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MINI REVIEW

Roles of autophagy in lymphocytes: reflections and directions

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Recent studies have revealed that autophagy, a fundamental intracellular process, plays many different roles in lymphocyte development and function. Autophagy regulates naive T-lymphocyte homeostasis, specifically by regulating mitochondrial quality and turnover, and is necessary for the proliferation of mature T cells. Autophagy also acts as a cellular death pathway in lymphocytes, both upon prolonged cytokine withdrawal and during acute antigen-receptor stimulation if improperly regulated. Furthermore, during HIV infection, hyperinduction of autophagy leads to massive T-cell death in uninfected CD4$^+$ T cells, and is rescued by inhibiting autophagic initiation. Constitutively high levels of autophagy in thymic epithelial cells are necessary for optimal processing and presentation of endogenous antigens, and required for proper positive and negative selection of developing thymocytes. Autophagy also promotes the survival of B lymphocytes, as well as the development of early B-cell progenitors. In B cells, autophagy is an alternative death pathway, as antigen-receptor stimulation in the absence of costimulation induces a potent autophagic death. Thus, autophagy plays a complex role in lymphocytes and is regulated during their lifespan to ensure a healthy immune system.
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INTRODUCTION

Autophagy, an intracellular program responsible for sequestering cytoplasmic components and delivering them to the lysosome, is a highly evolutionarily conserved process that dates back to eukaryotic origins. Although initially thought to be simply responsible for the degradation of long-lived proteins and maintenance of high levels of cytoplasmic amino acids, many more intricate functions are now attributed to autophagy. There are three classes of autophagy, all of which play crucial roles in healthy cellular homeostasis. The first, macroautophagy, is by far the best characterized, involving the *de novo* synthesis of a double-membrane bound compartment to engulf large portions of cytoplasm, and hijacking the endosomal maturation pathway to deliver its contents to the lysosome. The second, microautophagy, is less well understood, and involves lysosomal membrane invagination to engulf very proximal portions of cytoplasm. Finally, chaperone-mediated autophagy involves direct import of soluble proteins into the lysosome without the need for complex membrane maturation, through the action of cytoplasmic chaperones and autolysosomal receptors. In all three cases, the resulting membranes and macromolecules are degraded by the panoply of lysosomal hydrolases and recycled back into the cytoplasm for future use.[1] In this way, cytoplasmic components can be recycled, energy conserved, and dangerous or aged organelles, or even whole pathogens, can be selectively removed.[2] Since most literature focuses on the consequences and substrates of macroautophagy, or components common to all three, this review will be largely limited to macroautophagy (hereafter 'autophagy').

Mammalian autophagy genes are conserved from yeast (termed *Apg* in yeast, and *Atg* in higher eukaryotic systems), and many of the mammalian homologues have been deduced.[3] Negative regulation of this system is often attributed to the action of mammalian target of rapamycin (mTOR), under nutrient-rich conditions. Under periods of starvation, serum withdrawal or other stresses, the GTPase activates protein TSC2 inhibits Ras homolog enriched in brain and mTOR activity, and consequentially activating the serine/threonine kinase Atg1.[4,5] However, the direct substrates of Atg1 remain to be determined. Also, in most models, including *Saccharomyces cerevisiae*, the action of the class III PI(3)K, Vps34 and Atg6 (Beclin-1 in mammals), is required to specify the membrane that will form the nascent autophagosome[6] and for the maturation of the autophagosome toward fusion with the lysosome.[7] After autophagy has been induced and membrane specified, two ubiquitin-like conjugation systems are required for the elongation, septation and maturation of autophagosomes. The first involves the action of the E1 ubiquitin ligase-like protein Atg7 priming and transferring Atg12 to Atg5 through the E2 ubiquitin ligase-like protein Atg10, and finally covalently attaching to Atg16L, which forms a tetramer on the autophagosomal membrane. The second again involves Atg7 conjugating Atg8 (LC3), through the E2 ubiquitin ligase-like Atg3 to phosphatidyl-ethanolamine in the expanding autophagosome.[8] Finally, the autophagosome matures, fuses with endosomes to become amphisomes, and acidifies or fuses with lysosomes to become autolysosomes.

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AUTOPHAGY IN T LYMPHOCYTES

Given that resting naive T lymphocytes have limited cytoplasm, it is a surprise to observe that these cells not only express autophagy genes, such as *LC3*, *Atg5*, *Atg7* and *Beclin-1*, but also have basal levels of autophagy.[9,10] This autophagy was strongly induced by stimulation through the T-cell antigen receptor (TCR), and to a lesser extent by common-gamma chain cytokine stimulation and serum starvation (Table 1).[9,10] This is relevant because lymphocytes split their time between cytokine-replete secondary lymphoid organs, serum-rich vasculature, and a lymphatic system that is relatively devoid of such homeostatic factors. T lymphocytes are especially sensitive to death signals and antiapoptotic molecules, such as Bcl-2 and Bcl-Xl, and Mcl-1 are highly regulated during lymphocyte development.[11–13] Therefore, it is not surprising that pathways, such as cytokine-receptor signaling, which are known to maintain lymphocyte homeostasis, also induce autophagy.[14,15] Autophagy, however, seems to be especially important for the homeostasis of T lymphocytes. In the absence of the core autophagy proteins Atg5 and Atg7, both $CD4^+$ and $CD8^+$ T lymphocytes still undergo full functional maturation. However, they rapidly succumb to apoptosis in the periphery, have reduced numbers in secondary lymphoid organs and fail to proliferate in response to TCR stimulation.[10,16]

Autophagy has been proposed to function as either a pathway to recycle macromolecules, or removing damaged organelles and unfolded proteins from the cell. These tasks are not mutually exclusive. Using a T-cell-specific deletion of the *Atg7* gene, we have shown that mitochondria, which are usually cleared when single-positive thymocytes egress and transition to secondary lymphoid organs, persist in autophagy deficient T cells.[16] In further supporting this observation, autophagy deficiency in T lymphocytes results in enrichment of genes encoding proteins associated with the mitochondrion.[17] This aggregation of mitochondria, a portion of which may be depolarized, leads to an increase in reactive oxidative species, which have been shown to be toxic to T cells.[16,18,19]

Interestingly, autophagy has been proposed as both a mechanism for cell survival and homeostasis,[20–22] and an alternative form of cell death.[23,24] In T lymphocytes there is evidence for both functions as well. Autophagic cell death appears to occur during cytokine withdrawal-induced cell death of resting cells,[9] and proliferating cells lacking Fas-associated protein with death domain die a potent autophagic cell death that is inhibited by a knockdown of Atg7 or pharmacological inhibition of Vps34.[25] Furthermore, in the absence of Fas-associated protein with death domain, a hyperactivated autophagy program without caspase activation, but dependent on receptor (TNFRSF)-interacting serine-threonine kinase 1, leads to marked T-lymphocyte death.[26] However, at steady state, naive lymphocytes critically depend on autophagy for homeostasis and resistance to cell death from a variety of stresses.[10,16,17]

Some of the paradoxical evidence about the role of autophagy in T-lymphocyte homeostasis comes from HIV models. Though HIV causes massive $CD4^+$ T-cell depletion of infected cells over an extended period of time, it is well established that the death of uninfected 'bystander' cells is also important for the pathogenesis of this disease.[27] From the perspective of the pathogen, this is entirely logical, as uninfected T cells could organize an effective response against HIV, but infected $CD4^+$ T cells provide an attractive citadel for the virus, in which it could integrate into the host genome and lay dormant. What is striking about this collateral death in uninfected cells is that it is induced by the envelope glycoprotein gp41, and coincides with a global induction of autophagy.[28] This cell death is also inhibited with pharmacological inhibition of autophagy by 3-MA or by knockdown of Beclin-1 or Atg7.[29,30] Additionally, this death requires binding of HIV glycoproteins to CD4 and CXCR4, but requires no signaling from CD4, as a truncated form of the receptor was sufficient to induce autophagy.[29] However, in many models of viral infection, autophagy is protective to the infected cell,[31] and provides a means to present endogenously derived viral antigens to immune surveillance.[32–35] Indeed, in HIV-infected macrophages, viral load is contained in those cells that have induced potent levels of autophagy, but still detectable in those macrophages that have only slightly increased autophagy.[36] Thus, autophagy in $CD4^+$ T lymphocytes in response to HIV infection remains a highly complex issue, since high levels of autophagy needed to effectively control the virus are effectively killing the cells that could truly combat this pathogen.

Most of the research so far has concentrated on the role of autophagy in naive, resting T cells, or survival in proliferating T cells. However, autophagy may also be involved in a functional T-cell response. Li *et al.* demonstrated a higher level of autophagy in Th2-polarized cells by analysis of green fluorescent protein tagged LC3 punctate formation.[9] Though the reason for this is still not well understood, it could underlie an unknown function in B-cell help, or represent a higher amount of restructuring that a naive $CD4^+$ cell must

Table 1 Initiation and function of autophagy in lymphocytes and TECs

Function	*Evidence*	*References*
Induction of autophagy in T cells	Autophagy induced through TCR stimulation, cytokine stimulation and serum withdrawal	9, 10
Induction of autophagy in B cells	Autophagy induced through BCR stimulation, inhibited by CD28 costimulation	46, 47
Autophagy in TECs	Autophagy is constitutively high in cTECs and mTECs	42
Autophagy functions as prosurvival	Atg5- and Atg7-deficient T lymphocytes, pre- and immature B cells, and B-1 B cells succumb to apoptosis and have proliferation defects	10, 16, 45
Autophagy functions as alternative death pathway	Autophagy contributes to RIPK, IFN-γ induced, BCR-induced and HIV envelope glycoprotein-induced cell death	9, 25, 28, 37, 46
Autophagy clears organelles and toxic metabolites	Atg5- and Atg7-deficient T lymphocytes have accumulated damage from excess mitochondria and ROS	16, 17
Autophagy functions in T- and B-cell costimulation	Autophagy induced through the BCR helps B-cell antigen presentation, which is necessary to obtain T-cell help	46, 47
Autophagy in TEC functions in T-cell selection	Atg5-deficient cTECs and mTECs allow development of self-reactive T lymphocytes, colitis and wasting disease	42

Abbreviations: BCR, B-cell receptor; cTEC, cortical thymic epithelial cell; IFN-γ; interferon-γ; mTEC, medullary thymic epithelial cell; RIPK, receptor-interacting serine/threonine protein kinase; ROS, reactive oxidative species; TCR, T-cell antigen receptor; TEC, thymic epithelial cell.

undergo to become a Th2 cell as opposed to a Th1 cell. It might also be protective, if Th1 and Th2 cells have different thresholds for stress or propensities for apoptosis.

Autophagy has been proposed as a protective measure during periods of stress and rapid change in environmental conditions.[22] Inflammation resulting from conditions that also engender a Th1 response is one such stress. It appears that regulation of autophagy has also evolved as a mechanism to protect differentiating Th1 cells under such conditions. Feng *et al.* have described a mechanism by which the interferon-inducible immunity-related GTPase family M member 1 protects effector $CD4^+$ T cells from interferon gamma-induced death.[15,37] The authors describe this death as autophagic in nature, that is, ablated when interferon-γ is genetically deleted.[15,37] This highlights the importance of a highly regulated autophagic response in activated T lymphocytes, as genetic deletion of immunity-related GTPase family M member 1 may mimic the disregulation of autophagy seen in 'bystander' HIV-induced $CD4^+$ autophagic cell death discussed above.

Much has been made of the signals that induce autophagy in many systems. Limited studies have shown several molecules that are important for autophagy induction in T cells. c-jun amino-terminal kinase and Vps34 are important for the induction of autophagy in response to TCR stimulation, as genetic deletion of c-jun amino-terminal kinases and pharmalogical inhibition of Vps34 by 3-MA reduced the amount of punctate green fluorescent protein tagged LC3 in Th1 cells.[9] However, in a study by Arsov *et al.*, high levels of Beclin-1 in developing thymocytes were shown to increase sensitivity to glucocorticoid-induced cell death, and very low levels of Beclin-1 did not sensitize thymocytes to death *in vitro*.[38] This might simply highlight Beclin-1's autophagy-independent role as a tumor suppressor by regulation of Bcl-2 function.[39] In concurrence with previous data, Beclin-1 is upregulated upon TCR stimulation in mature T lymphocytes.[38] However, given that low expression of Beclin-1 has little effect on cell survival of T-lineage cells, and that autophagy is critical for the survival of naive T lymphocytes, this suggests that most autophagy is Beclin-1-independent in T lymphocytes.

AUTOPHAGIC CONTROL OF THYMOCYTE EDUCTION

Through thymocyte-independent mechanisms, autophagy plays a role in education of thymocytes to affect tolerence. Thymic epithelial cells (TECs) are essential to support thymic development. While cortical TECs are important for positive selection of double-positive thymocytes,[40] medullary TECs are the lynchpin for negative selection by expression of tissue specific antigens through the expression of AIRE.[41] When Atg5 null TECs were engrafted into nude mice, thymocyte development was seemingly normal in terms of thymocyte number. However, these thymocytes had defects in both positive and negative selection, despite normal expression of autoimmune regulator and T cells from these mice could recapitulate a massive wasting disease with colitis and lung infiltration.[42] Thus, it appears that autophagy is important for optimal expression of MHC–peptide complexes in TECs for proper positive and negative selection of MHC-restricted thymocytes. The authors propose that the unusually high levels of constitutive autophagy in TECs are important for delivering the necessary levels of endogenously derived peptides to MHCs for the selection processes.[42–44] This function might augment the already unusual repertoire of proteases seen in cortical TECs, showcasing autophagy's function as a method for generating peptides, as well as its role in MHC loading through endogenous presentation.[44]

AUTOPHAGY IN B LYMPHOCYTES

Recent studies have also revealed important roles for autophagy in B lymphocytes. Using Atg5-deficient fetal liver chimeras, we found that splenic B-cell compartments are reduced in adult mice. Additionally, these Atg5-deficient splenic B cells had higher rates of apoptosis than wild-type counterparts, though not nearly to the level seen in $Atg5^{-/-}$ T cells.[10] Interestingly, this apoptosis, and hence the need for autophagy in B cells, was highly developmentally stage-specific. Although there was no significant defect in pro-B cells in an $Atg5^{-/-}$ chimeric system, the levels of pre- and immature B cells were drastically impacted.[45] These reductions were, as with T cells, linked to an increased apoptotic phenotype, although B cells were somewhat able to populate the peripheral lymphoid organs. Also, B-1 B cells in the peritoneum were markedly reduced.[45] The stage-specific requirements for this autophagy, or perhaps even autophagy-independent function of Atg5, could give important insights into the cytoplasmic remodeling that occurs during B-cell development. When Atg5 was specifically deleted in developing B cells using a *CD19-cre* transgene, the outcome was drastically different. There was no detectable phenotype in either developing or mature B cells, and only self-renewing B-1 cells were impacted by the loss of Atg5.[45] This would seem to indicate that autophagy is really only necessary at the earliest stages of B-cell development. This is very different from what has been observed in T cells, in which a constitutive autophagy is strictly necessary for naive homeostasis.[10] Further research is needed to investigate the roles of autophagy in a functional B-cell response *in vivo*.

There has also been some investigation as to signals that regulate autophagy in B cells. In studies using green fluorescent protein tagged Beclin-1, expression of Beclin-1 was very high in pro-B cells, but highly downregulated at the pro- to pre-B cells transition.[38] This is correlated with the fact that Atg5 is dispensable when deleted later in B-cell development, but required for early stages and self-renewing populations of B cells.[45] Furthermore, Beclin-1 was expressed in mature B cells, but less so in immature (IgD^{lo}) cells.[38] More recently, Watanabe *et al.* have shown that autophagy is induced in splenic B cells and B-cell lines after prolonged-B-cell receptor (BCR) stimulation through IgM.[46] This autophagy was concurrent with activation-induced apoptosis, and was readily inhibited by CD40 ligation, perhaps through the induction of Bcl-2.[46] Interestingly, this autophagy was also largely inhibited by the addition of wortmannin, such that it appears that the Vps34/Beclin-1 complex has a role in autophagy induction in B lymphocytes. An interesting hypothesis was supplied by the authors, linking BCR activation, autophagy and priming for T-cell 'help'. Since BCR activation is linked to autophagosome formation, and autophagosome formation is necessary for B-cell antigen presentation to T cells, which in turn provide CD40L costimulation, then autophagy links antigen-receptor stimulation to costimulation in B cells.[47] It remains to be seen if more conventional autophagy inducers, such as serum withdrawal or rapamycin, have any effect on autophagy in B cells. Also, do G-protein-coupled receptors or cytokine receptors affect autophagy in a prosurvival way, or is autophagy in B lymphocytes more geared towards a death-effector pathway?

CONCLUDING REMARKS

Autophagy in lymphocytes, especially in T cells, is an indispensable pathway for both immune homeostasis and the generation of a functional immune response. The fact that the autophagic pathway is not only intact, but so vital for homeostasis in immune cells displays the versatility of autophagy. So far, it appears that all of the core components of autophagy are intact in lymphocytes. Further research needs

to be done to determine whether different signals, such as cytokine signaling, serum and amino-acid deprivation, and antigen-receptor stimulation all integrate to initiate autophagosome formation. Perhaps most importantly, is autophagy during periods of rapid change, such as during the initiation of an immune response, a pro-survival pathway or a death pathway to select for the most fit cells?

Much work that has been done seems to point to autophagy being a double-edged sword. Under homeostatic conditions, regulated autophagy can ensure the survival of naive lymphocytes. However, during transition from naive to effector phase in a functional immune response, autophagy is regulated from a host of signals, many of which are still being elucidated. In this case, autophagy induction seems to put lymphocytes one step closer to death, perhaps until further pro-survival signals are received. In the case of B lymphocytes, this would be CD40L ligation. In this manner, autophagy seems to play a role in preventing aberrant immune responses by priming cells for autophagic death in the absence of costimulation.

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