2008

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[Autophagy 4:3, 309-314; 1 April 2008]; ©2008 Landes Bioscience

Research Paper

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

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Key words: B cells, cell differentiation and development, transgenic/knockout mice, ATG5

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 pro- 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.[4] 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 [*ATG5*$^{flox/flox}$].[6]

Autophagy 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.[23] 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

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Submitted: 11/13/07; Revised: 12/21/07; Accepted: 12/24/07

Previously published online as an *Autophagy* E-publication: www.landesbioscience.com/journals/autophagy/article/5474

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 *ATG5$^{flox/flox}$* mice have been described.[5,6] C.129P2-*Cd19$^{tm1(cre)Cgn}$*/J (CD19-*cre*) mice, B6.129S7-*Rag1^{tm1Mom}*/J (*RAG1-/-*) mice, and B6.SJL-*Ptprc^{a} Pepc^{b}*/BoyJ (CD45.1) mice were obtained from The Jackson Laboratory (Bar Harbor, ME). We compared *ATG5$^{flox/flox}$*-CD19-*Cre* mice to CD19-*Cre* mice and littermate *ATG5$^{flox/flox}$* mice throughout these experiments. Mice were genotyped as described,[6] with the *ATG5* gene detected with the primers exon3-1 (GAATATGAAGGCACACCCCTGAAATG), short2 (GTACTGCA TAATGGTTTAACTCTTGC), and check2 (ACAACGTCGAGCA CAGCTGCGCAAGG) 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 (TCGACCAGTTTAGTTACCC) 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 generated as described.[22] For the generation of *RAG1$^{-/-}$* chimeric mice, day 15.5–18.5 *ATG5$^{+/-}$* x *ATG5$^{+/-}$* fetuses were harvested and genotyped with the REDExtract-N-Amp^{TM} 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 thymi while peritoneal cells were harvested by lavage.[28] Cells were stained and data 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 as per the manufacturer's instructions except that DMEM was used in place of 1x Binding Buffer. All samples were gated by forward and side scatter on lymphocyte populations for analysis.

Immunoblots. 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[3] (1:1000 dilution), LC3 (Novus Biologicals, Littleton, CO) (1:3000 dilution), and β-actin (Sigma-Aldrich, St. Louis, MO) (1:5000 dilution). Immunoblots were developed with HRP-conjugated secondary antibodies (Jackson Immunoreseach 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.[29] 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 repopulation 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[22] 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 (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 a knock-in *ATG5* gene in which LoxP sites were inserted flanking the third exon [*ATG5$^{flox/flox}$*].[6] B cell specific expression of Cre was obtained using the B-cell specific CD19 promoter [CD19-*Cre*].[30] Although the expression of Cre from the CD19 promoter effectively deletes loxP-flanked DNA in more than 90–95% of mature B cells, it results in incomplete DNA rearrangement in pre-B cells.[30] Given that the CD19-*Cre* construct replaces the wild type *CD19* gene and could therefore have an effect on B cells independent of the deletion of *ATG5*,[31] all mice were bred to have only one copy of CD19-*Cre*. Furthermore, we used *ATG5* wild type CD19-*Cre* mice (herein CD19-*Cre* mice) as a control for subsequent experiments.

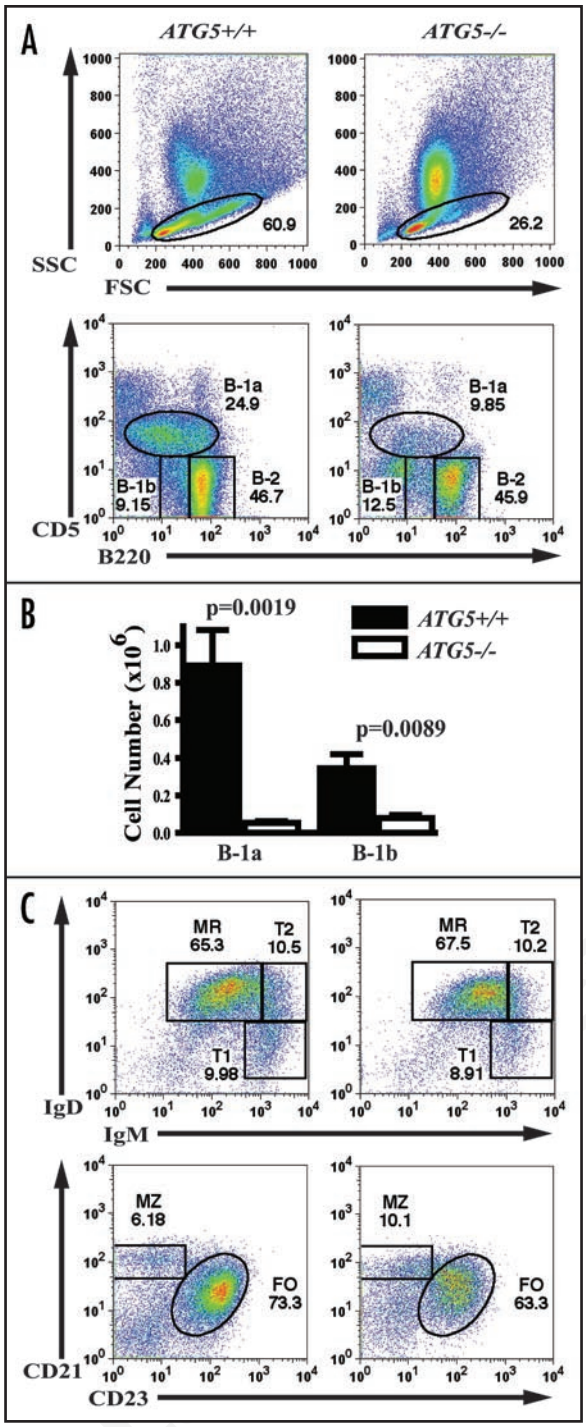

Figure 1. B-1 B cells are deficient in *ATG5*$^{-/-}$ chimeric mice. (A) Staining of peritoneal cells from *ATG5*$^{+/+}$ and *ATG5*$^{-/-}$ *RAG1*$^{-/-}$ chimeric mice to identify B cell subsets: B-1a (CD5+, B220^{lo}); B-1b (CD5-, B220^{lo}); B-2 (CD5-, B220^{hi}). Numbers indicate percentage of cells in each gate. Data representative of at least seven mice from four independent experiments. (B) Quantitation of B-1 B cells from the peritoneum of *ATG5*$^{+/+}$ and *ATG5*$^{-/-}$ *RAG1*$^{-/-}$ chimeric mice (*ATG5*$^{+/+}$: n = 7; *ATG5*$^{-/-}$: n = 6). (C) IgM/IgD and CD21/CD23 staining of B220^{+} lymphocytes from the spleens of *ATG5*$^{+/+}$ and *ATG5*$^{-/-}$ *RAG1*$^{-/-}$ chimeric mice to identify B cell subsets: M, mature B cells (IgM^{lo}, IgD^{hi}); T1, transitional type 1 (IgM^{hi}, IgD^{lo}); T2, transitional type 2 (IgM^{hi}, IgD^{hi}) ; MZ, marginal zone B cells (CD21^{hi}, CD23^{lo}); FO, follicular B cells (CD21^{lo}, CD23^{hi}). T1 gating includes marginal zone and splenic B-1 B cells. Data representative of at least eight mice from four independent experiments.

We confirmed rearrangement of the *ATG5*$^{flox/flox}$ allele specifically in CD19^{+} B cells from *ATG5*$^{flox/flox}$-CD19-*Cre* mice using PCR (data not shown). In addition, B cells from *ATG5*$^{flox/flox}$-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 *ATG5*$^{flox/flox}$ 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,[4] 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 *ATG5*$^{flox/flox}$-CD19-*Cre* mice (data not shown). There was a two fold decrease in fraction F cells in *ATG5*$^{flox/flox}$-CD19-*Cre* mice compared with *ATG5*$^{flox/flox}$ controls (*ATG5*$^{flox/flox}$: 1.6 ± 0.27 x 10^6; *ATG5*$^{flox/flox}$-CD19-*Cre*: 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, *ATG5*$^{flox/flox}$-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 *ATG5*$^{flox/flox}$, *ATG5*$^{flox/flox}$-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,[32] 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 *ATG5*$^{-/-}$ 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-1a B cells.** In contrast to these findings for B-2 B cells, *ATG5*$^{flox/flox}$-CD19-*Cre* mice showed a 5-fold decrease in the number of B-1a B cells in the peritoneum (Fig. 4A; *ATG5*$^{flox/flox}$: 4.5 ± 1.2 x 10^5; *ATG5*$^{flox/flox}$-CD19-*Cre*: 0.76 ± 0.19 x 10^5; p = 0.0079). Although there was a trend towards decreased B-1b and B-2 B cell numbers in *ATG5*$^{flox/flox}$-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 B220^{lo}, IgM^{hi}, IgD^{lo}, CD11b+, CD43+, and CD23-.[32] We therefore analyzed expression of IgD, IgM, and CD23 on peritoneal B cells (Fig. 4B and C). We found that *ATG5*$^{flox/flox}$-CD19-*Cre* mice contained fewer B-1 B cells in the peritoneum than *ATG5*$^{flox/flox}$ 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

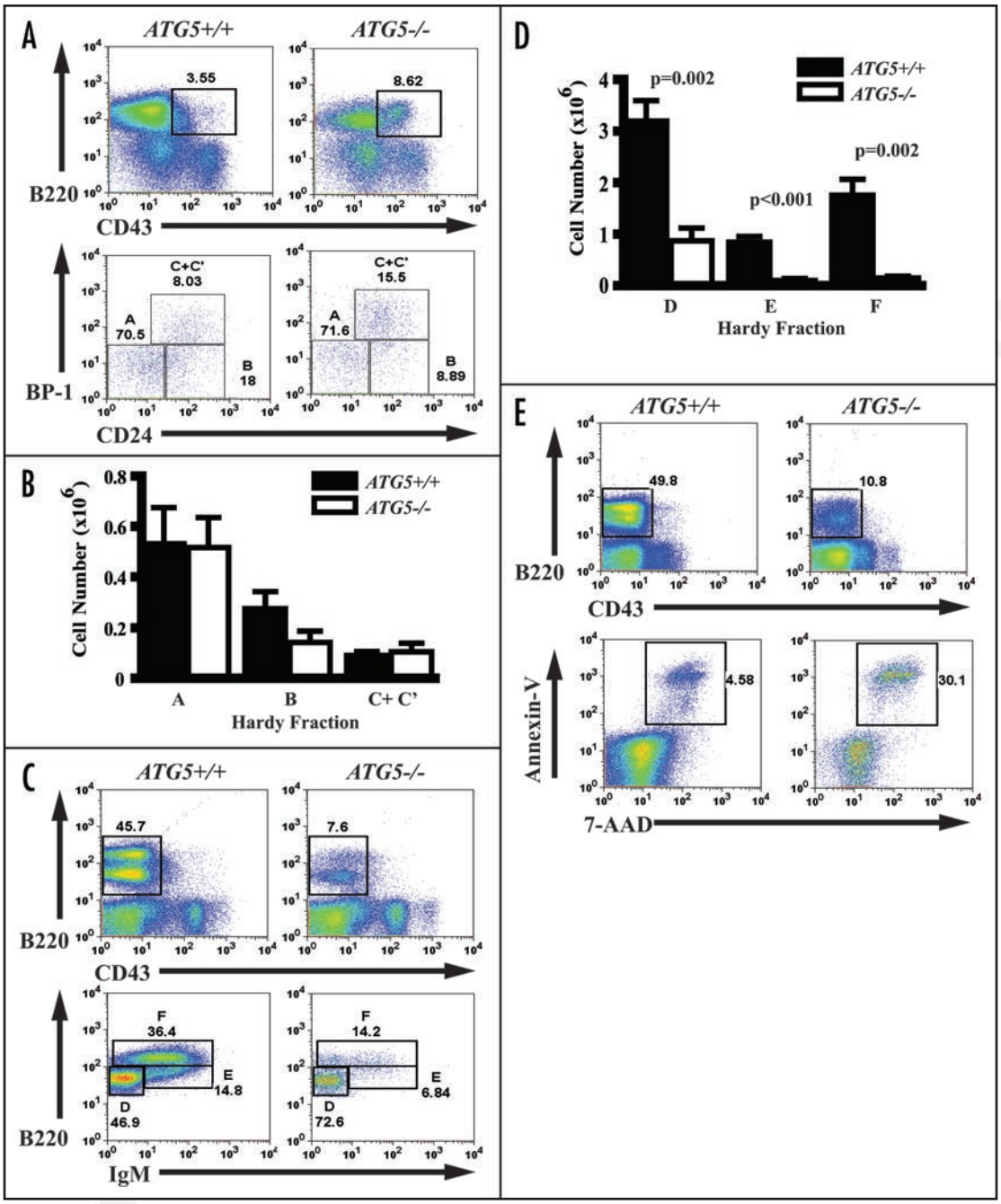

Figure 2. *ATG5* is required for the normal development of pre-B cells in the bone marrow. (A) Flow cytometry of bone marrow cells from *ATG5*$^{+/+}$ and *ATG5*$^{-/-}$ CD45.1 chimeric mice. Cells were gated on CD45.2^{+}, GR1^{-}, Mac1^{-} and 7-AAD^{-}. B220^{+}, CD43^{+} cells were further analyzed for expression of BP-1 and CD24. Data representative of at least four mice from four independent experiments. (B) Quantitation of Hardy fractions A-C' in *ATG5*$^{+/+}$ and *ATG5*$^{-/-}$ CD45.1 chimeric mice (n = 4 for each group). (C) Flow cytometry of bone marrow cells from *ATG5*$^{+/+}$ and *ATG5*$^{-/-}$ *RAG1*$^{-/-}$ chimeric mice. Cells were gated on B220^{+}, CD43^{-} and analyzed for expression of IgM. Data representative of at least eight mice from four independent experiments. (D) Quantitation of Hardy fractions D–F in *ATG5*$^{+/+}$ and *ATG5*$^{-/-}$ *RAG1*$^{-/-}$ chimeric mice (n = 8 for each group). (E) Flow cytometry of freshly isolated bone marrow cells from *ATG5*$^{+/+}$ and *ATG5*$^{-/-}$ *RAG1*$^{-/-}$ chimeric mice. Cells were gated on B220^{+}, CD43^{-} and analyzed for cell death by Annexin-V and 7-AAD. Data representative of at least six mice from three independent experiments.

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

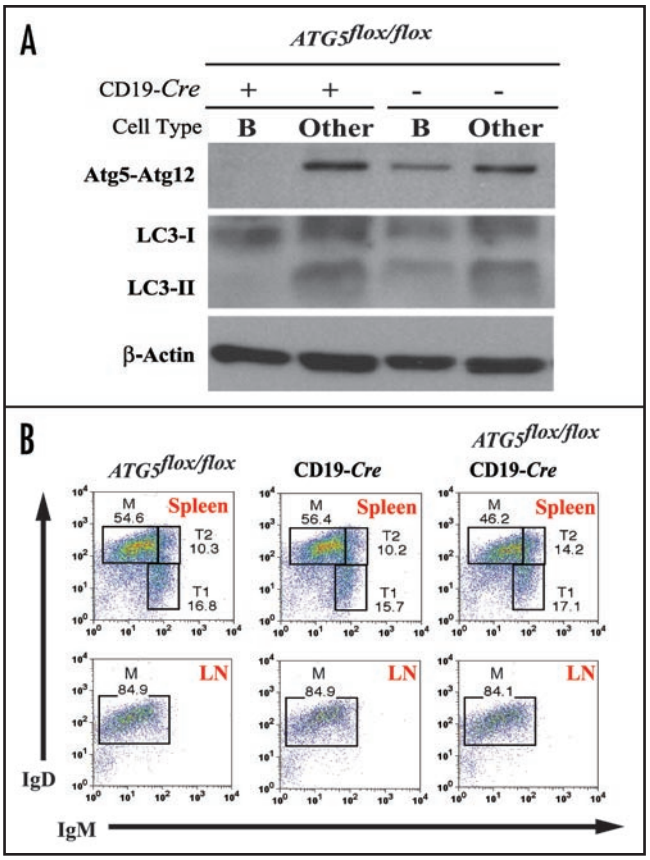

Figure 3. B-2 B cells from *ATG5*$^{flox/flox}$-CD19-*Cre* mice do not express ATG5 but are present in normal numbers. (A) Immunoblotting against ATG5 and LC3 from CD19^{+} (B) and CD19^{-} (Other) bead-purified splenocytes. Representative blot of three independent experiments. (B) IgM and IgD staining of B220^{+} lymphocytes from the spleen and lymph nodes. M, mature B cells (IgM^{lo}, IgD^{hi}); T1, transitional type 1 (IgM^{hi}, IgD^{lo}); T2, transitional type 2 (IgM^{hi}, IgD^{hi}). T1 gating includes marginal zone and splenic B-1 B cells. Data representative of at least ten mice from five independent experiments.

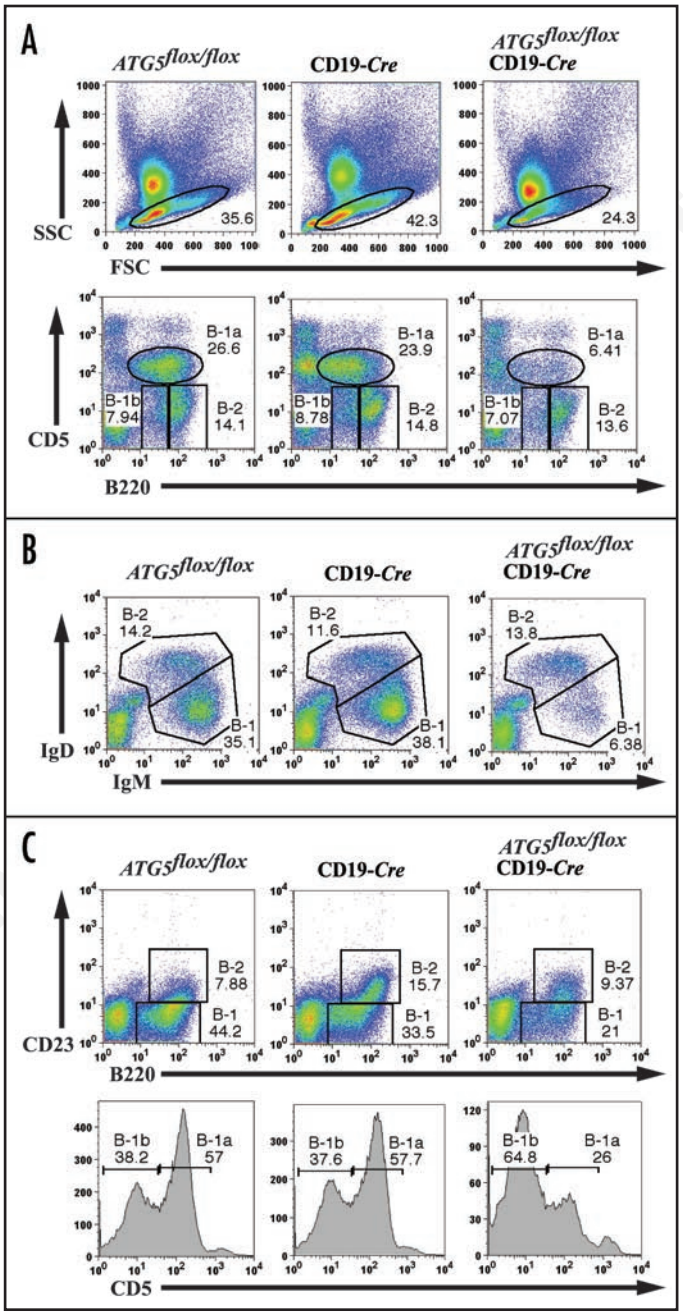

Figure 4. B-1a B cells are deficient in *ATG5*$^{flox/flox}$-CD19-*Cre* mice. Staining of peritoneal cells to identify B cell subsets: B-1a (CD5+, B220^{lo}, CD23-, IgM^{hi}, IgD^{lo}); B-1b (CD5-, B220^{lo}, CD23-, IgM^{hi}, IgD^{lo}); B-2 (CD5-, B220^{hi}, CD23^{lo}, IgM^{lo}, IgD^{hi}). All data is representative of at least five mice from three independent experiments. (C) B-1 B cells are gated by B220^{lo}, CD23- and separated into B-1a and B-1b B cells by expression of CD5.

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 *ATG5*$^{flox/flox}$-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.[36] 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.[7]

Acknowledgements

This work was supported by NIH grants CA74730 and U54 AI057160 Project 6 to H.W.V.; AI06107703 and AI06302402 to W.S.; 5-T32-AI07163-27 to L.M.S.; CA92123 to Y.W.H.; AI064705 to A.I.; Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology, Japan, and the Toray Science Foundation to N.M. H.K.L. is a recipient of the Anna Fuller Fellowship.

We thank David Strong and Boris Calderon for assistance with the generation of chimeric mice.

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