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The autophagy gene ATG5 plays an essential role in B lymphocyte development

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Macroautophagy (herein autophagy) is an evolutionarily conserved process, requiring the gene ATG5, by which cells degrade cytoplasmic constituents and organelles. Here we show that ATG5 is required for efficient B cell development and for the maintenance of B-1a B cell numbers. Deletion of ATG5 in B lymphocytes using Cre-LoxP technology or repopulation of irradiated mice with ATG5−/− fetal liver progenitors resulted in a dramatic reduction in B-1 B cells in the peritoneum. ATG5−/− progenitors exhibited a significant defect in B cell development at the pre- to pre-B cell transition, although a proportion of pre-B cells survived to populate the periphery. Inefficient B cell development in the bone marrow was associated with increased cell death, indicating that ATG5 is important for B cell survival during development. In addition, B-1a B cells require ATG5 for their maintenance in the periphery. We conclude that ATG5 is differentially required at discrete stages of development in distinct, but closely related, cell lineages.

Introduction

Autophagy is a cellular process in which cytoplasm and cytoplasmic organelles are enveloped in double-membrane bound vesicles (autophagosomes) for delivery to the lysosome and subsequent degradation.1,2 Formation of the autophagosome requires the action of two evolutionarily conserved ubiquitin-like conjugation systems, both of which require the ATG5 gene.3,4 One system efficiently generates ATG5-ATG12 conjugates that associate with the elongating isolation membrane during autophagosome formation, the other modifies the free C-terminal glycine of ATG8/LC3 with phosphatidylethanolamine.5 The unconjugated form of ATG8/LC3 (LC3-I) can be distinguished from the lipidated form (LC3-II) by mobility in electrophoretic gels, providing one commonly used assay for autophagic activity. As mice lacking ATG5 die shortly after birth,5 conditional knockout alleles of ATG5 have been generated [ATG5flaxfl].6 Automophagy and autophagy genes have a role in multiple aspects of eukaryotic biology including survival during stress and growth factor withdrawal,1,5,7 tumor suppression,8-11 development,1,12,13 neurodegeneration,6,14 innate host defense,15-18 antigen presentation,19-21 and T cell homeostasis.22 Because there are well-defined stages of lymphocyte development and function, the immune system presents a unique opportunity to study the role of autophagy and autophagy genes in normal cellular physiology. Pua et al. demonstrated that ATG5 is essential for T cell survival and proliferation.22 The requirement for ATG5 in B cell development remains to be addressed. B cells are classified into two subsets, B-1 and B-2, based on cell surface marker expression, developmental origin, and functional properties.23 B-2 B cells develop from hematopoietic stem cells in the bone marrow, undergoing a series of maturational stages that can be defined by expression of cell surface markers (Hardy fractions A-F).24 B-1 B cells are part of the innate immune system and are subdivided into B-1a and B-1b subsets based on expression of the cell surface protein CD5.25 The developmental origin of B-1 B cells is still controversial, with two competing models under study. According to the selection model, B-1 and B-2 B cells are derived from a common progenitor that differentiates into one of the two lineages based on antigen selection at the transitional stage.25 Alternatively, the lineage model proposes that B-1 and B-2 B cells originate from distinct progenitors. The lineage model is supported by the recent identification of a B-1 B cell progenitor in fetal and adult tissues.26 It has also recently been suggested that B-1a and B-1b B cells have distinct progenitors and lineages.27

In this paper we evaluated the role of ATG5 in B cell development. While B-2 B cells can be generated from ATG5−/− precursor cells and populate peripheral lymphoid organs, ATG5 was required for efficient development from pro- to pre-B cells in the bone marrow. The absence of ATG5 was associated with a significant increase in the death of Hardy fraction D-F B cells. In addition to its function during development, ATG5 is important for the maintenance of B-1a B cells in peripheral tissues. The autophagy gene ATG5 and an intact
autophagy pathway are required at specific stages in B cell development and differentially required for distinct, but closely related, cell lineages.

Materials and Methods

Mice. ATG5−/− and ATG5fl/fl mice have been described. C57BL/6J (CD19−/−), CD19−/− mice were obtained from The Jackson Laboratory (Bar Harbor, ME). We compared ATG5fl/fl mice with CD19−/− mice to CD19−/−Cre mice and littermate ATG5fl/fl mice throughout these experiments. Mice were genotyped as described, with the ATG5 gene detected with primers exon3-1 (GAATTGAGGCCACCCCTGAAATG), short2 (GTACTGCA TAAATGCTTTAATCTTTGC), and check2 (ACAACGGTGCAGC ACGCTGCGCAAGG) using PCR [94°C (4 min); 30 cycles of 94°C (30 sec), 60°C (30 sec), 72°C (1 min); 72°C (5 min)]. The Cre gene was detected with primers cre1 (AGGTTCGTTCACTCATGGA) and cre2 (TCGACCAGGTATAGTATCC) using PCR [94°C (4 min); 25 cycles of 94°C (30 sec), 60°C (30 sec), 72°C (1 min); 72°C (5 min)]. CD45.1 chimeric mice were genotyped as described. For the generation of RAG1−/− chimeric mice, day 15.5–18.5 ATG5fl/fl x ATG5−/− fetuses were harvested and genotyped with the REDExtract-N-AmpTM Tissue PCR Kit (Sigma-Aldrich, St. Louis, MO). Sublethally irradiated (600 rad) RAG1−/− hosts were injected with cells from one-fourth of a fetal liver. Chimeric mice were analyzed at least 6 weeks after reconstitution. All animal studies were performed in accordance with institutional policies for animal care and usage.

Flow cytometry. Single-cell suspensions were prepared from the bone marrow, lymph nodes, spleens, and thyroids of peritoneal cells were harvested by lavage. Stained cells were stained and collected on a FACSCalibur cytometer (BD Biosciences, San Jose, CA) for analysis using FlowJo software (TreeStar, Ashland, OR). Antibodies against IgM, IgD, TCRβ, B220, BP-1, CD4, CD5, CD8, CD19, CD21, CD23, CD24 and CD43 were from BD Biosciences (San Jose, CA), eBioscience (San Diego, CA), or Southern Biotech (Birmingham, AL). Biotinylated antibodies were detected with streptavidin-PE-Cy7 or streptavidin-APC (BD Biosciences, San Jose, CA). Annexin-V and 7-AAD staining (BD Biosciences, San Jose, CA) was carried out as per the manufacturer’s instructions except that DME (donor) was used in place of 1x Binding Buffer. All samples were gated by forward and side scatter on lymphocyte populations for analysis.

Immunoblotting. B cells were purified using CD19-conjugated microbeads (Miltenyi, Auburn, CA). CD19-purified B cells and CD19 negative cells that did not bind to CD19-conjugated magnetic beads were lysed in 2x Laemmli buffer and subjected to western blotting using antibodies specific for ATG5 (1:1000 dilution), LC3 (Novus Biologicals, Littleton, CO) (1:3000 dilution), and β-actin (Sigma-Aldrich, St. Louis, MO) (1:5000 dilution). Immunoblotting was developed with HRP-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA) (1:10,000 dilution) and visualized by chemiluminescence (Amersham Biosciences, Pittsburgh, PA).

Statistical analyses. All data were analyzed with Prism software (GraphPad, San Diego, CA) using two-tailed unpaired t tests. Error bars represent SEM.

Results

ATG5 is required to maintain normal numbers of peripheral B-1 B cells. To study the role of the autophagy gene ATG5 in B cells, we generated ATG5−/− chimeric mice. ATG5−/− or wild type fetal liver cells were used to reconstitute irradiated RAG1−/− hosts. In the peritoneum, ATG5−/− reconstituted mice had decreased numbers of B-1a B cells (16 fold), B-1b B cells (4 fold), and B-2 B cells (6 fold) (Fig. 1A and B). Lymph node B-2 B cell reconstitution was equivalent between wild type and ATG5−/− chimeras, while both B-1 and B-2 B cell numbers were decreased in the spleen approximately 50% (B-2 B cells: p = 0.017; B-1 B cells: p = 0.035). The proportions of transitional, mature follicular, and marginal zone B-2 B cells in the spleen were normal, however (Fig. 1C). These data indicate that ATG5 has a role in maintaining both B-1 and B-2 B cell numbers in the periphery, with a more dramatic role in B-1a B cells. ATG5 is required for the survival of pre-B cells. The observed decrease in peripheral B cells in ATG5−/− chimeras could be due to a decrease in B cell production in the bone marrow or an increase in cell death in the periphery. To test the first hypothesis, we evaluated the stages of bone marrow B cell development in ATG5−/− versus wild type fetal-liver reconstituted irradiated CD45.1+/- and RAG1−/− hosts (Fig. 2). FACS analysis of donor pro-B cells from CD45.1 chimeras revealed no significant differences in cell numbers of fraction A–C, developing B cells (Fig. 2A and B). Analysis of ATG5−/− RAG1−/− chimeric mice revealed a decrease in Hardy fractions D (3.7 fold), E (8.3 fold), and F (11.8 fold) (Fig. 2C and D). CD45.1 chimeras had a similar deficiency of fractions D, E, and F (data not shown). These data indicate that ATG5 has a critical role in the final stages of bone marrow B cell development. We hypothesized that ATG5 may be required for B cell survival after the pro-B cell stage of development. FACS analysis of freshly isolated bone marrow cells revealed a greater percentage of fraction D-F cells were dead or dying in ATG5−/− RAG1−/− chimeric mice compared with control mice as measured by Annexin-V and 7-AAD staining (Fig. 2E; ATG5−/−: 3.2 ± 0.8%; ATG5−/−: 22.2 ± 5.3%; p = 0.0056). We conclude that ATG5 is required as a survival factor after the pro-B to pre-B cell transition in the bone marrow.

B-2 B cells survive normally in the periphery without ATG5. Although the role of ATG5 in B cell development could explain the deficiency in peripheral ATG5−/− B cells, we could not rule out that ATG5 is also required for B cell survival in the periphery. To determine if ATG5 is required for B cell homeostasis without the confounding role of ATG5 in B cell development, we expressed Cre recombinase in the B cells of mice containing two copies of the ATG5 flox/flox gene detected with the primers exon3-1 and check2 (GAATTGAGGCCACCCCTGAAATG, TAAATGCTTTAATCTTGCA, and ACGCTGCGCAAGG) using PCR [94°C (4 min); 30 cycles of 94°C (30 sec), 60°C (30 sec), 72°C (1 min); 72°C (5 min)]. The Cre gene was detected with primers cre1 (AGGTTCGTTCACTCATGGA) and cre2 (TCGACCAGGTATAGTATCC) using PCR [94°C (4 min); 25 cycles of 94°C (30 sec), 60°C (30 sec), 72°C (1 min); 72°C (5 min)]. CD45.1 chimeric mice were genotyped as described. For the generation of RAG1−/− chimeric mice, day 15.5–18.5 ATG5−/− x ATG5−/− fetuses were harvested and genotyped with the REDExtract-N-AmpTM Tissue PCR Kit (Sigma-Aldrich, St. Louis, MO). Sublethally irradiated (600 rad) RAG1−/− hosts were injected with cells from one-fourth of a fetal liver. Chimeric mice were analyzed at least 6 weeks after reconstitution. All animal studies were performed in accordance with institutional policies for animal care and usage.

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Statistical analyses. All data were analyzed with Prism software (GraphPad, San Diego, CA) using two-tailed unpaired t tests. Error bars represent SEM.

ATG5 in B cell development

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We confirmed rearrangement of the ATG5flx/flx allele specifically in CD19+ B cells from ATG5flx/flx-CD19-Cre mice using PCR (data not shown). In addition, B cells from ATG5flx/flx-CD19-Cre mice did not express detectable ATG5-ATG12 conjugate and failed to convert LC3-I to LC3-II (Fig. 3A). Splenic B cells from control ATG5flx/flx mice expressed ATG5-ATG12 conjugate and converted LC3-I to LC3-II (Fig. 3A). We conclude that ATG5 is specifically deleted from peripheral CD19+ B lymphocytes in our mice and that, as expected, loss of ATG5 results in defective autophagy as shown by loss of the capacity to generate LC3-II from LC3-I.

We next determined whether ATG5 is required to maintain normal numbers of B cells in peripheral tissues of adult mice. Importantly, we observed no difference in B cell development in fractions A through E in ATG5flx/flx-CD19-Cre mice (data not shown). There was a two fold decrease in fraction F cells in ATG5flx/flx-CD19-Cre mice compared with ATG5flx/flx controls (ATG5flx/flx: 1.6 ± 0.27 x 10^6; ATG5flx/flx: 0.805 ± 0.164 x 10^6; p = 0.0243). This was consistent with, although less severe than, the decrease in fraction F cells observed in chimeric mice. There was no decrease in fraction F cells in CD19-Cre mice. In the periphery, ATG5flx/flx-CD19-Cre mice had normal numbers of B220+ B lymphocytes in the spleen and lymph nodes, and IgM and IgD were expressed normally on these cells. The percentage and number of splenic and lymph node mature recirculating B cells and transitional B cells was comparable between ATG5flx/flx, ATG5flx/flx-CD19-Cre, and CD19-Cre mice (Fig. 3B). As expected, there were no differences in the numbers of T cell subsets in the thymus or periphery in these mice (data not shown). As more than 95% of splenic and nearly 100% of lymph node B cells are B-2 B cells, we observed that B-2 B cell numbers are normal in the spleen and lymph node in the absence of ATG5. To determine if ATG5 is required for the in vivo survival of mature B cells, we evaluated Annexin-V and 7-AAD staining of freshly isolated CD19-Cre mice. In the periphery, ATG5flx/flx-CD19-Cre mice had normal numbers of B220+ B lymphocytes in the spleen and lymph nodes, and IgM and IgD were expressed normally on these cells. The percentage and number of splenic and lymph node mature recirculating B cells and transitional B cells was comparable between ATG5flx/flx, ATG5flx/flx-CD19-Cre, and CD19-Cre mice (Fig. 3B). As expected, there were no differences in the numbers of T cell subsets in the thymus or periphery in these mice (data not shown). As more than 95% of splenic and nearly 100% of lymph node B cells are B-2 B cells, it follows that B-2 B cell numbers are normal in the spleen and lymph node in the absence of ATG5. To determine if ATG5 is required for the in vivo survival of mature B cells, we evaluated Annexin-V and 7-AAD staining of freshly isolated splenic and lymph node B220+ cells and observed no differences in the numbers of dead or dying B cells (data not shown). These data indicate that ATG5 is not essential for the maintenance of B-2 B cell populations in the periphery and suggest that the decrease in splenic B-2 B cells in ATG5flx/flx chimeric animals is due to a requirement of ATG5 for efficient development.

ATG5 is required in a cell-intrinsic manner to maintain normal numbers of B-1 B cells. In contrast to these findings for B-2 B cells, ATG5flx/flx-CD19-Cre mice showed a 5-fold decrease in the number of B-1a B cells in the peritoneum (Fig. 4A; ATG5flx/flx: 4.5 ± 1.2 x 10^6; ATG5flx/flx-CD19-Cre: 0.76 ± 0.19 x 10^6; p = 0.0079). Although there was a trend towards decreased B-1b and B-2 B cell numbers in ATG5flx/flx-CD19-Cre mice, there was no statistically significant difference in the number of B-1b or B-2 B cells in the peritoneum. There was also no decrease in B-1a B cell numbers in CD19-Cre mice (Fig. 4A). One explanation for this finding would be that ATG5 is required for expression of CD5, a marker of B-1a B cells. In the peritoneum, both B-1a and B-1b B cells are B220lo, IgMhi, IgDlo, CD11b+, CD43+, and CD23-. We therefore analyzed expression of IgD, IgM, and CD23 on peritoneal B cells (Fig. 4B and C). We found that ATG5flx/flx-CD19-Cre mice contained fewer B-1 B cells in the peritoneum than ATG5flx/flx or CD19-Cre mice, confirming that the deficiency of B-1a B cells shown using CD5/B220 staining was not an artifact due to lack of CD5 expression in ATG5 deficient cells. In addition, we confirmed
that this deficiency is specific for B-1a B cells but not B-1b B cells (Fig. 4C). We conclude that \( ATG5 \) is required for the maintenance of B-1a B cells but not B-1b or B-2 B cells in peripheral tissues, providing a surprising differential requirement for an autophagy gene between three closely related cell lineages. In addition, the requirement for \( ATG5 \) to maintain B-1a B cell numbers is cell-intrinsic since only B cells lack \( ATG5 \) in \( ATG5^{flox/flox} \)-CD19-Cre mice. This suggests that the observed results in \( ATG5^{-/-} \) chimeric mice may also be due to cell intrinsic effects of \( ATG5 \) deficiency.

**Discussion**

In this paper we demonstrate a critical role for the autophagy gene \( ATG5 \) in specific B cell developmental stages and lineages. \( ATG5 \) is required for the efficient development and survival of pre-B cells in
the bone marrow. A few B-2 B cells survive this transition and are able to populate peripheral lymphoid tissues. In the absence of ATG5 there is also a dramatic reduction in peripheral B-1a B cells.

The requirement for ATG5 in autophagy is clear.3,4 Therefore, we can safely conclude from our studies in ATG5flox/flox-CD19-Cre mice that autophagy is not required for the maintenance of peripheral mature B-2 B cell numbers. However, it has recently been reported that ATG5 may have other roles in addition to its primary role in autophagy.33 ATG5 can be cleaved by calpain and gain pro-death activity.34 The protein can also interact with Fas-associated protein with death domain (FADD) to trigger autophagic cell death.35 We believe it unlikely that these pro-death roles of ATG5 are related to our findings in which ATG5 is required for the survival of B cells.

Our results, in conjunction with the ATG5−/− T cell characterization by Pua et al.,22 demonstrate that ATG5 is differentially required in T cells versus B cells, two cell types from a common developmental lineage. We have shown that ATG5 is required for the maintenance of B-1a B cells, but not B-1b or B-2 B cells. Recently it has been suggested that B-1a, B-1b, and B-2 B cells may develop from distinct cell lineages.26,27 If so, then our results suggest that these distinct lineages have differential requirements for ATG5 and ATG5-dependent autophagy. Additional studies assessing the basal levels of autophagy in wild type B-1a B cells would be useful. In addition, our data suggest that ATG5-dependent autophagy is required at very specific stages in lymphocyte development. This is perhaps surprising for a cellular process that is observed in essentially all cells and that is conserved through evolution.

It is intriguing to speculate that the critical role for ATG5 in the maintenance of B-1a B cells in the periphery and for efficient pro-B cell to pre-B cell transition during B-2 B cell development in the bone marrow are related phenomena. B-1a B cells maintain their
numbers in the adult by self-renewal. We speculate that B-1a B cells lacking ATG5 may not be able to self-renew efficiently resulting in decreased peripheral numbers. In this case the effects of ATG5 on B-2 and B-1a B cells may reflect a critical role for ATG5 in survival during specific stages of cellular differentiation. Given the role of cytokines and growth factors in B cell development, this might be due to a role for ATG5 in cytokine-driven differentiation or cell survival after growth factor withdrawal.

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