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Lack of autophagy in the hematopoietic system leads to loss of hematopoietic stem cell function and dysregulated myeloid proliferation

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The regulated lysosomal degradation pathway of autophagy prevents cellular damage and thus protects from malignant transformation. Autophagy is also required for the maturation of various hematopoietic lineages, namely the erythroid and lymphoid ones, yet its role in adult hematopoietic stem cells (HSCs) remained unexplored. While normal HSCs sustain life-long hematopoiesis, malignant transformation of HSCs or early progenitors leads to leukemia. Mechanisms protecting HSCs from cellular damage are therefore essential to prevent hematopoietic malignancies. By conditionally deleting the essential autophagy gene *Atg7* in the hematopoietic system, we found that autophagy is required for the maintenance of true HSCs and therefore also of downstream hematopoietic progenitors. Loss of autophagy in HSCs leads to the expansion of a progenitor cell population in the bone marrow, giving rise to a severe, invasive myeloproliferation, which strongly resembles human acute myeloid leukemia (AML).

Key words: acute myeloid leukemia, autophagy, myeloproliferation, hematopoietic stem cells, mitophagy, reactive oxygen species, hematopoietic progenitors, Atg7, hematopoietic malignancy

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Autophagy is the cell's major regulated pathway for lysosomal degradation. Autophagy takes place at basal levels but is also regulated developmentally and/or by environmental stimuli, such as nutrient/energy availability, hypoxia and reactive oxygen species (ROS). We had previously shown that mice conditionally knocked out for the essential autophagy gene *Atg7* in the hematopoietic system (*Vav-iCre*$^{+/-}$*; Atg7*$^{Flox/Flox}$: named Vav-Atg7$^{-/-}$ mice) develop progressive anemia and lymphopenia accompanied by splenomegaly and lymphadenopathy before dying at an average age of 12 weeks. Although the anemia of Vav-Atg7$^{-/-}$ mice is caused by the absence of mitochondrial autophagy (mitophagy), the mechanism proven responsible for the degradation of mitochondria in developing red blood cells, the progressive nature of their anemia and the ultimate cause of death remained unexplained. We hypothesized that a defect in the maintenance of primitive hematopoietic cells could be responsible for the cytopenias of Vav-Atg7$^{-/-}$ mice worsening over time. We therefore set out to study the integrity of *Atg7*$^{-/-}$ HSCs and investigate the cause of death of Vav-Atg7$^{-/-}$ mice.

After confirming uniform *Atg7* mRNA levels throughout the wild-type (WT) hematopoietic stem and progenitor cell (HSPC) compartment, the function of *Atg7*$^{-/-}$ HSPCs was investigated by serial replating of colony-forming cell assays, which revealed the inability of autophagy-deficient HSPCs to form secondary colonies. As this suggested a self-renewal defect in *Atg7*$^{-/-}$ HSPCs, a series of in vivo competitive and noncompetitive repopulation assays were performed to further investigate the function of *Atg7*$^{-/-}$ HSPCs. In competitive repopulation assays *Atg7*$^{-/-}$ bone marrow (BM) cells were outcompeted by WT BM cells and did not participate in the hematopoietic reconstitution of lethally irradiated recipients. Vav-Atg7$^{-/-}$ BM cells also failed to rescue

lethally irradiated hosts in noncompetitive repopulation assays. In order to prevent confounding effects from the underlying anemia found in the BM of Vav-Atg7$^{-/-}$ mice, Lin$^-$Sca-1$^+$c-Kit$^+$ (LSK) cells—containing HSPCs—were purified from either WT or Vav-Atg7$^{-/-}$ mice and used in noncompetitive repopulation assays. This led to death of the lethally irradiated recipients of *Atg7*$^{-/-}$ LSK cells within weeks of transplantation, suggesting that the loss of HSC functions in the absence of Atg7 is cell intrinsic. Finally, noncompetitive repopulation assays with fetal liver (FL) cells revealed that *Atg7*$^{-/-}$ FL cells, unlike *Atg7*$^{-/-}$ BM cells, can rescue lethally irradiated recipients in half of the cases, suggesting that Atg7 is less essential for fetal than adult HSC functions or that the HSC defect resulting from the loss of autophagy becomes exacerbated over time. The differential requirement of autophagy in fetal and adult hematopoiesis therefore remains to be resolved.

To understand the lack of HSC function observed in Vav-Atg7$^{-/-}$ mice, their HSPC compartment was also investigated immunophenotypically. Consistent with the functional readouts, this revealed that true HSC (LSK CD150$^+$ CD48$^-$) numbers are severely reduced, despite an overall expansion of the Vav-Atg7$^{-/-}$ LSK compartment. The myeloid and lymphoid progenitor compartments are also significantly reduced in the BM of Vav-Atg7$^{-/-}$ mice. Notably, as all of the clearly defined progenitor populations were investigated, it remains unclear what cell type accounted for the expanded BM LSK compartment of Vav-Atg7$^{-/-}$ mice. As found previously in the erythrocytes and T lymphocytes, it is is not surprising that the expanded *Atg7*$^{-/-}$ LSK compartment displays an increased mitochondrial load with higher mitochondrial membrane potential as a consequence of impaired mitophagy. This mitochondrial accumulation in turn leads to increased mitochondrial ROS and DNA damage along with higher levels of both apoptosis and proliferation.

To investigate the cause of death of Vav-Atg7$^{-/-}$ mice histopathological analyses were performed and revealed the presence of a myeloid cell mass in one of the Vav-Atg7$^{-/-}$ mice examined, severe myeloid dysplasia in hematopoietic organs and widespread infiltration of myeloid blast cells in non-hematopoietic organs in all Vav-Atg7$^{-/-}$ mice. This myeloproliferation can also be detected by flow cytometry where, unlike all other mature lineages, CD11b$^+$Gr1$^+$ cells are significantly increased in the blood and BM of Vav-Atg7$^{-/-}$ mice. These cells are highly proliferative and have increased expression of the myeloid leukemia marker CD47 on their surface. Importantly, histopathological analysis of the lethally-irradiated hosts reconstituted with Vav-Atg7$^{-/-}$ BM, FL and LSK cells also reveals the myelodysplastic/myeloproliferative features originally found in Vav-Atg7$^{-/-}$ mice, suggesting that this myeloproliferation is transplantable and that the population responsible for it is found within the expanded LSK compartment of Vav-Atg7$^{-/-}$ mice. The myeloproliferation could also be reproduced by transplantation of Vav-Atg7$^{-/-}$ BM cells into immunocompromised hosts, however, without any appearance of symptoms or lethality in these secondary hosts.

Overall, histological analyses of Vav-Atg7$^{-/-}$ mice strongly suggested that they suffer from myeloid leukemia and consequently die of BM failure. This would be consistent with the fact that Vav-Atg7$^{-/-}$ mice show a range in disease progression and time of death, which in turn suggests that a stochastic event is responsible for the final steps of their disease. Such an event could be speculated to be the malignant transformation of a progenitor cell, found within the LSK compartment and induced by the phenotypic characteristics resulting from the lack of autophagy/mitophagy, namely mitochondrial accumulation leading to oxidative stress, DNA damage, uncontrolled proliferation and dysregulated activation of cell death.

In conclusion, this study is the first to establish the requirement for autophagy in the maintenance of adult HSCs, as well as the first to provide evidence suggesting that autophagy protects against leukemia development. Future studies will have to be performed to understand why the *Atg7*$^{-/-}$ myeloid leukemia does not kill secondary hosts. Yet, one could speculate that autophagy is required by the malignant cells, once the leukemia is established, for survival and proliferation in stressful conditions, which could explain the nonaggressive nature of the autophagy-deficient leukemia. It has been proposed that inhibitors of autophagy, such as chloroquine, should be used in cancer treatment, and it would therefore be of great interest to uncover whether altered autophagy levels are found in human cancer stem cells. Finally, it remains to be shown whether a role for autophagy in the maintenance of healthy stem cells can be extended to stem cells in tissues other than the hematopoietic system.