organelles, specific proteins, protein aggregates, or bulk cytoplasm. Under cellular stress such as nutrient or growth factor deprivation, autophagy promotes cell survival by providing a source of metabolites that can be used for maintaining bioenergetics (13, 14). In the case of DNA damage or oxidative stress, autophagy is able to maintain genomic integrity by eliminating dysfunctional mitochondria and toxic protein aggregates (14). As such, it has been proposed that autophagy functions as a cellular protective mechanism in mammalian cells.

Autophagy plays a key role in naive T cell homeostasis and is essential for in vitro T cell activation (15–21). Bone marrow chimera studies found that deletion of Atg5 resulted in the reduction of peripheral T and B cells (21) whereas Arg5 or Arg7 knockout impaired T cell development due to hyperaccumulation of mitochondria and reactive oxygen species (ROS) (18, 22, 23). In the absence of autophagy, both CD8+ and CD4+ T cells have a profound defect in early activation and reduced proliferative capacity following in vitro TCR stimulation (16, 21). In CD4+ T cells, autophagy provides bioenergetics to support the demands of activated T cells (16, 21). It has also been shown that B cells...
defective for autophagy have normal primary responses but failed to generate protective immunity and memory B cells upon a secondary challenge (24). More recently, two reports provide evidence that autophagy is important for CD8+ T cell survival and is crucial for memory development (25, 26). However, there are many unanswered questions about how autophagy is activated in effector T cells and what autophagy provides to suppress cell death after in vivo exposure to viruses.

Several studies have shown that the regulation of metabolism by mammalian target of rapamycin (mTOR) and AMP-activated protein kinase (AMPK) can have profound influences on T cell differentiation. Stimulation of AMPK with metformin leads to enhanced CD8+ T cell memory via fatty acid oxidation (27), whereas AMPK knockout T cells have defective CD8+ T memory responses (28). Similarly, rapamycin can increase the magnitude and quality of memory CD8+ T cells after lymphocytic choro-meningitis virus infection (29). Furthermore, mTOR can modulate mitochondrial homeostasis and production of ROS in T cells, two factors that are essential for proper T cell function (30). Given the importance of the mTOR/AMPK signaling axis on autophagy regulation, these reports provide strong evidence that autophagy may have a more direct contribution to T cell homeostasis beyond the early stages of T cell activation.

In this study, we developed a unique inducible Cre mouse model to examine the requirement of autophagy in CD8+ T cells during the course of influenza infection. Our data demonstrate that cell-intrinsic expression of Atg5 prevents effector T cells from undergoing premature cell death. We found a significant level of hypoxia in tissues of infected animals and this was associated with an accumulation of ROS in effector CD8+ T cells, activation of p53, and the loss in cell viability. Taken together, our study suggests that modulating autophagy to improve effector T cell survival may lead to enhanced efficacy of vaccines or T cell–based immunotherapies.

Materials and Methods

Generation of mice with inducible deletion of Atg5

Atg55/− mice have been previously described (31) and were crossed onto the C57BL/6 (The Jackson Laboratory, no. 664) background at least 10 generations or until congenicity was reached as determined by genome scanning performed by The Jackson Laboratory. Congenic Atg55/− mice were bred with OTI (The Jackson Laboratory, no. 3831) and Thy1.1 (The Jackson Laboratory, no. 406) mice and finally to Cre-ERT2 mice (Taconic, no. 10471). The resulting transgenic mice were referred to as Atg55<i>−/−</i> or Atg5<i>5/5</i> and were used as donors for adoptive transfer experiments. Only female mice between 8 and 12 wk old were used. All in vivo experiments were blinded and randomized. The animals were housed and treated in accordance with the Canadian Council for Animal Care guidelines and protocols approved by the University of Victoria Animal Care Committee.

Adoptive transfer, influenza infection, and tamoxifen administration

Splenocytes from transgenic mice were isolated by filtration and treatment with ACK buffer (Lonza). Naïve CD8+ T cells were selected with the CD8a+ T cell isolation kit II (Miltenyi Biotec) and bio- tinylated CD44 Ab (BD Biosciences). Ten thousand CD8+CD44+ T cells were adoptively transferred into the tail vein of C57BL/6 mice. The following day, mice were injected intranasally with 40 µl PBS solution containing influenza A/PR8 (H1N1, 15 plaque-forming units) or A/HKx31 (H3N2, 5,000 plaque-forming units) expressing the OVA peptide (aa 257–264). Viruses were generated as described previously (32) and provided by Dr. Tania Watts (University of Toronto). Animals experiencing >15% weight loss were supplied additional nutrition (i.e., injection of Ringer solution or feeding of Napa Nectar, Lenderking). Mice were injected i.p. with 1.5 mg tamoxifen (Sigma-Aldrich; dissolved in ethanol and diluted in peanut oil) for 4 consecutive days starting 5 d postinfection.

Pimonidazole treatment and immunohistochemistry

Pimonidazole was administered i.p. at 100 mg/kg. After 2 h, lungs were perfused with PBS, harvested into 10% neutral buffer formalin (Sigma-Aldrich), and embedded in paraffin blocks the next day. Sections were de- sectioned and incubated at 37°C overnight before deparaffinization in a series of xylene and degrading ethanol washes followed by three rinses in dH2O. Ags were retrieved using Rodent Decloaker (Biocare Medical) diluted 1:10 in dH2O. Slides were placed in a decloaking chamber for one cycle (110°C, 29 min) and allowed to cool for 10 min. The buffer was decanted with dH2O and slides were washed. Peroxidase blocking was performed using one drop of Peroxidized 1 (Biocare Medical) and incubated for 5 min at room temperature followed by a rinse with dH2O. Samples were blocked using two drops of Rodent Block M (Biocare Medical) and incubated for 30 min at room temperature. Slides were then washed with dH2O/0.2% Tween 20 and the anti-pimonidazole primary Ab from rabbit (Hypoxyprobe-1) was incubated for 30 min at room temperature. Samples were washed with dH2O/0.2% Tween 20 and one drop of rabbit alkaline phosphatase polymer (Biocare Medical) was applied. Samples were incubated for 30 min at room temperature and washed with dH2O/0.2% Tween 20. Anti-pimonidazole Abs were detected using Warp Red chro- mogen or Ferengi Blue (Biocare Medical) and incubated for 7 min at room temperature. Slides were then washed with dH2O, and prewarmed denaturing solution (25 mM glycine, 1% SDS [pH 2]) was applied and incubated with shaking for 60 min at 55°C. Slides were washed with dH2O and rinsed with H2O2/0.2% Tween 20. The second primary anti-rabbit CD3 Ab (Spring Bioscience) was diluted in Ab diluent (1% BSA, 0.085% Tween 20, 0.5 M 10X TBS) and incubated for 30 min at room temperature. Tissues were then washed with dH2O/0.2% Tween 20. One drop of anti-rabbit horseradish peroxide secondary Ab solution (Biocare Medical) was applied to each slide and incubated for 30 min at room temperature. Slides were then washed with dH2O/0.2% Tween 20 and CD3 was detected with 3,3′-diaminobenzidine (DAB) and incubated for 5 min at room temperature. Slides were washed with dH2O and allowed to air dry overnight before mounting with EcoMount (Biocare Medical). The following day, they were analyzed using the Nuance multispectral imaging system in combination with an Olympus BX53 microscope.

Cell sorting

Splenocytes were presorted with a CD8α+ T cell isolation kit II (Miltenyi Biotec) and stained for 20 min with CD8α (BD Pharmingen), Thy1.1 (eBioscience), CD44 Abs (eBioscience), and a Live/Dead fixable yellow dead cell stain kit (Life Technologies) before sorting with an Influx cell sorter (BD Pharmingen) (Supplemental Fig. 2). All steps before, during, and after sorting were performed on ice or within a cooled unit. Cells were pelleted and subjected to immunofluorescence with RNA or DNA isolation.

Flow cytometry, apoptosis detection, and BrdU cell proliferation assay

All investigations were performed on an Influx (BD Pharmingen), FACS Calibur (BD Pharmingen), or Guava easyCyte 8HT (Millipore) and analyzed by Spigot, FlowJo, or Guavasoft, respectively. Peripheral blood was collected from the saphenous vein and incubated for 20 min at room temperature with CD8α and Thy1.1 Abs before ACK buffer treatment and flow cytometry. Spleens, lung, and mediastinal lymph nodes were harvested at the indicated time points and processed into a single-cell suspension. Cells were stained with CD8α, Thy1.1, CD25, CD27, or CD44 Abs. Absolute cell numbers were quantified using 123count eBeads (eBioscience) according to the manufacturer’s instructions. Mitotracker Green or 2′,7′- dichlorodihydrofluorescein diacetate (H2-DCFDA) (Life Technologies) staining was performed at 37°C for 30 and 15 min, respectively. Flow cytometry was analyzed with the Annexin V apoptosis detection kit (eBioscience) and propidium iodide (Life Technologies). For BrdU incorporation, cell cultures were supplemented with 3 µg/ml BrdU (Sigma-Aldrich) for 1 h prior to pelleting and resuspension in 100% ethanol for fixation and storage at 4°C. After washing with PBS plus 0.05% Triton X-100, cells were incubated for 30 min at room temperature with 2 N HCl in PBS plus 0.5% Triton X-100. Cells were washed again, stained with BrdU Ab (Phoenix Flow Systems) overnight at 4°C, and investigated by flow cytometry.

Immunofluorescence

Sorted cells were fixed with 4% paraformaldehyde for 1 h and permeabilized for 20 min in PBS with 0.1% saponin at room temperature. After several washes in blocking buffer (PBS plus 1% FBS plus 0.01% saponin), cells were probed with an LC3-reactive Ab (gift of C.B. Thompson) and

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Alexa Fluor 488-conjugated secondary Ab (Life Technologies). Cells were investigated in a blinded fashion. Z-stack images were taken with a confocal microscope (Nikon) using a ×100 magnification objective and subjected to maximum projection with EZ-CI software.

**In vitro cell culture, T cell stimulation, and inducible Arg5 knockout**

For all cell culture, RPMI 1640 medium (HyClone) was used with the following supplements: 10% FBS (HyClone), 0.2 mM glutamine (HyClone), 1% penicillin-streptomycin (HyClone), and 0.5 µM 2-ME (Sigma-Aldrich). Splenocytes were stimulated at a density of 10 million cells/ml with 50 µM OVA peptide (AnaSpec) for 2 h at 37˚C. After two wash steps with media, cells were cultured in 7% oxygen at 37˚C in a hypoxia glove box (Coy Laboratory Products). One day after stimulation, activated splenocytes were continuously supplemented with 100 U/ml IL-2 (PeproTech). Cells were seeded to 200,000 CDS+ cells/ml every day or every second day. Three days after stimulation, Arg5 knockout was induced by addition of 3 µM tamoxifen for 3 consecutive days. For hypoxia and ROS scavenging experiments, at 6 d poststimulation cells were cultured at 1% O2 in the presence or absence of N-acetylcysteine (NAC) (OmniPur).

**LC3 flux assay and Western blotting**

Cell cultures were incubated in the presence or absence 50 µM chloroquine (Sigma-Aldrich) for 12 h under hypoxic (1% O2) or atmospheric (20% O2) conditions and subjected to SDS-PAGE and Western blotting using anti–LC3 Ab. For Western blot analysis, cells were pelleted, washed with PBS, and boiled for 10 min in denaturing lysis buffer containing protease inhibitor (Roche) and phosphatase inhibitor (Sigma-Aldrich). Cellular lysates were separated on a 4–12% Bis-Tris gel (Life Technologies) before LC3 Ab. For Western blot analysis, cells were pelleted, washed with PBS, and boiled for 10 min in denaturing lysis buffer containing protease inhibitor (Roche) and phosphatase inhibitor (Sigma-Aldrich). Cellular lysates were separated on a 4–12% Bis-Tris gel (Life Technologies) before LC3 Ab. For Western blot analysis, cells were pelleted, washed with PBS, and boiled for 10 min in denaturing lysis buffer containing protease inhibitor (Roche) and phosphatase inhibitor (Sigma-Aldrich). Cellular lysates were separated on a 4–12% Bis-Tris gel (Life Technologies) before LC3 Ab.

**PCR analysis**

To investigate Cre recombinase of genomic DNA, we generated 1 million splenocytes with a RedExtract-N-Amp blood PCR kit (Sigma-Aldrich) according to manufacturer information. PCR was run with 0.5 µM of the following primers: Short2 (5'-GTACTGATAATGT-TTAACCTTGTG-3'), Check2 (5'-ACAACGTGAGCAGACCTGCCC-AAGG-3'), and 5L2 (5'-CAGGGATGCGTCCACCAAGAA-3'), and 5L2 (5'-CAGGGATGCGTCCACCAAGAA-3'). PCRs were performed with the following program: 50˚C for 3 min, 60 cycles of 95˚C for 30 s, followed by 60˚C for 30 s and 72˚C for 5 s. A standard curve for each gene was built by running a serial dilution of plasmid encoding each gene of interest and at the same time. Copy number of Cox1 was quantified relative to ASPG.

**Microarray**

RNA from sorted cells was investigated with the mouse GE 44K v2 microarray (Agilent Technologies) according to the one-color Low Input Quick Amp Labeling Kit (Agilent Technologies) at the Laboratory for Advanced Genome Analyses at the Vancouver Prostate Centre. All samples from two independent experiments were run on the same chip. Arrays were scanned with the Agilent Technologies DNA microarray scanner at a 3 µm scan resolution, and data were processed with Agilent Feature Extraction 11.0.1.1. Data were quantile normalized with Agilent Gene Spring 12.0. Genes that were below the detection threshold or compromised were excluded from the analysis. Only genes that were differentially expressed >1.5-fold were considered for the in silico analysis.

**Quantitative real-time PCR**

RNA from cultured cells or sorted cells was isolated with the RNeasy Plus Mini Kit and transcribed into cDNA with the qScript cDNA synthesis kit (Quanta BioSciences). Quantification of gene expression was performed with the MyiQ real-time PCR detection system (Bio-Rad) using PerfeCTa SYBR Green SuperMix for iQ (Quanta Biosciences). All samples were investigated with GeneX software and normalized to 18S RNA levels. Primers were purchased from Integrated DNA Technologies, including: PumA (5'-ACAGCTTCAACCGGATAGC-3'), 5'-GAGGATCCCTTTCGAGG-3'), Bax (5'-CAGGATCCGCTCCCAAGAA-3'), 5'-AGAGTCAGCTATTTGACGTT-3'), and Atg5 Exon3 (5'-TGCCA-AGAGTCAGCTATTTGACGTT-3'). PCRs were performed with the following program: 50˚C for 3 min, 95˚C for 3 min, 60 cycles of 95˚C for 30 s, followed by 60˚C for 30 s and 72˚C for 30 s. PCRs were validated by investigation of melt curves and gel electrophoresis.

**Mitochondrial DNA content quantification**

Total nucleic acid was isolated using the AllPrep DNA/RNA Mini Kit. Briefly, cells were homogenized with a QIAshredder spin column, and the DNA was eluted in 100 µl kit elution buffer. For mitochondrial DNA content, a mitochondrial gene, cytochrome c oxidase subunit I (Coxl), was quantified by quantitative real-time PCR on a LightCycler 480 (Roche) using the LightCycler 480 SYBR Green Master (Roche). Cox1 copy number was normalized to that of the single nuclear DNA gene accession subunit of DNA polymerase γ (ASPG). The following primers were used for Cox1 (5'-CTGTTGAATTCTCTCAAATCTGA-3', 5'-GCCCTCATATATTGTAAATATGA-3') and ASPG (5'-GGAGGAGATGCTTTCACGC-3', 5'-GGAGAAGCTCTGTTCG-3'). The amplification protocol consisted of one cycle at 95˚C for 10 min followed by 45 cycles alternating between 95˚C for 5 s, 60˚C for 10 s, and 72˚C for 5 s. A standard curve for each gene was built by running a serial dilution of plasmid encoding each gene of interest and at the same time. Copy number of Cox1 was quantified relative to ASPG.

**Results**

Induction of Arg5 knockout leads to complete abrogation of autophagy in T cells

To overcome the requirement for autophagy during T cell activation (21), we created a novel system whereby mice expressing a Cre recombinase/estrogen receptor fusion protein (CreER<sup>T2</sup>) were crossed to the conditional Arg5<sup>fl/fl</sup> knockout mouse (herein denoted Atg5<sup>fl/fl</sup>). These mice also express the conditioning marker Thy1.1 as well as the SIINFEKL (OVA)-specific TCR OTI. This allows donor T cells to be tracked when adoptively transferred into wild-type recipients after their specific activation via infection with OVA-expressing influenza viruses. When tamoxifen was administered to the mice, we observed complete Cre-mediated loss of Arg5 expression in Atg5<sup>fl/fl</sup> animals (Fig. 1A, 1B). The deletion of Arg5 following in vitro OVA activation of splenocytes was achieved within 2–3 d after tamoxifen treatment (Fig. 1C). Next, we confirmed in vivo deletion of Arg5 after adoptive transfer of Arg5<sup>fl/fl</sup> donor T cells into wild-type recipients and primary infection with influenza virus A/PR8-OVA. Five days postinfection, mice were given 4 consecutive days of tamoxifen. On day 7 postinfection, donor cells sorted from the spleens of mice showed complete loss of Arg5 expression (Fig. 1D). Additionally, there were low to undetectable levels of LC3<sup>+</sup> autophagosomes in the donor Arg5<sup>fl/fl</sup> cells (Fig. 1E). Based on these initial optimizations, all subsequent experiments focused on days 5–8 for tamoxifen administration to allow us to study immune responses during the effector phase and prior to the onset of the contraction phase. This schema also allowed sufficient time to elapse to complete Cre-mediated deletion of Arg5 in the donor T cells.
Atg5 expression is essential for survival of effector CD8+ T cells

We next examined whether deletion of Atg5 after intranasal influenza infection affects the survival of effector T cells following the schema outlined in Fig. 2A. The frequency of congenic Atg5 knockout cells was monitored using flow cytometry 7–9 d after the initial infection and on the third day after tamoxifen administration. As expected, the initial stages of activation and expansion were similar between Atg5−/− and Atg5−/− CD8+ T cells. In contrast to Atg5−/− CD8+ T cells, there was a significant loss of Atg5−/− CD8+ T cells on day 9 in the lungs of infected mice (Fig. 2B, 2C). This survival defect was also seen in Atg5−/− CD8+ T cells from the spleen (Fig. 2D, 2E). We also investigated the kinetics of influenza response between day 7 and day 28 in autophagy defective cells in the peripheral blood during the course of the infection (Fig. 2F). In this analysis, the peak response to influenza infection was diminished in Atg5−/− CD8+ T cells despite similar levels of activation based on the level of CD27 and CD44 expression (Supplemental Fig. 1A) (36, 37). We observed this substantial loss in all of the tissues that we examined, including the lung, mediastinal lymph nodes, and spleen, where there were 5- to 10-fold fewer Atg5−/− CD8+ T cells that survived (Fig. 2H). Neither tamoxifen nor the activity of Cre itself impaired T cell expansion or cell survival (Supplemental Fig. 1B). These data indicated that autophagy is critical for effector CD8+ T cells to survive the transition phase between effector/memory cell differentiation. Additionally, these data are consistent with findings from two recent reports showing that autophagy is essential for CD8+ memory generation (26, 38).

Atg5 is crucial for the generation of memory responses to influenza infection

The profound loss of effector T cell survival prompted us to investigate whether defective autophagy in CD8+ T cells impairs the generation of memory T cells. Mice that had received tamoxifen during the primary influenza infection were given a heterologous challenge on day 70 with the influenza strain A/HKx31-OVA. On day 7 following this rechallenge, Atg5−/− donor CD8+ T cells failed to mount a recall response (Fig. 3A). The compromise in effector-memory T cell survival led to a severe reduction in the generation of long-term memory T cells in all lymphoid tissues examined (Fig. 3B, 3C). Overall, these data provide evidence that effector T cells transitioning into functional memory T cells require autophagy for survival.

Atg5−/− CD8+ T cells activate p53 during influenza infection

To elucidate possible reasons for the survival defect in Atg5−/− CD8+ T cells, we assessed the global gene expression profile of sorted donor cells from pooled mice on days 7–8 of the immune response to influenza (Supplemental Fig. 2A, 2B). Two independent in silico bioinformatic approaches, DAVID (34, 39) and TFcalc (33), identified p53 as the most significantly enriched pathway in Atg5−/− cells compared with Atg5+/− control cells, consistent with the published role of Atg7 in the regulation of p53 (Fig. 4A, 4B) (35). We also found changes in the expression of several other enriched pathways, including cytokine–cytokine receptor signaling genes and genes involved in intermediary metabolism. We validated the upregulation (>1.5-fold) of the seven p53-regulated genes in Atg5−/− CD8+ donor T cells (Supplemental Fig. 2C).

Influenza infection promotes widespread hypoxia in the lung that is associated with high levels of ROS in Atg5−/− T cells

Several key metabolic and DNA damage response pathways converge on the activation of p53 such as irradiation, nutrient shortages, and high levels of ROS (40, 41). Given the known role of autophagy in protecting against these forms of oxidative stress, we hypothesized that hypoxia-induced elevations in ROS could potentially occur as part of the inflammatory response to influenza infection. Hypoxia-mediated ROS may therefore account for the high levels of p53 activation in Atg5−/− T cells (41) and consequently cause a reduction in cell viability. To examine this possibility, we performed experiments as described in Fig. 2A where pimonidazole, a hypoxia marker, was administered to recipient mice on day 7 after influenza infection. We used CD3 as a proxy to reveal the presence of host and donor T cells in hypoxic areas of the lung from infected animals (Fig. 5A). There was clear evidence of strong pimonidazole positivity in the lung tissue, indicating that influenza causes widespread hypoxia at the site of infection (Fig. 5B). In contrast to mock-infected mice, dual staining with anti-CD3 Abs and pimonidazole in the lung of infected mice revealed the presence of T cells in hypoxic regions (Fig. 5C–F). We also observed hypoxic staining with pimonidazole and CD3 in both the spleen and lymph nodes of infected mice (Supplemental Fig. 3). The presence of hypoxia coincided with...
the timing of p53 upregulation and is consistent with in vitro work showing that autophagy contributes to protecting cells against oxidative damage (42). Owing to the lack of a suitable Ab to detect donor T cells, we were unable to distinguish and quantify OVA-specific Thy1.1+ donor cells from endogenous T cells. However, these data indicate that hypoxia may be important for

FIGURE 2. Atg5 expression is essential for survival of effector CD8+ T cells in response to influenza infection. (A) Experimental scheme for T cell preparations and in vivo infection. Frequency and absolute number of effector CD8+ donor T cells in the (B and C) lung and (D and E) spleen of day 8–9 infected mice. The flow cytometry plots are representative of three independent experiments with at least seven mice per group. Quantification plots are means of two independent experiments with at least six mice per group. (F) Kinetics of donor cell response in the peripheral blood as the percentage of total PBMCs. Shaded area indicates tamoxifen administration; dotted line indicates detection limit defined as the mean of false-positive cells in mice that did not receive adoptive transfer (mean ± SEM, representative of two independent experiments with at least seven mice per group). (G) Total donor cells in the peripheral blood at day 8 after primary infection (six to seven mice per group). (H) Absolute donor T cells in lung, mediastinal lymph node, and spleen of mice at day 28 after primary infection (mean ± SD, four to six mice per group). **p < 0.01, ***p < 0.001, ****p < 0.0001.

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FIGURE 3. Autophagy is critical for the generation of memory T cell responses. (A) Mice were rechallenged after day 70 of the primary infection with influenza A/HKx31-OVA. Frequency of donor cells in the peripheral blood at day 7 after rechallenge (mean ± SEM, data from three independent experiments). **p < 0.01, Mann–Whitney U test. (B) Representative flow cytometry of donor T cells from the lung, mediastinal lymph node (mdLN), and spleen on day 8 following rechallenge. (C) Absolute number of donor T cells on day 8 following rechallenge (mean ± SD, three to six mice per group and per tissue). *p < 0.05, **p < 0.005.
stimulating autophagy when effector T cells are transitioning to memory during influenza infection.

We next asked whether tissue hypoxia coincides with changes in the level of ROS. To test this, donor T cells were isolated from the spleen on day 8 postinfection and stained with the ROS marker H2-DCFDA. The entire Atg5\(^{-/-}\) donor population of CD8\(^{+}\) T cells displayed an increase in ROS when compared with control cells (Fig. 5G, 5H). In addition to the presence of hypoxia, another possible explanation for the observed increase in ROS is an accumulation of mitochondria due to the inability to execute mitophagy (22, 23, 42). However, we detected no difference in the total amount of mitochondria or mitochondrial DNA between the Atg5\(^{-/-}\) CD8\(^{+}\) T cells and control cells (Fig. 5I, 5J).

p53-mediated cell death under hypoxia in Atg5\(^{-/-}\) CD8\(^{+}\) T cells is rescued by ROS scavenging

Given the above results and to determine whether ROS contributes to the impaired survival of Atg5\(^{-/-}\) T cells under hypoxia, we activated CD8\(^{+}\) T cells with OVA peptide in vitro and treated cultures with tamoxifen on day 3 to delete Atg5. On day 6 after activation, Atg5\(^{-/-}\) or Atg5\(^{+/+}\) cells were cultured at 1% oxygen for an additional 3 d in the presence or absence of the ROS scavenger NAC. An autophagy flux assay confirmed that deletion of Atg5 resulted in the abrogation of autophagy (Supplemental Fig. 4A, 4B). Atg5\(^{-/-}\) CD8\(^{+}\) T cells had higher levels of ROS compared with control cells (Fig. 6A, 6B). There was also no discernible change in overall mitochondrial content between Atg5\(^{-/-}\) and Atg5\(^{+/+}\) cells (Fig. 6C, 6D). Similar to the in vivo response to influenza, conditioning Atg5\(^{-/-}\) CD8\(^{+}\) T cells under hypoxia resulted in impaired expansion, a higher degree of apoptosis, and a significant compromise in cell proliferation (Fig. 6E–G). These effects appear to be specific to hypoxia, as we observed no change in proliferation when Atg5\(^{-/-}\) or Atg5\(^{+/+}\) cells were cultured under physiological oxygen concentration (Supplemental Fig. 4C). Under conditions of hypoxia, treatment with the anti-oxidant NAC fully restored the defect in cell proliferation and, to a large extent, protected the cells from apoptosis (Fig. 6E–G).

We next determined whether there was a link between the accumulation of ROS and the activation of p53-mediated targets. Splenocytes from Atg5\(^{-/-}\) or Atg5\(^{+/+}\) cells were cultured under hypoxia in the presence or absence of 0.3 or 1 mM NAC. In the absence of NAC, hypoxia induced p53 activation as shown by the dramatic increase in phosphorylated p53 (Fig. 7A). In contrast, NAC caused a dose-dependent inhibition of p53 activation and

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**FIGURE 4.** Differential gene expression analysis of Atg5\(^{-/-}\) and Atg5\(^{+/+}\) CD8\(^{+}\) T cells following influenza infection. (A) At day 7 and 8 postinfection, Atg5-transgenic donor cells were sorted from spleens (\(n = 5–8\) mice, see Supplemental Fig. 2A and 2B for schema) and microarray analysis of mRNA was performed. Graphs show KEGG pathways that contain enriched amounts of genes that are upregulated in Atg5\(^{-/-}\) cells (DAVID database). The top ranked pathways include p53, cytokine–cytokine receptors, as well as lipid and amino acid metabolism. (B) TFactS database analysis found upregulated transcription factors that are known to regulate genes in the above pathways, including p53 (fatty acid metabolism), myc (amino acid metabolism), and STAT (cytokine signaling).

**FIGURE 5.** Hypoxia is a feature of lung tissue following influenza infection and is associated with high levels of ROS in Atg5\(^{-/-}\) T cells. Infections and adoptive transfer of donor T cells were performed as described in Fig. 2A. (A and B) Single stain controls of lung tissues on day 7 postinfection for CD3 (brown) and pimonidazole (blue). Endogenous CD3\(^{+}\) T cells are present in infected lungs and the lung tissue is positive for p53. (C and D) Dual staining with both CD3 and pimonidazole in mock-infected or influenza-infected animals. (E and F) Higher magnification of dual CD3 and pimonidazole staining. Scale bars, 50 \(\mu\)m. (G) Representative flow cytometry assessment of ROS on day 8 postinfection via H2-DCFDA. (H) Quantification of ROS levels (mean ± SD with three to nine mice per group). (I) Representative flow cytometry of the mitochondrial content via MitoTracker Green (representative of two independent experiments with three to nine mice per group). (J) Quantification mitochondrial DNA content measurement (mean ± SEM of two independent experiments with three to nine mice per group). MFI, mean fluorescence intensity.
reduced the cleavage of PARP-1, a marker for apoptosis. Moreover, the addition of NAC prevented the activation of several major p53 targets (Fig. 7B). Many of these targets were ones that we found to be highly upregulated in autophagy-defective T cells following influenza infection (Supplemental Fig. 2C). Taken together, these results demonstrate that Atg5 is essential for T cell expansion under conditions of oxidative stress and to protect effector CD8+ T cells from ROS-mediated cell death.

**Discussion**

Several published studies have shown an essential role for autophagy in T cell activation, which, until recently, has prevented the in vivo evaluation of autophagy into the events following activation and changes associated with survival of activated CD8+ T cells (21). To overcome this obstacle, an inducible Atg5 knockout mouse system was generated where inactivation of autophagy in CD8+ T cells can be induced several days after the initial phases of T cell activation. Using this novel mouse system, we investigated the kinetics of CD8+ T cell responses to a natural viral infection. In this study, we demonstrate that cell-intrinsic Atg5 is essential for the survival of effector CD8+ T cells. Based on our data it is possible that in addition to regulating cell survival, autophagy is required for T cell activation because autophagy-deficient effector CD8+ T cells did not reach the primary peak response. However, the expression of CD44 and CD27, markers of activated and Ag-experienced T cells (36, 37), was similar between knockout and control cells, which suggests that autophagy may have a more prominent role in controlling cell survival. Nonetheless, as a consequence of the loss in effector cell survival, Atg5−/− T cells fail to generate a recall response. It may be tempting to speculate that memory precursors as well as fully differentiated memory T cells are also dependent on autophagy for their survival. Future studies will need to clarify the impact of autophagy on these specific subsets of cells where Atg5 is deleted at later time points following the effector phase.

Our data are consistent with two recently published studies showing that mice lacking Atg7 or Atg5 have defective effector responses to lymphocytic choriomeningitis virus and influenza infection (25, 26). Although these other studies also show impaired ability to generate memory T cells in autophagy knockout T cells, the present study provides new insight into the potential role of hypoxia to activate autophagy during CD8+ T cell effector responses. During a typical acute viral infection, different intracellular signaling events cooperate to support CD8+ T cell activation, expansion, contraction, and the development of immunological memory. Excessive ROS can impede T cell responses to viral infections, highlighting the need for tight control of intracellular ROS production and detoxification (43, 44). We found a significant amount of ROS accumulation in autophagy-deficient effector CD8+ T cells. This increase in ROS was associated with higher p53 activity and trans-activation of several p53-dependent proapoptosis regulators such as Puma, Perp, TNFrs10b, and Bax. These results are consistent with previous studies showing that autophagy abrogation in fibroblasts and malignant cells results in p53-dependent cell death (45, 46). We show that the observed cell death of autophagy-deficient T cells is due to an accumulation of ROS because treatment with the antioxidant NAC reversed cell death and p53 activation.

Owing to limitations in oxygen as the final electron acceptor, hypoxia can cause a dramatic increase in ROS generation (47). During an in vivo viral infection, activated T cells may experience hypoxia due to inflammation and the hyperproliferation of lymphocytes (48, 49), a hypothesis that is supported by our data showing hypoxia in the lung, spleen, and lymph nodes of influenza-infected mice. Thus, we provide in vivo evidence that hypoxia may be a crucial activator of T cell autophagy during viral infection. It is also known that influenza hemagglutinin and matrix proteins can directly or indirectly stimulate autophagy of infected cells (50). Although this could account for the activation of autophagy in the lung, it is unlikely that intranasal inoculation...
of influenza would impact autophagy in T cells that are present in other non-target lymphoid tissues. In line with the potential role of hypoxia-induced autophagy, our in vitro studies showed that an acute accumulation of ROS arises under hypoxia in autophagy-deficient CD8+ T cells. Under hypoxia, autophagy has been reported to limit mitochondria-dependent generation of ROS by the selective degradation of damaged mitochondria through mitophagy in Atg7−/− B cells as well as in vitro studies with autophagy knockout T cells (22, 24). However, this is unlikely the reason for the increased amount of ROS that we observed in Atg5-deficient CD8+ T cells because the mitochondrial mass was comparable to that of Atg5-expressing control cells. Besides the mitochondria, the peroxisome and the endoplasmic reticulum could also serve as potential sources of ROS (17, 51, 52). Additionally, impaired ROS scavenging via the glutathione pathway may also explain why autophagy-deficient CD8+ T cells displayed higher levels of ROS. Therefore, our findings reveal a plausible model whereby Atg5-deficient T cells activate autophagy via hypoxia. This, in turn, leads to accumulation of ROS and activation of p53-dependent cell death.

Our work also sheds some light on the outstanding question of what does autophagy provide to T cells to allow continued survival under cellular stress. One major possibility is that knockout of autophagy in T cells might interfere with the liberation of key intermediate metabolites such as glutamine that serve as substrates for glutathione-dependent ROS scavenging. The induction of autophagy may have several beneficial roles for effector CD8+ T cells by providing requisite fuels to preserve mitochondrial metabolism. This is consistent with recent work showing that the induction of fatty acid oxidation through the metabolic activities of mTOR and AMPK can increase the generation of memory T cells (27, 29, 53). Additionally, the transcription factors Foxo1 and Foxo3a have intrinsic roles in controlling the survival of primed CD8+ T cells and the transition from effector to memory. Interestingly, the Foxo transcriptional factors are key regulators of ROS and autophagy, further implicating ROS as an important regulator of effector T cell survival (54–56). Finally, overexpression of Atg5 extended the lifespan of mice due to an enhanced tolerance to oxidative stress–induced cell death (57). Thus, an important area of future investigation is the metabolic consequences of autophagy inhibition on effector CD8+ T cells.

Some early metabolomic analysis by Xu et al. (26) suggests that mitochondria fatty acid oxidation was impaired in Atg7-deficient T cells, and unpublished data from our own studies corroborate these findings. Thus, the loss in cell survival of autophagy-deficient CD8+ T cells may be a consequence of an inability to switch to oxidative metabolism and energy maintenance. We speculate that autophagy-dependent breakdown of lipid stores for fatty acid oxidation may not be sufficient for T cells to transition from effector to memory. Although some NADH/flavin adenine dinucleotide may be generated when lipids are oxidized to acetyl-CoA, most usable NADH/flavin adenine dinucleotide for ATP generation is formed in the tricarboxylic acid cycle. However, oxygen restriction in tissues of infected mice could limit the function of complex IV of the electron transport chain and subsequent ATP production. Furthermore, the high levels of ROS in Atg5−/− T cells (Fig. 5G, 5H) would lead to inactivation of numerous iron-sulfur–containing proteins. Iron-sulfur proteins include NADH dehydrogenases and several electron transport complex proteins. Thus, it is likely that autophagy is involved in

**FIGURE 7.** p53 activation is dependent on ROS. Splenocytes from Atg5-transgenic mice were activated by the OVA-peptide and expanded for 6 d followed by incubation under hypoxia (1% O2) for an additional 3 d, in the presence or absence of NAC. Atg5 knockout was induced by tamoxifen on day 3 after activation. The analyses were performed at 16–20 h after the start of hypoxia. (A) Lysates of cells were subjected to SDS-PAGE and Western blots were probed for phosphorylation of p53 at Ser18 and for the apoptosis marker cleaved PARP-1 (representative of four independent experiments). Loading control is β-actin. (B) Quantitative real-time PCR of p53 responsive genes (mean ± SEM of three to four independent experiments). *p < 0.05, **p < 0.01, ***p < 0.001.
other yet to be identified metabolic pathways used to support cell survival. Many questions remain unanswered and will be the subject of intense future investigations to uncover the consequences of autophagy inhibition on the metabolic reprogramming of effector T cells.

In summary, our results raise the possibility that modulation of autophagy during the effector phase may be an attractive approach to improve vaccines or T cell–based immunotherapy treatments where facilitating survival of effector T cells and the establishment of immunological memory are of critical importance.

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Disclosures

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