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***i*NKT cells require autophagy to coordinate proliferation and survival signals during differentiation**

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Abstract

Autophagy regulates cell differentiation, proliferation and survival in multiple cell types, including cells of the immune system. Herein we examined the effects of a disruption of autophagy on the differentiation of invariant natural killer T cells (*i*NKT cells). Using mice with a T lymphocyte specific deletion of *Atg5* or *Atg7*, two members of the macroautophagic pathway, we observed a profound decrease in the *i*NKT cell population. The deficit is cell-autonomous, and it acts predominantly to reduce the number of mature cells, as well as the function of peripheral *i*NKT cells. In the absence of autophagy, there is reduced progression of *i*NKT cells in the thymus through the cell cycle, as well as increased apoptosis of these cells. Importantly, the reduction in Th1-biased *i*NKT cells is most pronounced, leading to a selective reduction in *i*NKT cell derived IFN γ . Our findings highlight the unique metabolic and genetic requirements for the differentiation of *i*NKT cells.

Keywords

Autophagy; T lymphocyte; thymus; differentiation; cytokines

Introduction

Autophagy is an evolutionarily conserved catabolic process in which cells sequester cytoplasmic components and deliver the cargo to lysosomes for degradation and recycling (1, 2). Three types of autophagy have been described: macroautophagy, microautophagy and chaperone mediated autophagy (1, 3). Of these forms, macroautophagy, hereafter referred to as autophagy, is the major pathway, and it is the most extensively investigated. Macroautophagy is characterized by the compartmentalization of cytoplasmic molecules or organelles in double-membrane vesicles, termed autophagosomes (1, 3–5). Over thirty autophagy-related gene products (ATG proteins) have been identified in yeast, with orthologs in mammals. The core autophagic machinery includes two ubiquitin-like

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conjugation systems that contribute to the elongation of autophagic vacuoles. One consists of ATG7, an E1 ubiquitin ligase-like enzyme that participates in the lipid modification of ATG8/LC3 (yeast protein/mammalian ortholog). The other is formed by ATG7-mediated catalysis of ATG12 conjugation to ATG5. The ATG5-ATG12 complex is further non-covalently associated with ATG16/ATG16L (yeast protein/mammalian ortholog) to form a macromolecular complex with E3 ligase-like activity (1, 2, 6–8).

Autophagy plays a role in recycling cytoplasmic materials and is an alternative source for energy production under conditions of nutrition shortage or energy loss. In addition, autophagy has been verified in different contexts to control cell growth, proliferation and differentiation, cell death and functional responses to various stimuli (4, 5, 9–12). In the immune system, naïve T cells maintain a basal level of autophagy that is further induced upon activation through the T cell antigen receptor (TCR) (13–17). Upon depletion of components of the autophagy pathway, conventional T cells exhibited impaired development and homeostasis, with decreased cell numbers and increased markers of cell death (13, 14, 16, 18–20).

Natural killer T (NKT) cells are a T lymphocyte sub-population that shares the properties of both natural killer (NK) cells and T lymphocytes (21–23). The majority of NKT cells in mice express a semi-invariant TCR, with an invariant V α 14-J α 18 chain coupled with a V β repertoire consisting mostly of V β 8, V β 7 and V β 2. This major population of NKT cells is often referred to as type I or invariant NKT cells (*i*NKT cells) (21, 22, 24, 25). *i*NKT cells recognize lipid antigens (Ag) presented by CD1d, a MHC class I-like Ag presenting molecule (26–29). *i*NKT cells have the capacity to rapidly secrete large amount of cytokines after activation, consequently amplifying innate and adaptive immune responses (21, 22, 27). The results from recent studies indicate that a functional subset of *i*NKT cells is dedicated to producing IL-17, and also that there are subsets more prone to the production of Th1 or Th2 cytokines (30–34).

*i*NKT cells develop in the thymus, but branch off from the main stream of T cell differentiation at the double positive (DP) stage (21, 22, 24, 35–37). Although many factors that contribute to the regulation of *i*NKT cell development have been identified, the determinants that direct *i*NKT cell development are still not fully understood (35, 36). It is notable that *i*NKT cell differentiation requires positive selection by CD1d expressing DP thymocytes rather than cortical epithelial cells (38). *i*NKT cells also require a number of cell surface molecules, kinases, adaptors, transcription factors and chromatin modifying proteins that are much less important for the differentiation of MHC class I and class II restricted thymocytes (39).

Considering the requirement for autophagy in cell growth and proliferation, we analyzed the development of *i*NKT cells in mice with a conditional knockout of *Atg5* or *Atg7*. Our results show that a lack of either of these two autophagy genes causes a severe defect in *i*NKT cells, with selective effects on the subset that produces IFN γ .

Materials and Methods

Mice and reagents

The generation of *Atg5^{fl/fl}* and *Atg7^{fl/fl}* has been previously described (40, 41). The *Atg5^{fl/fl}* mice were a kind gift from Dr. Noboru Mizushima (Tokyo Medical and Dental University, Japan) and the *Atg7^{fl/fl}* mice were kindly provided by Dr. Masaaki Komatsu (Tokyo Metropolitan Institute of Medical Science, Japan). CD4-Cre transgenic mice, C57BL/6J (B6) and CD45.1 congenic B6.SJL strains were purchased from The Jackson Laboratory and Lck-Cre transgenic mice were obtained from Taconic Farms. The *Atg5^{fl/fl}* mice and *Atg7^{fl/fl}* mice were bred to CD4-Cre or Lck-Cre mice and littermates were used for analysis and comparison. B6 mice were crossed with the B6.SJL mice to generate CD45.1⁺CD45.2⁺ heterozygotes. Mice were maintained under specific pathogen-free conditions, and the experiments were approved by the Institutional Animal Care and Use Committee of the La Jolla Institute for Allergy & Immunology.

Antibodies and reagents

The following antibodies, with clone designation in parentheses, were from BD PharMingen: CD1d-PE (1B1), CD4-APC (RM4-5), CD8-PerCP-Cy5.5 (53-6.7), CD24-FITC (M1/69), CD45.1-FITC (A20), Fas-FITC (Jo2), anti-BrdU-Alexa Fluor 488 (3D4), IFN γ -PE-Cy7 (XMG1.2), IL-4-Alexa Fluor 647 (11B11), Ki-67-PE (B56), NK1.1-PE-Cy7 (PK136), GATA3-PE-Cy7 (L50-823), phospho-Akt(pS473)-PE (M89-61), phospho-Akt(pT308)-PE (J1-223.371) and purified antibody anti-active caspase 3 (C92-605). CD45.2-APC (104), ROR γ t-PE (B2D) and TCR β -APC-eFluor 780 (H57-597) were bought from eBioscience (San Diego, CA). CD44-Alexa Fluor 700 (IM7) and CD69 Alexa Fluor 647 (H1.2F3) were obtained from BioLegend (San Diego, CA). p21^{cip1}-Alexa Fluor 647 (C-19), T-bet-Alexa Fluor 488 (4B10) and PLZF-Alexa Fluor 647 (D-9) were from Santa Cruz Biotechnology (Santa Cruz, CA). CD19-PE-Texas Red (6D5) and second antibody goat anti-rabbit IgG (H+L)-AF488 were from Invitrogen (Carlsbad, CA). Purified antibodies recognizing cleaved caspase 8 (D5B2) or phospho-4E-BP1(pT37/pT46) (236B4) were purchased from Cell Signaling Technology (Danvers, MA). Cytotfix/Cytoperm buffer, Perm/Wash buffer and Transcription Factor Buffer Set were all from BD Biosciences. α GalCer was kindly provided by Kyowa Hakko Kirin. Live/Dye (Yellow) was obtained from Invitrogen (Carlsbad, CA), and 5-Bromo-deoxyuridine (BrdU) and Annexin V-APC were from BD PharMingen.

Flow cytometry

Thymus, spleen and liver were collected and single cell suspensions were prepared. For cell surface staining, after blocking in staining buffer (PBS, 2% BSA, 10 mM EDTA, and 0.1% sodium azide) containing anti-FcR antibody (2.4G2) for 30 min at 4°C, cells were stained with fluorophore-conjugated antibodies and fixed with fixation buffer (PBS, 1% paraformaldehyde, and 0.1% sodium azide). To stain intracellular cytokines and transcription factors, after cell surface staining, cells were treated with Cytotfix/Cytoperm buffer and Transcription Factor buffer, respectively, followed by staining with corresponding fluorophore-conjugated antibodies in Perm/Wash buffer. The data were acquired on an LSRII flow cytometer (BD Biosciences) and analyzed using FlowJo software

(Tree Star). The *i*NKT cell population was gated as CD19⁻, TCRβ⁺ and CD1d-tetramer⁺ cells.

Mixed bone marrow chimeras

Bone marrow cells were prepared from tibias and femurs, and Thy1⁺ cells were removed by MACS microbeads (Miltenyi Biotec, Auburn, CA). 10×10^6 bone marrow cells with a 1:1 ratio of B6.SJL (CD45.1⁺) and either wild type controls (CD45.2⁺) or *Atg5^{ff}* CD4-Cre or *Atg7^{ff}* CD4-Cre (CD45.2⁺) were injected i.v. to eight-to-ten-week old B6 mice (CD45.1⁺CD45.2⁺) that had been subjected to twice to 600 Rads irradiation with an X-Ray Irradiator, with 3h between doses. Mice were analyzed 11–12 weeks post bone marrow transfer.

In vitro culture and apoptosis analysis

Thymocytes were purified and single cell suspensions were prepared. 20×10^6 cells were inoculated in 1 ml RPMI-1640 medium supplemented with 10% fetal bovine serum, 50 μM 2-mercaptoethanol as well as antibiotics, and cultured overnight at 37°C in a humidified atmosphere of 5% CO₂. Cells were collected and stained with cell surface markers to gate either DP thymocytes for some experiments or *i*NKT cells in others, as well as with Annexin V and Live/Dead Dye Yellow (Invitrogen, Carlsbad, CA) following the manufacturer's instructions.

Semi-quantitative PCR

Single cell suspensions of thymocytes were prepared and stained. DP thymocytes were sorted with the αGalCer CD1d-tetramer positive cells removed. Total mRNA was purified by using TriZOL (Invitrogen) and reverse transcribed to cDNA. PCR was carried out with titrated cDNA templates at concentrations of 200 ng, 60 ng and 20 ng. The primer pairs used were the following: forward primer for Vα14-Jα18: 5'-GTGTCCCTGACAGTCCTGGT-3' and reverse: 5'-CAAATGCAGCCTCCCTAAG-3'; forward primer for Cα: 5'-CCTCTGCCTGTTACCGACTT-3' and reverse: 5'-TGGCGTTGGTCTCTTTGAAG-3'. The PCR program consisted of an initial denaturation step at 95 °C, 4 min; 38 cycles for Vα14-Jα18 or 28 cycles for Cα at 95 °C for 15 sec, 55 °C for 15 sec, 72 °C for 15 sec; and a final extension for 7 min at 72 °C. The PCR products were analyzed by electrophoresis on a 2% agarose gel.

Autophagy and mitochondrial mass detection

After staining with cell surface markers, thymocytes were washed twice with PBS, resuspended with pre-warmed PBS containing 0.1 μM MitoTracker Green FM (Invitrogen, Carlsbad, CA) or DMSO as a control, and incubated at 37°C for 30 min. The cells were washed with warm PBS twice prior to flow cytometry analysis. Similar procedures were carried out for detection of mitochondrial superoxide, except staining was done with freshly made 5 μM MitoSOX Red (Invitrogen, Carlsbad, CA) in pre-warmed HBSS/Ca²⁺/Mg²⁺ medium. Thymocytes were stained with the Cyto-ID[®] Autophagy detection kit (Enzo Life Sciences, Farmingdale, NY) according to the manufacturer's instructions.

Proliferation and cell cycle analysis

To measure cell turnover in vivo, six-week-old female mice were injected i.v. with 2 mg BrdU and 5 h after injection thymus tissue was collected and a single cell suspension of thymocytes was prepared. Cells were stained with surface markers, followed by staining for BrdU and Ki-67 according to the manufacturer's manual. For analysis of cell cycle, 2 mg BrdU was introduced into each mouse intravenously. 2 h later, single cell suspensions of thymocytes were prepared and stained to evaluate BrdU incorporation and DNA content with 7-aminoactinomycin D (7-AAD, Invitrogen, Carlsbad, CA)

Lipid Ag immunization

Eight-week-old mice were injected i.v. with 1 µg αGalCer. 2 h after injection, spleens were collected and single cell suspensions were prepared. Cells were stained with surface markers and then for intracellular cytokines, followed by analysis by flow cytometry.

Statistical analysis

Two-tailed Student's *t* test was used for analysis of statistical significance. *p* values < 0.05 were considered statistically significant.

Results

Deficiency in autophagy genes caused decreased iNKT cells

Disruption of either ATG5 or ATG7 expression effectively eliminates the majority of autophagic processes (42–44). While a germ line deletion causes neonatal lethality, mice with cell type-specific deletions of *Atg5* or *Atg7* have been used as models to evaluate the role of autophagy in various physiological processes. Herein, *Atg5^{fl/fl}* or *Atg7^{fl/fl}* mice were crossed with either CD4-Cre or Lck-Cre mice, producing mice with the gene deletions specifically restricted to T lymphocytes. T lymphocytes continuously undergo autophagy, and previous results showed that when autophagy gene deficient mice were crossed to Lck-Cre transgenic mice the amount of LC3-II formed in T cells was greatly decreased, indicating that autophagy was highly impaired (14, 18).

We analyzed *i*NKT cells from thymus, spleen and liver of eight-to-nine-week old mice. Mice with deletion of either *Atg5* or *Atg7* had a dramatic reduction in both the percentage and absolute number of *i*NKT cells compared with controls that do not express Cre recombinase (Fig. 1 and data not shown), although there were subtle differences in the extent of the reduction, depending on the *Atg* gene deleted and the promoter controlling T cell-specific Cre expression. The effect of *Atg5* or *Atg7* deficiency on *i*NKT cells also was somewhat organ-specific, with a relatively smaller decrease in cell frequency in the thymus and the most profound defect occurring in liver (Table 1).

In *Atg5^{fl/fl}* CD4-Cre and in *Atg7^{fl/fl}* CD4-Cre mice, lack of either of these two autophagy genes did not cause a significant decrease in total thymocyte cellularity (Supplemental Fig. 1). However, consistent with some previous reports (13, 19), there was a reduction in peripheral CD4 and CD8 T cells in *Atg5^{fl/fl}* CD4-Cre mice, but this was much smaller in magnitude than the decrease in *i*NKT cells (Supplemental Fig. 1 and Table 1). In Lck-Cre mice, however, in

agreement with earlier reports (18, 20), the total cellularity of the thymus was reduced, which might reflect the earlier expression of the Cre recombinase under the control of this promoter. Similarly, the frequencies of peripheral CD4 and CD8 T cells exhibited more pronounced decreases in Lck-Cre mice. However, in both the CD4-Cre and Lck-Cre strains the reductions in *i*NKT cells caused by either *Atg5* or *Atg7* deletion were much more pronounced than the reductions in CD4 and CD8 T cells (Table 1).

Wild type thymocytes were analyzed for autophagy using a dye that detects autophagic vacuoles. By this analysis, *i*NKT cells exhibited increased autophagy compared to either DP, CD4 single positive or CD8 single positive cells (Supplemental Fig. 2). Therefore, an increased requirement for autophagy correlated with increased autophagy in these cells.

Autophagy related gene deficiency interfered with mature *i*NKT cell stages

Differentiation of *i*NKT cells has been divided into four developmental stages, with the most immature or stage 0 cells expressing CD24, but lacking both expression of NK1.1 and a high level of CD44 (45). Stage 1 cells lose CD24 expression (CD24⁻, CD44^{low}, NK1.1⁻), while stage 2 cells become CD44^{high}, and finally, stage 3 is characterized by acquisition of NK1.1 expression (CD24⁻, CD44^{high}, NK1.1⁺) (21, 24). The final maturation of *i*NKT cells to stage 3 is not confined to the thymus, however, as cells with a stage 2 phenotype are the majority of *i*NKT cell recent thymic emigrants, and NK1.1 expression can be acquired in the periphery (46, 47).

In either *Atg5* or *Atg7* deficient mice thymic and splenic *i*NKT cells exhibited a dramatic decrease in the number of stage 3 or CD44^{high}, NK1.1⁺*i*NKT cells, and a significant but less dramatic decrease for stage 2 cells in *Atg5* but not *Atg7* deficient mice (Fig. 2). Unless indicated, the data shown in the following figures were derived from CD4-Cre mice, but the results were similar for Lck-Cre strains. While the percentages of *i*NKT cells in the early stages (stage 0 and 1) were higher in mice deficient for either *Atg5* or *Atg7*, the absolute cell numbers were not significantly different (Fig. 2). These results indicate that ablation of autophagy genes greatly reduced the transition of immature *i*NKT cells to the more mature stages 2 and 3.

Autophagy gene mutation caused a cell autonomous defect in *i*NKT cells

A defect in autophagy genes in T cells could have affected CD1d surface expression or the presentation of positively selecting lipid ligands by thymocytes. However, deletion of either *Atg5* or *Atg7* did not significantly affect the amount of CD1d on the surface of thymocytes (Supplemental Fig. 3).

We carried out mixed bone marrow chimera experiments to determine if the decrease in *i*NKT cells in the autophagy gene deficient mice was cell intrinsic, meaning the problem is located with the *i*NKT cell progenitor. Alternatively, it could be cell extrinsic, for example due to impaired presentation of a positively selecting ligand, in which case the co-transfer of wild type bone marrow cells should rescue the defect. CD45.2⁺ marrow cells derived from either autophagy gene deficient mice or controls were transferred together with CD45.1⁺ wild type bone marrow cells to lethally irradiated recipient mice. After at least eleven

weeks, *i*NKT cells in thymus, spleen and liver were analyzed by flow cytometry. Wild type CD45.1⁺ and CD45.2⁺*i*NKT cells developed equally in the thymus and periphery in the mixed chimeras, while autophagy gene deficient *i*NKT cells manifested a 4- to 10-fold reduction compared to their wild type counterparts in all three organs tested (Fig. 3 and *Atg7* data not shown). These results indicate that the defect in *i*NKT cells caused by autophagy gene deficiency is cell-intrinsic.

Equivalent viability of DP thymocytes

Rearrangements of J α gene segments distal to the V α locus, including J α 18, require secondary TCR α rearrangements in DP thymocytes that are undergoing positive selection. The frequency of these secondary rearrangements is dependent on a normal DP thymocyte lifespan (48), and therefore a defect in DP thymocyte survival will cause a cell intrinsic decrease in *i*NKT cells, due to the negative impact on the frequency of rearrangement of the J α 18 gene segment (48, 49). To determine if an alteration in DP lifespan was the cause of the reduction in *i*NKT cells, we cultured thymocytes overnight, followed by analysis of cell death by flow cytometry. The data show that the proportions of cells undergoing apoptosis (Annexin V⁺, Live/Dead Dye not stained) and cells that were already dead, as detected with a vital dye (Live/Dead Dye stained), were very similar for either *Atg5* or *Atg7*-deficient thymocytes and wild type controls (Fig. 4A–C).

If the DP thymocyte lifespan were decreased *in vivo* in *Atg5* or *Atg7* deficient mice, we would expect a decrease in the rearrangement of V α 14 to the distal J α 18 locus in DP thymocytes. Therefore we measured V α 14-J α 18 rearrangements in DP thymocytes, which had not yet undergone positive selection, utilizing semi-quantitative PCR. We found that amplified copies of the V α 14-J α 18 rearrangement were not diminished in autophagy gene deficient DP thymocytes (Fig. 4D). In summary, the *in vitro* and *in vivo* experiments are consistent in showing that the deletion of either *Atg5* or *Atg7* did not alter the number of thymic *i*NKT cell progenitors by reducing the lifespan of DP progenitor thymocytes.

Arrested cell cycle in *Atg5* deficient *i*NKT cells

We determined if the decrease in *i*NKT cells in autophagy gene deficient mice could be attributed to decreased proliferation. Surprisingly, autophagy gene disrupted *i*NKT cells exhibited a higher rate of incorporation of BrdU and an augmented level of Ki-67, suggesting they are more proliferative (Fig. 5A).

An increase in BrdU and Ki-67 staining was observed in NK1.1 negative *i*NKT cells, but the increase in the absence of *Atg5* was even more striking when analyzing the NK1.1⁺ cells, a population that is normally quiescent in wild type mice (50). This may reflect the fact that this residual NK1.1⁺ population is not fully differentiated, and the remnant is more similar to the less mature *i*NKT cells. The results obtained from BrdU and Ki-67 analyses do not exclude an arrest in the cell cycle after chromosome replication, and therefore we analyzed cell cycle progression of *Atg5* deficient thymic *i*NKT cells. Fig. 5B shows an analysis following a short pulse with BrdU and measurement of DNA content with 7-AAD. The results show that *Atg5* deficient *i*NKT cells accumulated in S-phase, with relatively fewer cells accomplishing the S to G2 transition and proceeding to the G2/M phases, indicative of

a blockade in the cell cycle in *Atg5* knockout *i*NKT cells. This was especially true for the NK1.1⁺ subset.

We further determined that a negative regulator of cell cycle progression, p21^{cip1} (51), was increased in *Atg5* deficient, either NK1.1⁻ or NK1.1⁺*i*NKT cells (Fig. 5C). Because the limited number of *i*NKT cells did not permit a biochemical analysis, especially in mice deficient for autophagy genes, in this and subsequent experiments we used flow cytometry to analyze signaling pathways in *i*NKT cells. *Atg5* deficient *i*NKT cells also had increased expression of p27^{kip1}, another cell cycle inhibitor (51) (data not shown). Activation of cyclin-dependent kinase 1 (CDK1) promotes cell cycle progression (52), and a lack of *Atg5* also led to an increase of inhibitory phosphorylation at Tyr15 on CDK1 (data not shown). These results indicate that intact autophagy machinery is required for a normal control of proliferation of *i*NKT cells and a smooth transition between cell cycle phases for *i*NKT cells in the thymus.

Autophagy gene deficient *i*NKT cells exhibited increased mitochondria and superoxide

The status of mitochondria, the cellular energy-producing organelle, is closely related to cell cycle and proliferation, although mitochondria also have a central role in apoptotic cell death. Recent studies have shown autophagy involves quality control of mitochondria, removing damaged organelles through mitophagy (53). Therefore, we analyzed mitochondrial mass in *Atg5* deficient *i*NKT cells utilizing MitoTracker Green, a lipophilic thiol-reactive dye that specifically labels mitochondria (54). Disruption of the autophagic machinery led to an increased mitochondrial mass (Fig. 6A) indicating that the organelle accumulated in thymic *i*NKT cells due to the loss of the autophagic clearance system.

Mitochondria are a major site for the production of reactive oxygen species (ROS). ROS positively regulates cellular functions, such as promoting cell growth and proliferation, but excessive ROS can induce apoptosis and cell death. We therefore measured superoxide in *i*NKT cells. *Atg5* deficiency not only led to accumulated mitochondria, but also to one of their products, excessive superoxide, in the NK1.1⁺ *i*NKT cells (Fig. 6B).

Autophagy genes are required for optimal survival of *i*NKT cells

Several recent studies have reported that ablation of ATG proteins caused increased apoptosis in T cells (13, 14, 16, 19, 20). We reasoned that cell death must be increased in *i*NKT cells from *Atg5* or *Atg7* deficient mice, given their dramatically decreased cell number, especially in the stage 3 cells. T lymphocytes have the ability to sense external signals by death receptors expressed on their cell surface, such as Fas/CD95 and death receptor 5 (DR5/CD262), which initiate a serial caspase cascade that eventually commits the cells to apoptosis (55). The expression of Fas, as well as DR5 and its ligand Trail (data not shown), were increased on the cell surface of both thymic and splenic *i*NKT cells from *Atg5* or *Atg7* deleted mice, more prominently for the NK1.1⁺ subset (Fig. 6C and *Atg7* data not shown).

In order to assess more directly if *i*NKT cells with either *Atg5* or *Atg7* deleted were undergoing apoptosis more than wild type controls, we measured the viability of the

autophagy gene deficient *i*NKT cells. Thymocytes derived from either *Atg5^{ff}* CD4-Cre, *Atg7^{ff}* CD4-Cre, or control strains without Cre recombinase, were cultured at 37°C overnight and then stained with Annexin V and Live/Dead Dye. Unlike the total population of *Atg5* or *Atg7* deficient DP thymocytes, which did not exhibit decreased survival (Fig. 4A–C), *i*NKT cells from *Atg5* or *Atg7* deficient mice exhibited increased proportions of dead cells, especially in the NK1.1⁺ population (Fig. 6D and *Atg7* data not shown).

Autophagy gene deficient thymic *i*NKT cells cultured overnight consistently exhibited a higher level of active caspase 8, an initiator caspase downstream of Fas and DR5 (Fig. 7A). They also had increased active caspase 3, an executioner caspase cleaved and activated by caspase 8 (Fig. 7C). The increases were not observed for the majority population of DP thymocytes (Fig. 7B and D). This is consistent with the result that DP thymocytes derived from the autophagy gene deficient mice did not exhibit reduced viability. Overall, these results demonstrate that the autophagy genes are required specifically for the survival of *i*NKT cells in the thymus.

Autophagy gene deficient *i*NKT cells exhibited an altered cytokine profile

Once activated by strong antigens, the majority of *i*NKT cells rapidly release large amounts of both Th1 and Th2 cytokines. We determined how *Atg5* or *Atg7* deficiency impacted the in vivo response of mature *i*NKT cells to α -galactosylceramide (α GalCer), a potent synthetic glycolipid Ag. 2 h after α GalCer injection, splenic *i*NKT cells were analyzed directly ex vivo at the single cell level. Compared to wild type controls, the percentage of responding *Atg5* deficient *i*NKT cells was decreased approximately two-fold for IL-4, while the IFN γ response was decreased more than four-fold (Fig. 8A,B). *Atg7* deficient mice showed similar results (data not shown). These data suggest that the autophagy machinery is required for optimal responses by mature cells, particularly for antigen-dependent Th1 cytokine secretion by *i*NKT cells.

Differential requirements of *Atg5* by *i*NKT cell subsets

Recent studies have highlighted functional heterogeneity within mature *i*NKT cells. During their differentiation in the thymus, immature *i*NKT cells become committed to cytokine polarized subsets: NKT1 (Th1-biased), NKT2 (Th2-biased) and NKT17 (Th17-biased) (30, 31, 34). These subsets can be distinguished on the basis of their expression of surface proteins and transcription factors. For example, NKT1 cells constitute the majority in C57BL/6 mice, and they are NK1.1⁺ and express a high level of T-bet (34). By contrast, both NKT2 and NKT17 cells are relatively minor in C57BL/6 mice, but more abundant in BALB/c mice (34). NKT2 and NKT17 cells are NK1.1⁻ and express IL-17RB (30), but NKT2 cells are GATA3^{high} while NKT17 cells express CCR6 along with other surface proteins, and they are characteristically ROR γ ^{high} (30, 34). PLZF (promyelocytic leukemia zinc finger) is a transcription factor required for *i*NKT cell maturation and acquisition of effector function (56, 57). Of the three subsets, NKT2 cells express the highest level of PLZF, followed by NKT17 cells, while PLZF is the lowest in NKT1 cells (34). These functional categories overlap to a degree with the developmental stages, as NKT2 and NKT17 cells have a phenotype similar to stage 2 thymocytes, while stage 3 cells are similar

to NKT1 cells. Therefore it is difficult to unambiguously distinguish a maturing, stage 2 *i*NKT cell from a committed NKT2 cell.

When analyzed by flow cytometry, total tetramer⁺ *i*NKT cells from the thymus of *Atg5^{ff}* CD4-Cre transgenic mice maintained a higher expression of PLZF, GATA3 and ROR γ t, but a lower level of T-bet compared to their counterparts from wild type mice (Fig. 8C and data not shown). The differences remain, although to a lesser degree, when NK1.1⁺ and NK1.1⁻ *i*NKT cells were analyzed separately (data not shown). This indicated that *i*NKT cell functional subsets were differently affected by disruption of the autophagic machinery. To confirm this hypothesis, we analyzed individual sub-populations of *i*NKT cells in *Atg5* knockout mice and wild type controls according to their expression level of signature transcription factors. Fig. 8D and E show that *Atg5* deficiency caused a severe loss of NKT1 phenotype cells, the majority population in this strain, with an approximate 40-fold reduction in cell number compared with the wild type controls. The absolute numbers of NKT17 and NKT2 phenotype cells were much less affected, with a 3-fold decrease and a marginal 1.3-fold decrease, respectively, although this reached statistical significance. These changes in the pattern of transcription factor expression by thymic *i*NKT cells are consistent with changes in the expression of surface proteins, including a decreased percentage of *i*NKT cells expressing NK1.1 (Fig. 2) and an increased percentage expressing IL-17RB (data not shown).

Atg5 deficiency leads to augmented mTOR (mechanistic target of rapamycin) signaling

During development and maturation, T lymphocytes experiencing positive and negative selection have an enormous demand of energy and material supply. mTOR, a highly conserved serine/threonine protein of the phosphatidylinositol kinase-related kinase family, functions as a master controller, regulating cellular metabolism to support cell growth and division (58, 59). There are two mTOR complexes both with mTOR as the catalytic subunit, mTORC1 and mTORC2, consisting of different accessory proteins that instruct their substrate specificity (58, 59). Upon activation, mTORC1 phosphorylates 4E-BP1 (eIF4E-binding protein 1) (60), while mTORC2 phosphorylates AKT at Ser473, which primes AKT for subsequent phosphorylation at Thr308 to achieve full activation of the kinase (61).

Fig. 9A shows *Atg5^{ff}* CD4-Cre NK1.1⁺ *i*NKT cells expressed a higher level of phosphorylated 4E-BP1, indicating an augmented mTORC1 activity in these cells compared with controls. Similarly, phosphorylation of AKT at Ser473 was also elevated in *Atg5^{ff}* deficient, NK1.1⁺ *i*NKT cells, which is consistent with a hyperactive mTORC2 in these cells (Fig. 9B). Furthermore, combined with the same case for phospho-AKT (pT308) shown in Fig. 9C, the results suggest AKT exhibits a higher activation in *Atg5^{ff}* deleted *i*NKT cells than in the controls.

Discussion

Our study demonstrates that *i*NKT cells depend on *Atg5* and *Atg7* for their differentiation to a far greater extent than mainstream CD4 or CD8 T lymphocytes. Removal of either *Atg5* or *Atg7* severely reduced both thymic and peripheral *i*NKT populations. Although some effects of *Atg5* deficiency on host defense from infection that do not involve autophagy have been

reported (62–64), the results of deletion of either *Atg5* or *Atg7* were in agreement, supporting the hypothesis that a defect in autophagy was responsible, rather than an effect on a different function of one of these proteins. The mouse strains with deletion mediated either by the CD4 or Lck promoter driven Cre transgenes had similar phenotypes, although a stronger effect in Lck-Cre transgenic mice might be due to the earlier deletion of *Atg* genes in this strain (65). Peripheral *i*NKT cells had an even greater reduction than did thymic *i*NKT cells, suggesting that in addition to effects on thymus differentiation, autophagy also may be required for optimal *i*NKT cell maintenance in the periphery.

Because *i*NKT cells are positively selected by DP thymocytes (37), the defect in *i*NKT cells could have been due to a defect in either the *i*NKT cell precursor and/or in the positively selecting DP thymocyte population, perhaps related to a problem with presentation of positively selecting lipid ligands. We found, however, that there was no reduction in CD1d expression on DP thymocytes from *Atg5* or *Atg7* deficient mice. Moreover, the analysis of mixed bone marrow chimeras demonstrated a cell intrinsic defect.

Autophagy is related to cell survival, and even a partial impairment in the survival of DP thymocytes could have caused a cell intrinsic diminution of *i*NKT cells in the thymus, due to decreased secondary rearrangements in the TCR α gene locus. However, we did not find a reduction in the survival of cultured DP thymocytes in CD4-Cre transgenic mice crossed to mice with floxed *Atg5* or *Atg7* alleles. In addition, there was no reduction in V α 14-J α 18 rearrangements in DP thymocytes, which would have not occurred if DP survival were impaired, and we observed a normal number of stage 0 *i*NKT cells in the thymus. Therefore, general effects on DP survival, causing decreases in the secondary TCR α rearrangements needed to form the invariant TCR α chain, do not account for the cell intrinsic *i*NKT cell deficit in mice deficient for autophagy genes.

Atg5 or *Atg7* deficient *i*NKT cells exhibited a selective defect in their maturation pathway, with the earliest stages 0 and stage 1 *i*NKT cells maintained, and the later stages depleted due to decreased cell survival. The differentiation of *i*NKT cells in the thymus along a single developmental pathway has been challenged, however, by recent studies showing there are functional subsets of *i*NKT cells analogous to T helper cell subsets, such as Th1, Th2 and Th17 cells. It is only the Th1 cytokine-biased NKT1 subset that expresses NK1.1 in C57BL/6 mice and the CD44⁺, NK1.1^{low} *i*NKT cell phenotype, defined as less mature stage 2, also includes mature NKT2 and NKT17 cells. Based on the finding of functional subsets, we explored the effects of *Atg5* and *Atg7* deficiency on *i*NKT cell function. We found that the production of IFN γ by antigen stimulated autophagy deficient *i*NKT cells was more severely affected than the production of IL-4. Additionally, based on an analysis of transcription factor expression, the NKT1 subset in the thymus was greatly decreased, and NKT2 and NKT17 cells much less so. There were, however, also defects in the other subsets including a reduced percentage of antigen activated *i*NKT cells producing IL-4 and decreased expression of key transcription factors, such as ROR γ t. Additionally, we found reduced expression of CD69 by autophagy deficient *i*NKT cells, either before or after activation (data not shown). Therefore, while NKT1 cells have by far the most severe survival and functional defects in the absence of either of *Atg5* or *Atg7*, there are deficiencies that affect the entire *i*NKT cell population.

Given the reduced *i*NKT cell population in mice with deleted autophagy genes, it would be reasonable to expect reduced proliferation of these cells. However, *Atg5* or *Atg7* deficient *i*NKT cells exhibited enhanced incorporation of BrdU and elevated Ki-67 staining. Despite this, there was a blockade in completing the cell cycle, with an arrest at the S-phase. As a consequence, *Atg5* deleted thymic *i*NKT cells accumulated a high DNA content, fewer cells went to the G2/M phases, and they also had decreased survival. Consistent with the decreased survival, *Atg5* or *Atg7* deficient *i*NKT cells had increased cell surface expression of several death receptors, and elevated levels of active caspase 8, implicating the cell extrinsic apoptotic pathway. Caspase 8 has been reported to negatively regulate autophagy (66, 67), while the induction of autophagy promotes a reduction in caspase 8 and other pro-apoptotic proteins (19). On the other hand, our data are also consistent with a role for the intrinsic pathway for apoptosis, as evidenced by the increase in mitochondria and ROS in the *i*NKT cells from *Atg5* deficient mice. An increase in mitochondria previously has been observed in autophagy deficient T cells (14, 18, 20). Overexpression of Bcl-X_L failed to rescue the defective *i*NKT cell differentiation in our *Atg5* deficient mice, although the frequency of the CD8⁺ T cells was normalized (Supplemental Fig. 4). This failure to restore the *i*NKT cell population is not consistent with a major role for the intrinsic apoptosis pathway, but the timing or expression level of the Bcl-X_L transgene could have been a mitigating factor preventing restoration. In summary, our data are consistent with the hypothesis that increased apoptosis, which could be induced in the autophagy gene deficient *i*NKT cells that are in proliferation and in the transition from stage 2 to 3 or in mature stage 3 cells, is responsible for the decrease in the *i*NKT population, with possible roles for both the cell extrinsic and cell intrinsic pathways.

What drives the increased entry of autophagy deficient *i*NKT cells into cell cycle? One candidate is mTOR, which is involved in T cell differentiation, including the formation of memory cells (68). There are two mTOR complexes: mTORC1 phosphorylates and activates S6K1 and 4E-BP1, which increase mRNA translation (58, 60, 69). mTORC2, acting in synergy with PDK1 (phosphoinositide-dependent Protein Kinase 1), catalyzes the dual phosphorylations of AKT for its full activity, facilitating cell survival and cell cycle progression (61). We showed that autophagy deficiency led to hyperactivity of mTORC1 and mTORC2 in *i*NKT cells. The T cell specific deletion of Raptor, an essential component of mTORC1, also impaired the maturation of *i*NKT cells (70), suggesting the need for a balance, with either too much or too little mTORC1 inhibiting *i*NKT cell development.

Our findings are consistent with the results from several recent studies indicating a role for proper regulation of metabolism in regulating *i*NKT cell differentiation. First, mice with a T cell specific deletion of *Vps34*, a class III PI3 kinase that is an initiator of autophagy, recently were shown to have an even more profound defect in *i*NKT cells than the one reported here (71), although the mechanism for the effect was not described. Furthermore, while the importance of VPS34 for autophagy in T lymphocytes has been found by several groups (13, 71), this enzyme is also important for IL-7R signaling (72), and therefore some of the deficit in *i*NKT cells in the absence of VPS34 could have been due to reduced IL-7 signals, or some other effects, including the severe inflammation these mice spontaneously developed. Second, mice with a T cell specific deletion of the gene encoding tuberous

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

4E-BP1	eIF4E-binding protein 1
7-AAD	7-aminoactinomycin D
αGalCer	α-galactosylceramide
ATG	autophagy-related gene
AMPK	AMP-activated protein kinase
CDK	cyclin dependent kinase
DP	CD4 and CD8 double positive
DR5	death receptor 5
Fnip1	folliculin-interacting protein 1
iNKT cells	invariant natural killer T cells
MDM2	mouse double minute 2 homolog
mTORC1 and mTORC2	mechanistic target of rapamycin complex 1 and 2
PDK1	phosphoinositide-dependent Protein Kinase 1
PLZF	promyelocytic leukemia zinc finger
ROS	reactive oxygen species
S6K	p70 ribosomal S6 kinase 1
TSC 1 and TSC 2	tuberous sclerosis 1 and 2

References

1. Mizushima N, Yoshimori T, Ohsumi Y. The role of Atg proteins in autophagosome formation. *Annual review of cell and developmental biology*. 2011; 27:107–132.
2. Weidberg H, Shvets E, Elazar Z. Biogenesis and cargo selectivity of autophagosomes. *Annual review of biochemistry*. 2011; 80:125–156.
3. Mizushima N, Komatsu M. Autophagy: renovation of cells and tissues. *Cell*. 2011; 147:728–741. [PubMed: 22078875]
4. Virgin HW, Levine B. Autophagy genes in immunity. *Nature immunology*. 2009; 10:461–470. [PubMed: 19381141]

5. Levine B, Mizushima N, Virgin HW. Autophagy in immunity and inflammation. *Nature*. 2011; 469:323–335. [PubMed: 21248839]
6. Yang Z, Klionsky DJ. An overview of the molecular mechanism of autophagy. *Current topics in microbiology and immunology*. 2009; 335:1–32. [PubMed: 19802558]
7. Nakatogawa H, Suzuki K, Kamada Y, Ohsumi Y. Dynamics and diversity in autophagy mechanisms: lessons from yeast. *Nature reviews. Molecular cell biology*. 2009; 10:458–467.
8. Xie Z, Klionsky DJ. Autophagosome formation: core machinery and adaptations. *Nature cell biology*. 2007; 9:1102–1109.
9. Dunkle A, He YW. Apoptosis and autophagy in the regulation of T lymphocyte function. *Immunologic research*. 2011; 49:70–86. [PubMed: 21128005]
10. Lu JV, Walsh CM. Programmed necrosis and autophagy in immune function. *Immunological reviews*. 2012; 249:205–217. [PubMed: 22889224]
11. Mizushima N, Levine B. Autophagy in mammalian development and differentiation. *Nature cell biology*. 2010; 12:823–830.
12. Walsh CM, Edinger AL. The complex interplay between autophagy, apoptosis, and necrotic signals promotes T-cell homeostasis. *Immunological reviews*. 2010; 236:95–109. [PubMed: 20636811]
13. Willinger T, Flavell RA. Canonical autophagy dependent on the class III phosphoinositide-3 kinase Vps34 is required for naive T-cell homeostasis. *Proceedings of the National Academy of Sciences of the United States of America*. 2012; 109:8670–8675. [PubMed: 22592798]
14. Jia W, He YW. Temporal regulation of intracellular organelle homeostasis in T lymphocytes by autophagy. *Journal of immunology*. 2011; 186:5313–5322.
15. Li C, Capan E, Zhao Y, Zhao J, Stolz D, Watkins SC, Jin S, Lu B. Autophagy is induced in CD4+ T cells and important for the growth factor-withdrawal cell death. *Journal of immunology*. 2006; 177:5163–5168.
16. Pua HH, Dzhagalov I, Chuck M, Mizushima N, He YW. A critical role for the autophagy gene Atg5 in T cell survival and proliferation. *The Journal of experimental medicine*. 2007; 204:25–31. [PubMed: 17190837]
17. Hubbard VM, Valdor R, Patel B, Singh R, Cuervo AM, Macian F. Macroautophagy regulates energy metabolism during effector T cell activation. *Journal of immunology*. 2010; 185:7349–7357.
18. Stephenson LM, Miller BC, Ng A, Eisenberg J, Zhao Z, Cadwell K, Graham DB, Mizushima NN, Xavier R, Virgin HW, Swat W. Identification of Atg5-dependent transcriptional changes and increases in mitochondrial mass in Atg5-deficient T lymphocytes. *Autophagy*. 2009; 5:625–635. [PubMed: 19276668]
19. Kovacs JR, Li C, Yang Q, Li G, Garcia IG, Ju S, Roodman DG, Windle JJ, Zhang X, Lu B. Autophagy promotes T-cell survival through degradation of proteins of the cell death machinery. *Cell death and differentiation*. 2012; 19:144–152. [PubMed: 21660048]
20. Pua HH, Guo J, Komatsu M, He YW. Autophagy is essential for mitochondrial clearance in mature T lymphocytes. *Journal of immunology*. 2009; 182:4046–4055.
21. Bendelac A, Savage PB, Teyton L. The biology of NKT cells. *Annual review of immunology*. 2007; 25:297–336.
22. Kronenberg M. Toward an understanding of NKT cell biology: progress and paradoxes. *Annual review of immunology*. 2005; 23:877–900.
23. Cohen NR, Brennan PJ, Shay T, Watts GF, Brigl M, Kang J, Brenner MB. Shared and distinct transcriptional programs underlie the hybrid nature of iNKT cells. *Nature immunology*. 2013; 14:90–99. [PubMed: 23202270]
24. Matsuda JL, Gapin L. Developmental program of mouse Valpha14i NKT cells. *Current opinion in immunology*. 2005; 17:122–130. [PubMed: 15766670]
25. Godfrey DI, MacDonald HR, Kronenberg M, Smyth MJ, Van Kaer L. NKT cells: what's in a name? *Nature reviews Immunology*. 2004; 4:231–237.
26. Cohen NR, Garg S, Brenner MB. Antigen Presentation by CD1 Lipids, T Cells, and NKT Cells in Microbial Immunity. *Advances in immunology*. 2009; 102:1–94. [PubMed: 19477319]

27. Brigl M, Brenner MB. CD1: antigen presentation and T cell function. *Annual review of immunology*. 2004; 22:817–890.
28. Silk JD, Salio M, Brown J, Jones EY, Cerundolo V. Structural and functional aspects of lipid binding by CD1 molecules. *Annual review of cell and developmental biology*. 2008; 24:369–395.
29. Kawano T, Cui J, Koezuka Y, Toura I, Kaneko Y, Motoki K, Ueno H, Nakagawa R, Sato H, Kondo E, Koseki H, Taniguchi M. CD1d–restricted and TCR-mediated activation of valpha14 NKT cells by glycosylceramides. *Science*. 1997; 278:1626–1629. [PubMed: 9374463]
30. Watarai H, Sekine-Kondo E, Shigeura T, Motomura Y, Yasuda T, Satoh R, Yoshida H, Kubo M, Kawamoto H, Koseki H, Taniguchi M. Development and function of invariant natural killer T cells producing T(h)2- and T(h)17-cytokines. *PLoS biology*. 2012; 10:e1001255. [PubMed: 22346732]
31. Constantinides MG, Bendelac A. Transcriptional regulation of the NKT cell lineage. *Current opinion in immunology*. 2013; 25:161–167. [PubMed: 23402834]
32. Coquet JM, Chakravarti S, Kyparissoudis K, McNab FW, Pitt LA, McKenzie BS, Berzins SP, Smyth MJ, Godfrey DI. Diverse cytokine production by NKT cell subsets and identification of an IL-17-producing CD4-NK1.1- NKT cell population. *Proceedings of the National Academy of Sciences of the United States of America*. 2008; 105:11287–11292. [PubMed: 18685112]
33. Michel ML, Keller AC, Paget C, Fujio M, Trottein F, Savage PB, Wong CH, Schneider E, Dy M, Leite-de-Moraes MC. Identification of an IL-17-producing NK1.1(neg) iNKT cell population involved in airway neutrophilia. *The Journal of experimental medicine*. 2007; 204:995–1001. [PubMed: 17470641]
34. Lee YJ, Holzapfel KL, Zhu J, Jameson SC, Hogquist KA. Steady-state production of IL-4 modulates immunity in mouse strains and is determined by lineage diversity of iNKT cells. *Nature immunology*. 2013; 14:1146–1154. [PubMed: 24097110]
35. Godfrey DI, Berzins SP. Control points in NKT-cell development. *Nature reviews. Immunology*. 2007; 7:505–518.
36. Godfrey DI, Stankovic S, Baxter AG. Raising the NKT cell family. *Nature immunology*. 2010; 11:197–206. [PubMed: 20139988]
37. Gapin L, Matsuda JL, Surh CD, Kronenberg M. NKT cells derive from double-positive thymocytes that are positively selected by CD1d. *Nature immunology*. 2001; 2:971–978. [PubMed: 11550008]
38. Wei DG, Lee H, Park SH, Beaudoin L, Teyton L, Lehuen A, Bendelac A. Expansion and long-range differentiation of the NKT cell lineage in mice expressing CD1d exclusively on cortical thymocytes. *The Journal of experimental medicine*. 2005; 202:239–248. [PubMed: 16027237]
39. Engel I, Kronenberg M. Making memory at birth: understanding the differentiation of natural killer T cells. *Current opinion in immunology*. 2012; 24:184–190. [PubMed: 22305304]
40. Hara T, Nakamura K, Matsui M, Yamamoto A, Nakahara Y, Suzuki-Migishima R, Yokoyama M, Mishima K, Saito I, Okano H, Mizushima N. Suppression of basal autophagy in neural cells causes neurodegenerative disease in mice. *Nature*. 2006; 441:885–889. [PubMed: 16625204]
41. Komatsu M, Waguri S, Ueno T, Iwata J, Murata S, Tanida I, Ezaki J, Mizushima N, Ohsumi Y, Uchiyama Y, Kominami E, Tanaka K, Chiba T. Impairment of starvation-induced and constitutive autophagy in Atg7-deficient mice. *The Journal of cell biology*. 2005; 169:425–434. [PubMed: 15866887]
42. Kuma A, Hatano M, Matsui M, Yamamoto A, Nakaya H, Yoshimori T, Ohsumi Y, Tokuhisa T, Mizushima N. The role of autophagy during the early neonatal starvation period. *Nature*. 2004; 432:1032–1036. [PubMed: 15525940]
43. Komatsu M, Waguri S, Chiba T, Murata S, Iwata J, Tanida I, Ueno T, Koike M, Uchiyama Y, Kominami E, Tanaka K. Loss of autophagy in the central nervous system causes neurodegeneration in mice. *Nature*. 2006; 441:880–884. [PubMed: 16625205]
44. Nishida Y, Arakawa S, Fujitani K, Yamaguchi H, Mizuta T, Kanaseki T, Komatsu M, Otsu K, Tsujimoto Y, Shimizu S. Discovery of Atg5/Atg7-independent alternative macroautophagy. *Nature*. 2009; 461:654–658. [PubMed: 19794493]

45. Benlagha K, Wei DG, Veiga J, Teyton L, Bendelac A. Characterization of the early stages of thymic NKT cell development. *The Journal of experimental medicine*. 2005; 202:485–492. [PubMed: 16087715]
46. Pellicci DG, Hammond KJ, Uldrich AP, Baxter AG, Smyth MJ, Godfrey DI. A natural killer T (NKT) cell developmental pathway involving a thymus-dependent NK1.1(-)CD4(+) CD1d-dependent precursor stage. *The Journal of experimental medicine*. 2002; 195:835–844. [PubMed: 11927628]
47. Benlagha K, Kyin T, Beavis A, Teyton L, Bendelac A. A thymic precursor to the NK T cell lineage. *Science*. 2002; 296:553–555. [PubMed: 11968185]
48. Guo J, Hawwari A, Li H, Sun Z, Mahanta SK, Littman DR, Krangel MS, He YW. Regulation of the TCRalpha repertoire by the survival window of CD4(+)CD8(+) thymocytes. *Nature immunology*. 2002; 3:469–476. [PubMed: 11967541]
49. Egawa T, Eberl G, Taniuchi I, Benlagha K, Geissmann F, Hennighausen L, Bendelac A, Littman DR. Genetic evidence supporting selection of the Valpha14i NKT cell lineage from double-positive thymocyte precursors. *Immunity*. 2005; 22:705–716. [PubMed: 15963785]
50. Coles MC, Raulet DH. NK1.1+ T cells in the liver arise in the thymus and are selected by interactions with class I molecules on CD4+CD8+ cells. *Journal of immunology*. 2000; 164:2412–2418.
51. Sherr CJ, Roberts JM. CDK inhibitors: positive and negative regulators of G1-phase progression. *Genes & development*. 1999; 13:1501–1512. [PubMed: 10385618]
52. Norbury C, Blow J, Nurse P. Regulatory phosphorylation of the p34cdc2 protein kinase in vertebrates. *The EMBO journal*. 1991; 10:3321–3329. [PubMed: 1655417]
53. Youle RJ, Narendra DP. Mechanisms of mitophagy. *Nature reviews Molecular cell biology*. 2011; 12:9–14.
54. Presley AD, Fuller KM, Arriaga EA. MitoTracker Green labeling of mitochondrial proteins and their subsequent analysis by capillary electrophoresis with laser-induced fluorescence detection. *Journal of chromatography. B, Analytical technologies in the biomedical and life sciences*. 2003; 793:141–150.
55. Pua HH, He YW. Autophagy and lymphocyte homeostasis. *Current topics in microbiology and immunology*. 2009; 335:85–105. [PubMed: 19802561]
56. Savage AK, Constantinides MG, Han J, Picard D, Martin E, Li B, Lantz O, Bendelac A. The transcription factor PLZF directs the effector program of the NKT cell lineage. *Immunity*. 2008; 29:391–403. [PubMed: 18703361]
57. Kovalovsky D, Uche OU, Eladad S, Hobbs RM, Yi W, Alonzo E, Chua K, Eidson M, Kim HJ, Im JS, Pandolfi PP, Sant' Angelo DB. The BTB-zinc finger transcriptional regulator PLZF controls the development of invariant natural killer T cell effector functions. *Nature immunology*. 2008; 9:1055–1064. [PubMed: 18660811]
58. Zoncu R, Efeyan A, Sabatini DM. mTOR: from growth signal integration to cancer, diabetes and ageing. *Nature reviews. Molecular cell biology*. 2011; 12:21–35.
59. Hung CM, Garcia-Haro L, Sparks CA, Guertin DA. mTOR-dependent cell survival mechanisms. *Cold Spring Harbor perspectives in biology*. 2012; 4:a008771. [PubMed: 23124837]
60. Ma XM, Blenis J. Molecular mechanisms of mTOR-mediated translational control. *Nature reviews Molecular cell biology*. 2009; 10:307–318.
61. Sarbassov DD, Guertin DA, Ali SM, Sabatini DM. Phosphorylation and regulation of Akt/PKB by the rictor-mTOR complex. *Science*. 2005; 307:1098–1101. [PubMed: 15718470]
62. Zhao Z, Fux B, Goodwin M, Dunay IR, Strong D, Miller BC, Cadwell K, Delgado MA, Ponpuak M, Green KG, Schmidt RE, Mizushima N, Deretic V, Sibley LD, Virgin HW. Autophagosome-independent essential function for the autophagy protein Atg5 in cellular immunity to intracellular pathogens. *Cell host & microbe*. 2008; 4:458–469. [PubMed: 18996346]
63. Hwang S, Maloney NS, Bruinsma MW, Goel G, Duan E, Zhang L, Shrestha B, Diamond MS, Dani A, Sosnovtsev SV, Green KY, Lopez-Otin C, Xavier RJ, Thackray LB, Virgin HW. Nondegradative role of Atg5-Atg12/ Atg16L1 autophagy protein complex in antiviral activity of interferon gamma. *Cell host & microbe*. 2012; 11:397–409. [PubMed: 22520467]

64. Selleck EM, Fentress SJ, Beatty WL, Degrandi D, Pfeffer K, Virgin HWt, Macmicking JD, Sibley LD. Guanylate-binding protein 1 (Gbp1) contributes to cell-autonomous immunity against *Toxoplasma gondii*. *PLoS pathogens*. 2013; 9:e1003320. [PubMed: 23633952]
65. Wolfer A, Wilson A, Nemir M, MacDonald HR, Radtke F. Inactivation of Notch1 impairs VDJbeta rearrangement and allows pre-TCR-independent survival of early alpha beta Lineage Thymocytes. *Immunity*. 2002; 16:869–879. [PubMed: 12121668]
66. Yu L, Alva A, Su H, Dutt P, Freundt E, Welsh S, Baehrecke EH, Lenardo MJ. Regulation of an ATG7-beclin 1 program of autophagic cell death by caspase-8. *Science*. 2004; 304:1500–1502. [PubMed: 15131264]
67. Bell BD, Leverrier S, Weist BM, Newton RH, Arechiga AF, Luhrs KA, Morrisette NS, Walsh CM. FADD and caspase-8 control the outcome of autophagic signaling in proliferating T cells. *Proceedings of the National Academy of Sciences of the United States of America*. 2008; 105:16677–16682. [PubMed: 18946037]
68. Zeng H, Chi H. mTOR and lymphocyte metabolism. *Current opinion in immunology*. 2013; 25:347–355. [PubMed: 23722114]
69. Hung CM, Garcia-Haro L, Sparks CA, Guertin DA. mTOR-dependent cell survival mechanisms. *Cold Spring Harbor perspectives in biology*. 4:a008771. [PubMed: 23124837]
70. Zhang L, Tschumi BO, Corgnac S, Ruegg MA, Hall MN, Mach JP, Romero P, Donda A. Mammalian Target of Rapamycin Complex 1 Orchestrates Invariant NKT Cell Differentiation and Effector Function. *Journal of immunology*. 2014
71. Parekh VV, Wu L, Boyd KL, Williams JA, Gaddy JA, Olivares-Villagomez D, Cover TL, Zong WX, Zhang J, Van Kaer L. Impaired autophagy, defective T cell homeostasis, and a wasting syndrome in mice with a T cell-specific deletion of Vps34. *Journal of immunology*. 2013; 190:5086–5101.
72. McLeod IX, Zhou X, Li QJ, Wang F, He YW. The class III kinase Vps34 promotes T lymphocyte survival through regulating IL-7Ralpha surface expression. *Journal of immunology*. 2011; 187:5051–5061.
73. Yang K, Neale G, Green DR, He W, Chi H. The tumor suppressor Tsc1 enforces quiescence of naive T cells to promote immune homeostasis and function. *Nature immunology*. 2011; 12:888–897. [PubMed: 21765414]
74. Wu J, Yang J, Yang K, Wang H, Gorentla B, Shin J, Qiu Y, Que LG, Foster WM, Xia Z, Chi H, Zhong XP. iNKT cells require TSC1 for terminal maturation and effector lineage fate decisions. *The Journal of clinical investigation*. 2014; 124:1685–1698. [PubMed: 24614103]
75. Park H, Tsang M, Iritani BM, Bevan MJ. Metabolic regulator Fnip1 is crucial for iNKT lymphocyte development. *Proceedings of the National Academy of Sciences of the United States of America*. 2014; 111:7066–7071. [PubMed: 24785297]
76. Salio M, Puleston DJ, Mathan TS, Shepherd D, Stranks AJ, Adamopoulou E, Veerapen N, Besra GS, Hollander GA, Simon AK, Cerundolo V. Essential role for autophagy during invariant NKT cell development. *Proc Natl Acad Sci U S A*. 2014; 111:E5678–E5687. [PubMed: 25512546]
77. Matsuda JL, Gapin L, Sidobre S, Kieper WC, Tan JT, Ceredig R, Surh CD, Kronenberg M. Homeostasis of V alpha 14i NKT cells. *Nat Immunol*. 2002; 3:966–974. [PubMed: 12244311]
78. Miller BC, Zhao Z, Stephenson LM, Cadwell K, Pua HH, Lee HK, Mizushima NN, Iwasaki A, He YW, Swat W, Virgin HWt. The autophagy gene ATG5 plays an essential role in B lymphocyte development. *Autophagy*. 2008; 4:309–314. [PubMed: 18188005]

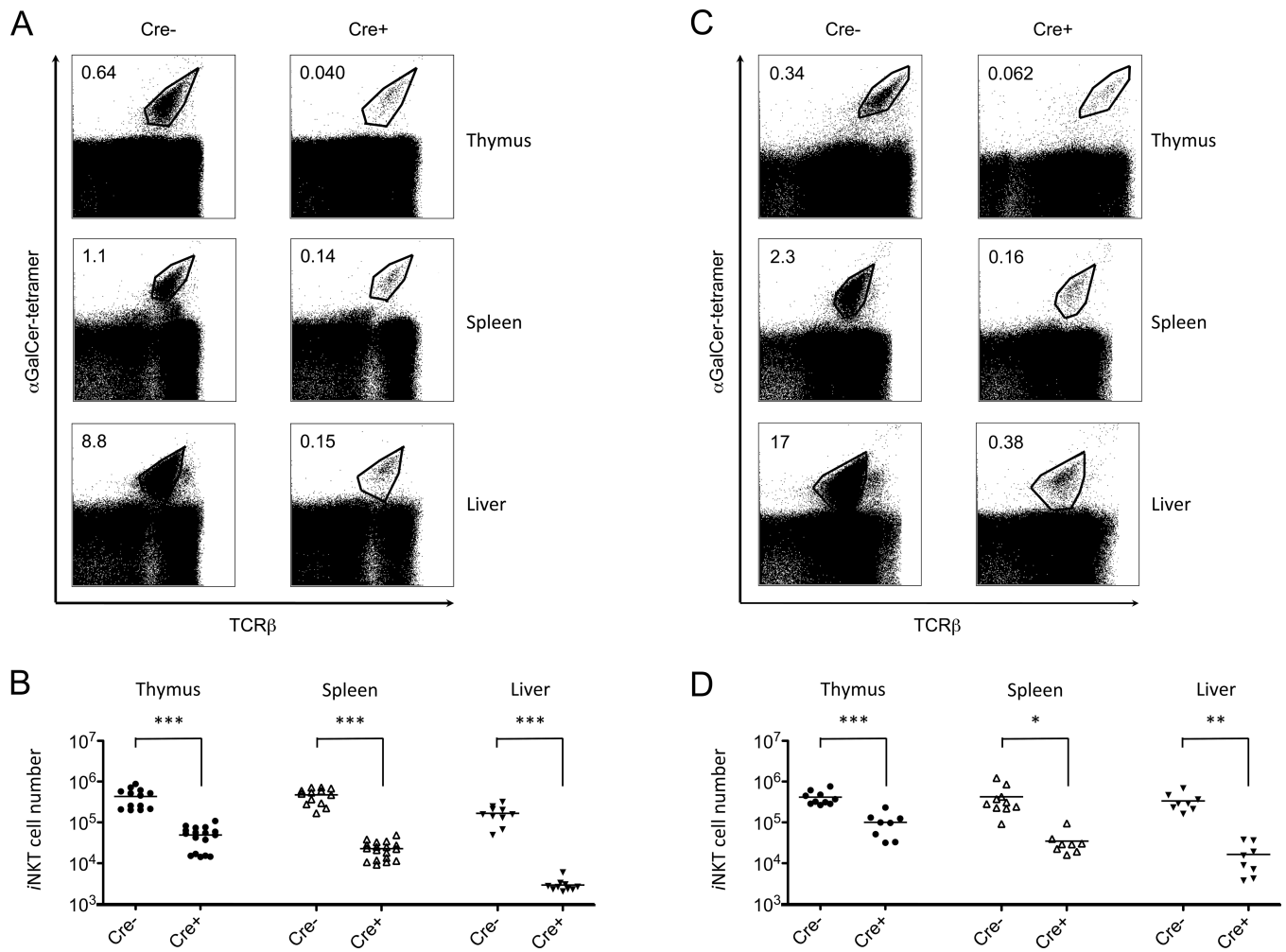


Figure 1.

Mice deficient for autophagy genes had reduced *i*NKT cells. *i*NKT cells were analyzed by gating on CD1d tetramer⁺ cells as described in Experimental Procedures. Depicted are the results from cells from the indicated organs from eight-to-nine-week old mice with a CD4-Cre mediated deletion of *Atg5* (A,B) or *Atg7* (C,D) and controls. Shown are the percentages (A,C) and absolute cell numbers (B,D) compared with *Atg5^{fl/fl}* or *Atg7^{fl/fl}* mice without the Cre transgene. Representative flow cytometry analyses are from one of at least three independent experiments. **p*<0.01, ***p*<0.005, ****p*<0.0001, *n* = 8.

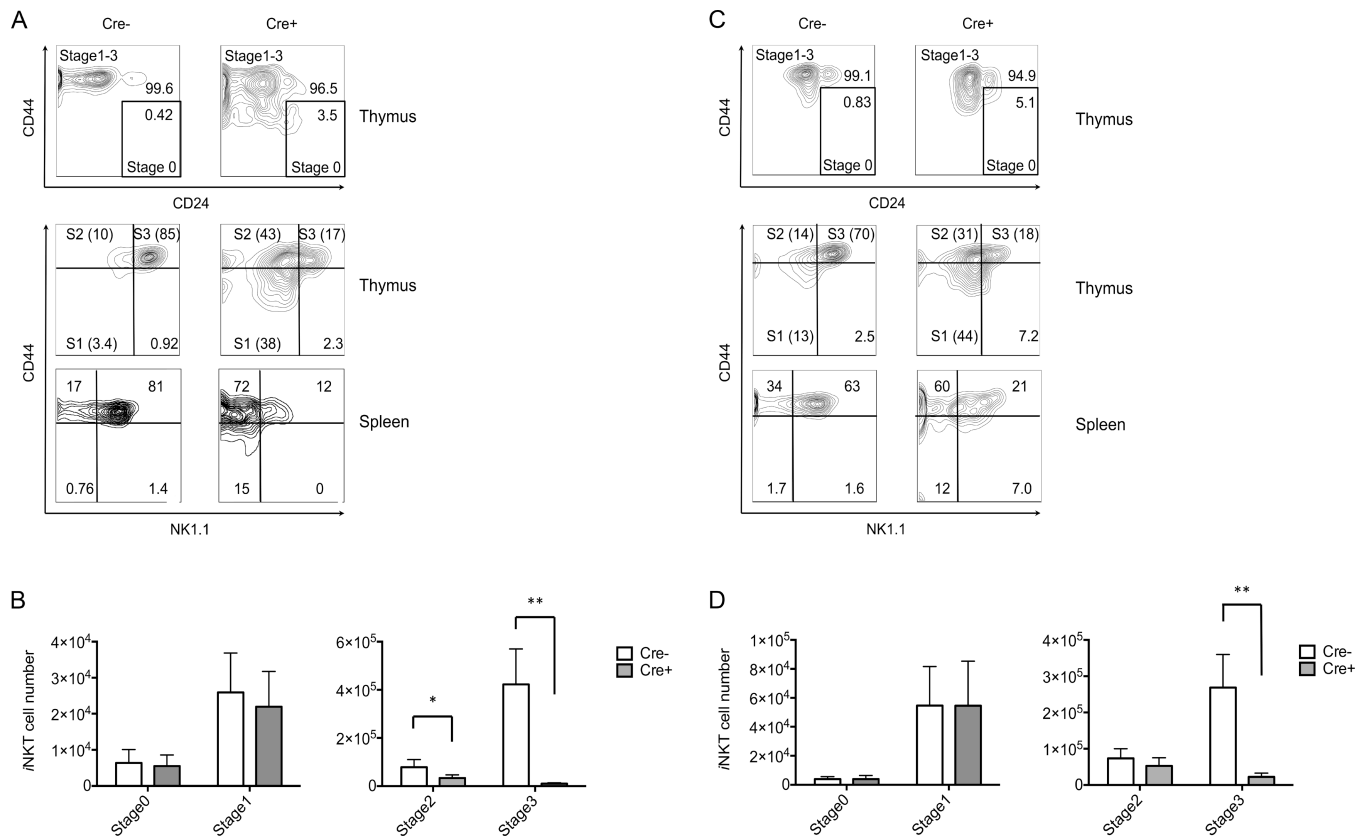


Figure 2. Deficiency for autophagy genes inhibited *i*NKT cell maturation. (A,C) Thymus and spleen cells from eight-to-nine-week old mice were analyzed for *i*NKT cell phenotype. Representative flow cytometry plots from one of four experiments showing gated *i*NKT cells from *Atg5^{ff}* CD4-Cre (A) or *Atg7^{ff}* CD4-Cre (C) mice and controls lacking the Cre transgene. Absolute cell numbers of thymic *i*NKT cells at different stages were shown in (B, *Atg5^{ff}* CD4-Cre and D, *Atg7^{ff}* CD4-Cre). The analysis of all stages was carried out simultaneously, but because stages 0 and 1 have much fewer cells, for visual clarity their numbers are plotted on a separate graph (left). * $p < 0.0005$, ** $p < 0.0001$, $n = 10$. Error bars are SD.

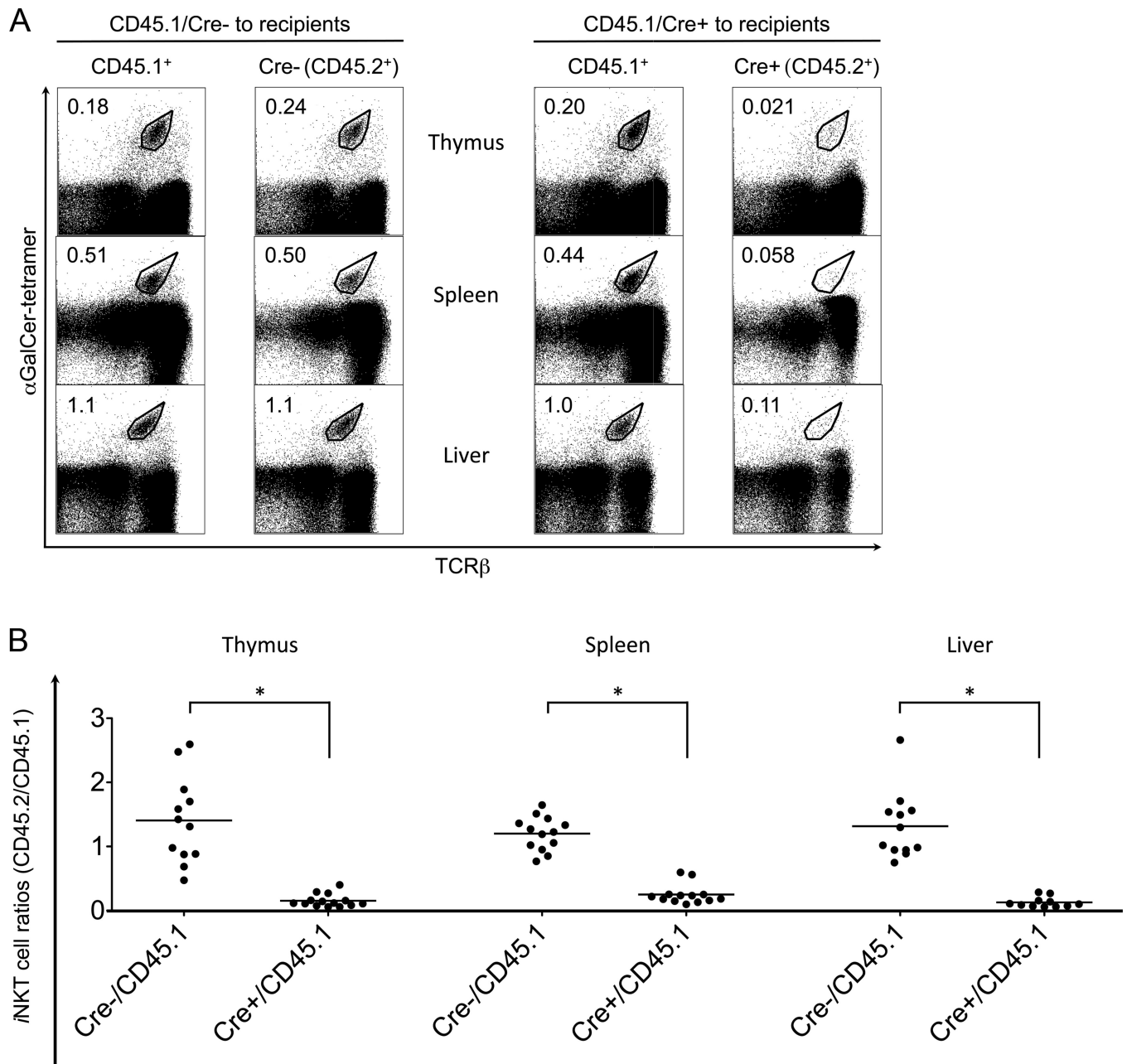


Figure 3.

Cell-intrinsic defects impaired *Atg5* deficient *i*NKT cell development. (A) Bone marrow cells from *Atg5^{ff}* CD4-Cre mice or wild type controls (both CD45.2⁺), together with CD45.1⁺, wild type bone marrow cells, were co-transferred into recipients. Reconstituted *i*NKT cells from the indicated organs were analyzed after 11–12 weeks with flow cytometry. Data are representative from one of at least three separate experiments. (B) The ratios of reconstituted *i*NKT cells (CD45.2⁺/CD45.1⁺). **p*<0.0001, *n*>10.

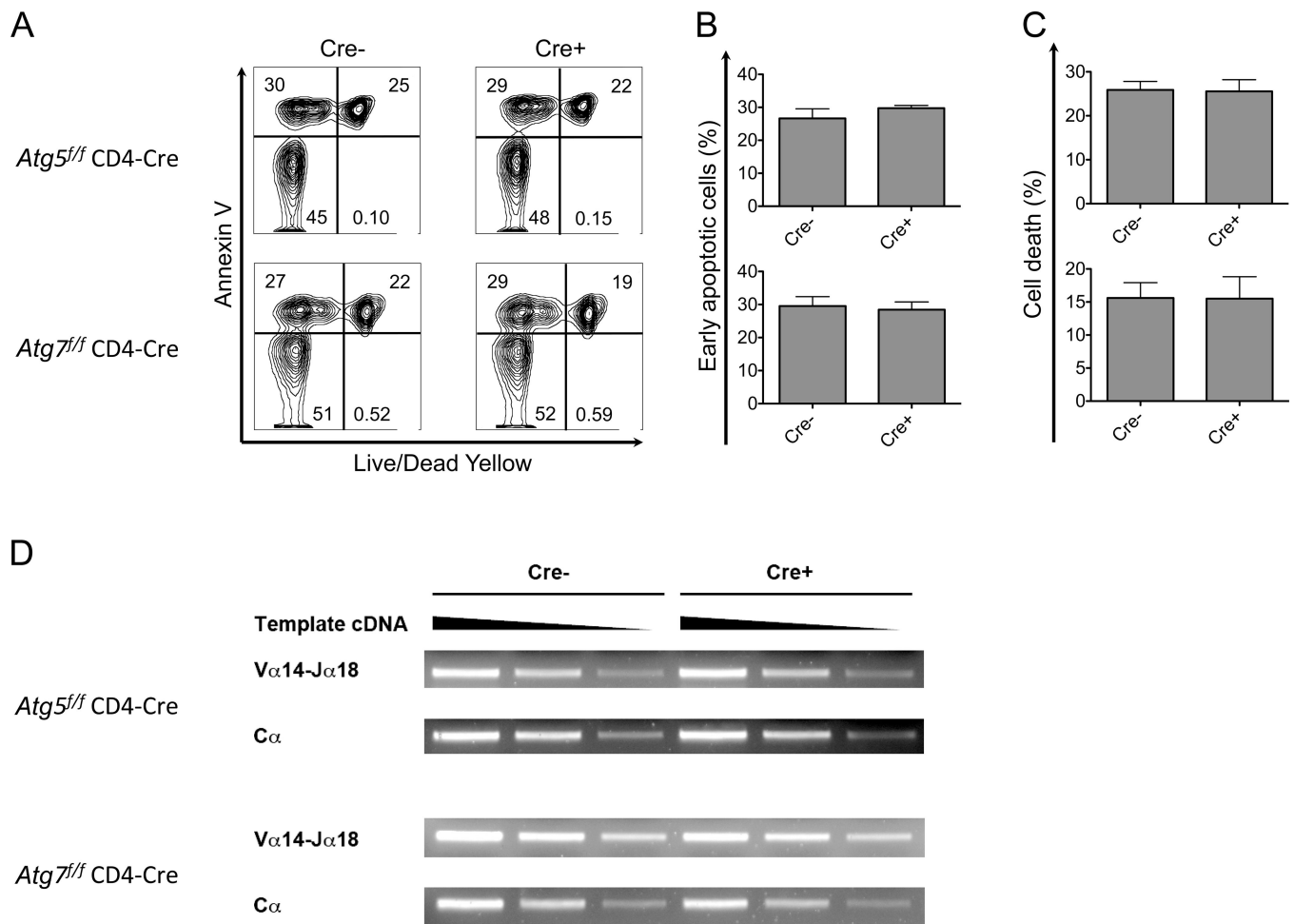
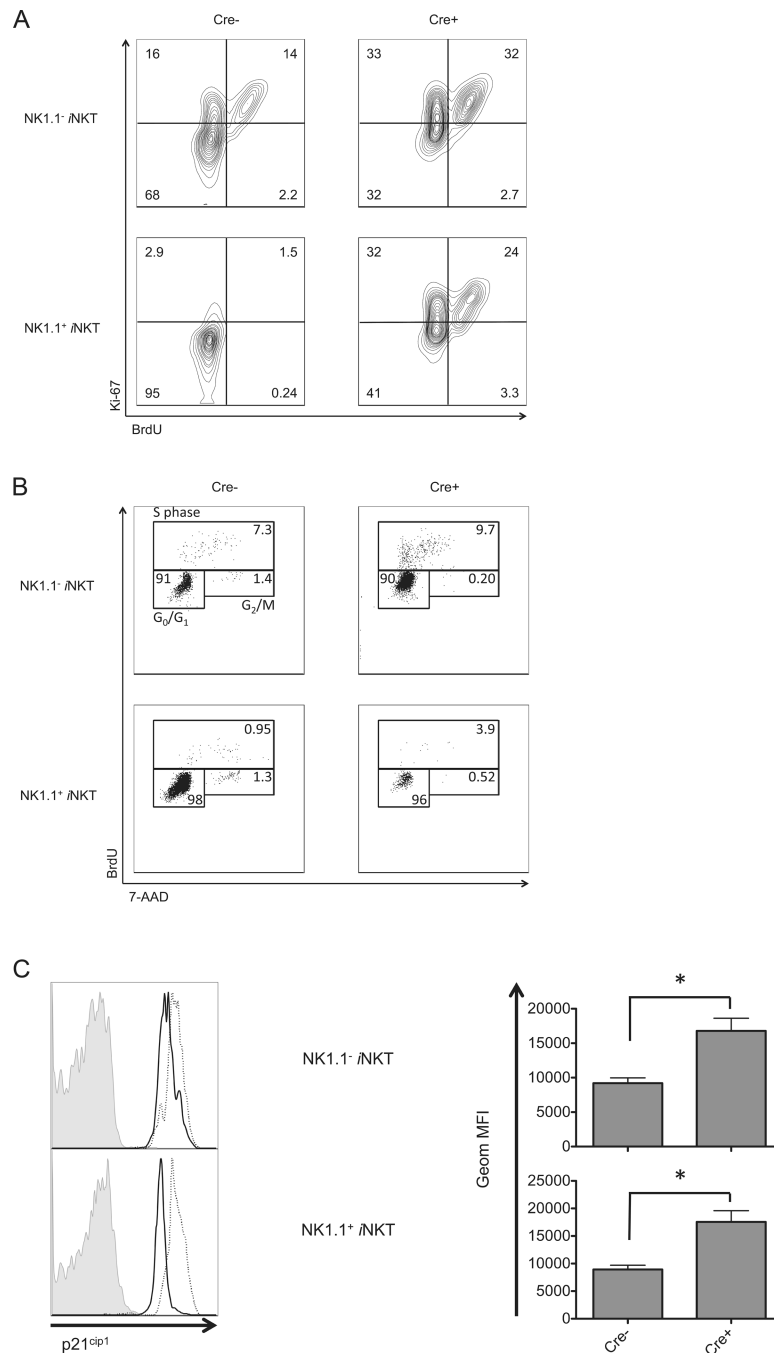


Figure 4.

Atg5 or *Atg7* deficiency did not cause significant DP thymocyte death and skewed TCR α rearrangements in thymocytes. (A-C) Thymocytes were cultured overnight and cells undergoing apoptosis and dead cells were identified, shown here as flow cytometry analyses (A) and the geometric MFI results indicating cells undergoing apoptosis (B) and dead cells (C). Data in (A) are representative of at least four independent experiments. (B and C) $n = 5$. Error bars are SD. (D). Equivalent TCR $V\alpha 14$ - $J\alpha 18$ rearrangements were observed in *Atg5* or *Atg7* deficient DP thymocytes. cDNA reverse transcribed from total RNA of DP thymocytes depleted of α GalCer-CD1d tetramer⁺ cells was used as template and titrated at serial dilutions (200 ng, 60 ng and 20 ng) for semi-quantitative PCR. Representative data are from three separate experiments.

**Figure 5.**

Loss of *Atg5* expression led to cell cycle arrest. (A) *Atg5* deficient *iNKT* cells exhibited enhanced incorporation of BrdU. 5 h after injection, thymic *iNKT* cells were collected and analyzed for BrdU incorporation and Ki-67 expression. (B) Thymic *iNKT* cells from *Atg5^{f/f}* CD4-Cre mice accumulated in S-phase. 2 h post injection of BrdU, thymic *iNKT* cells were stained with 7-AAD and anti-BrdU antibody to evaluate cell cycle progression. (C) Increased expression of p21^{cip1} in *Atg5* deficient *iNKT* cells. Shown are the representative flow cytometry analysis (left) and geometric MFI (right) of data pooled from at least three

independent experiments gating on either NK1.1 or NK1.1⁺, CD1d tetramer⁺ thymocytes. Filled histogram: isotype control; solid line: wild type mice; dotted line: *Atg5^{fl/fl}* CD4-Cre mice. * p<0.01, n = 4. Error bars are SD.

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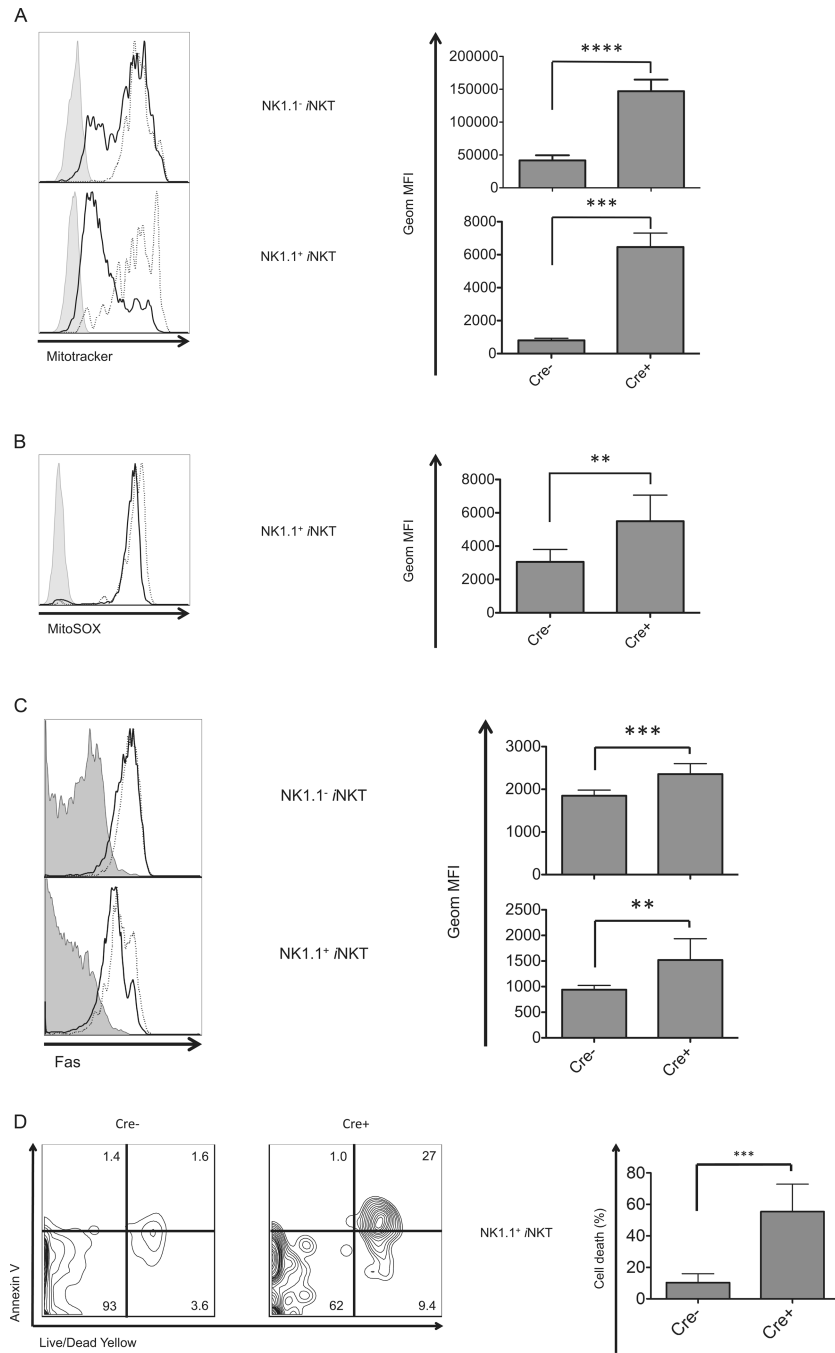


Figure 6. *Atg5* deletion resulted in increased mitochondria, super oxide and cell death. Augmented mitochondrial mass (A) and mitochondrial superoxide production (B) in *Atg5* deleted, thymic, NK1.1 and NK1.1⁺iNKT cells. (C) Higher expression of Fas on *Atg5* deficient iNKT cells compared to wild type controls. At least four independent experiments were performed with consistent results, both representative flow cytometry plots (left) and geometric MFI values (right) are shown. Filled histogram: isotype control; solid line: wild type mice; dotted line: *Atg5^{fl/fl}* CD4-Cre mice. (D) Dramatically increased apoptosis leading

to cell death for NK1.1⁺ thymic *i*NKT cells devoid of *Atg5*. Cells were analyzed after overnight culture and compared with wild type controls. Representative flow cytometry analyses from three separate experiments (left) and geometric MFI (right) of dead cells (Annexin V⁺, Live/Dead Yellow⁺) are shown. **p*<0.05, ***p*<0.01, ****p*<0.001, *****p*<0.0001, n = 4. Error bars are SD.

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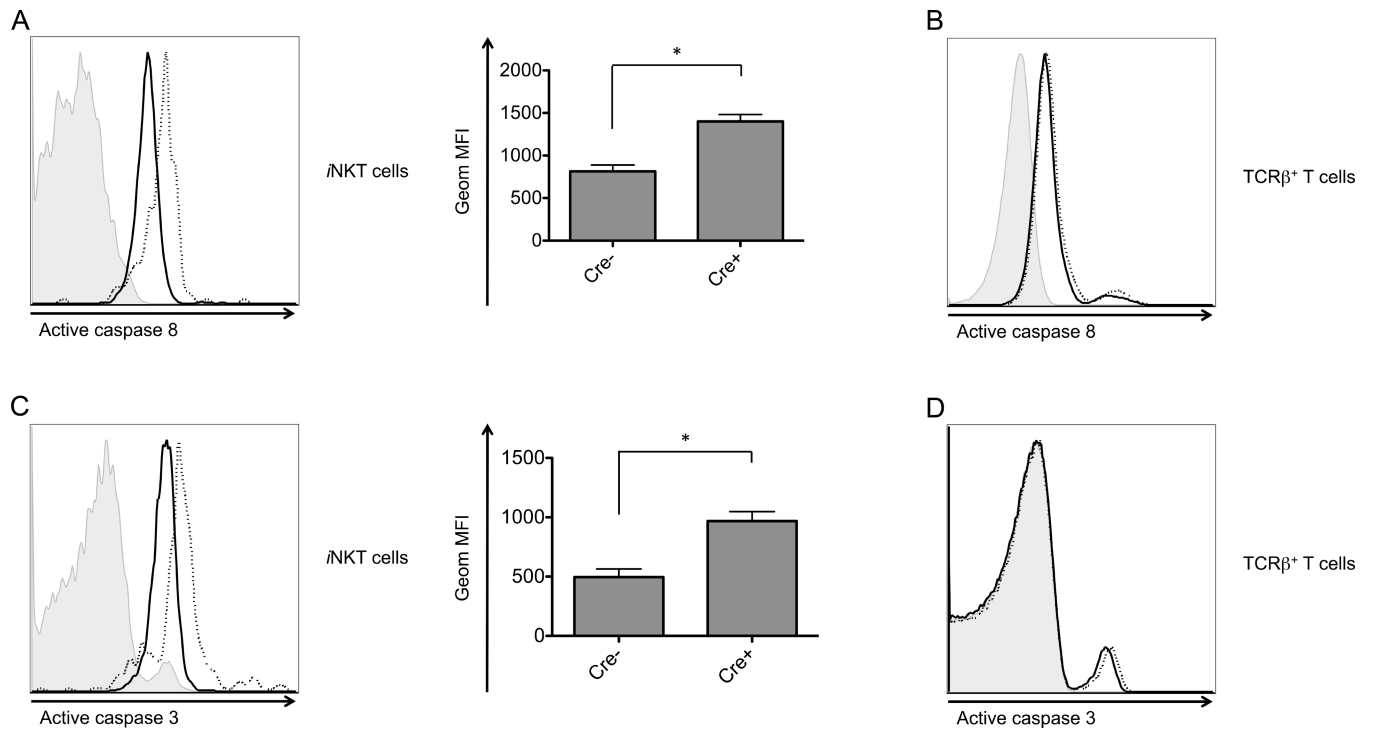


Figure 7. *Atg5* deficient thymic *i*NKT cells exhibited an increase in active caspase 8 and caspase 3. *Atg5* deleted thymocytes were cultured overnight, followed by analysis by flow cytometry of active caspase 8 (A and B) and active caspase 3 (C and D) in thymic *i*NKT cells (A and C) and TCR β^+ thymocytes (B and D). Shown here are both flow cytometry plots (left) and geometric MFI (right). Data are representative of at least two independent experiments. Filled histogram: isotype control; solid line: wild type cells; dotted line: *Atg5* deficient. * $p < 0.05$, n = 3. Error bars are SD.

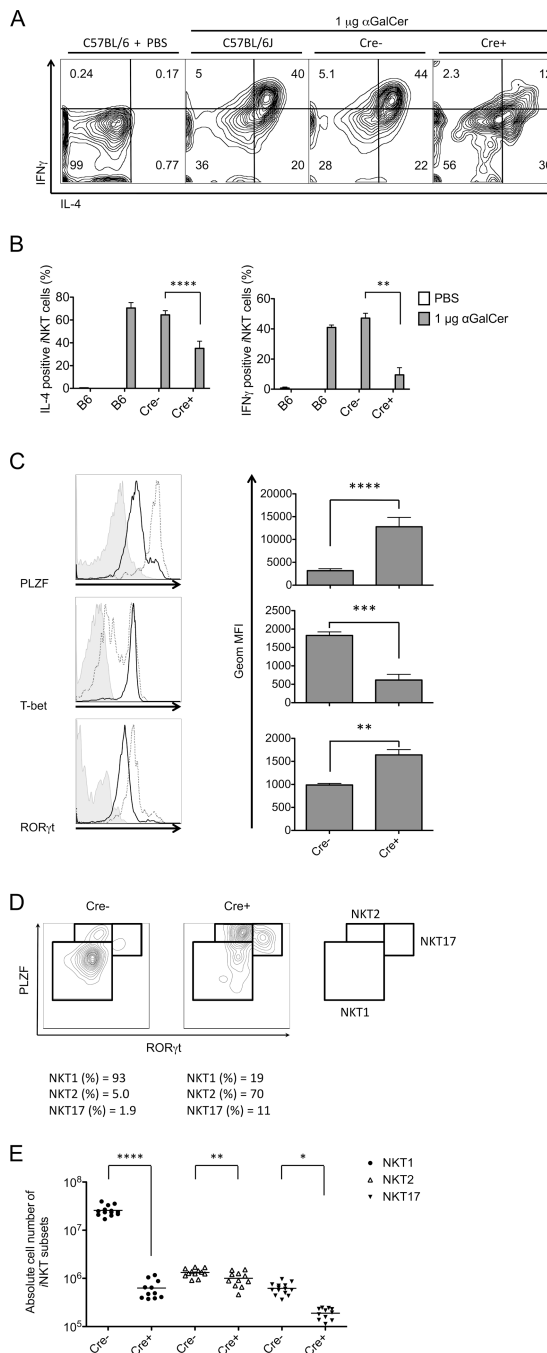


Figure 8. *Atg5* deficiency differentially affected *i*NKT cell functional subsets. (A and B) Impaired *i*NKT cell activation and polarized cytokine outcome in *Atg5^{f/f}* CD4-Cre mice after α GalCer immunization. Intracellular cytokine production in splenic *i*NKT cells was analyzed 2 h after α GalCer injection. Flow cytometry plots (A), geometric MFI of intracellular IL-4 and IFN γ (B) are representative of three independent experiments. (C) Altered expression profile of transcription factors in autophagy deficient thymic *i*NKT cells. Representative flow cytometry plots from three independent experiments and geometric MFI are indicated. Filled

histogram: isotype control; solid line: wild type mice; dotted line: *Atg5^{ff}* CD4-Cre mice. (D) *i*NKT cell subset analysis in autophagy deficient mice compared with wild type controls. Representative flow cytometry plots of three independent experiments are shown. (E) Absolute cell number of thymic *i*NKT cell functional subsets defined by transcription factor expression levels in *Atg5^{ff}* CD4-Cre mice and controls. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, n = 4. Error bars are SD.

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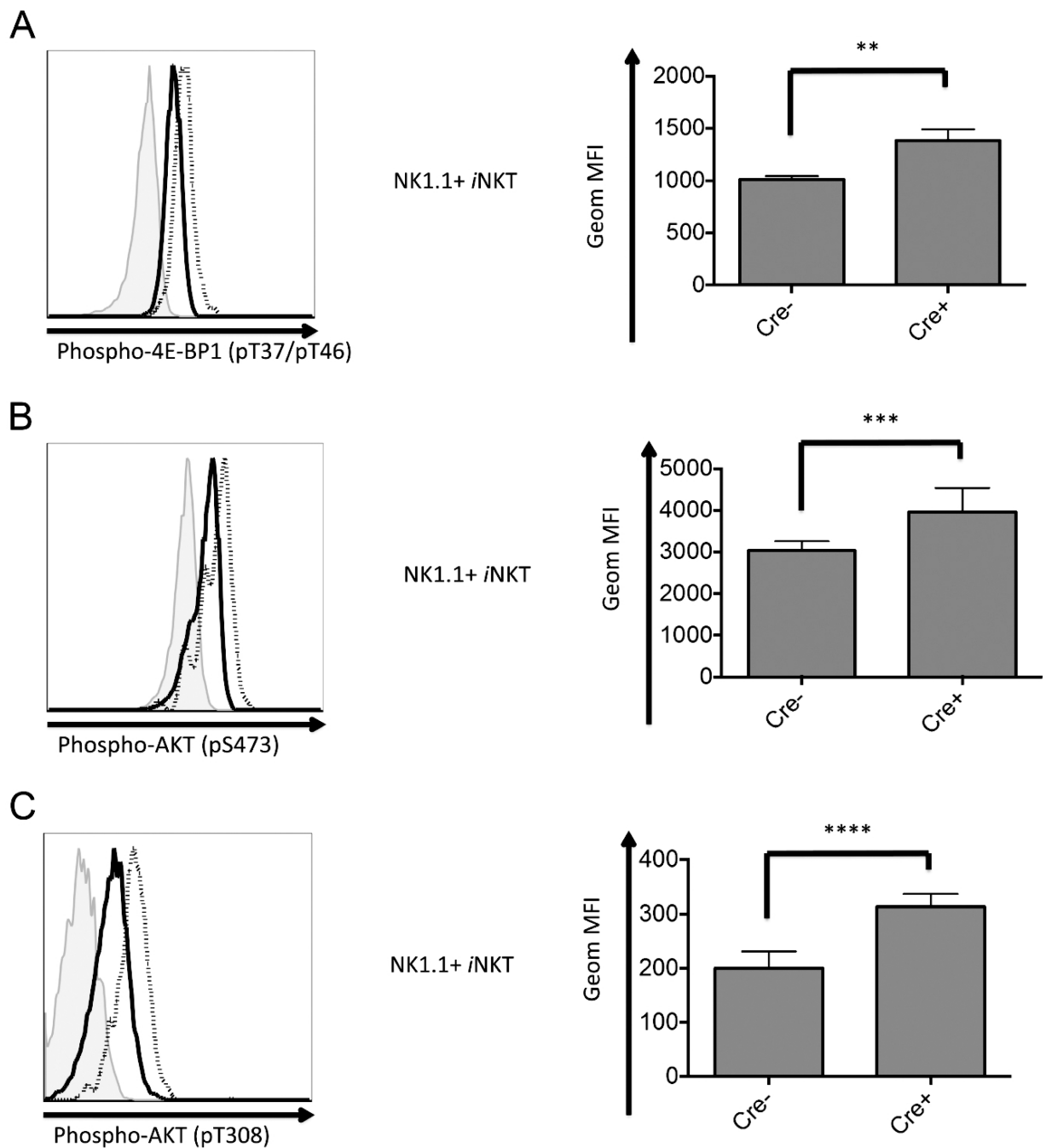


Figure 9.

Unbalanced proliferative and apoptotic signaling in *Atg5* deficient *i*NKT cells. Thymic *i*NKT cells were analyzed ex vivo for activation of mTOR signaling pathway.

Phosphorylation of mTORC1 substrate, 4E-BP1 (pT37/pT46) (A), and phosphorylation of mTORC2 substrate, AKT(pS473) (B), were analyzed. (C) Phosphorylation of AKT at T308 was measured to evaluate the PI3K/PDK1 signaling pathway. Both Cre⁻ and Cre⁺ were pooled from at least four samples. Shown here is the representative flow cytometry analysis of three independent experiments. Filled histogram: isotype control; solid line: wild type

mice; dotted line: *Atg5^{ff}* CD4-Cre mice. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$, **** $p < 0.0005$,
n = 4. Error bars are SD.

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Table 1

Effects on the population size of iNKT cells and conventional T cells in autophagy deficient mice compared with wild type controls.

<i>Arg5^{fl/fl}</i> CD4-Cre												
	iNKT cells			DP thymocytes			CD4 T cells			CD8 T cells		
	Thymus	Spleen	Liver				Thymus	Spleen	Liver	Thymus	Spleen	Liver
Reduction folds (Cre ⁻ /Cre ⁺)	8.8 ^a	20 ^a	56 ^a	1.1			1.2	2.9 ^a	1.7 ^c	1.1	3.1 ^a	1.5 ^c
<i>Arg5^{fl/fl}</i> CD4-Cre												
	iNKT cells			DP thymocytes			CD4 T cells			CD8 T cells		
	Thymus	Spleen	Liver				Thymus	Spleen	Liver	Thymus	Spleen	Liver
Reduction folds (Cre ⁻ /Cre ⁺)	4.1 ^a	12 ^d	20 ^c	1.1			1.1	1.2	1.4	1.0	1.5 ^e	0.97
<i>Arg5^{fl/fl}</i> Lck-Cre												
	iNKT cells			DP thymocytes			CD4 T cells			CD8 T cells		
	Thymus	Spleen	Liver				Thymus	Spleen	Liver	Thymus	Spleen	Liver
Reduction folds (Cre ⁻ /Cre ⁺)	15 ^a	21 ^a	35 ^a	2.2 ^d			2.8 ^c	3.9 ^b	3.4 ^b	2.7 ^c	3.7 ^b	2.2 ^c
<i>Arg5^{fl/fl}</i> Lck-Cre												
	iNKT cells			DP thymocytes			CD4 T cells			CD8 T cells		
	Thymus	Spleen	Liver				Thymus	Spleen	Liver	Thymus	Spleen	Liver
Reduction folds (Cre ⁻ /Cre ⁺)	13 ^a	51 ^a	65 ^c	3.7 ^b			3.5 ^b	3.1 ^b	2.7 ^e	3.6 ^b	7.3 ^b	3.6 ^c

Arg5^{fl/fl} or *Arg5^{fl/fl}* crossed with either CD4-Cre or Lck-Cre transgenic mice were analyzed and the average cell numbers of the indicated T cell populations were calculated.

Shown here are the ratios of wild type controls (Cre⁻) to autophagy deficient iNKT cells and conventional T cells (Cre⁺). n = 8 in all cases. The differences that reach significance compared to mice wild type for autophagy are indicated

^a p<0.0001

^b <0.0005

^c p<0.005

^d p<0.01

^e <0.05