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LC3-associated phagocytosis in bone marrow macrophages suppresses acute myeloid leukemia progression through STING activation

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Running Title: LAP suppresses AML tumour burden

The authors declare no competing interests

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Abstract
The bone marrow (BM) microenvironment regulates acute myeloid leukemia (AML) initiation, proliferation and chemotherapy resistance. Following cancer cell death, a growing body of evidence suggests an important role for remaining apoptotic debris in regulating the immunologic response to, and growth of, solid tumors. Here we investigated the role of macrophage LC3-associated phagocytosis (LAP) within the BM microenvironment of AML. Depletion of BM macrophages increased AML growth in-vivo. We showed that LAP is the predominate method of BM macrophage phagocytosis of dead and dying cells in the AML microenvironment. Targeted inhibition of LAP led to accumulation of apoptotic cells (AC) and apoptotic bodies (AB) resulting in accelerated leukemia growth. Mechanistically, LAP of AML derived-AB by BM macrophages, resulted in STING pathway activation. We identified that AML derived mitochondrial damage associated molecular patterns were processed by BM macrophages via LAP. Moreover, depletion of mitochondrial DNA (mtDNA) in AML derived-AB showed that it is this mtDNA which was responsible for the induction of STING signalling in BM macrophages. Phenotypically we found that STING activation suppressed AML growth through a mechanism related to increased phagocytosis. In summary, we report that macrophage LAP of apoptotic debris in the AML BM microenvironment suppressed tumor growth.
Introduction

Acute Myeloid Leukemia (AML) is a genetically, biologically and clinically heterogeneous set of diseases, which share in common the malignant proliferation of clonal haematopoietic stem and progenitor cells within the bone marrow (BM) microenvironment (1). Cure can be achieved in a minority of patients with AML, medically effected through either intensive cytotoxic chemotherapy or alternatively via an allo-immune mediated graft versus leukemia mechanism. Curiously for such a clinically aggressive, and often chemo-refractory disease, ex-vivo AML exhibits a high level of spontaneous apoptosis (2-6). Furthermore, relapse for patients who achieve therapy induced remission occurs from minimal residual disease sequestered within the BM microenvironment (7-9). Taken together, these observations illustrate the fundamental importance of the BM microenvironment in leukemia initiation, proliferation, and chemo-resistance, across the broad set of AML subtypes.

The BM microenvironment is a complex, highly organised organ evolved to support the life-long production of blood cells from hematopoietic stem cells (HSC). HSC reside within niches whereby their fate is regulated through interactions with multiple hematopoietic and non-hematopoietic cells, regulated by direct cell-cell contact, growth factors and cytokines (10-14). BM macrophages (BMM) have long been recognised to have diverse and indispensable, tissue specific roles in host defence and tissue homeostasis (15, 16). In the BM, macrophages contribute to the maintenance of the HSC niche, and their depletion results in egress of HSC into the peripheral blood (17). In addition, BMM are involved in processes regulating HSC quiescence and have been reported to negatively regulate the HSC pool in response to infection (18, 19). In cancer, pro-tumoral, tumor associated macrophages (TAM) appear to be fundamentally involved in cancer progression and metastasis, linking to poorer clinical outcomes, across a diverse spectrum of malignancies and tumor microenvironments (20-26). In AML, macrophages have been shown to interact with leukemic cells to promote AML progression, through mechanisms of action linked to phagocytosis and immune modulation (27-29). The expression of the transmembrane protein CD47, correlates with poor prognosis in AML, in part through inhibition of mononuclear phagocytosis of leukemia stem cells (LSC). In-vivo experiments using monoclonal antibody to block AML cell surface CD47 interaction with SIRPα on macrophages, enables phagocytosis of LSCs (30). This work which has since been extended into clinical trials (NCT04778397 and NCT04912063) (31, 32), provides a proof-of-concept paradigm for the therapeutic modulation of macrophage phagocytic function in the AML microenvironment.
Phagocytosis by mononuclear cells occurs by either an LC3 dependent, or independent, mechanism. LC3-associated phagocytosis (LAP) is physiologically triggered by pathogens and cellular debris via interaction with phagocyte cell surface receptors, including T-cell membrane protein 4 (TIM-4) and FcR. This results in a rapid response to phagosomal maturation and degradation of cargo (33). Though LAP shares components similar to canonical autophagy, LAP has been shown to be a distinct process (34). Impairment of LAP in myeloid cells has been shown to suppress solid tumor growth (35), however, the tumor specific roles and functions of LAP in blood cancers have not been defined. In the present study we investigate the role of LC3 mediated phagocytosis in the context of AML. We examine the mechanisms regulating BMM LAP in the leukemia microenvironment, and the outcomes of targeting the LAP pathway in models of AML.
Results

Depletion of phagocytes increases AML tumor burden

To establish the role played by phagocytes in AML we used clodronate liposomes (CL) to deplete phagocytic macrophages in a series of experiments using two AML in-vivo models: MEIS1/HOXA9 driven AML (36) containing a GFP and a luciferase reporter or MN1 driven AML (37) containing a GFP reporter which allowed for detection using in vivo imaging as well as measuring tumour burden by measuring GFP positive cells (Figure 1A). We used flow cytometry to determine frequency of BMM as previously described (38) (Figure 1B). Figure 1C shows successful BMM depletion in response to CL compared to control liposomal (CNT) treated animals. In vivo imaging of MEIS1/HOXA9 engrafted cells showed that animals depleted of phagocytic macrophages by CL had increased AML burden compared to CNT 23 days following MEIS1/HOXA9 injections (Figure 1D). No bioluminescence signal was detected in apoptotic MEIS1/HOXA9 (Figure 1E). Using a second model of mouse AML (MN1-GFP) we confirmed the observation that CL treatment results in increased AML tumor burden in the BM compared to control treated animals (Figure 1F-H and supplementary figure 1A and 1B). To determine if AML associated BMM exhibit increased phagocytosis compared to control BMM, we isolated the BMM (F4/80+ cells) from WT or MN1 engrafted animals and cultured them with Zymosan A bioparticles (Figure 1I). We found that BMM from AML engrafted animals had increased phagocytosis of the bioparticles compared to BMM from WT animals (Figures 1J and 1K). To evaluate whether phagocytosis involved LC3, we examined LC3 recruitment to phagosomes in BMM from MN1 engrafted animals, after incubation with Zymosan A bioparticles. Figures 1L and 1M show that phagosomes generated in BMM from MN1 engrafted animals recruited LC3.

AML disease progression is accelerated in LAP deficient animals

The recruitment of LC3 to phagosomes suggests a role for LAP during phagocytosis by BMM. To understand the role of LAP in the tumor microenvironment of AML, LAP-/- mice lacking the linker and WD domains of Atg16L1 (Supplementary Figure 2) (39), which are required for LAP but not for canonical autophagy (Atg16L1E230-), were injected with MN1 or MEIS1/HOXA9 cells. Animals were monitored for levels of tumor growth (Figure 2A). AML disease burden (by measuring GFP+ cells in the BM) was accelerated in Atg16L1E230- mice compared to WT controls (Atg16L1E230+) as determined at 14 and 20 days post injection with MN1 driven AML (Figure 2B). As expected, increased tumor growth in the LAP deficient animals was associated with decreased survival (Figure 2C). Similar results were seen using our MEIS/HOXA9 (by measuring GFP+ cells in the BM) driven AML model. There were more AML blasts in the BM of Atg16L1E230- animals, as well as decreased survival in Atg16L1E230-
mice compared Atg16L1<sup>E230+</sup> animals (Figure 2D-2F). To engraft MN1 we condition the mice with sublethal dose of busulfan. To determine if busulfan changes BM cellularity we treated Atg16L1<sup>E230+</sup> mice compared Atg16L1<sup>E230+</sup> animals with busulfan alone. No significant changes in BM cellularity were observed (supplementary figure 3). As a second approach we used conditional targeting in macrophages and granulocytes using lysozyme M-Cre-lox recombination to generate Atg16L1<sup>E230f/f</sup> Cre<sup>+</sup> animals. MN1 cells were injected into Atg16L1<sup>E230f/f</sup> Cre<sup>+</sup> or Atg16L1<sup>E230f/f</sup> Cre<sup>+</sup> mice. AML tumour burden was increased in Atg16L1<sup>E230f/f</sup> Cre<sup>+</sup> mice compared to Atg16L1<sup>E230f/f</sup> Cre<sup>-</sup> (Figure 2G, 2H and supplementary figure 1C). To determine if LAP is important in human AML, we examined LC3 density in isolated CD14<sup>+</sup> cells. Data show that LC3 density is higher in CD14<sup>+</sup> cells from AML patient compared to control (Figure 2I). These data suggest that LAP is an important process in reducing AML disease burden.

Next, we assessed macrophage phenotype within the BM of AML engrafted mice. AML tumor associated macrophages (TAM) have been characterised as CD45<sup>+</sup>, Lys6G<sup>-</sup>, CD11b<sup>+</sup> macrophages and are called AML associated macrophages (AAM) (28), whilst tissue resident BMM express CD45<sup>+</sup>, GR1<sup>-</sup>, F4/80<sup>+</sup> CD115<sup>INT</sup> (Supplementary Figure 4A) (38). C57BL/6 were injected with MN1 cells, and the BM analysed 14 days post injection. The percentage of AAM in the CD45 population were increased in animals engrafted with MN1 compared to controls. Furthermore, AAM had decreased CD86 expression (an indicator of M1 phenotype) compared to controls. The M2 marker, CD206, showed no differences between treatment groups (Supplementary Figure 4B). BMM were also increased in mice injected with MN1 cells, with CD86 and CD206 expression increased compared to controls (Supplementary Figure 4B). Next, we examined AAM and BMM phenotypes in Atg16L1<sup>E230-</sup> mice compared to Atg16L1<sup>E230+</sup> mice engrafted with MN1. Data show no observable differences between immunophenotype of AAM or BMM when MN1 cells were injected into Atg16L1<sup>E230-</sup> mice compared to Atg16L1<sup>E230+</sup> mice (Figure 2J and 2K). This shows that whilst AML changes the phenotype and number of macrophages in the BM, deficiency in LAP does not alter this response.

**BMM LAP mediates clearance of AML apoptotic cells**

As the recognition of apoptotic tumor cells has previously been reported to promote anti-tumor immunity (40-42), we hypothesised a link between reduced clearance of AML apoptotic cells (AC) and apoptotic bodies (AB) with increased leukemia progression. Next, we measured AC in LAP deficient animals with AML and compared that to AC in WT controls with AML. Gating strategy for this is shown in supplementary figure 1A. We found that the percentage of Annexin V<sup>+</sup> staining was higher in LAP deficient Atg16L1<sup>E230-</sup> animals
with AML compared to wild type Atg16L1<sup>E230+</sup> animals with AML (Figure 3A). Furthermore, Atg16L1<sup>E230-</sup> AML engrafted animals had increased Annexin V<sup>+</sup> debris compared to WT Atg16L1<sup>E230+</sup> controls with AML, indicating a defect in clearance of AB and AC in the BM of Atg16L1<sup>E230-</sup> animals (Figure 3B). To determine if LAP was important in clearance of AML AB, we induced apoptosis in MN1 cells in vitro and isolated the AB (Supplementary Figure 5). Isolated AB were labelled with pHrodo, to create pHrodo-AB, which when phagocytosed cause the AB to fluoresce. pHrodo-AB were cultured with BM-derived macrophages (BMDM) from Atg16L1<sup>E230+</sup> and Atg16L1<sup>E230-</sup> mice and results show Atg16L1<sup>E230+</sup> BMDM had increased phagocytosis of AB compared to Atg16L1<sup>E230-</sup> BMDM (Figure 3C). Next, we examined if LC3-localised phagosomes occur more frequently in Atg16L1<sup>E230+</sup> BMDM compared to Atg16L1<sup>E230-</sup> BMDM. Co-staining the pHrodo-AB phagosome with anti-LC3-GFP showed that Atg16L1<sup>E230+</sup> BMDM had significantly more LC3-localised phagosomes compared to Atg16L1<sup>E230-</sup> BMDM (Figure 3D). To confirm that BMDM from Atg16L1<sup>E230+</sup> mice deliver apoptotic bodies to the lysosomes, and that this does not happen in Atg16L1<sup>E230-</sup> BMDM, we used the lysosomal inhibitor baflomycin A. Figure 3E shows that baflomycin A inhibits the delivery of apoptotic bodies to lysosomes in Atg16L1<sup>E230+</sup> mice, and that this does not happen in Atg16L1<sup>E230-</sup> BMDM. These data demonstrate that LAP enhances clearance of apoptotic AML cells and debris in the bone marrow.

**LAP induces STING in BMM which suppresses AML growth**

To investigate the impact of LAP in regulating AML progression we induced apoptosis in MN1 cells and isolated the AB and co-cultured them with BMDM from Atg16L1<sup>E230+</sup> and Atg16L1<sup>E230-</sup> animals for 24 hours. Cell supernatant was assayed for cytokine profile using Proteome Profiler Mouse XL Cytokine Arrays (Figure 3F and supplementary figure 6). Pathway analysis before and after AB treatment revealed cytokines and chemokines related to STING activation were present in the supernatant from Atg16L1<sup>E230+</sup> BMDM, but absent from Atg16L1<sup>E230-</sup> BMDM (Figure 3G). BMM isolated from Atg16L1<sup>E230+</sup> and Atg16L1<sup>E230-</sup> animals engrafted with AML were therefore examined for STING activation by measuring Gbp2, Irf7 and Ifit3 gene expression (Figure 4A) (35, 43). BMM from AML engrafted Atg16L1<sup>E230+</sup> animals showed pronounced activation of STING indicated by increased expression of Gbp2, Irf7 and Ifit3 compared to BMM from AML engrafted Atg16L1<sup>E230-</sup> animals (Figure 4B). To confirm the increased cytokine expression resulted from activation of STING, C57/BL6 mice engrafted with MN1 cells were treated over 7 days with the STING inhibitor H-151. The animals were then sacrificed, the BM was isolated and the BMM FACS purified and analysed for Gbp2, Irf7 and Ifit3 gene expression (Figure 4C). When compared with control animals, BMM from H-151 treated animals had decreased expression of STING markers, Gbp2, Irf7 and Ifit3 (Figure 4D), but increased proinflammatory cytokines Il1-ß and
Furthermore, animals treated with H-151 had increased AML tumor burden (Figure 4F). To confirm the role of LAP we used the STING inhibitor H-151 in the Atg16L1^{E230} model (Figure 4G). Figure 4H shows that the STING inhibitor enhanced tumour burden in the Atg16L1^{E230+} mice but not in the Atg16L1^{E230-} mice.

In solid tumors, STING activation promotes recognition and killing of cancer cells via mechanisms which include both enhancing cancer antigen presentation and regulation of CD8^{+} T cell trafficking and infiltration into tumors (44-46). To understand the anti-tumoral effects of STING in AML, we first looked at T-cell migration into the BM and subsequent activation. Post engraftment analysis showed that CD4^{+} cells were increased in MN1 engrafted animals compared to control animals, but no changes were observed in CD8^{+} cells or their IFN-γ status (Supplementary figure 7). Since we observed increased phagocytosis in AML primed BMM compared to naive BMM we examined phagocytic capacity of F4/80^{+} BMM following STING inhibition by H-151. Figure 4I and J show that inhibition of STING reduced phagocytosis in BMM of pHrodo bioparticles compared to control BMM. Therefore, unlike solid tumors, the anti-tumoral effects of BMM STING activation in AML functions via upregulation of the phagocytic potential.

**AML derived apoptotic bodies contain mitochondria which are processed by BMM**

As self-DNA (nuclear and/or mitochondrial) has been shown to stimulate STING in autoinflammatory and malignant disease (47-50), and both AML growth and chemotherapy induced DNA damage dysregulate the BM apoptotic response (51, 52), we hypothesised that AML specific BMM STING activation was mediated by local tumoral apoptosis and cellular debris. To address this question, AML derived-AB were isolated from MN1 cells and non-malignant LSK cells (as controls, surrogate of HSPCs). These were cultured ex-vivo with BMDM for 24 hours before analysis of STING induced gene expression by qPCR (Figure 5A). MN1 derived-AB caused upregulation in BMDM STING related genes when compared to control LSK derived-AB (Figure 5B). Activation of STING has been shown to occur in response to mitochondrial damage associated molecular patterns (mtDAMPs), including mitochondrial DNA (mtDNA) (53, 54). Previously, we and others have shown that AML contain a higher mitochondrial mass than non-malignant LSK cells (Supplementary Figure 8) (55, 56). Therefore, we assessed whether AB from AML contain mitochondria. We first measured mitochondrial content in AML derived-AB and LSK AB, by MitoTracker Green and VybrantDil membrane stain and analysed AB via image flow cytometry. MN1 derived-AB had increased mitochondrial content compared to LSK derived-AB (Figure 5C). Additionally, we looked at mitochondrial association with the AB, we found that MitoTracker Green and VybrantDil association was increased in MN1 derived-AB compared to LSK derived-AB.
(Figure 5D). Furthermore, by confocal microscopy we directly visualised cell membrane blebbing containing mitochondria (Figure 5E). This data demonstrates that AML-derived-AB contain mitochondria.

Secondly, to understand if this process is unique to the mouse models used or translates to human disease, we stained 5 separate human AML bone marrow samples, MN1 and non-malignant human CD34+ cells with MitoTracker Red and isolated AB. Human AML and MN1 derived-AB had significantly more MitoTracker Red staining compared to non-malignant human CD34+ derived-AB (Figure 5F). To determine if human AML mitochondria are processed by BMM in vivo we engrafted human AML (supplementary figure 9A and B), or human AML transduced with mCherry mito9 lentivirus (mCh-AML) to visualise mitochondria, into NSG mice (Figure 5G). At day 35 post tumor injection (prior to the disease associated terminal end point), animals were sacrificed and analysis of the BMM from mCh-AML engrafted animals showed increased mCherry fluorescence compared to BMM from control animals transplanted with non-malignant CD34+ cells (Figure 5H). To confirm this BMM mediated phenotype, we co-cultured mCh-AML cells ex vivo with either BMDM or BMSC and analysed the macrophages and stromal cells for mCherry uptake via fluorescent microscopy. BMDM had increased mCherry fluorescent compared to BMSC (Figure 5I). This data shows that AML derived-AB containing mitochondria are phagocytosed by BMM.

**AML derived apoptotic bodies containing mtDNA activate STING in BMM via LAP dependent mechanism**

Next, as we have seen that AML derived-AB induce STING regulated gene activation, and that mitochondria containing AB are processed by BMM, we investigated if LAP is required for phagocytosis of AB containing mitochondria. We first isolated AB from mCh-AML (mCh-AB) and cultured them ex vivo with BMDM from Atg16L1E230+ and Atg16L1E230- animals for 24 hours (Figure 6A). After 4 hours both Atg16L1E230+ and Atg16L1E230- BMDM showed an increase in mCherry uptake. This reduced after 24 hours in the Atg16L1E230+ BMDM, but not the Atg16L1E230- BMDM, suggesting that Atg16L1E230- BMDM take up the mCh-AB but are unable to deliver them to lysosomes for degradation (Figure 6B). Since mtDNA activates STING, we generated MN1 cells depleted of mtDNA ($\rho^0$ MN1) by long term culture in ethidium bromide and 2’3’-dideoxycytidine (Figure 6C). We isolated MN1 $\rho^0$ derived-AB, as well as FACS purified mitochondria containing AB from MN1 and LSK cells and co-cultured the AB with BMDM from C57/BL6 mice for 24 hours before preforming qPCR for gene expression (Figure 6D). While there was increase in the expression of STING related genes in BMDM treated with MN1 mitochondria containing AB, there was no increase in STING related genes from BMDM co-cultured with MN1 $\rho^0$ AB (Figure 6E). To
understand if this response was LAP dependent, we isolated mitochondria containing AB from MN1 and non-malignant LSK cells (LSK is used as a control that makes AB with low levels of mitochondria – see Figure 5D) by FACS and incubated these cells with Atg16L1\textsuperscript{E230-} or Atg16L1\textsuperscript{E230+} BMDM for 24 hours (Figure 6F). We then analysed the BMDM for gene expression relating to STING. Whilst there was an increase in STING related gene expression in Atg16L1\textsuperscript{E230+} BMDM treated with mtAB from MN1 cells, there were no such increases in Atg16L1\textsuperscript{E230-} BMDM (Figure 6G). Together, this data shows that LAP is required in BMM to process AML derived-AB containing mtDAMPs and results in STING related activation.
Discussion

The presence of tumor associated macrophages (TAM) is generally related to a poorer prognosis in solid tumors (57-62). In contrast, we observed that in-vivo models of AML, generalised bone marrow macrophage depletion accelerated AML growth. This occurred because BMM phagocytosis of apoptotic cellular debris in the BM microenvironment results in suppression of AML growth. Specifically, we found that phagocytosis of mtDAMP induces STING activation in the BMM, conferring an anti-tumoral phenotype. The activation of STING results in an increased phagocytic capacity of BMM and inhibited AML progression, independent of T-cell activation.

The data describing the role of macrophages in the progression of AML appears at first look contradictory. In some contexts, macrophages in the tumor microenvironment have been found to promote AML progression. Specifically, displacement of resident macrophages or invasion of tumor supporting macrophages has been shown to correlate with low survival in patients with AML (29). Additionally, macrophages have the ability to protect AML cells from apoptosis (28). Furthermore, the immunosuppressive environment created by leukemic cells alters BMM to reduce their phagocytic capacity and avoidance of immune regulation (63). Contrary to these studies, we and others have shown that depletion of macrophages using clodronate liposomes increases AML engraftment (64). Moreover, we find that LAP in macrophages negatively regulates AML progression by altering the phagocytic potential of macrophages to promote increased phagocytic clearance. Furthermore, our results show that deficiencies of LAP in macrophages leads to accumulation of apoptotic AML debris, which results in a tumor supporting environment. The spectrum of apparently diverse roles of BMM, may be explained in part through observations leading to experimental sub-categorisation of macrophages by phenotypic polarization. Through this, distinct transcriptional programs are activated resulting in defined patterns of cytokine response and protein expression profiles (20). A simplified conceptual framework has been developed to broadly divide macrophages into M1 and M2 subtypes (21), with ‘classically activated’ macrophages (M1 macrophages) generally considered to be primed for pathogen and anti-tumor response (20) and ‘alternatively activated’ macrophages (M2 macrophages) regarded to have immunoregulatory and pro-tumoral functions (65). However, macrophage polarity likely represents a dynamic continuous spectrum of phenotypes, within which M1 and M2 polarity is regulated by microenvironment cues (20, 23, 24). How these largely in-vitro defined phenotypes relate to in-vivo pathophysiology and explain the spectrum of macrophage functions remains to be explained.
Ecologically, tumors exhibit dynamic and synchronous cell death and proliferation. Apoptosis has previously been thought of as immunologically silent or even tolerogenic death modality (66-68). However, a growing body of evidence suggests an important role for uncleared apoptotic debris in stimulating immunologic responses in malignancies (40, 41, 69-71). We identify LAP mediated BMM clearance of AML apoptotic debris as an important regulator of AML disease progression. The suppression of AML by BMM through a LAP dependent mechanism is a phenotype not seen in solid tumors. Studies using models of adenocarcinoma and melanoma models (35), report recruitment of cytotoxic T-cells into tumors in the context of LAP-deficient myeloid cells, resulting in an antitumoral response. In contrast, our results show no increased recruitment or activation of cytotoxic T-cells into the leukemic BM in LAP deficient animals.

The identification of the importance of LAP in AML suppression has been revealed in this study by using a mouse model that lacked the linker and WD domains of ATG16L1 from E230 in the amino acid sequence (supplementary figure 2) (39, 72). The conventional Atg16L1<sup>E230</sup>-is not limited to the myeloid lineage which may allow for non-myeloid cells to be implicated in generating the results observed in this study. To overcome this limitation, we generated a myeloid specific Atg16L1<sup>E230</sup>- which when engrafted shows increased tumour burden compared to control mice. Other studies have used deletion of <i>RUBCN/Rubicon</i> (<i>rubcn<sup>-/-</sup></i>) (73, 74), which can be targeted to the myeloid cells. <i>RUBCN</i> is part of a complex upstream of ATG16L1 containing UVRAG, Beclin 1 and VPS34. <i>RUBCN</i> is essential for LAP because it increases the class III PI3 kinase activity of VPS34 to generate Pi(3)P on phagosomes to stabilise the NOX2 complex for the production of ROS, and recruit the ATG5-ATG12:ATG16L1 complex to conjugate LC3 to the phagosome membrane (74). Rubcn<sup>-/-</sup> mouse models show inefficient clearance of pathogens and apoptotic cells as well as elevated inflammation leading to the development of autoimmune disease like systemic lupus erythematosus (SLE). Additionally, rubcn<sup>-/-</sup> mice were shown to induce a type I IFN responses in tumor infiltrating macrophages (35). Rubcn<sup>-/-</sup> models are LAP deficient and could have been used to determine the role of LAP in AML progression.

We and others have shown the mitochondrial content of AML is increased compared to non-malignant haematopoietic and progenitor cells (55, 56). Unlike many tumors which rely on the Warburg effect for energy production, AML primarily uses oxidative phosphorylation (OXPHOS) for ATP production, hence the increased mitochondrial mass (75). The increased mitochondrial mass and the rapid growth of the AML cells leads to the generation of dysfunctional mitochondria and ultimately to an increase in reactive oxygen species as a by-product of OXPHOS (76). Dysfunctional mitochondria also initiate signal cascades for apoptosis (77-79). In tumor evolution, increasing cell turnover rate slows tumor growth but
accelerates the rate of evolution for both proliferation and migration (80). Increasing cell turnover also results in increased numbers of apoptotic cells. Apoptotic cells generate membrane blebs that release from the cell in apoptotic bodies and contain cellular components including mtDAMPs (54). mtDNA has been shown to activate STING via cyclic GMP-AMP synthase (cGAS) to cause downstream affects, such as type I IFN response (53). Previous investigation into AML STING activation has shown that AML type I IFN response is not triggered when compared to solid tumors, where STING activation is responsible for maturation of dendritic cells (81). Here, we have shown that mtDNA from AML derived-AB is processed by macrophages in a LAP dependent manner. Furthermore, we see that inhibition of STING in BMM leads to decreased phagocytic potential, increased AML progression and no changes in T-cell activation. Accordingly, AML rapid cell proliferation is driven by ATP, primarily from OXPHOS (over glycolysis), but occurs with the metabolic cost of increased ROS, apoptosis and dysfunctional mitochondria released into the BM microenvironment. These observations reveal tumor specific vulnerabilities and present strategic opportunities when considering novel approaches to managing patients with AML. Clinically, shifting the balance between cell turnover and tumor growth may be possible through the regulation of processing of ROS and apoptotic cells in the BM microenvironment.

Recently, CD47 has been identified as a “do not eat me” signal, which is overexpressed in myeloid malignancies (30, 82, 83). Blockade of CD47 leads to engulfment of leukemic cells, with pre-clinical studies demonstrating anti-malignant activity in AML and myelodysplastic syndrome (32, 84). Subsequently, clinical studies have been initiated with CD47 targeting agents in both AML and MDS as monotherapy and in chemotherapy combination (31). Others have shown that STING activation using 5,6-Dimethylxanthene-4-acetic Acid (DMXAA) can significantly extend survival of in-vivo models of AML (81). In our model, LAP dependent activation of STING increased phagocytosis of AML cells. In mouse melanoma cells and several other cell lines, the anthracycline drug doxorubicin induced activation of STING. Therefore, as anthracycline chemotherapy has long been the standard of care for younger patients with AML treatment (85) these data combined lead us to hypothesise that chemotherapy induced STING activation in macrophages may enhance the anti-AML effects of CD47 inhibition.

These experimental data, set within the context of the existing literature, highlight the diversity of macrophage phenotypic function in AML. This, is dynamic and entirely dependent on the cellular, tissue, treatment and disease specific context induced by the tumor and its microenvironment. Accordingly, the appreciation of the relationship between proliferation and apoptosis, set within the broader chemotherapy context, will be vital when planning the drug
sequence and timing of treatment strategies looking to harness the therapeutic potential of macrophages in the management of AML.
Materials and methods

Animals
Non-obese diabetic (NOD) severe combined immunodeficiency (SCID) Il2rg knockout (NOD.Cg.Prkdc<sup>scid</sup>Il2rg<sup>tm1Wji</sup>/SzJ (NSG) mice were purchased from The Jackson Laboratory (Bar Harbour, ME, USA). C57BL/6J mice were purchased from Charles River (Massachusetts, United States). The generation of LAP deficient mice, Atg16L1<sup>E230<sub>f</sub></sup>, has been previously described. Atg16L1<sup>E230<sub>f</sub></sup>Cre<sup>+</sup> were generated by crossing mice containing the Lysozyme-M-Cre promoter with mice containing floxed sites flanking exon 2 of ATG16L1 gene. Animals were housed in a specific pathogen-free facility. All animal work used in this study were carried out in accordance with regulations set by the UK Home Office and the Animal Scientific Procedures Act 1986. Mice used were at age 8-12 weeks of age and both genders were used for experiments.

Primary cells
Non-malignant and malignant haematopoietic cells were collected at the Norfolk and Norwich University Hospital. Studies were performed following approval from the United Kingdom Health Research Authority research ethics committee (ref 07/H0310/146). CD34<sup>+</sup> haematopoietic stem cells were isolated via density gradient centrifugation and CD34<sup>+</sup> microbeads (Miltenyi Biotec). To engraft non-malignant CD34<sup>+</sup> cells into NSG mice we used 3 doses of 25mg/kg of busulfan on day -3, day -2 and day -1 and then injected non-malignant CD34<sup>+</sup> on day 0. Primary AML blasts were obtained from the bone marrow of AML patients after informed consent and under approval from the UK National Research Ethics Service (LRCERef07/H0310/146) and Table 1. AML cell isolation was performed by density gradient centrifugation using Histopaque (Sigma-Aldrich). AML cells were cultured in Dulbecco’s Modified Eagle Media (DMEM) containing 10% fetal bovine serum (FBS) plus 1% penicillin-streptomycin. To generate mCherry mitochondria in primary AML cells, cells were seeded at a density of 5 × 10<sup>4</sup> in 500μl of DMEM supplemented with 10% FBS and transduced with 0.5μl of rLV.EF1.mCherry-Mito-9 lentivirus (Clontech Takara Bio Europe, Saint-Germain-en-Laye, France). AML cells were cultured for an additional week to ensure that no residual lentivirus remained. Transduction was confirmed by the detection of mCherry fluorescence in AML cells by fluorescent microscopy. To engraft human AML into NSG mice we did not use busulfan but injected the AML without conditioning into NSG mice.

Bone marrow isolation
Isolation of bone marrow was achieved by isolating the femur and tibia from each mouse. Each bone was cut centrally and placed in a 0.5ml microcentrifuge tube containing a hole allowing the removal of the bone marrow from the bone. This was placed in a 1.5ml microcentrifuge and centrifuged for 6 seconds to allow collection of bone marrow cells. The bone marrow pellet from each mouse was collected and washed in MACS buffer (1XPBS, 0.5% BSA, 2mM EDTA, filtered) before being passed through a 40µm CellTrics filter (Sysmex, Japan). The bone marrow was isolated from one tibia and one femur and the cells were counted via an automated cell counter (Cellometer Auto T4, Nexcelom Bioscience LLC Lawrence, MA, USA). Absolute live cell numbers and AML cell numbers were calculated by multiplying the frequencies of cells by the total numbers of cells per tibia and femur.

**LSK cell isolation**
Mouse LSK cells were isolated from mouse bone marrow using Lin- microbeads followed by CD117 microbeads (Miltenyi Biotec, Germany) and then sorted for SCA1-APC (Miltenyi Biotec) on a SH800S Cell Sorter (Sony). The LSK cells were expanded in DMEM containing 10% FBS plus 1% penicillin-streptomycin supplemented with mSCF (100ng/ml), mIL3 (10ng/ml) and hIL6 (10ng/ml) (Peptrotech, NJ, USA).

**Bone marrow macrophages**
Mouse bone marrow derived macrophages (BMDM) were isolated via cultured mouse bone marrow in RPMI-1640 (Gibco) containing 20% FBS (Gibco) plus 1% penicillin-streptomycin (Gibco) supplemented with 20ng/ml of macrophage CSF (Peptrotech, NJ, USA). Briefly, 1-2x10^7 bone marrow cells were plated onto non-tissue cultured treated 10cm plastic dishes, with fresh media added at day 3. At day 6-7, cells were washed with 1xPBS, cold PBS was added, and cells were removed by cell scrapping. After cell number was established, BMDM were plated in RPMI-1640 containing 20% FBS plus 1% penicillin-streptomycin for 24 hours before experimental use. For longer survival, BMDM were culture in RPMI-1640 containing 20% FBS plus 1% penicillin-streptomycin supplemented with macrophage CSF (10ng/ml). Mouse BMDM markers were confirmed by flow cytometry for F4/80^+, GR1^-. Isolation of F4/80^+ from BM samples were isolated using positive selection for F4/80 microbeads (Miltenyi Biotec, Germany).

**Bone marrow stromal cell isolation**
Mouse bone marrow stromal cells (BSMC) were isolated from the mouse bone marrow by adherence to tissue culture plastic and then cultured in MEM containing 20% FBS plus 1% penicillin-streptomycin. Mouse BMSC markers were confirmed via flow cytometry for CD45-, CD31-, Ter119-, CD105+, CD140a+.
MN1 and MEIS1/HOXA9 cells
MN1 and MEIS1/HOXA9 cells were generated as previously described (86) and maintained in DMEM containing 10% FBS plus 1% penicillin-streptomycin supplemented with mSCF (100ng/ml), mIL3 (10ng/ml) and hIL6 (10ng/ml) (Peptrotech, NJ, USA). MEIS1/HOXA9 cells were infected with pCDH-luciferase-T2A-mCherry for in vivo imaging, kindly provided by Professor Irmela Jeremias, (Helmholtz Zentrum München, Munich, Germany) (87). Transduced MEIS1/HOXA9 cells (MEIS1/HOXA9-luci) were sorted using mCherry fluorescence on a BD FACSMelody (BD Bioscience). Culture of cells was carried out in 5% CO₂ at 37°C. To engraft AML into WT and Atg16lE230- mice we intraperitoneal (IP) injected 2 doses of 25mg/kg of busulfan on day -2 and day -1 and then injected MN1 and MEIS1/HOXA9 cells by intravenous (IV) tail vein injection on day 0.

Clodronate liposome experiment
C57/BL6 mice were treated with 25mg/kg of IP busulfan on day -2 and day -1 prior to tail vain injections of 1x10⁵ MEIS/HOXA9-luci cells or MN1-GFP cells on day 0. 19 days post injection animals were imaged via in vivo bioluminescent imaging (Bruker, Coventry) and then injected IP with either 150µl of clodronate liposomes or control liposomes (Stratech, UK). Animals were imaged and scarified at day 23 post injection. Bioluminescence levels of MEIS/HOXA9-luci cells was analysed via ImageJ (Fiji). Macrophage number (CD45⁺, GR1⁻, CD115LOW/INT, F4/80⁺) and engraftment was analysed via flow cytometry.

Flow Cytometry and Cell Sorting
The following antibodies were used CD45-BV510 (Biolegend) clone 30-F11, CD45-Alexa Fluor® 700 (Biolegend) clone I3/2.3, Ly6G-PerCP (Biolegend) clone 1A8, CD11b-BV510 (Biolegend) clone M1/70, GR1-FITC (Biolegend) clone RB6-8C5, F4/80-PE-Cy7 (Biolegend) clone BM8, F4/80-APC (Biolegend) clone BM8, CD115-APC/Fire™ 750 (Biolegend) clone AFS98, CD86-BV421 (Biolegend) clone GL-1, CD206-PE (Biolegend) clone C068C2, NK1.1-APC-Cy7 (Biolegend) clone PK136, B220-BV421 (Biolegend) clone RA3-6B2, CD4-PE-Cy7 (Biolegend) clone GK1.5, CD8-PerCP (Biolegend) clone 53-6.7, IFN-γ-APC (Biolegend) clone XMG1.2, Annexin V-PE-Cy7 (Fisher Scientific), Annexin V-APC (Biolegend), CD31-Pe-Cy5 (Biolegend) clone WM59, CD45-APC (Biolegend) clone 30-F11, CD34-BV421 (Biolegend) clone 561, Lineage Cocktail-Pacific Blue (Biolegend) clones 17A2; RB6-8C5; RA3-6B2; Ter-119; M1/70 and CD33-PE (Biolegend) clone P67.6. Antibody cocktails were prepared in MACS buffer as previously described and incubated with bone marrow cells for at least 30min at 4°C. In experiments using MitoTracker™ Green or MitoTracker™ Red (Invitrogen), the cells were incubated at room temperature for 30 min, washed 2x in PBS, and centrifuged at 1200rpm for 5 min before adding any additional...
antibodies. In experience using Vybrant™ Dil Cell-Labeling (Invitrogen), the cells were incubated at room temperature for 20 minutes, washed 2x in PBS, and centrifuged at 1200rpm for 5 min. For sorting of AML associated bone marrow macrophages, bone marrow cells were re-suspended in antibody mix and cells were sorted directly into lysis buffer. Sorted BMM were also sorted into RPMI-1640 containing 20% FBS plus 1% penicillin-streptomycin supplemented with macrophage CSF (10ng/ml). Sorted mitochondrial containing AB were sorted directly in PBS. Compensations and fluorescence minus one (FMO) controls were run for each panel.

Flow cytometry was carried out using FACSCanto II flow cytometer (BD Bioscience) and cell sorting was performed on a BD FACSMelody (BD Bioscience) or a SH800S Cell Sorter (Sony). Image flow cytometry was carried out using Amnis ImageStream® Mk II (Luminex). Data was analysed using FlowJo (TreeStar, USA).

**Real-time qPCR**

RNA from cells was isolated using the ReliaPrep RNA cell miniprep system (Promega, Southampton, UK). RT-qPCR assay was performed with the one-step SYBR-green technology (PCR biosystems, UK) with QuantiTect Primer Assays (Qiagen, Germany). After a reverse transcription step (45°C for 10 minutes), PCRs were then amplified for 45 cycles (polymerase activation at 95°C for 2 minutes followed by cycles of 95°C/10 seconds, 60°C/10 seconds, 72°C/30 seconds), on a Roche 384-well LightCycler480. Messenger RNA (mRNA) expression was normalized against glyceraldehyde 3-phosphate dehydrogenase (GAPDH) using the comparative cycle threshold method and calculations were achieved using the ΔΔCt method.

**ρ₀ MN1 generation**

5x10⁶ MN1 cells were cultured in DMEM containing 10% FBS plus 1% penicillin-streptomycin supplemented with mSCF (100ng/ml), mIL3 (10ng/ml), hIL6 (10ng/ml), ethidium bromide (1μg/ml), 2’,3’-dideoxyctydine (ddC; 200μM), sodium pyruvate (100μg/ml) and uridine (50μg/ml). Every 7 days, cells were centrifuged at 1200rpm for 5 minutes and resuspended in fresh media. At 20 days mitochondrial DNA detection was performed using qPCR as previously described (11). Briefly, DNA was extracted from MN1 and ρ₀ MN1 cells using the GenElute Mammalian Genomic DNA Miniprep Kit (Sigma Aldrich, St Louis, MO, USA) according to the manufacturer’s protocols. Purified DNA was then analysed via Taqman® probes ND3 for mitochondrial DNA and normalised against Tert for genomic DNA (ThermoFisher, Waltham, MA, USA). Relative mtDNA/gDNA ratio was calculated using the ΔΔCt method.
Apoptosis induction and apoptotic body isolation

To induce apoptosis cells were treated with 5μM cytosine arabinoside (ara-C) for 24 hours. Apoptosis was determined by Annexin V-PE-Cy7 (Invitrogen) by flow cytometry. The AB were isolated via centrifugation as previously described (88). Briefly, cells were spun at 2000rpm for 10 minutes. The supernatant was spun at 10000rpm for 10 minutes and the pellet was resuspended in either PBS or appropriate media.

Microscopy

Isolated F4/80+ BMM were plated at a density of 2x10^5 on μ-Plate 24 well black plate (ibidi) in 500μl of RPMI-1640 containing 20% FBS plus 1% penicillin-streptomycin supplemented with macrophage CSF (10ng/ml) until required or FluoroBrite™ DMEM containing 10% FBS plus 1% penicillin-streptomycin for immediate use. Zymosan A (S. cerevisiae) BioParticles™, Texas Red™ conjugate (Invitrogen) were incubated with the BMM according to the manufacturer’s instructions for 2 hours. Cells were washed 2x with PBS before being fixed and permeabilised (ThermoFisher, MA, USA) during which LC3-fitc and DAPI was incubated with the BMM for 20 minutes. Cells were washed 2x with PBS and 500μl of FluoroBrite™ DMEM added. Cells were observed under EVOS™ M5000 Imaging System under 40x magnification (Invitrogen). BMDM were plated at a density of 2x10^5 on μ-Plate 24 well black plate (ibidi) in 500μl of RPMI-1640 containing 20% FBS plus 1% penicillin-streptomycin for 24 hours. MN1 AB were generated and isolated as previously described with the exception that AC were incubated with pHrodo™ Red, SE (Invitrogen) according to the manufacturer’s instructions for 30 minutes before AB isolation. The media was removed from the BMDM and 500μl of FluoroBrite™ DMEM added, AB were cultured with the BMDM for 3 hours. Cells were fixed and permeabilised (ThermoFisher, MA, USA) during which LC3-fitc and DAPI was incubated with the BMDM for 20 minutes. Cells were washed 2x with PBS and 500μl of FluoroBrite™ DMEM added. Cells were observed under EVOS™ M5000 Imaging System under 40x magnification (Invitrogen). Sorted BMM were plated at a density of 1.5x10^5 on μ-Plate 24 well black plate (ibidi) in in 500μl of RPMI-1640 containing 20% FBS plus 1% penicillin-streptomycin for 24 hours. The media was changed to 500μl of FluoroBrite™ DMEM containing 10% FBS plus 1% penicillin-streptomycin and BMM were cultured with the STING inhibitor H-151 (10μM, InvivoGen) or vehicle (PBS with 0.1% TWEEN80) for 2 hours. BMM were then incubated with pHrodo™ Red E. coli BioParticles™ (Invitrogen) for 2 hours before being imaged on Zeiss LSM 800 Axio Observer.Z1 confocal microscope with a 63x water objective (Carl Zeiss).
Human AML cells that were transduced with a rLV.EF1.AcGFP-Mem9 lentivirus (Clontech Takara Bio Europe, Saint-Germain-en-Laye, France) for plasma membrane expression of GFP (AML-GFP). AML-GFP cells were stained with MitoTracker™ Red (Invitrogen) according to manufactures protocol and Hoechst 33342 (Thermo Scientific, MA, USA). Images were captured on Zeiss LSM 800 Axio Observer.Z1 confocal microscope with a 63x water objective (Carl Zeiss). For May-Grunwald Giemsa stained bone marrow smear slides we used an Olympus BX51 light microscope (Olympus UK, Southend-on-sea, UK).

**Cytokine Array**

BMDM were isolated from Atg16\textsuperscript{E230+} and Atg16\textsuperscript{E230-} mice as previously described and plated at a density of 2.5x10\textsuperscript{5} in 24 well plates in 200μl of RPMI-1640 containing 10% FBS plus 1% penicillin-streptomycin. Vehicle (PBS) or AB from MN1 cells were generated as previously described and added to the BMDM for 24 hours. The supernatant was removed, pooled, and centrifuged at 10,000rpm for 10mins to remove any debris. Cell supernatant was used for Proteome Profiler Mouse XL Cytokine Arrays (R&D Systems) following the manufacture’s protocol. Cytokine membranes were analysed using the G:BOX Chemi XRQ (Syngene) and quantified using ImageJ (Fiji).

**In vivo STING inhibition**

Mice were treated with 25mg/kg of busulfan on day -2 and day -1 prior to tail vain injections of 1x10\textsuperscript{6} MN1-GFP cells on day 0. At day 7, 9, 11 and 13 post MN1-GFP injection, mice were intraperitoneally injected with 200μl of H-151 (750nmol, Invivogen) or vehicle (PBS with 0.1% TWEEN80). The animals were sacrificed at day 14 post injection and the BM isolated as previously described. The BM was analysed using flow cytometry for MN1 engraftment and T-cell activity. The BMM were sorted and the RNA was extracted for qPCR analysis.

**Engraftment of AML in to NSG mice**

1x10\textsuperscript{6} primary AML blasts, with and without rLV.EF1.mCherry-mito9 lentivirus transduction, were injected intravenously into non-irradiated 6-8 week old NSG mice. At pre-defined humane end points animals were sacrificed, bone marrow isolated and engraftment determined using expression of human CD45. Mouse bone marrow cells were examined via flow cytometry for BMSC (CD45\textsuperscript-/CD105\textsuperscript{+}), BMM (CD45\textsuperscript{+}, GR1\textsuperscript{-}, CD115\textsuperscript{LOW/INT}, F4/80\textsuperscript{+}) and mature lymphoid cells (CD45\textsuperscript{+}/CD3\textsuperscript{+}). The MFI mCherry intensity of each population was analysed via FlowJo (TreeStar, Ashland).

**Statistics**
For statistical analysis of two groups, unpaired Mann-Whitney test was used. When more than two groups were compared, a Kruskal-Wallis test (one-way ANOVA) was performed followed by a Dunn’s post-hoc test for significance using Prism version 7.0a for Mac OS X (GraphPad, La Jolla, CA, USA). Differences among group means were considered significant when the p value was less than 0.05* 0.01** 0.001*** 0.0001****.

Study approval
All animal work used in this study were carried out in accordance with regulations set by the UK Home Office and the Animal Scientific Procedures Act 1986. Non-malignant and malignant haematopoietic cells were collected at the Norfolk and Norwich University Hospital. Studies were performed following approval from the United Kingdom Health Research Authority research ethics committee (ref 07/H0310/146).

Author Contribution

Acknowledgements
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Declaration of interests

The authors declare no competing interests
References


Figure 1. Depletion of phagocytes increasing AML tumor burden.

(A) MEIS/HOXA9 cells transduced with rLV.EF1.mCherrymito9 lentivirus (MEIS/HOXA9-luci) or MN1-GFP cells were injected (1x10⁶) into busulfan treated C57/BL6 mice. Animals were treated with either control liposomes (CNT) or clodronate liposomes (CL) at day 19 post injection and scarified at day 23. (B) Representative flow plot and gating strategy for BMM (CD45⁺, GR1⁻, CD115LOW/INT and F4/80⁺). (C) The number of BMM macrophages were analysed via flow cytometry (n=5 mice). (D) In vivo imaging analysis of animals engrafted with MEIS/HOXA9-luci at day 19 and day 23 representing before and after the clodronate and control liposome treatment. Bioluminescence analysis between clodronate and control liposome treatment groups (n=5 mice). (E) Bioluminescence analysis of apoptotic and live MEIS/HOXA9-luci cells. (F) Representative flow plot for MN1-GFP engraftment between clodronate and control liposome treatment groups. (G) BM was extracted and analysed for MN1-GFP engraftment and (H) and annexin V staining of MN1-GFP (n=5 mice). (I) MN1 cells (1x10⁶) or vehicle (PBS) were injected into busulfan treated C57/BL6 mice, and the BM harvested 14 days post injection. BMM F4/80⁺ cells were isolated via magnetic separation and incubated with Zymosan A bioparticles for 2 hours and imaged via fluorescence microscopy. (J and K) The number of bioparticles (red) were counted per macrophage for control and MN1 associated BMM via microscopy (n=25 BMM). (L and M) The number of bioparticles (red) and LC3 (green) were counted per MN1 associated BMM and compared to the number of bioparticles without LC3 (n=25 BMM). Data shown are means ± SD *p<0.05 **p<0.01 ****p<0.0001 using the Mann-Witney U test (Two-tailed).
Figure 2. AML disease progression is accelerated in LAP deficient animals.
(A) MN1 cells were injected (1x10^6) into busulfan treated Atg16L1^{E230+} or Atg16L1^{E230-} mice. (B) The BM was extracted, and the cells were analysed by flow cytometry for engraftment at day 14 and day 20 (n=5 mice). (C) Kaplan-Meier curve showing the survival of Atg16L1^{E230+} and Atg16L1^{E230-} mice post injection (n=5 mice). (D) MEIS/HOXA9 cells were injected (1x10^6) into busulfan treated Atg16L1^{E230+} or Atg16L1^{E230-} mice. (E) The BM was extracted, and the cells were analysed by flow cytometry for engraftment at day 20 (n=5 mice). (F) Kaplan-Meier curve showing the survival of Atg16L1^{E230+} and Atg16L1^{E230-} mice post injection (n=5 mice). (G and H) MN1 cells were injected (1x10^6) into busulfan treated Atg16L1^{E230+/f} Cre^+ or Atg16L1^{E230+/f} Cre^- mice. The BM was extracted, and the cells were analysed by flow cytometry for engraftment at day 14 (n=4 mice). (I) CD14^+ cell where isolated from blood samples from AML patients and controls. Cells were then fixed, permeabilised and stained for LC3 and analysed by microscopy (n=5 control patients and n=8 AML patients). (J) MN1 cells (1x10^6) or vehicle (PBS) were injected into busulfan treated Atg16L1^{E230+} or Atg16L1^{E230-} mice, and the BM harvested 14 days post injection. The percentage of AAM (CD45^+, Lys6G^- and CD11b^+) in the BM as well as the percentage of CD86 and CD206 expressing AAM cells was analysed via flow cytometry (n=3 mice). (K) The percentage of BMM (CD45^+, GR1^-, CD115^{LOW/INT} and F4/80^+) in the BM as well as the percentage of CD86 and CD206 expressing BMM cells was analysed via flow cytometry (n=3 mice). Data shown are means ± SD. *p<0.05, **p<0.01 using the Mann-Witney U test.
Figure 3. LC3-associated phagocytosis in BM macrophage mediates AML apoptotic cell clearance and activates STING pathway.

(A) MN1 cells (1x10^6) or vehicle (PBS) were injected into busulfan treated Atg16L1^{E230+} and Atg16L1^{E230-} mice. The BM was harvested 14 days post injection and the percentage of Annexin V positive cells in the BM was analysed via flow cytometry (n=5 mice). (B) The percentage of Annexin V positive debris found in Atg16L1^{E230+} and Atg16L1^{E230-} mice engrafted with MN1 cells via flow cytometry (n=5 mice). (C) MN1 derived AB were isolated via centrifugation and cultured with Atg16L1^{E230+} and Atg16L1^{E230-} BMDM for 3 hours. Fluorescent microscopy representative images of BMDM cultured with pHrodo labelled AB. The number of phagosomes were counted per Atg16L1^{E230+} and Atg16L1^{E230-} BMDM (n=10). (D) MN1 derived AB were isolated via centrifugation and cultured with Atg16L1^{E230+} and Atg16L1^{E230-} BMDM for 3 hours. Fluorescent microscopy representative images of BMDM cultured with pHrodo labelled AB and stained for LC3. The number of LAPosomes were counted per Atg16L1^{E230+} and Atg16L1^{E230-} BMDM (n=25). (E) MN1 derived AB were isolated via centrifugation and cultured with Atg16L1^{E230+} and Atg16L1^{E230-} BMDM for 3 hours with and without baflomycin treatment (1µM). Fluorescent microscopy representative images of BMDM cultured with pHrodo labelled AB. The number of phagosomes were counted per Atg16L1^{E230+} and Atg16L1^{E230-} BMDM (n=10) (F) MN1 AB were isolated and cultured with Atg16L1^{E230+} and Atg16L1^{E230-} BMDM for 24 hours. The supernatant was removed and centrifuged at 10,000rpm for 10mins to remove any debris before performing a cytokine array. (G) Quantification of cytokine array results segmented in inflammatory, regulatory and STING related cytokines. Data shown are means ± SD. **p<0.01, ***p<0.001 using the Mann-Witney U test (B, C and D) or the Kruskal-Wallis test (A and E).
Figure 4. LAP activates STING in BMM reducing AML engraftment

(A) MN1 cells (1x10^6) or vehicle (PBS) were injected into busulfan treated Atg16L1^{E230+} and Atg16L1^{E230-} mice. BMM F4/80^+ cells were isolated via magnetic separation and the RNA extracted to be analysed via qPCR. (B) The relative gene expression between untreated, engrafted Atg16L1^{E230+} and Atg16L1^{E230-} isolated BMM (n=4 mice). (C) MN1 cells (1x10^6) were injected into busulfan treated C57/BL6 mice. At day 7, 9, 11 and 13 post injection, mice were injected I.P with 200μl of H-151 (750nmol) or vehicle and sacrificed at day 14 post injection. The BMM were sorted, and the RNA extracted to be analysed via qPCR. (D) The relative gene expression from AAM between animals engrafted with MN1 and MN1 treated with H-151. (E) The relative gene expression of IL1β and IL6 (n=4 mice). (F) The BM was extracted, and the cells were analysed by flow cytometry for engraftment at day 14 (n=4 mice). (G and H) MN1 cells (1x10^6) were injected into busulfan treated Atg16L1^{E230+} and Atg16L1^{E230-} mice. At day 7, 9, 11 and 13 post injection, mice were injected I.P with 200μl of H-151 (750nmol) or vehicle and sacrificed at day 14 post injection. The BM was extracted, and the cells were analysed by flow cytometry for engraftment at day 14 (n=4 mice). (I) Schematic diagram of experimental design. F4/80^+ BMM were sorted from C57/BL6 mice and cultured with H-151 (10μM) or vehicle for 2 hours. BMM were then incubated with pHrodo E.coli bioparticles for 2 hours. (J) Representative microscopy images. The number of bioparticles (red) were counted per BMM for control and H-151 treated BMM (n=25 BMM). Data shown are means ± SD. *p<0.05 ***p<0.001 using the Mann-Witney U test (D, E, F, H and J) or the Kruskal-Wallis test (B).
Figure 5. AML derived apoptotic bodies contain mitochondria which are processed by BMM.
(A) AB were isolated from MN1 and non-malignant LSK cells and cultured with BMDM from C57/BL6 mice for 24 hours and the RNA extracted to be analysed via qPCR. (B) The relative gene expression of gbp2, irf7 and ifit3 of BMDM cultured with MN1 and LSK AB (n=5). (C) Representative images of non-malignant LSK and MN1 cells that were stained with MTG and VybrantDil, the AB isolated and analysed via image flow cytometry. (D) The percentage of AB that were positive for MTG and VybrantDil between LSK and MN1 cells (n=5). (E) Representative image of human AML cells that were transduced with a GFP-membrane virus and stained with MTR and Hoechst. AML cells were observed under confocal microscopy, arrows indicate blebs containing mitochondria. (F) Non-malignant CD34, MN1 and human AML cells were stained with MTR before isolating the AB and analysing them via flow cytometry for the percentage of MTR containing AB (n=5). (G) Schematic diagram of experimental design. Primary AML cells were transduced with rLV.EF1.mCherry mito9 lentivirus (mCh-AML) and injected into NSG mice for 35 days (n=3). (H) The BM was extracted and BMM were analysed by flow cytometry for mCherry fluorescence. (I) mCh-AML cells were co-cultured with BMSC and BMDM and analysed via microscopy for mitochondrial uptake via MFI of mCherry (n=25). Data shown are means ± SD. *p<0.05, **p<0.01 using the Mann-Witney U test (B, D, and I) or the Kruskal-Wallis test (F and H).
Figure 6. AML derived apoptotic bodies containing mtDNA activates STING in BMM via LAP dependent mechanism.

(A). Primary AML cells were transduced with rLV.EF1.mCherry mito9 lentivirus (mCh-AML) and the AB isolated (mCh-AB). mCh-AB were cultured with BMDM from Atg16L1\textsuperscript{E230+} and Atg16L1\textsuperscript{E230-} mice for 24 hours. (B) The mCherry intensity was analysed between Atg16L1\textsuperscript{E230+} and Atg16L1\textsuperscript{E230-} BMDM at 4 and 24 hours via confocal microscopy (n=5). (C) The relative mitochondrial DNA levels of MN1 cells and \(p^0\) generated MN1 cells normalised to DNA levels using Taqman PCR and Tert and ND3 probes (n=5). (D). Non-malignant LSK cells and MN1 cells were stained for MTR and the AB isolated before being sorted based on positive MTR signal. \(p^0\) MN1 cells AB were also isolated. The AB were cultured with BMDM from C57/BL6 mice for 24 hours and the RNA extracted to be analysed via qPCR. (E) The relative gene expression of \textit{gbp2}, \textit{irf7} and \textit{ifit3} of BMDM cultured with sorted mitochondria containing AB from MN1 and LSK and \(p^0\) MN1 cells AB (n=4). (F) Non-malignant LSK cells and MN1 cells were stained for MTR and the AB isolated before being sorted based on positive MTR signal. The AB were cultured with BMDM from Atg16L1\textsuperscript{E230+} and Atg16L1\textsuperscript{E230-} mice for 24 hours and the RNA extracted to be analysed via qPCR. (G) The relative gene expression of \textit{gbp2}, \textit{irf7} and \textit{ifit3} of Atg16L1\textsuperscript{E230+} or Atg16L1\textsuperscript{E230-} BMDM cultured with sorted mitochondria containing AB from MN1 and LSK cells (n=5). Data shown are means ± SD. *\(p<0.05\), **\(p<0.01\) using the Mann-Witney U test (B and C) or the Kruskal-Wallis test (E and G).
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<td>37</td>
<td>M</td>
<td>AML without maturation</td>
</tr>
<tr>
<td>AML8</td>
<td>61</td>
<td>M</td>
<td>AML with mutated NPM1</td>
</tr>
<tr>
<td>AML9</td>
<td>54</td>
<td>M</td>
<td>AML with mutated NPM1</td>
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<tr>
<td>AML10</td>
<td>67</td>
<td>M</td>
<td>AML with mutated NPM1</td>
</tr>
<tr>
<td>AML11</td>
<td>58</td>
<td>M</td>
<td>AML with biallelic mutations of CEBPA</td>
</tr>
<tr>
<td>AML12</td>
<td>78</td>
<td>M</td>
<td>Acute monoblastic and monocytic leukaemia</td>
</tr>
<tr>
<td>AML13</td>
<td>54</td>
<td>F</td>
<td>Acute myeloid leukaemia with t(9;11)(p21.3;q23.3); KMT2A-MLLT3</td>
</tr>
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</table>

**Table 1.** AML patient information.