A Role for Autophagic Protein Beclin 1 Early in Lymphocyte Development

Ivica Arsov, Adeola Adebayo, Martina Kucerova-Levisohn, Joanna Haye, Margaret MacNeil, F. Nina Papavasiliou, Zhenyu Yue and Benjamin D. Ortiz

*J Immunol* published online 14 January 2011
http://www.jimmunol.org/content/early/2011/01/14/jimmunol.1002223

**Supplementary Material**
http://www.jimmunol.org/content/suppl/2011/01/14/jimmunol.1002223.3.DC1

**Subscription**
Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

**Permissions**
Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**
Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
A Role for Autophagic Protein Beclin 1 Early in Lymphocyte Development

Ivica Arsov,* Adeola Adebayo,*1 Martina Kucerova-Levisohn, † Joanna Haye,*2 Margaret MacNeil,* F. Nina Papavasiliou,‡ Zhenyu Yue,§ and Benjamin D. Ortiz‡

Autophagy is a highly regulated and evolutionarily conserved process of cellular self-digestion. Recent evidence suggests that this process plays an important role in regulating T cell homeostasis. In this study, we used Rag1−/− (recombination activating gene 1−/−) blastocyst complementation and in vitro embryonic stem cell differentiation to address the role of Beclin 1, one of the key autophagic proteins, in lymphocyte development. Beclin 1-deficient Rag1−/− chimeras displayed a dramatic reduction in thymic cellularity compared with control mice. Using embryonic stem cell differentiation in vitro, we found that the inability to maintain normal thymic cellularity is likely caused by impaired maintenance of thymocyte progenitors. Interestingly, despite drastically reduced thymocyte numbers, the peripheral T cell compartment of Beclin 1-deficient Rag1−/− chimeras is largely normal. Peripheral T cells displayed normal in vitro proliferation despite significantly reduced numbers of autophagosomes. In addition, these chimeras had greatly reduced numbers of early B cells in the bone marrow compared with controls. However, the peripheral B cell compartment was not dramatically impacted by Beclin 1 deficiency. Collectively, our results suggest that Beclin 1 is required for maintenance of undifferentiated/early lymphocyte progenitor populations. In contrast, Beclin 1 is largely dispensable for the initial generation and function of the peripheral T and B cell compartments. This indicates that normal lymphocyte development involves Beclin 1-dependent, early-stage and distinct, Beclin 1-independent, late-stage processes. The Journal of Immunology, 2011, 186: 000–000.

Autophagy is essential for maintaining cellular energy homeostasis during nutritional stress, and it also appears to play important roles in development and differentiation. During nutrient starvation-induced autophagy, portions of the cytoplasmic material are sequestered within double-membrane vesicles and then delivered to the lysosome for degradation. Beclin 1, the mammalian ortholog of yeast Atg6, is a critical component of a complex containing class III PI3K and other proteins, including UVRAG, Ambra-1, Bif-1, and Atg14L, that stimulates autophagy by initiating the isolation membrane formation (1, 2). Beclin 1 also interacts with antiapoptotic Bcl-2 family members, Bcl-2 and Bcl-

xL (3, 4), and it is believed that this interaction could represent an important link between autophagy and apoptosis. Several studies have demonstrated that Beclin 1 can affect the apoptotic pathway in different types of eukaryotic cells (5–7). In addition, Bcl-2 has been shown to inhibit Beclin 1-mediated autophagic death induced by nutrient starvation in cultured cells (4).

The analyses of several genetically modified mouse strains and early embryonic stem cell (ESC)-derived mouse embryos deficient in proteins essential for autophagy, including Beclin 1, indicate that autophagy is critical for maintaining cellular energy homeostasis and normal development (8–12). Beclin 1-deficient mice die during early embryonic development (8, 13), which prevents the analysis of Beclin 1 deficiency in specific tissues. In our previous report using Beclin 1-GFP transgenic mice, we provided evidence that Beclin 1 could be involved in T cell development in the thymus (14). In this study, we used a recombination activating gene 1 (Rag1) blastocyst complementation approach (15) and in vitro ESC differentiation to T cells to analyze the role of Beclin 1 in T and B cells in more detail. Our studies indicate that Beclin 1 plays a unique role in maintaining normal thymic cellularity and early B cells in the bone marrow. However, it appears to be largely dispensable for initial generation and proliferation of peripheral T and B cells. The absence of Beclin 1 does not appear to cause any specific block in the development of T or B lineage cells. Rather, Beclin 1 deficiency results in impaired maintenance of lymphoid progenitors in vivo and in vitro. Our results thus reveal a selectively early role for Beclin 1 in lymphocyte development distinct from that observed for other autophagic proteins, such as Atg5 and Atg7.

Materials and Methods

Generation of Rag1−/− chimeric mice

Rag1−/− mice of both sexes were purchased from The Jackson Laboratory. Rag1−/− blastocysts harvested from pregnant Rag1−/− female mice (3.5 postcoitum) were injected with 8–15 of either Beclin 1−/− or Beclin 1+/− ESCs

The online version of this article contains supplemental material.

Abbreviations used in this article: CLP, common lymphoid progenitor; DLL1, Delta-like 1; DN, double negative; DP, double positive; ESC, embryonic stem cell; HSC, hematopoietic stem cell; Rag1, recombination activating gene 1; RT, room temperature.

Copyright © 2011 by The American Association of Immunologists, Inc. 0022-1767/11/S16.00

www.jimmunol.org/cgi/doi/10.4049/jimmunol.1002223
and implanted into pseudopregnant foster mothers. Both ESC clones used in this study were described previously (8). All chimeric mice were maintained under specific pathogen-free conditions at either The Mount Sinai Animal Care Facility or The Rockefeller University Animal Care Facility and used at 4–8 wk of age. All studies and procedures were approved by Animal Care and Use Committees at both institutions.

**Abs and Flow Cytometry**

Fluorescently labeled mAbs against mouse CD4 (clone GK1.5), CD8 (clone 53-6.7), CD43 (clone IM7), IgM (R6-60.2), CD43 (clone S7), B220 (clone RA3-6B2), CDS (53-7.3), CD229.1 (Ly9.1, 30C7), CD21 (7G6), CD23 (B34), and CD117 (c-KIT, 2B8), and aliphophycocyanin-conjugated lineage mixture were purchased from BD Biosciences (San Jose, CA). PE-conjugated anti-mouse IgD (clone 11-26) was from Southern Biotech (Birmingham, AL). Abs against mouse CD69 (clone H1.2F3), CD25 (PC61.5), CD44 (IM7), CD127 (IL-7Rα, clone A7R34), and Ly-6A/E (Sca-1, clone D7) were purchased from eBioscience (San Diego, CA). Single-cell suspensions were generated from the spleen, bone marrow, and thymus, and stained for 30 min on ice using saturating concentrations of directly conjugated mAbs. Cells were washed three times with PBS containing 2% FBS, and data were acquired using either FACScan or LSR II flow cytometers (BD Biosciences, San Jose, CA). Flow cytometry data were analyzed using FlowJo software (Tree Star, Ashland, OR).

**In vitro differentiation of Beclin 1−/− and Beclin 1+/− ESCs into T cells**

In vitro differentiation of ESCs to T cells was done as previously described (16). In brief, both ESC clones, Beclin 1−/− and Beclin 1+/−, were maintained in an undifferentiated state at mitomycin C arrested mouse embryonic fibroblasts (Millipore/Chemicon, Billerica, MA) in ESC media (16) with addition of 103 U/ml LIF (ESGRO from Millipore/Chemicon). Coculture experiments were initiated by seeding 5 × 104 ESCs on top of 800-μm confluent monolayer of the OP-9 stromal cell line in OP9 media (16). Mesoderm colonies were trypsinized on day 5 of coculture, and 5 × 104 cells were plated on a new OP-9 stromal monolayer with additional supplement of Fli-3 ligand (R&D Systems, Minneapolis, MN) to a final concentration of 5 μg/ml in the OP9 media. The formation of hematopoietic stem cells (HSCs) was observed by day 8 of coculture, and HSCs were transferred to an OP9-DL1 monolayer of cells that were virally transduced to express the Notch ligand Delta-like 1 (DLL1) (17). From this point, the OP9 medium was supplemented with Fli-3L and IL-7 (Pepro-Tech, Rocky Hill, NJ) to a final concentration of cytokines of 5 and 1 ng/ml, respectively. Cocultures were subjected to alternating media change and no-trypsin passages in 2-d intervals. Differentiating lymphocytes were analyzed on days 12 and 19/20 by flow cytometry.

For T cell cloning, flat-bottom 96-well plates were precoated with 10 μg/ml 2C11 Ab in PBS (BD Biosciences) for 4 h in a tissue culture incubator at 37°C or for 12 h at 4°C. Plates were washed three times with ice-cold PBS, and splenocytes (2 × 106 cells/well) were incubated in RPMI 1640 media supplemented with 10% FBS. For B cell proliferation assays, naive splenic B cells were purified by depletation-type magnetic separation using CD43 (Ly-48) MACS beads (Miltenyi Biotec, Auburn, CA) and gated on the CD45+ population of cells. For day 12 analyses, the differentiated lymphocytes were further stained with rat anti-mouse CD44 conjugated to fluorescein (FITC) and rat-anti mouse CD25 conjugated to aliphophycocyanin (both from CalTag/InVitrogen Laboratories). On day 19/20, differentiated lymphocytes were analyzed with rat anti-mouse CD4 conjugated to aliphophycocyanin (CalTag/InVitrogen Laboratories) and rat anti-mouse CD8α conjugated to FITC (BD Pharmingen, Franklin Lakes, NJ).

**Lymphocyte proliferation assays**

For T cell stimulation, flat-bottom 96-well plates were precoated with 10 μg/ml 2C11 Ab in PBS (BD Biosciences) for 4 h in a tissue culture incubator at 37°C or for 12 h at 4°C. Plates were washed three times with ice-cold PBS, and splenocytes (2 × 106 cells/well) were incubated in RPMI 1640 media supplemented with 10% FBS. For B cell proliferation assays, naive splenic B cells were purified by depletation-type magnetic separation using CD43 (Ly-48) MACS beads (Miltenyi Biotec, Auburn, CA) and gated on the CD45+ population of cells. For day 12 analyses, the differentiated lymphocytes were further stained with rat anti-mouse CD44 conjugated to fluorescein (FITC) and rat-anti mouse CD25 conjugated to aliphophycocyanin (both from CalTag/InVitrogen Laboratories). On day 19/20, differentiated lymphocytes were analyzed with rat anti-mouse CD4 conjugated to aliphophycocyanin (CalTag/InVitrogen Laboratories) and rat anti-mouse CD8α conjugated to FITC (BD Pharmingen, Franklin Lakes, NJ).

For CFSE-based T cell proliferation assays, spleen cells were labeled with 0.5 μM CFSE in PBS (Invitrogen) for 8 min at room temperature (RT). To quench the reaction, we added FCS immediately to the final concentration of 20%, and washed cells three times in PBS and resuspended them in RPMI 1640 media supplemented with 10% FBS. The CFSE-labeled spleen cells were then incubated with plate-bound 2C11 Ab (10 μg/ml) in a tissue culture incubator at 37°C in a humidified atmosphere containing 5% CO2 for 72 h and analyzed using FACScan flow cytometer.

**Confocal microscopy**

Spleen T cells were isolated using Dynal mouse T cell-negative isolation kit (Invitrogen) and stimulated in 24-well plates in the presence of T cell-activating magnetic beads coupled with anti-CD3 and anti-CD28 Abs (BD Biosciences) and IL-2 (0.5 ng/ml) in a CO2 incubator for 7 d. After incubation, stimulating beads were removed with a magnet, and cytokines were prepared using Shandon cytosip (Thermo Fisher Scientific, Waltham, MA) with ~1 × 106 T cell on each microscope slide. T cells were subsequently fixed for 20 min in 4% paraformaldehyde in PBS on ice, washed twice with PBS/1% BSA, and permeabilized 5 min at RT with 0.5% saponin/0.03% Suroccle/1% BSA in PBS. The cells were washed once with PBS/1% BSA and blocked using 5% normal goat serum in PBS/1% BSA for 15 min at RT. After a single wash with PBS/1% BSA, T cells were incubated with polyclonal anti-IC3 Ab PM036 (MBL International Corporation, Woburn, MA) for 60 min at RT. After this incubation, T cells were three times and then blocked with 5% normal goat serum PBS/1% BSA. After subsequent wash with PBS/1% BSA, T cells were incubated with FITC-conjugated goat anti-rabbit Ab for 30 min at RT. T cells were finally washed three times in PBS/1% BSA, nuclei counterstained with DRAQ5 (Biostatus Limited, Leicestershire, U.K.) DNA stain for 5 min at RT, and mounted using SlowFade Gold antifade reagent (Molecular Probes, Eugene, OR). The cells were visualized using an Olympus Fluoview300 confocal microscope (Olympus, Center Valley, PA) at a magnification of ×600.

**Statistical analysis**

Statistical analysis was performed using two-tailed Student t test. A P value <0.05 was considered statistically significant.

**Results**

**Loss of thymocytes in the absence of Beclin 1**

Targeted disruption of Beclin 1 in mice results in early embryonic lethality before generation of a lymphoid system, thus preventing the analysis of in situ lymphoid development (8). To circumvent this obstacle, we generated Beclin 1−/−→Rag1−/− chimeric mice and control Beclin 1+/−→Rag1−/− chimeras using Beclin 1+/− and Beclin 1+/− ESC clones, respectively. Because Rag1−/− mice cannot produce mature T and B cells because of deficient V(DJ) recombination (18), any mature lymphocytes in chimeric mice must be derived from microinjected ESCs, indicating that they have the potential to complement Rag1 deficiency in lymphopoiesis. The injection of Beclin 1−/− and Beclin 1+/− ESC clones into Rag1−/− blastocyst mostly produced mice with extensive agouti-black coat-color chimerism (>80%), which confirmed the developmental potential of Beclin 1−/− and Beclin 1+/− cells. Because ESC clones used in this study are of 129/1Sv origin, any lymphocytes derived from these cells can be easily distinguished from Rag1−/−-derived cells, which are of C57BL/6 origin, based on the expression of the Ly9.1 alloantigen. This alloantigen, present on ES but absent on Rag1−/−-derived cells, is a surface glycoprotein expressed by most thymocytes, peripheral B and T cells, bone marrow lymphoid cells, and hematopoietic progenitors.

To determine T cell development in Beclin 1-deficient chimeric mice, we analyzed thymocytes using flow cytometry. Virtually all double positive (DP) and single positive thymocytes in chimeric mice expressed the Ly9.1 surface marker, indicating that they developed from injected ESCs (data not shown). In 20% of Beclin 1+/−→Rag1−/− chimeras, a complete reconstitution of the major thymocyte populations was observed compared with control mice.
Beclin 1+/− → Rag1−/− chimeras (Fig. 1A). However, in about 80% of chimeras, we have detected various degrees of loss of Beclin 1-deficient DP thymocytes (Fig. 1B, 1C). This loss ranged from 20% to almost complete depletion of DP thymocytes in some Beclin 1−/− → Rag1−/− chimeras (Fig. 1C) but was never observed in control animals. Enumeration of thymocytes and spleen cells by trypan blue exclusion also revealed a dramatic reduction of thymocyte numbers in Beclin 1−/− → Rag1−/− chimeras (Fig. 1D). Different levels of chimerism in individual mice could potentially explain the high variability of thymocyte numbers observed in control chimeras (Fig. 1D). Nevertheless, the differences between Beclin 1-deficient and control chimeras are significant (17.7 × 10⁶ versus 72.8 × 10⁶; p = 0.0002).

The dramatic loss of thymocytes in Beclin 1−/− → Rag1−/− chimeras could potentially arise from a specific block during double negative (DN) thymocyte development. To address this possibility, DN thymocytes expressing the Ly9.1 marker were stained with CD44 and CD25 Abs, and analyzed by flow cytometry (Fig. 1E). Compared with control mice, Beclin 1-deficient chimeras displayed perturbed DN thymocyte populations with accumulation of both DN1 cells (CD44⁺CD25⁻⁻) and the intermediate DN1-DN2 (CD44⁺CD25lo) cells. Our results indicate that Beclin 1 deficiency
results in the loss of thymocytes and apparent abnormalities in the DN thymocyte compartment. However, from these data alone, it is not possible to discern whether the abnormalities observed in Beclin 1-deficient DN thymocytes represent a true developmental block or are a result of competition between Beclin 1<sup>-/-</sup> and Rag1<sup>-/-</sup>-derived DN cells.

**Beclin 1<sup>-/-</sup> ESCs fail to maintain normal T cell development in vitro**

To further investigate the thymocyte abnormalities discovered in Beclin 1<sup>-/-</sup> chimeric animals, we used in vitro ESC differentiation system with OP9 stroma cells expressing the DL1 protein (17) to generate T cells in the absence of Rag1<sup>-/-</sup> cell competition. After 12 d of coculture, both Beclin 1<sup>-/-</sup> and control ESCs were capable of generating normal DN thymocyte subsets, as indicated by similar CD25 versus CD44 profiles, as well as similar numbers of c-Kit<sup>hi</sup> DN1 (CD25<sup>-</sup>CD44<sup>+</sup>) cells containing early T-lineage progenitors up to day 16 (Fig. 2A and data not shown). We also observed that the rate of DN2/3 cell generation from CD45<sup>+</sup> precursors (as indicated by the ratio of CD25<sup>+</sup>/CD45<sup>+</sup> cells) was equivalent in both knockout and control cocultures (Fig. 2A). This would rule out an intrinsic developmental block at this stage of thymocyte development caused by Beclin 1 deficiency. Remarkably, however, day 19/20 cocultures derived from Beclin 1<sup>-/-</sup> ESCs displayed sharp declines in DN thymocyte populations compared with control Beclin 1<sup>+/–</sup> ESC cocultures (10.44 ± 3.1 versus 1.66 ± 0.52; p < 0.05; Fig. 2C, 2D). We also observed impaired output of more mature CD8<sup>+</sup> T cells that is likely secondary to the loss of their progenitors in the DN compartment (Fig. 2C, 2D). The coculture-derived Beclin 1-deficient DP thymocytes exhibited normal sensitivity to apoptosis and normal TCR β chain expression (data not shown). Because earlier coculture time points, including days 12 (Fig. 2A) and 16, indicated no difference in the ability of Beclin 1<sup>-/-</sup> and control ESCs to initially generate T cells in vitro (unpublished observation), our results from Rag1<sup>-/-</sup> chimeras, as well as in this ESC differentiation system, strongly suggest that the failure of Beclin 1<sup>-/-</sup> ESC coculture to sustain normal T cell generation in vitro at later time points is caused by impaired maintenance of thymocyte precursors at, or prior to, the DN stage.

**Normal proliferation of Beclin 1-deficient peripheral T cells**

Despite the dramatic loss of thymocytes in Beclin 1<sup>-/-</sup>-Rag1<sup>-/-</sup> chimeras, we did not observe any change in peripheral, spleen CD4<sup>+</sup> and CD8<sup>+</sup> T cells numbers (Fig. 1A–C, right dot plot panels, 3A), suggesting that Beclin 1 is not essential for maintaining normal numbers of T cells in the periphery. The analysis of peripheral T cell subsets did not indicate any imbalance among naive CD44<sup>lo</sup>CD62L<sup>hi</sup>, effector CD44<sup>hi</sup>CD62L<sup>lo</sup>, and memory CD44<sup>hi</sup> CD62L<sup>hi</sup> T cells in Beclin 1-deficient chimeras (Fig. 3B). Surprisingly, peripheral T cells exhibited normal proliferative response in vitro compared with control T cells (Fig. 3C, 3D), as determined by both BrdU incorporation (Fig. 3C) and CFSE (Fig. 3D) assays, and also displayed normal induction of activation markers, CD69 and CD25 (Fig. 3F). Because previous studies have suggested that autophagy is required for peripheral T cell proliferation (19, 20), we analyzed whether Beclin 1-deficient T cells contained autophagosomes by anti-LC3 staining. The confocal imaging of LC3-stained, activated T cells revealed a reduced number of fluorescent puncta in Beclin 1-deficient versus control T cells (Fig. 4). Beclin 1<sup>-/-</sup> T cells contained, on average, 0.72 ± 0.09 fluorescent puncta per cell compared with 1.70 ± 0.12 puncta in control Beclin 1<sup>-/-</sup> T cells (p = 0.002). Because the presence of LC3 puncta has been considered a universal marker of autophago-

---

**FIGURE 2.** Beclin 1-deficient ESCs fail to maintain normal T cell development in vitro. T cell differentiation from Beclin 1<sup>-/-</sup> and control ESCs was carried out in the presence of OP9-DL1 cells, as described in Materials and Methods. The coculture aliquots were taken at days 12 (A) and 19/20 (C), stained with indicated Abs, and analyzed using flow cytometry. The experiments were repeated three times with similar results. A, The ratio of CD25<sup>+</sup>/CD45<sup>+</sup> cells in Beclin 1<sup>-/-</sup>- and control cocultures at day 12 indicating equivalent rates of DN2/3 cell generation from CD45<sup>+</sup> precursors in both cocultures. D, Distribution of T cell subsets based on the expression of CD4 and CD8 surface markers in Beclin 1<sup>-/-</sup>- and control cocultures at day 19/20. Data represent average percentages ± SD of cells from three independent experiments.
panels contained only a small number of ESC-derived Ly9.1 + cells (Fig. 5). B cells (B220 lowCD43+) from Beclin 1−/− Rag1−/− ESCs (Fig. 5) contained only a small number of ESC-derived Ly9.1 + cells (Fig. 5). B cells (B220 lowCD43+) from Beclin 1−/− Rag1−/− ESCs (Fig. 5) contained only a small number of ESC-derived Ly9.1 + cells (Fig. 5). B cells (B220 lowCD43+) from Beclin 1−/− Rag1−/− ESCs (Fig. 5) contained only a small number of ESC-derived Ly9.1 + cells (Fig. 5).

To analyze the peripheral B cell compartment in chimeric mice, we stained spleen cells using fluorescently labeled Abs and analyzed them by flow cytometry. We found that Beclin 1−/− Rag1−/− chimeras had slightly reduced populations of IgM+IgD+ B cells (12.6 ± 4.5 versus 19.1 ± 5.3; p < 0.05; n = 7; Fig. 6A, top panel). The frequencies of CD21+CD23+IgM+ T2, as well as CD21+CD23+ IgM+ marginal zone B cells, were generally unchanged (Fig. 6A, middle and bottom panels). However, the most developmentally early population of CD21−CD23− IgM+ (T1) peripheral B cells (representing recent bone marrow emigrants) was significantly diminished in Beclin 1−/− Rag1−/− chimeras (5.6 ± 1.7 versus 19.9 ± 3.6; p < 0.05; n = 3; Fig. 6A, middle panel). Importantly, the analysis of peritoneal B1 cells using CD5 and IgM marker expression revealed normal development of the B1 cell population in Beclin 1−/− Rag1−/− chimeric mice (Fig. 6B). To determine whether peripheral Beclin 1−/− B cells were functional, we analyzed the proliferative response of purified spleen B cells stimulated in vitro with LPS and IL-4. Our results indicate that in vitro proliferative response of Beclin 1−/− B cells is not significantly different from the response of control Beclin 1+/− B cells (Fig. 6C). In summary, we find that Beclin 1 deficiency has far more dramatic effects at early stages of B cell development than at later stages. Thus, similar to T cells, Beclin 1 appears to be largely dispensable for normal numbers and proliferation of B cell populations in the periphery.

**Impaired generation of Beclin 1−/− lymphoid progenitors**

The early-stage defects in T and B cell lineage cells of Beclin 1−/− deficient chimeras prompted an analysis of early lymphoid precursors in the bone marrow. We analyzed Beclin 1−/− Rag1−/− chimeric mice for potential alterations in common lymphoid progenitor (CLP) and HSC populations, as previously described (22). We observed that Ly9.1+ CLPs were substantially reduced in Beclin 1−/− Rag1−/− chimeras compared with control mice, indi-
eral T cells. In the case of thymocytes, these defects involve re-
tion in thymocytes (14), prompted the current study of the role of
combination with our previous analyses of Beclin 1 gene regula-
crease autophagic activity in CD4+ T cells (26). These reports, in
process can be triggered in T cells by extracellular cues different
creased frequency (over time) of hematopoietic progenitors seeding
in Beclin 1-deficient chimeras. This could be the result of a de-
duced autophagy-dependent removal of damaged or aging mitochondria in peripheral T cells (20, 27). It is therefore clear that Atg5 and Atg7 autophagy genes are required at both early and late stages of T cell development.

We found that Rag1 blastocyst chimeras lacking Beclin 1, similar to Atg5 and Atg7-deficient mice, show reduced thymocyte numbers (Fig. 1). We also detected an altered distribution of DN thymocytes in these chimeras with accumulation of cells in the DN1 population (Fig. 1E). This, however, likely does not reflect a genuine developmental block. Rather, it would seem that Beclin 1-deficient DN thymocytes are outcompeted by Rag1−/−-derived DN cells. This is because, using an in vitro ESC differentiation system for generating T cells, we observed a normal rate of initial DN subset generation and did not detect significant DN subset aberrations at early time points in the coculture (Fig. 2A, 2B). However, we did observe a dramatic reduction in the DN compartment at later time points (Fig. 2C, 2D). Importantly, we observed this reduction in the absence of competition from Beclin 1-sufficient cells that exist in the chimeric mice. Thus, the defects in thymocyte progenitor maintenance seen in vitro (and likely the related progenitor maintenance defects seen in vivo) are intrinsic to Beclin 1 deficiency. These in vitro data help illuminate the likely basis for the reduction of total thymocyte numbers observed in Beclin 1-deficient chimeras. This could be the result of a decreased frequency (over time) of hematopoietic progenitors seeding the thymus. Collectively, these results point to the impaired maintenance of thymocyte progenitors in the absence of Beclin 1.

In sharp contrast with the peripheral T cell defects previously reported for Atg5- and Atg7-deficient T cells (19), we could not detect a significant impact of Beclin 1 deficiency on peripheral T cell numbers and their proliferative response in vitro (Fig. 3). Considering the essential roles of Atg5 and Atg7 in autophagy, their apparent requirement for T cell proliferation is commonly regarded as a general requirement for autophagy in peripheral T cells. However, we were able to detect LC3 puncta in Beclin 1-deficient T cells (Fig. 4), suggesting that a noncanonical, Beclin 1-independent autophagy appears to be sufficient to sustain normal peripheral T cell proliferation. Similar examples of Beclin 1-independent autophagy have been observed in other cell types under certain conditions (28–31). The fact that Beclin 1-deficient T cells still retain some autophagic activity could thus potentially explain different outcomes of Beclin 1 and Atg5/Atg7 deficiencies in peripheral T cell phenotype and proliferation. In addition, we did not observe any significant accumulation of mitochondria in Beclin 1−/− T cells (unpublished observation), as seen in Atg5−/− T cells (20, 27). In summary, our data indicate that the Beclin 1 autophagy gene plays an essential, but selectively early, role during T cell development. This is because, unlike Atg5 and Atg7, Beclin 1 function seems largely dispensable in peripheral T cells.

Although previous studies indicate an important role for autophagic protein Atg5 in both early- and late-stage T cells, the data describing the impact of Atg5 deficiency in B cells are less clear. Atg5-deficient fetal liver chimeric mice display defects that suggest reduced lymphoid progenitor activity (19) including a significant reduction in the number of peripheral B cells. In contrast, a subsequent study in which the Atg5 gene was conditionally targeted in B cells (using CD19-cre) showed partial deficiencies at the pro-B to pre-B cell transition in the bone marrow, but a largely

Discussion

Several studies have recently addressed the role of autophagy in lymphocytes. First, autophagy has been found to play a role in CD4+ T cell death induced by HIV (25), indicating that this process can be triggered in T cells by extracellular cues different from starvation. In addition, T cell activation in vitro induces increased autophagic activity in CD4+ T cells (26). These reports, in combination with our previous analyses of Beclin 1 gene regulation in thymocytes (14), prompted the current study of the role of the Beclin 1 autophagy gene in T cell development.

Mice with T cell-specific inactivation of Atg5 or Atg7 genes exhibited a variety of intrinsic defects in thymocytes and peripheral T cells. In the case of thymocytes, these defects involve re-

![FIGURE 4. Reduced number of LC3 puncta in activated, Beclin 1-deficient T cells. Confocal microscopy images of immunofluorescent staining with anti-LC3 Ab reveals a punctate localization of LC3 in activated T cells from Beclin 1−/− (A), as well as Beclin 1−/−→Rag1−/− chimera (B). A and B, Right panels represent enlarged areas defined by red boxes in left panels. The average numbers of LC3 puncta in activated T cells from Beclin 1−/− versus Beclin 1−/−→Rag1−/− chimeras counted by confocal microscopy from 12 fields, each 52,430 μm² in area ± SD (C). Original magnification ×400.](http://www.jimmunol.org/003)
normal peripheral B cell compartment (29). The differences observed in the severity of the Atg5-deficient B cell phenotype undoubtedly stem from the disparate experimental systems used in these two studies. Nevertheless, these prior data suggest at least an early-stage role for Atg5 in the B cell compartment, even if its role in peripheral B cells remains nebulous.

FIGURE 5. Compromised reconstitution of B cell development in the bone marrow of Rag1−/− mice by Beclin 1-deficient ESCs. A, Frequency of Ly9.1+ cells in the bone marrow of age and sex-matched Beclin 1−/− →Rag1−/− and control chimeras as determined by flow cytometry. All mice were used between 4 and 8 wk of age. B, Bone marrow cells were stained with fluorescently labeled Abs against indicated B cell markers, as well as Ly9.1, and analyzed by flow cytometry. Bottom histogram represents the expression of Ly9.1 on pro- and pre-B cells as defined by CD43 and B220 surface markers. Gate positions are indicated with rectangles. The analysis was repeated three times with similar results.

FIGURE 6. Reduced early bone marrow emigrant B2 cell numbers but otherwise preserved peripheral B2 and B1 B cell subsets in the absence of Beclin 1. A, Spleen cells from chimeric mice were stained with indicated fluorescently labeled Abs and analyzed by flow cytometry. The applied gates are indicated above each pair of contour plots. Note the reduction in CD21−CD23− IgM+ cell subset representing the subset of recent bone marrow emigrants (bottom pair of plots). B, Peritoneal exudate cells from chimeric mice were stained with Abs against CD5, IgM, and CD23. The expression of CD5 and IgM on CD23− cells is shown. C, Purified splenic B cells (2 × 10^5/well) from chimeric mice were incubated in 96-well plates in the presence of LPS (25 µg/ml) and IL-4 (5 ng/ml), and proliferation assay carried out as described in Materials and Methods. Bars represent means of triplicate cultures ± SD. Data are representative from three independent experiments.
In concordance with the Atg5 knockout fetal liver chimera data, we detect deficiencies in lymphoid progenitors in the absence of Beclin 1. Our data also show that Beclin 1 deficiency causes significant defects at early stages of B cell development in the bone marrow, and a lesser deficit in the earliest peripheral B cell subset representing recent bone marrow emigrants. In contrast, Beclin 1-deficient B cells at later stages of development appear largely normal. Taken together, the data indicate that both Beclin 1 and Atg5 proteins are required in lymphoid progenitors and early-stage T and B cell development. Although Atg5 may be required in peripheral B cells (19), our data suggest that, as in peripheral T cells, Beclin 1 activity is not essential for most peripheral B cells. Although peripheral lymphocytes seem largely normal in our Beclin 1<sup>−/−</sup> chimeric mice, we cannot rule out the possible emergence of long-term deficits over time. It is conceivable that Beclin 1 activity will prove significant in preventing age-related decline in lymphocyte homeostasis and function. Long-term monitoring of Beclin 1<sup>−/−</sup> chimeric and control mice will be required to test this hypothesis.

Our findings of an essential role for Beclin 1 at early stages of both T and B cell development suggest that the reduction of early T and B cell numbers in Beclin 1-deficient chimeras could result from a reduced pool of lymphoid progenitors in the bone marrow. We examined this possibility by analyzing CLPs and HSC in both control and Beclin 1-deficient chimeras (32). Our results indicate that Beclin 1<sup>−/−</sup>→Rag1<sup>−/−</sup> chimeras have significantly reduced Ly9.1<sup>+</sup>CLPs and also a severely reduced population of Ly9.1<sup>+</sup> HSCs compared with control chimeras (Fig. 7). The impaired maintenance of lymphoid progenitors in the bone marrow may therefore explain the defects seen in the early T and B cell compartments in the absence of Beclin 1. It is not yet clear whether the problem in progenitor maintenance is selectively at a single stage (such as the HSC stage) or is a more general defect in maintenance of multiple types of undifferentiated progenitors. Nevertheless, using an in vitro ESC differentiation system to generate T cells, we found a depletion of the DN thymocyte compartment over time resulting in reduced maintenance of T cell generation in the cocultures at later time points. Because this depletion occurs in the absence of competition from Beclin 1-sufficient cells in the chimeras, this defect would be consistent with impaired progenitor activity/maintenance issues intrinsic to cells lacking Beclin 1. In future studies, this in vitro system may enable a comprehensive assessment of the generation, maintenance, and function of thymocyte progenitors at multiple stages to determine which populations are dependent on Beclin 1 function and why.

In summary, Beclin 1 deficiency results in severe depletion of T and B cell precursors, but has limited impact on peripheral T and B cells. Furthermore, loss of Beclin 1 function has no discernible effect on peripheral T cell proliferation despite reduced autophagy. Together with other recent reports, our results thus indicate distinct roles for various known autophagic proteins in T cells. Although recent studies in Atg5- and Atg7-deficient mice indicate a clear role for autophagy, in general, throughout lymphocyte development, we propose that Beclin 1-dependent processes are selectively required in progenitor cells and early stages of lymphocyte development. It remains to be seen, however, whether these processes reflect the role of Beclin 1 in autophagy or could involve other autophagy-independent function(s) of this protein in cell survival/differentiation. Importantly, our results reveal that noncanonical, Beclin 1-independent autophagy is sufficient to support later-stage peripheral T and B cell development and proliferation.

**FIGURE 7.** Reduced CLP and HSC compartments in the bone marrow of Beclin 1<sup>−/−</sup>→Rag1<sup>−/−</sup> chimeric mice. Frequencies of Beclin 1-deficient ESC-derived (A) CLPs and (B) HSCs were determined based on the expression of Ly9.1 (32). CLPs are defined as Lin<sup>−</sup>CD127<sup>−</sup>c-Kit<sup>−</sup>Sca-1<sup>−</sup> and HSC as Lin<sup>−</sup>CD127<sup>−</sup>c-Kit<sup>+</sup>Sca-1<sup>+</sup> cells (22, 33). Bone marrow cells were stained with indicated Abs and analyzed using LSR II flow cytometer. Gate positions were indicated with rectangles. The percentage of positive cells within each subset defined by corresponding gates within contour plots and histograms are indicated with small numbers within each gate. Data are representative from three independent experiments.
Acknowledgments
We thank Kevin Kelley (Mount Sinai School of Medicine) and Rada Norinsky (Rockefeller University) for expert Rag1+− chimeric mice generation, Chingwen Yang (Rockefeller University) for ESC work, Tatyana Leonova (Rockefeller University) for help with B cell assays, Joon Kim (Hunter College) for flow cytometry, and Derek Sant’Angelo (Memorial Sloan-Kettering Cancer Center) for helpful comments on the manuscript. We are indebted to Roxanne Holmes and Juan-Carlos Zúñiga-Pflucker for invaluable discussions and the ongoing advice and reagents that enabled us to establish in vitro T cell differentiation at City University of New York.

Disclosures
The authors have no financial conflicts of interest.

References