LAMP-5 is an essential inflammatory-signaling regulator and novel immunotherapy target for mixed lineage leukemia-rearranged acute leukemia

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ABSTRACT

Although great advances have been made in understanding the pathobiology of mixed lineage leukemia-rearranged (MLL-r) leukemias, therapies for this leukemia have remained limited, and clinical outcomes remain bleak. In order to identify novel targets for immunotherapy treatments, we compiled a lineage-independent MLL-r leukemia gene signature using publicly available data sets. Data from large leukemia repositories were filtered through the in silico human surfaceome, providing a list of highly predicted cell surface proteins overexpressed in MLL-r leukemias. LAMP5, a lysosomal associated membrane protein, is expressed highly and specifically in MLL-r leukemia. We found that LAMP5 is a direct target of the oncogenic MLL-fusion protein. LAMP5 depletion significantly inhibited leukemia cell growth in vitro and in vivo. Functional studies showed that LAMP-5 is a novel modulator of innate-immune pathways in MLL-r leukemias. Downregulation of LAMP5 led to inhibition of NF-κB signaling and increased activation of type-1 interferon signaling downstream of Toll-like receptor/interleukin 1 receptor activation. These effects were attributable to the critical role of LAMP-5 in transferring the signal flux from interferon signaling endosomes to pro-inflammatory signaling endosomes. Depletion of IRF7 was able to partially rescue the cell growth inhibition upon LAMP5 downregulation. Lastly, LAMP-5 was readily detected on the surface of MLL-r leukemia cells. Targeting surface LAMP-5 using an antibody-drug conjugate leads to significant cell viability decrease specifically in MLL-r leukemias. Overall, based on the limited expression throughout human tissues, we postulate that LAMP-5 could potentially serve as an immunotherapeutic target with a wide therapeutic window to treat MLL-r leukemias.

Introduction

Translocations in the mixed lineage leukemia (MLL) gene account for 10% of all human leukemias and are associated with pediatric, adult, and therapy-related cases. In infants, around 80% of acute lymphoid leukemia (ALL) and 35%-50% of acute myeloid leukemia (AML) cases carry a translocation in the MLL gene.1 However, despite improvements in conventional chemotherapy treatments for leukemia, patients with MLL-rearranged leukemia (MLL-r) have a poor response to treatment
and poor prognosis. Immunotherapy strategies have proven effective in multiple blood cancers, mainly targeting lineage-specific proteins like CD19 (blinatumomab, tisagenlecleucel) and CD33 (gemtuzumab), abundantly expressed in ALL and AML patients, respectively. However, mounting evidence in recent clinical trials and case reports have shown that patients with MLL-rearrangements frequently relapse after treatment with CD19 immunotherapies, arising as AML or mixed phenotype acute leukemia (MPAL). The exact mechanism of lineage switch induced by CD19 immunotherapies is still unclear.

One approach to overcome the lineage switching is to develop MLL-r specific immunotherapies targeting cell surface proteins essential for the survival of MLL-r leukemias. Recently, NG2/CSPG4 and CD133/PROM1 have been shown to be promising MLL-r specific immunotherapy targets, however, these targets are restricted to lymphoid lineage, increasing the potential for lineage switching within the leukemic population.

Gene-expression profiling based on underlying cytogenetic mutations is a way to identify proteins that are overexpressed and thus might be essential for the propagation of the specific leukemia. Both AML and ALL with MLL-rearrangements share a common gene signature that is distinct from that of MLL-germline (MLL-G) leukemias. Most of the well-studied and validated MLL-r gene targets however are DNA binding proteins like the HOXA gene cluster and its co-factor MEIS1, which are not suitable targets for immunotherapy.

In several of the published gene-expression studies, we found LAMPs significantly and specifically overexpressed in MLL-r leukemias. LAMP-5 is a member of the lysosome-associated membrane protein (LAMP) family. In contrast to other LAMP proteins which show widespread expression, LAMP5 expression in mice is confined to several regions of the postnatal brain. In neurons, the protein was found to recycle between the plasma membrane and a non-classical endosomal vesicle. In humans, aside from its conserved expression in the brain, LAMP5 is specifically expressed in plasmacytoid dendritic cells (pDC). Upon activation of pDC, LAMP-5 aids in the transport of Toll-like receptor 9 (TLR9) from early endosomal to lysosomal signaling vesicles, thereby regulating type 1 interferon (IFN-1) and pro-inflammatory signaling respectively, downstream of TLR9 activation. Importantly, results of in silico modeling predict LAMP-5 as a cell surface protein. In this report, we demonstrate LAMP5 as being highly expressed and essential for MLL-r leukemias through the regulation of innate immune signaling and describe its potential as a target for MLL-r specific immunotherapy.

**Methods**

**Cell lines and primary patient-derived xenografts**

Human leukemia cell lines were maintained in Iscove’s Modified Dulbecco Medium (IMDM) or Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% fetal bovine serum (FBS), 1% penicillin, and 1% streptomycin. Fully de-identified primary cells were obtained from the Cincinnati Children’s Hospital Medical Center Biorepository. Cells were cultured in IMDM supplemented with 20% FBS and 10 ng/mL human cytokines including SCF, FLT3-ligand, thrombopoietin, IL-3, and IL-6.

**Animal experiments**

All animal experiments were carried out in accordance with the guidelines of the Institutional Animal Care and Use Committee (IACUC). For xenograft experiments with MV4;11 and MLL-AF10 primary patient cells, immunocompromised NOD- Rag2<sup>−/−</sup> Il2rg<sup>−/−</sup> (NRG) (Jackson Laboratories, stock no. 007799) recipient mice were conditioned with busulfan and transplanted 24 hours later. In xenograft experiments bone marrow samples were collected 4 weeks after transplantation as well as when signs of leukemia were present; bone marrow aspirates were analyzed via flow cytometry for the presence of human CD45<sup>+</sup> cells and the presence of short hairpin RNA (shRNA)-transduced Venus+ cells.

**Retroviral and lentiviral transductions**

Retroviral and lentiviral supernatants were generated by transfection of HEK293T cells using the FuGENE 6 reagent (Promega) or Lipofectamine 3000 (Thermo Fisher Scientific) according to the manufacturer’s recommendations. All lentiviral shRNA constructs were purchased from Millipore Sigma. Cells transduced with constructs containing a fluorescent marker (Venus) were sorted 4-5 days after transduction by using MoFlo XDP (Beckman Coulter), FACSAria (BD Biosciences), or a SONY SH800S (Sony Biotechnology).

**Flow cytometry**

For apoptosis assays, cells were incubated with allophycocyanin (APC)-conjugated annexin V (BD Biosciences) for 15 minutes at room temperature in 1X annexin V binding buffer (BD Biosciences) followed by staining with 7-aminoactinomycin D (7-AAD) (eBioscience). For surface LAMP-5 detection, cells were incubated with 3 µg anti-human LAMP-5 therapeutic antibody overnight and then stained with anti-mouse IgG1-PE. Data were acquired on a BD FACSCanto analyzer and results were analyzed using FlowJo Version 10 (BD Biosciences).

** Colony-forming unit assays**

Transduced human cells were sorted 4-5 days after transduction and were cultured in methylcellulose-containing media (StemCell Technologies, H4434). Colonies were scored 10-14 days after plating.

**Real-time and quantitative real-time polymerase chain reaction**

Total RNA was extracted from human puromycin-selected or sorted Venus’ cells using the RNeasy Mini kit (QiAGEN). For quantitative real-time polymerase chain reaction (RT-PCR) 5-10 ng of cDNA was analyzed using iTaq Universal SYBR Green Supermix (Bio-Rad) or PowerUP SYBR Green (Thermo Fisher Scientific) in a StepOnePlus RT-PCR machine (Applied Biosystems).

**Cell viability**

MOLM-13, RS4;11, THP-1, and Kasumi-1 cells were plated at 10,000 cells per well in a 96-well plate. Cells were incubated with LAMP-5 therapeutic antibody (Creative Biolabs) and anti-mouse immunoglobulin G (IgG) Fc-DM1 antibody with non-cleavable linker (Moradec) at 5 ng/µl and 1 ng/µl final concentrations, respectively. In order to measure cell viability CellTiter-Glo® 2.0 Cell Viability Assay (Promega) was used following the manufacturer’s protocol.

**Immunofluorescence**

Cells seeded on alician blue-treated coverslips were fixed with 3.5% paraformaldehyde and permeabilized with 0.05% saponin. Cells were stained overnight with primary antibodies against...
LAMP-5, MYD88, and LAMP-1. Immunofluorescence and confocal microscopy were performed with a Zeiss LSM580 63x objective and accompanying imaging software.

Statistics

The statistical methodology used, and sample sizes are described in the individual Figure legends. t-tests were two-tailed unless otherwise stated. Results are presented as mean ± standard error of the mean (SEM) unless otherwise stated. A two-sided time-stratified Cochran-Mantel-Haenszel was used for the Kaplan-Meier Survival analysis. ROC curves were used to determine the diagnostic utility of LAMP5 mRNA. The sensitivity and specificity were identified at the optimal cutoff point that was chosen at which Youden’s index was maximal. A significance level cutoff of 0.05 was used unless otherwise stated. Statistical analysis was performed using GraphPad Prism.

More detailed information on the materials and methods used can be found in the Online Supplementary Appendix.

Results

LAMP5 is highly expressed in mixed lineage leukemia-rearranged leukemias and is a direct target of the mixed lineage leukemia-fusion protein

In order to determine genes that are highly expressed in AML and ALL with MLL-rearrangements, we compared recently published RNA sequencing (RNA-seq) studies that identified differentially expressed genes between MLL-r and MLL-G leukemias in both AML and B-ALL samples30,31 (Figure 1A). Twenty-seven genes were commonly overexpressed in MLL-r ALL and AML (Online Supplementary Table S1). Using the in silico human surfaceome tool (http://wlab.ethz.ch/surfaceome) five of these 27 genes were predicted to be expressed on the cell surface32,33 (Online Supplementary Figure S1A and B). We further validated the specificity of LAMP5 expression in MLL-r leukemias by analyzing the 1,109 pediatric leukemia-rearranged leukemias by analyzing the 1,109 pediatric leukemia patient samples from the St. Jude PeCan Portal and revealed LAMP5 mRNA. The sensitivity and specificity were identified at the optimal cutoff point that was chosen at which Youden’s index was maximal. A significance level cutoff of 0.05 was used unless otherwise stated. Statistical analysis was performed using GraphPad Prism.

More detailed information on the materials and methods used can be found in the Online Supplementary Appendix.

LAMP-5 is required for in vitro and in vivo leukemia cell survival

The ideal immunotherapy target should be essential for the survival of MLL-r leukemias. In order to test the functional role of LAMP-5 in MLL-r leukemia, we transduced both MLL-r leukemia (MOLM-13, MV4;11, RS4;11, THP-1) and MLL-G leukemia cells (Kasumi-1 and REH) with lentiviral shRNA vectors targeting LAMP5. We obtained efficient knockdown of LAMP5 with two independent hairpins as compared to non-targeting control (NT) (Online Supplementary Figure S4A). Upon LAMP5 depletion, we observed a significant reduction of cell growth in MLL-r leukemia cell lines (Figure 2A), while no effect was seen in Kasumi-1 and REH (Figure 2B). Additionally, LAMP5 knockdown led to a significant decrease in colony-forming units (CFU) in the MLL-r leukemia cell lines (Figure 2C), suggesting an effect on the clonogenicity of these cells. Furthermore, LAMP5 knockdown led to apoptosis in MLL-r leukemia cells, as evident by a significant increase in annexin V and 7-AAD double-positive staining (Figure 2D; Online Supplementary Figure S4B). We next sought to determine the role of LAMP-5 in leukemia propagation in vivo. MV4;11 cells were transduced with shLAMP5-2 or NT control followed by transplantation into immunocompromised NOD-rag1<sup>−/−</sup> Il2rg<sup>−/−</sup> (NRG) mice (Figure 2E). In the bone marrow, both groups showed similar human cell engraftment based on human CD45 expression. On the other hand, the transduced Venus<sup>+</sup> fraction was significantly reduced in shLAMP5-2 compared to shNT mice 4 weeks after transplantation (Figure 2F, left panels). We repeated this experiment using cells from an AML PDX with MLL-r (MLL-AF10) leukemia. We again observed a significant reduction in the proportion of Venus<sup>+</sup> cells with LAMP5 knockdown compared to NT control (Figure 2F, right panels). Overall, these data underscore a critical role for LAMP-5 in the growth of MLL-r leukemia cells.
LAMP-5 is required for activation of Toll-like receptor/interleukin 1 receptor signaling in leukemia

Acute leukemias exhibiting constitutive activation of innate immune signaling pathways have been characterized as having a pro-inflammatory profile which is required for their survival. These physiologic cellular systems involve TLR/IL-1R signaling and culminate in the release of pro-inflammatory cytokines via NF-κB and/or type I interferons (IFN-1). Recent studies reveal heightened activation of NF-κB signaling in MLL-r leukemia compared to other leukemias. Furthermore, MLL-r leukemias have been shown to require the TLR/IL-1R signaling pathway to survive, through degradation of the wild-type MLL protein, allowing the MLL-FP to bind to its target genes without restriction. Recently, Combes et al. showed that LAMP-5 plays an important role in controlling the subcellular location of TLR9 after activation in human pDC. Upon activation of TLR9, LAMP-5 shuttles TLR9 from the VAMP3-interferon response factor signaling endosome (IRF-SE), to the LAMP-1 pro-inflammatory-signaling endosome (PI-SE). This transition of TLR localization in turn acts as a negative regulator of IFN-1 signaling. Based on the known role of LAMP-5 in TLR9 localization in pDC, we first examined the localization of intracellular LAMP-5 in MOLM-13 cells. We performed co-staining of MOLM-13 cells with antibodies against LAMP-5, LAMP-1, and myeloid differentiation primary response 88 (MYD88), a scaffold protein that is required for TLR and IL-1R signaling. Confocal microscopy showed that in MOLM-13 leukemia cells, LAMP-5 local-
ized to LAMP-1+ vesicles. As suspected, we found MYD88 accumulating highly in the periphery of LAMP-1+ vesicles in MLL-r leukemia, suggestive of TLR/IL-1R activation (Figure 3A). Conversely, in Kasumi-1 cells, MYD88 does not co-localize with LAMP-1+ vesicles. However, overexpression of wild-type LAMP-5 in this cell line led to the relocation of MYD88 around LAMP-1+ vesicles (Figure 3B).

We subsequently hypothesized that LAMP-5 loss may dampen TLR/IL-1R signaling in MLL-r leukemias. We thus analyzed known effector proteins downstream of TLR/IL-1R activation by western blot. Upon LAMP5 knockdown, we observed a reduction in phosphorylated IRAK1, NF-kB, p38, and JNK, key players in the signal transduction downstream of TLR/IL-1R (Figure 3C). In order to further determine the impact of LAMP5 depletion in TLR-medi- ated NF-kB activation, we measured NF-kB activity using the THP-1 NF-kB-SEAP cell line, which contains an NF-kB inducible secreted embryonic alkaline phosphatase (SEAP) reporter. Robust activation of NF-kB was evident in control cells upon incubation with PAM3CSK4 (TLR2 agonist) or LPS (TLR4 agonist). Knockdown of LAMP5 led to a near-complete blockade of this activation, suggesting that TLR-induced NF-kB signaling is disrupted upon LAMP5 depletion (Figure 3D). Correspondingly, in Kasumi-1 cells, overexpression of LAMP5 led to increased phosphorylation of p38, JNK, and NF-kB along with increased cell growth (Figure 3E and F).

A previous study showed that NF-kB plays a critical role in MLL-r leukemias.39 We thus hypothesized that NF-kB activation would rescue the cell growth defect seen by LAMP5 as an essential target of MLL-r leukemias.

Figure 1. LAMP5 is highly expressed in mixed lineage leukemia-rearranged leukemias and is a direct target of the mixed lineage leukemia-fusion protein. (A) The intersection of published gene expression signatures composed of genes overexpressed in mixed lineage leukemia-rearranged (MLL-r) acute myeloid leukemia (AML) and acute lymphoid leukemia (ALL) when compared to MLL-germline (MLL-G) leukemias. (B) Log2 FPKM expression of LAMP5 in AML and ALL pediatric patients with MLL-rearrangement (AML MLL-r, n=36 and ALL MLL-r, n=70) compared to MLL-G (AML MLL-G, n=270) (ALL MLL-G, n=727) patients. Data obtained from the St. Jude PeCan Portal and presented as median value with quartiles (t-test, ***P<0.0001). (C) Receiving operating curve (ROC) analysis showing the capacity of LAMP5 to discriminate acute leukemia patients with MLL-G or MLL-r leukemias. Data obtained from GSE13159 and St. Jude Pecan Portal. (D) Western blot analysis of LAMP-5 expression in pediatric primary ALL and AML samples. Actin or vinculin was used as a loading control. (E) Relative expression of LAMP5 in MLLr leukemia (MV4;11, MOLM-13, THP-1, and RS4;11) and MLL-G leukemia (K562, HL-60, Kasumi-1, REH, RCH-AO1) cell lines. The graph represents the relative expression of LAMP5 normalized to β-actin. Data are from three biological replicates. LAMP5 expression in cord blood cells was set as 1.0. Bars show mean ± standard error of the mean (SEM). (F) Quantitative real-time polymerase chain reaction (RT-PCR) analysis of LAMP5 and MLL-AF9 gene expression in CD34+ cord blood cells were used as control. Actin was used as a loading control. (G) Representative chromatin immunoprecipitation sequencing (ChIP-seq) tracks at the LAMP5 locus from different MLL-r cell lines. ChIP-seq data were obtained from GSE95511 for MLL-2, GSE79899 for MV4;11 and THP-1, GSE38403 for RS4;11, GSE38338 for SEM, GSE84116 for CB CD34+ MLL-AF4, and GSE83671 for primary patient MLL-AF4.
affected by LAMP5 depletion (Online Supplementary Figure S5A and B). A potential mechanism underlying this essentiality was proposed by Wang et al., where they suggested that loss of LAMP5 in MLL-r leukemia led to degradation of the MLL-FP due to increased autophagy. However, in our experiments, we did not observe any change in the levels of the MLL-FP or LC3 A/B in THP-1 and MOLM-13 cells upon LAMP5 depletion (Online Supplemental Figure 6A and B). Overall, these results underscore a critical role for LAMP-5 in the activation of TLR/IL-1R signaling in MLL-r leukemia, while also indicating the presence of additional attributes that are also essential.
**LAMP-5 is a negative regulator of interferon-1 signaling in mixed lineage leukemia-rearranged leukemias**

Since activation of NF-κB was not sufficient to rescue the cell growth inhibition seen upon LAMP5 depletion, we next sought to understand the mechanistic significance of the inflammatory-signal-regulation function of LAMP-5 in MLL-r leukemia. In pDC, the carboxy-terminal YKHM domain of LAMP-5 was found to be required for normal localization of LAMP-5 and transportation of TLR9 from the early endosome vesicle to the pro-inflammatory vesicle.24,27,28 We thus overexpressed wild-type LAMP5 (LAMP5-WT), a Y276A mutant LAMP5 (LAMP5-mut), or control vector (EV) in MV4;11 and THP-1 cells, followed by selective knockdown of endogenous LAMP5 using an shRNA targeting the 3'UTR region of LAMP5 (Online Supplementary Figure S7A). Overexpression of LAMP5-WT completely prevented cell growth inhibition and apoptosis upon knockdown of endogenous LAMP5, validating LAMP5 as the main target of the shRNA. In contrast, LAMP5-mut was unable to rescue cell growth or apoptosis in MV4;11 (Figure 4A and B). In pDC, LAMP5 knockdown or overexpression of LAMP5-mut induced IFN-1 activation upon TLR9-stimulation, due to retention of TLR9 in the IRF-SE. In order to determine the effect of LAMP5 on IFN-1 signaling in MLL-r leukemia, we turned to THP-1-ISG-SEAP cells containing an interferon-stimulated gene (ISG) inducible-SEAP reporter. Upon TLR activation by PAM3CSK4, IFN-1 signaling activation was evident only in the LAMP5-depleted cells but not in the control condition (Figure 4C). Furthermore, gene set enrichment analysis of RNA-seq from MOLM-13 cells transduced with shNT or shLAMP5-2 showed enrichment of IFN gene signatures (Online Supplementary Figure S8). Additionally, we validated the increase in IFN-1 signaling in several MLL-r cell lines by demonstrably increased expression of interferon α2 (IFNA2) and interferon β (IFNB) upon depletion of LAMP5 (Figure 4D). In order to assess the role of LAMP5-depletion mediated IFN-1 activation on cell growth, we performed knockdown of interferon regulatory factor 7 (IRF7), a known regulator of IFN signaling downstream of TLR/IL1R activation, along with LAMP5 in MV4;11 and THP-1 cells. We found that loss of IRF7 alone had no significant effect on MLL-r leukemia cell growth but importantly, its depletion prevented the growth inhibition observed upon LAMP5 knockdown (Figures 4E and F; Online Supplementary Figure S9A and B).

Collectively, these results demonstrate that a critical function of LAMP-5 in MLL-r leukemias is to promote the transfer of TLR/IL-1R from the IFN-1–activating signaling pathway.
Figure 4. Continued on following page.
cascade to the pro-inflammatory signaling cascade. Depletion of LAMP5 thus leads not only to loss of NF-κB activation but also to activation of IFN-1-signaling, the latter inducing cell death.

**Surface LAMP-5 can be detected and targeted with antibody drug conjugate therapy**

LAMP-5 has been found to briefly localize in the plasma membrane of cortical neurons in mice and is highly predicted to reach the cell membrane based on the human surfaceome.\(^2^{4,29}\) We thus sought to confirm if LAMP-5 was expressed on the surface of MLL-r leukemia cells. Using an antibody targeting the N-terminus of LAMP-5, we were able to detect LAMP-5 on the surface of MLL-r leukemia cell lines, while none was detected in the MLL-G leukemias (Figure 5A and B). In order to validate the specificity of the antibody, we overexpressed LAMP5 or control empty vector (EV) in Kasumi-1 cells. We detected surface LAMP-5 only in the cells that express high levels of LAMP5 (Figure 5C).

As a proof-of-concept for potential therapeutic use, we used a secondary antibody conjugated to the tubulin-toxin Mertansine, targeting the surface-LAMP-5 antibody. We observed that a 72-hour treatment with this antibody-sandwich comprised of the surface LAMP-5 antibody along with the secondary antibody drug conjugate (ADC) antibody is sufficient to reduce cell viability in MLL-r leukemia cell lines MOLM-13, RS4;11 and THP-1, while no effect was seen in Kasumi-1 cells (Figure 5D). These results suggest that LAMP-5 could be exploited as an MLL-r specific biomarker and could potentially be used as a target for immunotherapy.

**Discussion**

Our findings further reaffirm LAMPs as a novel and essential core gene in MLL-r leukemias, directly upregulated by the MLL-4P. Additionally, we found that one of the critical functions of LAMP-5 is to regulate innate-immune signaling in MLL-r leukemias, specifically directing the flux of activity away from IRF-SE towards the PI-SE, leading to constant activation of NF-κB. Recent discoveries have highlighted how the specific subcellular location and timing of TLR activation affect signaling outcomes in normal immune cells.\(^43\) Combes et al. showed that LAMP-5 is a negative regulator of IFN-1 signaling in pDC wherein it transports activated TLR9 from the IRF-SE to the PI-SE. Although dispensable for pDC cell survival, LAMP5 depletion led to unrestricted activation of IFN-1 signaling. Furthermore, aberrant expression of LAMP-5 can lead to diminished activation of pDC in tumors and contribute to their immunomodulatory phenotype by decreasing the IFN-1 production capacity.\(^39\) However, how these mechanisms function in leukemia is still poorly understood. Innate immune signaling and inflammation have been shown to play a crucial role in acute leukemias.\(^37\) MLL-r leukemias rely on activation of NF-κB downstream of TLR/IL-1R to main the MLL-FP gene signature and block cell differentiation.\(^39\) Furthermore, it has been shown that treatment with IFN-1 or activation of IFN-1 signaling is deleterious for MLL-r leukemias.\(^44\) In our study, we describe a novel role for LAMP-5 in maintaining NF-κB activation and blocking IFN-1 signaling downstream of TLR/IL-1R in MLL-r leukemias. We show that LAMP-5 acts as a molecular switch to maintain active TLR/IL-1R signaling in the pro-inflammatory endosome leading to NF-κB activation, whereas LAMP5 depletion leads to activation of IFN-1 signaling and cell death. This suggests that both the LAMP-5-mediated induction of pro-inflammatory signaling and inhibition of IFN-1 signaling contribute to the pathogenesis of MLL-r leukemias. We confirmed that activation of IFN-1 signaling upon LAMP-5 depletion was deleterious for leukemia propagation, and that by depleting IRF7, cell growth and clonogenicity were rescued in LAMP5-depleted cells. This suggests that increased IFN-1 signaling is at least partly responsible for inducing cell death upon LAMP5 depletion. Additionally, overexpression of LAMP5 in MLL-G leukemia led to increased acti-
vation of NF-κB, p38, and JNK, and increased cell growth, which suggests that this signaling pathway might be contributing to the therapy-resistant phenotype of MLL-r leukemias.

In humans, LAMP5 expression is generally restricted to the brain and blood. In blood, LAMP5 is exclusively expressed on nonactivated pDC, wherein LAMP-5 resides in the ERGIC compartment and is transported to endo-lysosomal vesicles upon TLR9 activation. We found that the aberrant increased expression of LAMP-5 in MLL-r leukemia leads to its accumulation in the plasma membrane, as demonstrated by a novel LAMP-5 antibody targeting the N-terminus of the protein. The detection of LAMP-5 on the surface of MLL leukemias provides the opportunity to potentially use it as a target for immunotherapy in this treatment-refractory malignancy. Furthermore, LAMP-5 is highly expressed in other cancers such as multiple myeloma (MM) and blastic plasmacytoid dendritic cell neoplasm (BPDCN). Therefore, LAMP-5 immunotherapies could benefit other blood diseases. Finally, total loss of Lamp5 had no major effects on the health or lifespan of mice, only causing minor behavioral effects like deficits in olfactory discrimination and increased startle response to auditory and tactile stimuli, suggesting that there could be a wide therapeutic window for LAMP-5-directed therapies in humans.

Figure 5. Surface LAMP-5 can be detected and targeted with antibody drug conjugate therapy. (A) Representative histogram plots showing LAMP-5 surface expression in mixed lineage leukemia-rearranged (MLL-r) leukemia (MOLM-13, RS4;11, MV4;11, and THP-1) and MLL-germline (MLL-G) leukemia (Kasumi-1, HL-60, and NB4) cell lines. (B) Graph showing mean fluorescence intensity (MFI) of LAMP-5 surface staining in MLL-r leukemias vs. MLL-G leukemias represented as mean and ± standard deviation (SD). t-test, **P<0.01. (C) Representative histogram of LAMP-5 staining in Kasumi-1 expressing empty vector (EV) or LAMP5, confirming the specificity of the antibody. (D) MOLM-13, RS4;11, THP-1, and Kasumi-1 cells were incubated with surface LAMP-5 antibody clone D1 and αMFC/N-CLM1 antibody drug conjugate (ADC) antibody for 72 hours. Bar graph represents cell viability from three biological replicates presented as mean and ± SEM. t-test, *P<0.05.
Similar to our observations, Wang et al. recently showed that LAMP-5 is essential for the survival of MLL-r leukemias in vitro and in vivo using shRNA knockdown. However, they propose that LAMP-5 is a negative regulator of autophagy leading to MLL-FP stabilization. They show that LAMP-5 and ATG5 co-localize in MLL-r leukemia cells and that blockade of autophagy is sufficient to rescue the increased levels of apoptosis after LAMP5 knockdown. We were unable to detect any significant change in the levels of the MLL-FP or LC3A/B upon LAMP5 knockdown. Since TLR-mediated innate immune signaling can regulate autophagy, the function of LAMP-5 in regulating autophagy as described by Wang et al. may be downstream of its impact on endosome-lysosome trafficking. On the other hand, it is also possible that these effects are not directly linked, and that LAMP-5 might exert its growth-promoting effects in MLL-r leukemia by multiple mechanisms. It is notable, however, that the role of autophagy in leukemia is controversial. In murine MLL leukemia models, heterozygous loss of Atg5 leads to increased leukemia cell proliferation in vitro and more aggressive leukemia in vivo, while homozygous loss is lethal to these cells. Additionally, while some studies suggest that Atg5-dependent autophagy may contribute to the development of MLL-AF9 driven leukemia but dispensable for propagation and chemosensitivity, others suggest that Atg5-dependent autophagy is dispensable altogether. Overall, our results show that LAMP-5 localizes both on the surface and in LAMP-1+ endosomes in leukemia, leading to constitutive activation of pro-inflammatory signaling, and dampening of interferon-signaling and that it can be used as a target for immunotherapy.

Disclosures
No conflicts of interest to disclose.

Contributions
GGM, LHL and ARK contributed to study conception and design; GGM, JC, MB and BG acquired data; GGM, JC, MW, DL, PF, EG and LHL analyzed and interpreted data; NS and LHL analyzed and interpreted RNA-seq data; GGM, JC, LHL and ARK wrote and revised the manuscript; MW, DL and JC provided administrative, technical, or material support.

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