Acute myeloid leukemia stem cell function is preserved in the absence of autophagy

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Autophagy is a complex cellular process that regulates the processing and degradation of intracellular proteins and organelles. Within the hematopoietic system, autophagy has diverse roles and is required for hematopoietic stem cell (HSC) function, differentiation into erythroid or T-lymphoid lineages and response to extracellular cytokine signaling. In blood cancers such as acute myeloid leukaemia (AML), modulating autophagy has been proposed as a novel therapy to treat AML. Leukemia stem cell (LSC) populations initiate and maintain leukemia in vivo. We have investigated autophagy in AML and demonstrate that autophagic flux is activated in the transformation from HSCs to LSCs. The essential autophagy effector Atg7 is required for the efficient initiation of AML. However, upon transformation, LSCs adapt to overcome this requirement and autophagy is not required for LSC function in vivo.

AML is an aggressive and rapidly lethal blood cancer, characterized by the accumulation of immature leukemic blasts in the bone marrow and blood, leading to organ infiltration and failure of normal hematopoietic function. AML is maintained by a discrete stem cell population, known as LSCs that initiate, maintain and serially propagate leukemia, while retaining the ability to partially differentiate into more committed progeny. Moreover, these cells may remain dormant during chemotherapy treatment, providing a resistant pool of self-renewing cells that give rise to relapse in patients. LSCs are highly enriched within the immunophenotypic granulocyte-macrophage progenitor (GMP) compartment, although this may vary between models. There has been a concerted effort to develop therapeutic agents that target LSC populations to reduce relapse and improve survival after treatment for AML.

Recent publications have highlighted the key role of autophagic signaling in cancer biology and treatment. Autophagy is a complex multistep process that facilitates the processing and recycling of cellular components such as proteins and organelles to maintain cellular homeostasis. Autophagy is induced by nutrient deprivation, cytokine-induced cellular stress and certain drugs (such as rapamycin). Upon membrane isolation, the central regulatory protein Atg7 activates a heterotrimeric complex of Atg5, Atg12 and Atg16L1 to facilitate lipidation of LC3 to LC3-II and incorporation into a vesicular autophagolysosome. The final step of autophagy involves lysosomal fusion, resulting in the degradation and recycling of cytoplasmic contents. Atg7 is an essential effector of canonical autophagy and genetic deletion of Atg7 results in loss of LC3 lipidation and paralysis of autophagic
flux. Similarly, loss of other critical regulators such as Atg5 result in impaired autophagic flux. Autophagy regulates several important processes in the hematopoietic system including HSC maintenance, cytokine responsiveness and T-cell homeostasis.

We first sought to document the change in autophagic flux on leukemic transformation of normal stem cell populations using a well characterized retroviral model of MLL-AF9-induced AML, the most common genetic mutation found in infant AML and associated with poor prognosis. MLL-rearranged leukemia is commonly associated with secondary AML, and is also associated with poor prognosis in that context. Leukemic bone marrow (BM) showed stabilization of Atg7 together with enrichment of LC3-II, indicative of activated autophagic flux. We have observed further stabilization of LC3-II with the in vitro use of Bafilomycin A or chloroquine (data not shown), although these agents are not effective in vivo. To determine whether autophagy was functionally relevant in AML, we utilized VavCre:Atg7fl/fl (deletion of autophagy in all hematopoietic cells including HSCs), LysMCre:Atg7fl/fl (deletion of autophagy in all myeloid cells commencing at the GMP stage) and Cre-negative Atg7fl/fl controls. All mice were backcrossed at least 10 generations to pure B6 background. Freshly transduced MLL-AF9 cells derived from VavCre:Atg7fl/fl BM showed fewer leukemic colonies in methylcellulose, however this difference was lost with serial passage. VavCre:Atg7fl/fl cells were more apoptotic. In all cases, LysMCre:Atg7fl/fl cells were similar to Cre-negative controls. These data suggest that Atg7 expression within stem cells is required for efficient leukemia initiation, but does not affect in vitro self-renewal. We therefore performed transplantation assays to test for a requirement of Atg7 on in vivo AML generation. Consistent with the in vitro data, VavCre:Atg7fl/fl cells, but not LysMCre:Atg7fl/fl cells had delayed AML latency compared to controls. In vivo, there was reduced expansion of the leukemic clone evidenced by lower AML cell count, however at the time of disease onset, all groups had similar leukemia burden.

Finally, we demonstrated active autophagy in LysMCre:Atg7fl/fl AML cells together with Atg7 protein expression. Conversely, Atg7 was not expressed in VavCre:Atg7fl/fl cells and there was complete abrogation of LC3-II generation, consistent with absent autophagic flux in these leukemia cells. No difference in DNA damage or cell cycle was noted in VavCre:Atg7fl/fl vs. Atg7fl/fl cells, confirming the results of Chen et al. These data are consistent with recent publications, and demonstrate that Atg7 is required for the efficient generation of AML in vivo, however we note that AML penetrance was 100% even in the complete absence of autophagic flux, suggesting that full leukemic crisis may be sufficient to overcome this requirement for autophagy. Moreover, although LysM expression is active in GMPs in normal hematopoiesis, LysMCre does not efficiently excise Atg7 within LSC populations and should not be used to test gene function in AML LSC models.

Most patients with AML respond to initial chemotherapy treatment, however clinical relapse is common and this is thought to be mediated by residual LSC populations that persist during treatment. Serial bone marrow transplantation of AML provides a model to test the function of these LSCs in vivo. We isolated viable AML from VavCre:Atg7fl/fl vs. controls and transplanted into sublethally irradiated (5.5Gy) syngenic B6 recipients. Recipients of VavCre:Atg7fl/fl AML cells had similar disease latency, in vivo expansion and leukemia burden to controls. To examine this in an independent system, HSC from Atg5-/- chimeras were co-transduced with BCR-ABL and NUP98-HOXA9 to model myeloid blast crisis of chronic myeloid leukemia. No difference was noted in LSC function as defined by secondary leukemia transplantation or leukemia burden in transplanted recipients. LSC numbers were similar in autophagy deficient leukemias. As mature myeloid cells may activate alternative pathways of autophagy, presumably to compensate and overcome a putative requirement for autophagy, we examined alternative autophagic signaling in VavCre:Atg7fl/fl recipients compared to controls. There were no differences in the expression of Ulk1, Atg6 or Vps34, effectors of alternative autophagy in VavCre:Atg7fl/fl leukemias. These data demonstrate that autophagic flux is not required for LSC maintenance in vivo and do not support the hypothesis that selective pressure activates alternative pathways of autophagy in AML.
These data demonstrate that the complete genetic loss of Atg7, resulting in paralysis of autophagic flux, contributes to induction of AML but is dispensable for LSC function in vivo. Other factors including impaired engraftment, proliferation or altered apoptosis may all contribute to the delayed induction of leukemia. These results add to, and clarify, recent publications detailing a potential requirement of autophagy in AML\textsuperscript{12}. In particular, some important technical differences between studies may potentially explain the apparent discrepancies. Conditional Atg7 deletion using tamoxifen to induce Cre expression (using the Rosa26-CreERT and compared to saline controls) slowed the progression of MLL-ENL AML and also had modest effects on secondary transplantation\textsuperscript{8}. This study used MLL-ENL, an oncogene that is most frequently associated with human acute lymphoblastic leukemia\textsuperscript{14}. We note that tamoxifen therapy has been previously shown to retard progression of AML in MLL-ENL, even in the absence of gene deletion\textsuperscript{15}. Liu \textit{et al.}, also demonstrate the requirement of autophagy in MLL-AF9 AML initiation through deletion of different pathway regulator, Atg5. In this work, autophagy loss did not affect LSC function as measured by secondary transplantation nor on response to chemotherapy\textsuperscript{7}. Furthermore, in an elegant set of experiments using human primary AML samples and AML cell lines, Piya \textit{et al.} demonstrated that ATG7 was not required for human AML maintenance, however autophagic flux was increased by chemotherapy and shRNA depletion of ATG7 enhanced the effects of chemotherapy\textsuperscript{16}. Importantly, these data support and externally validate the recent findings of Chen \textit{et al.}, demonstrating that autophagy is dispensable for LSC function in AML\textsuperscript{12}. It is not clear whether these findings are generalizable to all molecular subtypes of AML, however we have observed similar findings in two independent model systems.

Altogether, these data caution against the use of agents targeting autophagy as a potential novel therapy to target LSCs in AML. However, the use of such agents to potentiate the effects of chemotherapy may be efficacious in some settings. Additionally, modulation of autophagy is likely to have several deleterious effects such as diminishing G-CSF response\textsuperscript{6}, impairment of normal HSC function\textsuperscript{2} and altering T-cell homeostasis\textsuperscript{5}.

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References

Figure legends:

**Figure 1. Autophagy is activated during leukemic transformation. Loss of autophagic flux causes a reduction in clonality and delays leukemia onset VavCre;Atg7^{fl/fl} MLL-AF9**

(A) Western blot comparison of Atg7 and LC3-I and LC3-II expression levels in primary bone marrow cells isolated from MLL-AF9 and WT mice (n=4)

(B) Colony forming assay of WT and VavCre; Atg7^{fl/fl} models of MLL-AF9. Represented as number of colonies per input of cells (%) represented as median. (n=7-8)

(C) Representative flow cytometry plots from in vitro cells using Annexin V and Sytox. (D) Early and (E) late stage apoptosis (n=3-4) of WT, LysmCre:Atg7^{fl/fl} and VavCre; Atg7^{fl/fl} models of MLL-AF9.

(F) Kaplan-Meier survival curve of recipient mice injected with MLL-AF9-GFP transduced bone marrow (input 8x10^5 cells/ mouse, n=8-9 recipients)

(G) Leukemia burden of transplanted mice represented as the number of GFP +ve white blood cells in the peripheral blood 6 or 8 weeks after transplantation (n=7-10)

(H) The spleen weight harvested from primary recipients at AML onset (n=3-8).

(I) Protein expression levels of Atg7, LC3-I and LC3-II isolated from MLL-AF9 spleen cells after in-vivo transplantation in mice (n=4).

(J) Quantification of the percentage of γH2AX positive cells in the G0/G1 stage of the cell cycle via flow cytometry (n=5-6)

(K) Analysis of the percentage of LSCs in each stage of the cell cycle using flow cytometry (n=5-6)

All data are mean plus individual dots represent biological replicates. Statistical significance calculated using non-parametric Kruskal Wallis one-way analysis of variance with Dunn’s correction for multiple testing corrections (Prism V7.0, Graphpad).

**Figure 2. Autophagy is not required for LSC function**

(A) Survival of mice injected with 1x10^5 AML cells derived from primary, transplanted mice (n=7-8)

(B) Leukemia burden of transplanted mice represented as the number of GFP +ve white blood cells in the peripheral blood at week 2 (n=5) and (C) week 3 after transplantation (n=7-8)

(D) The spleen weight and bone marrow cellularity (two femur and tibia) of mice at end point analysis (n=7-8)

(E) Survival analysis of secondary transplantation of 5x10^5 BCR-ABL/NUP98-HOXA9 transduced cells harvested from primary recipients (n=4-5)

(F) The percentage of GFP(+) cells and WBC in the peripheral blood of BCR-ABL/NUP98-HOXA9 secondary recipients

(G) The frequency of LSC’s in the bone marrow of mice injected with primary MLL-AF9 at time of disease onset (n=7)

(H) Relative expression levels of alternative autophagy genes by quantitative real time PCR (n=6-7) and (I) western blot n=3

Data represent 2 independent experiments with pooled data. Each experiment produced similar results. Statistical significance calculated using non-parametric Kruskal Wallis one-way analysis of variance with Dunn’s correction for multiple comparisons, except where noted.