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Invariant NKT Cells Require Autophagy To Coordinate Proliferation and Survival Signals during Differentiation

Bo Pei,*1 Meng Zhao,* Brian C. Miller,†,2 Jose Luis Véla,*3 Monique W. Bruinsma,‡ Herbert W. Virgin,† and Mitchell Kronenberg*†

Autophagy regulates cell differentiation, proliferation, and survival in multiple cell types, including cells of the immune system. In this study, we examined the effects of a disruption of autophagy on the differentiation of invariant NKT (iNKT) 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 iNKT 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 iNKT cells. In the absence of autophagy, there is reduced progression of iNKT cells in the thymus through the cell cycle, as well as increased apoptosis of these cells. Importantly, the reduction in Th1-biased iNKT cells is most pronounced, leading to a selective reduction in iNKT cell–derived IFN-γ. Our findings highlight the unique metabolic and genetic requirements for the differentiation of iNKT cells. The Journal of Immunology, 2015, 194: 000–000.

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. Macropathophagy is characterized by the compartmentalization of cytoplasmic molecules or organelles in double-membrane vesicles, termed autophagosomes (1, 3–5). More than 30 autophagy-related gene (ATG) products (ATG proteins) have been identified in yeast, with orthologs in mammals. The core autophagic machinery includes two ubiquitin-like 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 noncovalently 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. Additionally, 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, naive T cells maintain a basal level of autophagy that is further induced upon activation through the 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).

NKT cells are a T lymphocyte subpopulation that shares the properties of both NK cells and T lymphocytes (21–23). Most NKT cells in mice express a semi-invariant TCR, with an invariant Vα14–Ja18 chain coupled with a Vβ8 repertoire consisting mostly of Vβ8, Vβ7, and Vβ2. This major population of NKT cells is often referred to as type I or invariant NKT (iNKT) cells (21, 22, 24, 25). iNKT cells recognize lipid Ags presented by CD1d, an MHC class I–like Ag-presenting molecule (26–29). iNKT cells have the capacity to rapidly secrete a 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 iNKT 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).

iNKT cells develop in the thymus, but they 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 iNKT cell development have been identified, the determinants that direct iNKT cell development are still not fully understood (35, 36). It is notable that iNKT cell differentiation requires positive selection by CD1d-expressing DP thymocytes rather than cortical epithelial cells (38). iNKT cells also require a number of cell surface molecules, kinases, adapters, 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 iNKT 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 iNKT cells, with selective effects on the subset that produces IFN-γ.

Materials and Methods

Mice and reagents

The generation of Atg5<sup>−/−</sup> and Atg7<sup>−/−</sup> has been previously described (40, 41). The Atg5<sup>−/−</sup> mice were a gift from Dr. Noboru Mizushima (Tokyo Medical and Dental University, Tokyo, Japan) and the Atg7<sup>−/−</sup> mice were provided by Dr. Masaaki Komatsu (Tokyo Metropolitan Institute of Medical Science, Tokyo, Japan). CD4-Cre transgenic mice, C57BL/6 (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<sup>−/−</sup> mice and Atg7<sup>−/−</sup> 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<sup>−/+</sup>CD45.2<sup>−/+</sup> 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 and Immunology.

Abs and reagents

The following Abs, with clone designation in parentheses, were from BD Pharmingen: CD1d−PE (1B1), CD4−allophycocyanin (RM-4-5), CD8−PerCP-Cy5.5 (53-67), CD24−FITC (M1/69), CD45−FITC (A20), Fas−FITC (Jo2), anti–BrdU−Alexa Fluor 488 (3D7), IFN-γ−PE-Cy7 (XM1G12), 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 (pT380)-PE (J-223.371), and purified Ab anti-caspase 3 (C92-605), CD45.2−allophycocyanin (104), retinoic acid–related orphan receptor (ORiR)-PE (B2D), and TCRβ−allophycocyanin−eFluor 780 (H57-597) were purchased 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<sup>−<sup>op1</sup></sup>−Alexa Fluor 647 (C-19), T-bet−Alexa Fluor 488 (4B10), and promyelocytic leukemia zinc finger (PLZF)−Alexa Fluor 647 (D-9) were from Santa Cruz Bio- technologies (Santa Cruz, CA). CD19−PE-Cy5.5 (6D5) and secondary Ab goat anti-rabbit IgG (H+L)−Alexa Fluor 488 were from Invitrogen (Carlsbad, CA). Purified Abs recognizing cleaved caspase 8 (DS2B) or phospho−4E-BP1 (eIF4E-binding protein 1; pT37/46) (236B4) were purchased from Cell Signaling Technology (Danvers, MA). Cytofix/Cytoperm buffer, Perm/Wash buffer, and a transcription factor buffer set were all from BD Biosciences. α-Galactosylceramide (α-GalCer) was provided by Kyowa Hakko Kirin. Live/Dead (Yellow) dye was obtained from Invitrogen, and BrdU and annexin V−allophycocyanin 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-<sup>i</sup>Cr Ab (2.4G2) for 30 min at 4˚C, cells were stained with fluorochrome-conjugated Abs 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 Cytofix/Cytoperm buffer and transcription factor buffer, respectively, followed by staining with corresponding fluorochrome-conjugated Abs in Perm/Wash buffer. The data were acquired on an LSR II flow cytometer (BD Biosciences) and analyzed using FlowJo software (Tree Star). The iNKT cell population was gated as CD19<sup>−</sup>, TCRβ<sup>−</sup>, and CD1d-tetramer<sup>+</sup> cells.

Mixed bone marrow chimeras

Bone marrow cells were prepared from tibia and femurs, and Thy<sup>1</sup> cells were removed by MACS microbeads (Miltenyi Biotec, Auburn, CA). Bone marrow cells (10<sup>5</sup> to 10<sup>6</sup>) were inoculated in 1 ml RPMI 1640 medium supplemented with 10% FBS, 50 µM 2-ME, as well as antibiotics and cultured overnight at 37˚C in a humidified atmosphere of 5% CO<sub>2</sub>. Cells were collected and stained with cell surface markers to gate either DP thymocytes for some experiments or iNKT cells in others, as well as with annexin V and Live/Dead Yellow dye (Invitrogen) following the manufacturer’s instructions.

Semi-quantitative PCR

Single-cell suspensions of thymocytes were prepared and stained. DP thymocytes were sorted with the BD FACS Aria II and tetramer−<sup>iG1</sup>−<sup>CDA</sup>−<sup>iG1</sup>−<sup>CDA</sup> 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 20, 60, and 200 ng. The primer pairs used were as follows: V<sub>i4-14-J1a1</sub> forward, 5′−GTTCTCTGACAGTCTCCTGGT−3′; reverse, 5′−CAAAATGCAGCTCCCTCAGG−3′; C<sub>a4</sub>, forward, 5′−CTTCTGCGCTGTTACCGACT−3′; reverse, 5′−TGCGTTGTCCTTCGTGAAG−3′. The PCR program consisted of an initial denaturation step at 95˚C, 4 min; 38 cycles for V<sub>i4-14-J1a1</sub> or 18 cycles for C<sub>a4</sub> at 15 s, 55˚C for 15 s, 72˚C for 15 s; 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 prewarmed PBS containing 0.1 µM MitoTracker Green FM (Invitrogen) 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) in prewarmed HBSS/Ca<sup>2+</sup>/Mg<sup>2+</sup> 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, 6-wk-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 i.v. Two hours later, single-cell suspensions of thymocytes were prepared and stained to evaluate BrdU incorporation and DNA content with 7-aminoactinomycin D (7-AAD; Invitrogen).

Lipid Ag immunization

Eight-week-old mice were injected i.v. with 1 µg α-GalCer. Two hours 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

A two-tailed Student t test was used for analysis of statistical significance. A p value <0.05 was considered statistically significant.

Results

Deficiency in autophagy genes caused decreased iNKT cells

Disruption of either ATG5 or ATG7 expression effectively eliminates most autophagic processes (42–44). Although 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. In this study, Atg5<sup>−/−</sup> or Atg7<sup>−/−</sup> 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 iNKT cells from thymus, spleen, and liver of 8- to 9-wk-old mice. Mice with deletion of either Atg5 or Atg7 had a dramatic reduction in both the percentage and absolute number of iNKT 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<sup>−/−</sup> or Atg7<sup>−/−</sup> deficiency on iNKT cells

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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 I).

In Atg5<sup>−/−</sup> CD4-Cre and in Atg7<sup>−/−</sup> 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<sup>−/−</sup> CD4-Cre mice, but this was much smaller in magnitude than the decrease in iNKT cells (Supplemental Fig. 1, Table I). 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 iNKT cells caused by either Atg5 or Atg7 deletion were much more pronounced than the reductions in CD4 and CD8 T cells (Table I).

Wild-type thymocytes were analyzed for autophagy using a dye that detects autophagic vacuoles. By this analysis, iNKT cells exhibited increased autophagy compared with 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 iNKT cell stages**

Differentiation of iNKT 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<sup>−</sup>, CD44<sup>low</sup>, NK1.1<sup>−</sup>), whereas stage 2 cells become CD44<sup>high</sup>, and, finally, stage 3 is characterized by acquisition of NK1.1 expression (CD24<sup>−</sup>, CD44<sup>high</sup>, NK1.1<sup>+</sup>) (21, 24). The final maturation of iNKT cells to stage 3 is not confined to the thymus, however, as cells with a stage 2 phenotype are the majority of iNKT cell recent thymic emigrants, and NK1.1 expression can be acquired in the periphery (46, 47).

In either Atg5- or Atg7-deficient thymic and splenic iNKT cells exhibited a dramatic decrease in the number of stage 3 or CD44<sup>high</sup> NK1.1<sup>+</sup> iNKT 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. Whereas the percentages of iNKT cells in the early stages (stages 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 iNKT cells to the more mature stages 2 and 3.

**Autophagy gene mutation caused a cell-autonomous defect in iNKT 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).

![Figure 1](http://www.jimmunol.org/)

**FIGURE 1.** Mice deficient for autophagy genes had reduced iNKT cells. iNKT cells were analyzed by gating on CD1d tetramer<sup>+</sup> cells as described in Materials and Methods. Depicted are the results from cells from the indicated organs from 8- to 9-wk-old mice with a CD4-Cre–mediated deletion of Atg5 (A and B) or Atg7 (C and D) and controls. Shown are the percentages (A and C) and absolute cell numbers (B and D) compared with Atg5<sup>+/+</sup> or Atg7<sup>+/+</sup> mice without the Cre transgene. Representative flow cytometry analyses are from one of at least three independent experiments. n ≥ 8. *p < 0.05, **p < 0.01, ***p < 0.0001.
We carried out mixed bone marrow chimera experiments to determine whether the decrease in iNKT cells in the autophagy gene-deficient mice was cell-intrinsic, meaning the problem is located with the iNKT cell progenitor. Alternatively, it could be cell-extrinsic, for example due to impaired presentation of a positively selecting ligand, in which case the cotransfer 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 11 wk, iNKT cells in thymus, spleen, and liver were analyzed by flow cytometry. Wild-type CD45.1+ and CD45.2+ iNKT cells developed equally in the thymus and periphery in the mixed chimeras, whereas autophagy gene-deficient iNKT cells manifested a 4- to 10-fold reduction compared with their wild-type counterparts in all three organs tested (Fig. 3 and Atg7 data not shown). These results indicate that the defect in iNKT cells caused by autophagy gene deficiency is cell-intrinsic.

Equivalent viability of DP thymocytes

Rearrangements of Jα gene segments distal to the Va locus, including Ja18, require secondary TCRα 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 iNKT cells owing to the negative impact on the frequency of rearrangement of the Ja18 gene segment (48, 49). To determine whether an alteration in DP lifespan was the cause of the reduction in iNKT 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

![Table I. Effects on the population size of iNKT cells and conventional T cells in autophagy-deficient mice compared with wild-type controls](http://www.jimmunol.org/)

<table>
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<th>iNKT Cells</th>
<th>CD4 T Cells</th>
<th>CD8 T Cells</th>
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<tr>
<td></td>
<td>Thymus</td>
<td>Spleen</td>
<td>Liver</td>
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<tr>
<td>Atg5f/f CD4-Cre (RF)</td>
<td>8.8*</td>
<td>20*</td>
<td>56*</td>
</tr>
<tr>
<td>Atg7f/f CD4-Cre (RF)</td>
<td>4.1*</td>
<td>13****</td>
<td>20***</td>
</tr>
<tr>
<td>Atg5f/f Lck-Cre (RF)</td>
<td>15*</td>
<td>21*</td>
<td>35*</td>
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<tr>
<td>Atg7f/f Lck-Cre (RF)</td>
<td>13*</td>
<td>51*</td>
<td>65***</td>
</tr>
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</table>

Atg5f/f or Atg7f/f 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 (Cre2) to autophagy-deficient iNKT cells and conventional T cells (Cre+); n ≥ 8 in all cases. The differences that reach significance compared with mice wild-type for autophagy are indicated as *p < 0.0001, **p < 0.0005, ***p < 0.005, ****p < 0.01, and *****p < 0.05. RF, reduction folds (Cre−/Cre+).

Figure 2. Deficiency for autophagy genes inhibited iNKT cell maturation. (A and C) Thymus and spleen cells from 8- to 9-wk-old mice were analyzed for iNKT cell phenotype. Representative flow cytometry plots from one of four experiments showing gated iNKT cells from Atg5f/f CD4-Cre (A) or Atg7f/f CD4-Cre (C) mice and controls lacking the Cre transgene are shown. Absolute cell numbers of thymic iNKT cells at different stages are shown in (B) Atg5f/f CD4-Cre and (D) Atg7f/f 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). n ≥ 10. Error bars are SD. *p < 0.0005, **p < 0.0001.
normally quiescent in wild-type mice (50). This may reflect the...thymocytes (Fig. 4D). In summary, the in vitro results obtained from BrdU and Ki-67 analyses do not exclude an increase in BrdU and Ki-67 staining was observed in NK1.1 + cells, a population that is striking when analyzing the NK1.1 + cells, but the increase in the absence of Atg5 was even more...NKT cells. This was especially true for the NK1.1+ subset.

We further determined that a negative regulator of cell cycle progression, p21<sup>cip1</sup> (51), was increased in Atg5-deficient, either NK1.1<sup>+</sup> or NK1.1<sup>+</sup>*, NKT cells (Fig. 5C). Because the limited number of iNKT 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 NKT cells. Atg5-deficient iNKT cells also had increased expression of p27<sup>kip1</sup>, another cell cycle inhibitor (51) (data not shown). Activation of cyclin-dependent kinase 1 promotes cell cycle progression (52), and a lack of Atg5 also led to an increase in inhibitory phosphorylation at Tyr<sup>15</sup> on cyclin-dependent kinase 1 (data not shown). These results indicate that intact autophagy machinery is required for a normal control of proliferation of iNKT cells and a smooth transition between cell cycle phases for iNKT cells in the thymus.

**Autophagy gene-deficient iNKT 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 iNKT 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 iNKT 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 regulate cellular functions, such as promoting cell growth and proliferation, but excessive ROS can induce apoptosis and cell death. We therefore measured superoxide in iNKT cells. Atg5 deficiency not only led to accumulated mitochondria, but also to one of their products, excessive superoxide, in the NK1.1+ iNKT cells (Fig. 6B).

**Autophagy genes are required for optimal survival of iNKT 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 iNKT 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 iNKT cells from Atg5- or Atg7-deleted mice, more prominently for the NK1.1+ subset (Fig. 6C and Atg7 data not shown).

To assess more directly whether iNKT cells with either Atg5 or Atg7 deleted were undergoing apoptosis more than wild-type controls, we measured the viability of the autophagy gene-deficient iNKT cells. Thymocytes derived from either Atg5<sup>fl/fl</sup>CD4-Cre, Atg7<sup>fl/fl</sup>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 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 iNKT cells. This was especially true for the NK1.1+ subset.

If the DP thymocyte lifespan were decreased in vivo in Atg5- or Atg7-deficient mice, we would expect a decrease in the rearangement of V<sub>14</sub>-J<sub>18</sub> loci in DP thymocytes. Therefore, we measured V<sub>14</sub>-J<sub>18</sub> rearrangements in DP thymocytes, which had not yet undergone positive selection, utilizing semiquantitative PCR. We found that amplified copies of the V<sub>14</sub>-J<sub>18</sub> 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 iNKT cell progenitors by reducing the lifespan of DP progenitor thymocytes.

**Arrested cell cycle in Atg5-deficient iNKT cells**

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

**Mitochondrial mass was not diminished in autophagy gene-deficient DP thymocytes (Fig. 4D).** A: Bone marrow cells from Atg5<sup>fl/fl</sup>CD4-Cre mice or wild-type controls (both CD45.2<sup>+</sup>), together with CD45.1<sup>+</sup>, wild-type bone marrow cells, were cotransferred into recipients. Reconstituted iNKT cells from the indicated organs were analyzed after 11–12 wk with flow cytometry. Data are representative from one of at least three separate experiments. (B) Ratios of reconstituted iNKT cells (CD45.2<sup>+</sup>/CD45.1<sup>+</sup>), n > 10. *p < 0.0001.

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).
NKT CELLS REQUIRE AUTOPHAGY

decreased survival (Fig. 4A–C), iNKT 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 iNKT 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 effector caspase cleaved and activated by caspase 8 (Fig. 7C). These increases were not observed for the majority population of DP thymocytes (Fig. 7B, 7D). 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 iNKT cells in the thymus.

Autophagy gene-deficient iNKT cells exhibited an altered cytokine profile

Once activated by strong Ags, most iNKT 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 iNKT cells to αGalCer, a potent synthetic glycolipid Ag. Two hours after αGalCer injection, splenic iNKT cells were analyzed directly ex vivo at the single cell level. Compared to wild-type controls, the percentage of responding Atg5-deficient iNKT cells was decreased ∼2-fold for IL-4, whereas the IFN-γ response was decreased ∼4-fold (Fig. 8A, 8B). 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 Ag-dependent Th1 cytokine secretion by iNKT cells.

Differential requirements of Atg5 by iNKT cell subsets

Recent studies have highlighted functional heterogeneity within mature iNKT cells. During their differentiation in the thymus, immature iNKT 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 B6 mice, and they are NK1.1+ and express a high level of T-bet (34). In contrast, both NKT2 and NKT17 cells are relatively minor in B6 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 GATA3high whereas NKT17 cells express CCR6 along with other surface proteins, and they are characteristically RORγt+ (30, 34). PLZF is a transcription factor required for iNKT 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, whereas 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, whereas stage 3 cells are similar to NKT1 cells. Therefore, it is difficult to unambiguously distinguish a maturing, stage 2 iNKT cell from a committed NKT2 cell.

When analyzed by flow cytometry, total tetramer+ iNKT cells from the thymus of Atg5f/f CD4-Cre transgenic mice maintained a higher expression of PLZF, GATA3, and RORγt, but a lower level of T-bet compared with 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− iNKT cells were analyzed separately (data not shown). This indicated that iNKT cell functional subsets were differently affected by disruption of the autophagic machinery. To confirm this hypothesis, we analyzed individual subpopulations of iNKT cells in Atg5 knockout mice and wild-type controls according to their expression level of signature transcription factors. Fig. 8D and 8E show that Atg5 deficiency caused a severe loss of NKT1 phenotype cells, the majority population in this strain, with an
∼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 iNKT cells are consistent with changes in the expression of surface proteins, including a decreased percentage of iNKT cells expressing NK1.1 (Fig. 2) and an increased percentage expressing IL-17RB (data not shown).

**Atg5 deficiency leads to augmented 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. Mechanistic target of rapamycin (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, mTOR complex (mTORC)1 and mTORC2, consisting of different accessory proteins that instruct their substrate specificity (58, 59). Upon activation, mTORC1 phosphorylates 4E-BP1 (60), whereas mTORC2 phosphorylates AKT at Ser473, which primes AKT for subsequent phosphorylation at Thr308 to achieve full activation of the kinase (61).

Fig. 9A shows that Atg5<sup>f/f</sup> CD4-Cre NK1.1<sup>+</sup> iNKT 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-deficient iNKT 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 that AKT exhibits a higher activation in Atg5-deleted iNKT cells than in the controls.

Discussion
In this study, we demonstrate that iNKT cells depend on Arg5 and Arg7 for their differentiation to a far greater extent than do mainstream CD4 or CD8 T lymphocytes. Removal of either Atg5 or Atg7 severely reduced both thymic and peripheral iNKT cell 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 iNKT cells had an even greater reduction than did thymic iNKT 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. n ≥ 4. Error bars are SD. **p < 0.01, ***p < 0.001, ****p < 0.0001.
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 iNKT cells in the thymus owing to decreased secondary rearrangements in the TCRα 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. Additionally, there was no reduction in Vα14-Jα18 rearrangements in DP thymocytes, which would have not occurred if DP survival were impaired, and we observed a normal number of stage 0 iNKT cells in the thymus. Therefore, general effects on DP survival, causing decreases in the secondary TCRα rearrangements needed to form the invariant TCR α-chain, do not account for the cell-intrinsic iNKT cell deficit in mice deficient for autophagy genes. Atg5- or Atg7-deficient iNKT cells exhibited a selective defect in their maturation pathway, with the earliest stages 0 and stage 1 iNKT cells maintained, and the later stages depleted due to decreased cell survival. The differentiation of iNKT cells in the thymus along a single developmental pathway has been challenged, however, by recent studies showing that there are functional subsets of iNKT cells analogous to Th cell subsets, such as Th1, Th2, and Th17 cells. It is only the Th1 cytokine-biased NKT1 subset that expresses NK1.1 in B6 mice, and the CD44+, IFN-γ+ 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 iNKT cell function. We found that the production of IFN-γ by Ag-stimulated autophagy-deficient iNKT cells was more severely affected than was 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 Ag-activated iNKT cells producing IL-4 and decreased expression of key transcription factors, such as RORγt. Additionally, we found reduced expression of CD69 by autophagy-deficient iNKT cells either before or after activation (data not shown). Therefore, whereas NK1 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 iNKT cell population.

Given the reduced iNKT cell population in mice with deleted autophagy genes, it would be reasonable to expect reduced proliferation of these cells. However, Atg5- or Atg7-deficient iNKT 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 iNKT 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 iNKT 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), whereas the induction of autophagy promotes a reduction in caspase 8 and other proapoptotic proteins (19). Alternatively, 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 iNKT cells from Atg5-deficient mice. An increase in mitochondria previously has been observed in autophagy-deficient T cells (14, 18, 20). Overexpression of Bcl-xL failed to rescue the defective iNKT cell differentiation in our Arg5-deficient mice, although the frequency of the CD8+ T cells was normalized (Supplemental Fig. 4). This failure to restore the iNKT cell population is not consistent with a major role for the intrinsic apoptosis pathway, but the timing or expression level of the Bcl-xL 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 iNKT 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 iNKT population, with possible roles for both the cell-extrinsic and cell-intrinsic pathways.

**FIGURE 7.** Atg5-deficient thymic iNKT 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 iNKT cells (A and C) and TCRβ+ thymocytes (B and D). Shown here are both flow cytometry plots (left) and geometric mean fluorescence intensity (MFI) (right). Data are representative of at least two independent experiments. Filled histogram indicates isotype control; solid line indicates wild-type cells; dotted line indicates Atg5 deficient. n ≥ 3. Error bars are SD. *p < 0.05.
FIGURE 8. *Atg5* deficiency differentially affected iNKT cell functional subsets. (A and B) Impaired iNKT cell activation and polarized cytokine outcome in *Atg5* KO CD4-Cre mice after αGalCer immunization. Intracellular cytokine production in splenic iNKT cells was analyzed 2 h after αGalCer injection. Flow cytometry plots (A) and geometric mean fluorescence intensity (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 iNKT cells. Representative flow cytometry plots from three independent experiments and geometric MFI are indicated. Filled histogram indicates isotype control; solid line indicates wild-type mice; dotted line indicates *Atg5* KO CD4-Cre mice. (D) iNKT 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 iNKT cell functional subsets defined by transcription factor expression levels in *Atg5* KO CD4-Cre mice and controls. *n* ≥ 4. Error bars are SD. *p* < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.
Atg5f/f mice; dotted line indicates isotype control; solid line indicates wild-type in independent experiments. Filled histogram indicates the representative flow cytometry analysis of three pooled from at least four samples. Shown here is the representative flow cytometry analysis of three independent experiments. Filled histogram indicates isotype control; solid line indicates wild-type mice; dotted line indicates Atg5f/f mice. 

**FIGURE 9.** Unbalanced proliferative and apoptotic signaling in Atg5-deficient iNKT cells. Thymic iNKT 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/phosphoinositide-dependent protein kinase 1 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 indicates isotype control; solid line indicates wild-type mice; dotted line indicates Atg5f/f CD4-Cre mice. n ≥ 4. Error bars are SD. **p < 0.01, ***p < 0.005, ****p < 0.0005.

What drives the increased entry of autophagy-deficient iNKT 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 p70 ribosomal S6 kinase 1 and 4E-BP1, which increase mRNA translation (58–60). mTORC2, acting in synergy with 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 iNKT cells. The T cell–specific deletion of Raptor, an essential component of mTORC1, also impaired the maturation of iNKT cells (69), suggesting the need for a balance, with either too much or too little mTORC1 inhibiting iNKT cell development.

Our findings are consistent with the results from several recent studies indicating a role for proper regulation of metabolism in regulating iNKT 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 iNKT cells than the one reported in the present study (70), although the mechanism for the effect was not described. Furthermore, although the importance of VPS34 for autophagy in T lymphocytes has been found by several groups (13, 70), this enzyme is also important for IL-7R signaling (71), and therefore some of the deficit in iNKT 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 develop. Second, mice with a T cell–specific deletion of the gene encoding tuberous sclerosis (TSC)1, an inhibitor of mTORC1, also were found to have fewer iNKT cells due to reduced cell survival (72). Because mTOR inhibits autophagy, the increased mTOR activity in the absence of the TSC1 inhibitor in these mice may have led to decreased autophagy in iNKT cells, and therefore a phenotype similar to the one we report in the present study, although this has not been determined. Consistent with this hypothesis are data from a subsequent study finding that the Tsc1-deficient mice retained the NKT17 population, similar to the Atg5- and Atg7-deficient strains (73). Tsc1 deficiency induced an activated intrinsic apoptotic pathway, however, which was rescued by overexpression of Bcl2. This is different from our findings that Atg5/Atg7 deficiency led to apoptosis through an extrinsic pathway as well as possibly an intrinsic pathway, which was not rescued by Bcl-xL overexpression (data not shown). Third, an investigation exploring the effect of unbalanced metabolic homeostasis on iNKT cell differentiation showed that folliculin-interacting protein 1 (Fnip1)–null mice had a reduction in mature iNKT cells. Deficiency of Fnip1 led to inability to shut off mTOR-mediated energy-consuming cellular processes, which could inhibit autophagy. However, inhibition of mTOR was not able to rescue the defect in iNKT cells, indicating that Fnip1 is required for iNKT cell development in both an mTOR-dependent and -independent manner (74), and a connection to decreased autophagy was not established in this study. While this manuscript was in revision, a study was published indicating that mice with a 4E-BP1 null phenotype driven by CD4 Cre have a reduction in iNKT cells in the thymus and periphery, consistent with the results in the present study (75).

In summary, in this study, we show that the disruption of two autophagy-related genes, Atg5 or Atg7, had a particularly severe effect on the later stages of differentiation of iNKT cells, an innate-like T lymphocyte population. The ability of differentiating iNKT cells in the thymus to progress through the cell cycle was decreased and their survival was impaired due to increased apoptosis. The requirement for the autophagy pathway is consistent
with the increased autophagy exhibited by thymic iNKT cells compared with other thymocytes populations. It likely reflects the rounds of proliferation that iNKT cells undergo following gene rearrangement and expression of a CD1d autoantoreactive TCR, which may have similar metabolic requirements to the expansion of Ag-stimulated mainstream T cells in lymphoid organs. In fact, findings from several groups have confirmed that systemic or tissue-specific deletion of genes encoding proteins in the autophagy pathway led to significant reductions in the homeostasis of peripheral T lymphocytes and their ability to respond and produce cytokines (13, 14, 16–20). Alternatively, rather than acting at the effector stage, after expansion, mature iNKT cells are similar to memory cells in their survival requirements (76), and they may have similar metabolic requirements that depend on autophagy. Regardless, whereas all the functional subsets of iNKT cells analyzed were decreased in number and function, the effect was most severe for the dominant NK1.1 population. Interestingly, considering B lymphocytes, a more severe defect in innate-like B1a B cells was observed when Arg5 was deleted throughout the B cell lineage (77). This suggests that different types of innate-like lymphocytes, which have a degree of self-reactivity and that undergo rounds of proliferation during their differentiation, may have a selective requirement for autophagy.

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