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► To cite this version:

Gabriel Gracia-Maldonado, Jason Clark, Matthew Burwinkel, Brenay Greenslade, Mark Wunderlich, et al.. LAMP-5 is an essential inflammatory-signaling regulator and novel immunotherapy target for Mixed Lineage Leukemia-Rearranged acute leukemia. *Haematologica*, 2020, 10.3324/haematol.2020.257451 . hal-03385563

HAL Id: hal-03385563

<https://amu.hal.science/hal-03385563v1>

Submitted on 19 Oct 2021

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LAMP-5 is an essential inflammatory-signaling regulator and novel immunotherapy target in MLL-r leukemia

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Running Title: The LAMP5 as a essential target of MLL-r leukemias

Keywords: Leukemia; MLL; LAMP5; innate Immunity

Conflict of Interest: The authors have declared that no conflict of interest exists.

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31 **Abstract:**

32 Although great advances have been made in understanding the pathobiology of MLL-
33 rearranged (MLL-r) leukemias, effective therapies for this leukemia have remained
34 limited, and clinical outcomes remain bleak. To identify novel targets for immunotherapy
35 treatments, we compiled a lineage-independent MLL-r leukemia gene signature using
36 publicly available data sets. Data from large leukemia repositories were filtered through
37 the In-silico Human Surfaceome, providing a list of highly predicted cell surface proteins
38 over-expressed in MLL-r leukemias. *LAMP5*, a lysosomal associated membrane protein,
39 is expressed highly and specifically in MLL-r leukemia. We found that *LAMP5* is a direct
40 target of the oncogenic MLL-fusion protein (MLL-FP). *LAMP5* depletion significantly
41 inhibited leukemia cell growth *in vitro* and *in vivo*. Functional studies showed that LAMP-
42 5 is a novel modulator of innate-immune pathways in MLL-r leukemias. Downregulation
43 of *LAMP5* led to inhibition of NF- κ B signaling and increased activation of type-1 interferon
44 (IFN-1) signaling downstream of TLR/IL1R activation. These effects were attributable to
45 the critical role of LAMP5 in transferring the signal flux from Interferon Signaling
46 Endosomes (IRF-SE) to Pro-Inflammatory Signaling Endosome (PI-SE). Depletion of
47 Interferon Response Factor 7 (IRF7) was able to partially rescue the cell growth inhibition
48 upon *LAMP5* downregulation. Lastly, LAMP-5 was readily detected on the surface of MLL-
49 r leukemia cells. Targeting surface LAMP-5 using an antibody drug conjugate lead to
50 significant cell viability decrease specifically on MLL-r leukemias. Overall, based on our
51 results and the limited expression throughout human tissues, we postulate that LAMP-5
52 could potentially serve as an immunotherapeutic target with a wide therapeutic window
53 to treat MLL-r leukemias.

54 **Introduction**

55 Translocations in the Mixed Lineage Leukemia (*MLL*) gene account for 10% of all human
56 leukemias and are associated with pediatric, adult, and therapy-related cases(1–3). In
57 infants, around 80% of acute lymphoid leukemia (ALL) and 35%-50% of acute myeloid
58 leukemia (AML) cases carry a translocation in the *MLL* gene (4–6). However, despite
59 improvements in conventional chemotherapies treatments for leukemia, patients with
60 *MLL*-rearranged leukemia (*MLL*-r) have poor response to treatment and poor prognosis
61 (7, 8). Immunotherapy strategies have proven effective in multiple blood cancers, mainly
62 targeting lineage specific proteins like CD19 (blinatumomab, tisagenlecleucel) and CD33
63 (gemtuzumab), abundantly expressed in Acute Lymphoid Leukemia (ALL) and Acute
64 Myeloid Leukemia (AML) patients respectively(9). However, mounting evidence in recent
65 clinical trials and case reports have shown that patients with *MLL*-rearrangements
66 frequently relapse after treatment with CD19 immunotherapies, arising as AML or Mixed
67 Phenotype Acute Leukemia (MPAL) (10–18). The exact mechanism of lineage switch
68 remains unclear, although several possible mechanisms have been proposed(11, 15, 18–
69 22).

70 One approach to overcome this immune escape is to develop *MLL*-r specific
71 immunotherapies targeting cell surface proteins essential for the survival of *MLL*-r
72 leukemias. Gene-expression profiling based on underlying cytogenetic mutations is one
73 way to identify proteins that are over-expressed and thus might be essential for the
74 propagation of the specific leukemia(30–32). Both AMLs and ALLs with *MLL*-
75 rearrangements share a common gene signature that is distinct from that of *MLL*-
76 Germline (*MLL*-Germ) leukemias(33–35). Most of the well studied and validated *MLL*-r

77 gene targets however are DNA binding proteins like the HOXA gene cluster and its co-
78 factor MEIS1(36–43), which are not suitable targets for immunotherapy.

79 Advances in labeling and mass spectrometry have provided new strategies to more
80 accurately identify cell surface proteins in cancer(23). However, the focus has been on
81 finding suitable targets for acute leukemias without distinguishing between underlying
82 cytogenetic mutations(24–27). The recently published in-silico human surfaceome
83 provides a highly accurate surface protein dataset based on the experimental evidence
84 from 41 human tissues within the Cell Surface Protein Atlas. This tool allows for further
85 characterization of proteins-of-interest, specifically surface-expression (28, 29). In several
86 of the published gene-expression studies, we found *LAMP5* significantly and specifically
87 over-expressed in MLL-r leukemias(34, 35, 44–46). LAMP-5 is a member of the
88 Lysosome-Associated Membrane Protein (LAMP) family. In contrast to other LAMPs,
89 which show widespread expression, *LAMP5* expression in mice is confined to several
90 regions of the postnatal brain. In neurons, the protein was found to recycle between the
91 plasma membrane and a non-classical endosomal vesicle (47–49). In humans, aside
92 from its conserved expression in the brain, LAMP5 is specifically expressed in
93 plasmacytoid dendritic cells (pDCs)(50, 51). Upon activation of pDCs, LAMP-5 aids in the
94 transport of Toll-Like Receptor 9 (TLR9) from early endosomal to lysosomal signaling
95 vesicles, thereby regulating type 1 Interferon (IFN-1) and pro-inflammatory signaling
96 respectively, downstream of TLR9 activation (52). Importantly, results of in silico modeling
97 predict LAMP-5 as a cell-surface protein(29). In this report, we demonstrate *LAMP5* as
98 being highly expressed and essential for MLL-r leukemias through the regulation of innate
99 immune signaling, and describe its potential as a target for MLL-r specific immunotherapy.

100 **Results**

101 ***LAMP5* is highly expressed in MLL-r leukemias and is a direct target of the MLL-FP**

102 To determine genes that are highly expressed in AML and ALL with MLL-rearrangements,
103 we compared recently published RNA-seq studies which identified differentially
104 expressed genes between MLL-rearranged and MLL-germline leukemias in both AML
105 and B-ALL samples(46, 53)(**Figure 1A**). Twenty seven genes were commonly over-
106 expressed in MLL-r ALL and AML (**Supplemental table 1**). Using the In-silico Human
107 Surfaceome tool (<http://wlab.ethz.ch/surfaceome/>) 5 of these 27 were predicted to be
108 expressed on the cell surface(29) (**Supplemental table 1**). Of the 5 predicted proteins,
109 Lysosomal Associated Membrane Protein 5 (*LAMP5*) stood out for being present in
110 multiple previous MLL-r leukemia gene expression studies(34, 35, 44, 45, 54)
111 (**Supplemental Figure 1A-B**). We further validated the specificity of *LAMP5* expression
112 in MLL-r leukemias by analysing the 1,109 pediatric leukemia patient samples from the
113 St.Jude PeCan Portal which again revealed *LAMP5* as significantly overexpressed in both
114 AMLs and ALLs with MLL-rearrangements (**Figure 1B**)(30, 31). To determine if *LAMP5*
115 expression could discriminate between MLL-r leukemia and MLL-Germ leukemia
116 patients, we performed a Receiving Operating Curve (ROC) analysis. *LAMP5* achieved a
117 statistically significant AUC (area under the curve) score in both the Microarray
118 Innovations in Leukaemia (MILE) (GSE13159) and the St. Jude PeCan datasets, with
119 high sensitivity and specificity at the optimal cutoff points (**Figure 1C**). Further, a Kaplan-
120 Meier survival analysis of B-ALL and AML patients correlated higher expression of
121 *LAMP5* with poor survival (**Supplemental Figure 2**). High levels of *LAMP5* mRNA
122 expression (**Figure 1D**) and protein (**Figure 1E**) were also observed in human myeloid

123 and lymphoid MLL-r leukemia cell lines (MOLM-13, MV4;11, THP-1, and RS4;11) as
124 compared to MLL-Germ leukemia cell lines (HL-60, Kasumi-1, K562, REH and RCH-
125 ACV) and normal human CD34-enriched cord blood cells (CB-CD34⁺ cells).

126 Translocations of the *MLL* locus generate MLL-fusion proteins (MLL-FP) which activate
127 transcription of downstream target genes(55–58). To determine if *LAMP5* expression was
128 dependent on the MLL-FP, we transformed CB-CD34⁺ cells with a retrovirus carrying a
129 tetracycline-repressible MLL-AF9 construct. Treatment of transformed cells with
130 Doxycycline led to a simultaneous reduction in the levels of both *MLL-AF9* and *LAMP5*.
131 **(Figure 1F)**. To determine if the MLL-FP directly activates the *LAMP5* gene locus, we
132 interrogated previously published MLL-AF4 chromatin immunoprecipitation sequencing
133 (ChIP-seq) datasets derived from the RS4;11 cell line, and those from CD34⁺ cells
134 transformed with FLAG-MLL-AF4. Coincident ChIP-seq signals of MLL N-terminus and
135 AF4 C-terminus showed MLL-FP binding at the promotor region of *LAMP5*(55, 56, 58).
136 Additionally, there was accompanying significant enrichment of H3K4me3 and
137 H3K79me2 at the gene locus, confirming that *LAMP5* is a direct target of the MLL-FP
138 complex **(Figure 1G)**. In mice, *Lamp5* is not expressed in blood, as it is in human
139 (Supplemental Figure 3A-B). Furthermore, we did not detect upregulation of *Lamp5* in
140 mouse models of MLL-AF9, E2A-HLF and AML1-ETO leukemia, hence we focused our
141 studies exclusively on human cells **(Supplemental Figure 3C)**.

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

143 The ideal immunotherapy target should be essential for the survival of MLL-r leukemias.
144 To test the functional role of LAMP-5 in MLL-r leukemia, we transduced both MLL-r
145 leukemia (MOLM-13, MV4;11, RS4;11, THP-1) and MLL-Germ leukemia cells (Kasumi-1

146 and REH) with lentiviral shRNA vectors targeting *LAMP5*. We obtained efficient
147 knockdown of *LAMP5* with 2 independent hairpins as compared to non-targeting control
148 (NT) (**Supplemental Figure 4A**). Upon *LAMP5* depletion, we observed a significant
149 reduction of cell growth in MLL-r leukemia cell lines (**Figure 2A**), while no effect was seen
150 in MLL-Germ/*LAMP5*-negative leukemia cell lines, Kasumi-1 and REH (**Figure 2B**).
151 Additionally, *LAMP5* knockdown led to a significant decrease in colony forming units
152 (CFU) in the MLL-r leukemia cell lines (**Figure 2C**) suggesting an effect on the
153 clonogenicity of these cells. Furthermore, *LAMP5* knockdown led to apoptosis in MLL-r
154 leukemia cells, as evident by a significant increase in Annexin V and 7-AAD double
155 positive staining (**Figure 2D;Supplemental Figure 4B**). We next sought to determine the
156 role of *LAMP5* in leukemia propagation *in vivo*. MV4;11 cells were transduced with
157 shRNAs targeting either *LAMP5* or a NT control followed by transplantation into
158 immunocompromised NOD-*Rag1*^{null} *IL2r γ* ^{null} (NRG) mice (8 mice per shRNA group). Viral
159 transduction was confirmed by flow cytometry for Venus expression. Four weeks after
160 transplantation, mice developed signs of leukemia and were sacrificed. In the bone
161 marrow, both groups showed similar human cell engraftment based on human CD45
162 expression. On the other hand, the transduced Venus⁺ fraction was significantly reduced
163 in sh*LAMP5*-2 compared to shNT mice. (**Figure 2E, left panels**). We repeated this
164 experiment using cells from a patient with MLL-r (MLL-AF10) leukemia. We again
165 observed significant reduction in the proportion of Venus⁺ cells with *LAMP5* knockdown
166 compared to NT control (**Figure 2E, right panels**). Overall, these data underscore a
167 critical role for *LAMP5* in the growth of MLL-r leukemia cells.

168

169 **LAMP-5 is required for activation of TLR/IL1R signaling in leukemia**

170 Acute leukemias exhibiting constitutive activation of innate immune signaling pathways
171 have been characterized as having a pro-inflammatory profile which is required for their
172 survival(59, 60). These physiologic cellular systems involve TLR/IL1R signaling and
173 culminate in the release of pro-inflammatory cytokines via NF-kB and/or of type I
174 interferons (IFN-1)(61). Recent studies reveal heightened activation of NF-kB signaling in
175 MLL-r leukemia compared to other leukemias(62). Furthermore, MLL-r leukemias have
176 been shown to require the TLR/IL1R signaling pathway to survive, through degradation
177 of the wild-type MLL protein, allowing the MLL-FP to bind to its target genes without
178 restriction(63). Recently, Combes et al. showed that LAMP-5 plays an important role in
179 controlling the subcellular location of TLR9 after activation in human pDC. Upon activation
180 of TLR9, LAMP-5 shuttles TLR9 from the VAMP3⁺-Interferon Response Factor signaling
181 endosome (IRF-SE), to the LAMP-1⁺-pro-inflammatory-signaling endosome (PI-SE). This
182 transition of TLR localization in turn acts as a negative regulator of IFN-1 signaling (52).
183 Based on the known role of LAMP-5 in TLR9 localization in pDCs, we first examined the
184 localization of intracellular LAMP-5 in MOLM-13 cells. We performed co-staining of
185 MOLM-13 cells with antibodies against LAMP-5, LAMP-1 and Myeloid Differentiation
186 Primary Response 88 (MYD88), a scaffold protein that is required for TLR and IL1R
187 signaling (63–65). Confocal microscopy showed that in MLL-r leukemia cells, LAMP-5
188 localized to LAMP-1⁺ vesicles. As suspected, we found MYD88 accumulating highly in
189 the periphery of LAMP-1⁺ vesicles in MLL-r leukemia, suggestive of TLR/IL1R activation
190 **(Figure 3A)**. Conversely, in the LAMP-5-negative MLL-Germ leukemia cells (Kasumi-1),

191 MYD88 does not co-localize with LAMP-1⁺ vesicles. However, overexpression of LAMP-
192 5 in this cell line led to relocation of MYD88 around LAMP-1⁺ vesicles (**Figure 3B**).

193 We subsequently hypothesized that LAMP5 loss may dampen TLR/IL1R signaling in
194 MLL-r leukemias. We thus analyzed known effector proteins downstream of TLR/IL1R
195 activation by western blot. Upon *LAMP5* knockdown, we observed a reduction in
196 phosphorylated NF-κB, p38, and JNK, key players in the signal transduction downstream
197 of TLR/IL1R (**Figure 3C**). To further determine the impact of *LAMP5* depletion on
198 activation of NF-κB downstream of TLR activation, we measured NF-κB activity using the
199 MLL-r cell line THP-1Blue-NF-κB, which contains an NF-κB inducible Secreted Embryonic
200 Alkaline Phosphatase (SEAP) reporter. Robust activation of NF-κB was evident in control
201 cells upon incubation with PAM3CSK4 (TLR2 agonist) or LPS (TLR4 agonist).
202 Knockdown of LAMP-5 led to near-complete blockade of this activation, suggesting that
203 TLR-induced NF-κB signaling is disrupted upon LAMP-5 depletion (**Figure 3D**).
204 Correspondingly, in the LAMP-5 negative cell line Kasumi-1, overexpression of *LAMP5*
205 led to increased phosphorylation of p38, JNK and NF-κB along with increased cell
206 growth(**Figure 3E-F**).

207 Previous studies have shown that NF-κB plays a critical role in MLL-r leukemias (62). We
208 thus hypothesized that NF-κB activation would rescue the cell growth defect seen by
209 LAMP5 depletion. We induced persistent activation of NF-κB in leukemia cells by
210 overexpressing a constitutively active version of Inhibitor Of Nuclear Factor Kappa B
211 Kinase Subunit Beta (IKBKB-EE) in these cells. The IKBKB-EE is a variant of IKBKB that
212 contains mutations at Ser¹⁷⁷ and Ser¹⁸⁸ leading to constant degradation of IκB-α kinase
213 (66, 67). Despite sustained NF-κB activation, knockdown of LAMP-5 in MOLM-13 and

214 RS4;11 cells still led to growth inhibition, suggesting that loss of NF- κ B is not the only
215 signaling event being affected by LAMP5 depletion (**Supplemental Figure 5A-B**). A
216 potential mechanism underlying this essentially was proposed by Wang et al, where they
217 suggested that loss of LAMP5 in MLL-r leukemia led to degradation of the MLL-FP due
218 to increased autophagy(68). However, in our experiments we did not observe any change
219 in the levels of the MLL-FP in THP1 and MOLM-13 cells upon LAMP-5 depletion
220 (**Supplemental Figure 4C**). Overall, these results underscore a critical role for LAMP-5
221 in the activation of TLR/IL1R signaling in MLL-r leukemia, while also indicating the
222 presence of additional attributes that are also essential.

223 **LAMP-5 is a negative regulator of IFN-1 signaling in MLL-r leukemias**

224 Since activation of NF- κ B was not sufficient to rescue the cell growth inhibition seen upon
225 LAMP-5 depletion, we next sought to understand the mechanistic significance of the
226 inflammatory-signal-regulation function of LAMP-5 in MLL-r leukemia. In pDCs, the
227 carboxy-terminal YKHM domain of LAMP-5 was found to be required for normal
228 localization of LAMP-5 and for transportation of TLR9 from the early endosome vesicle to
229 the pro-inflammatory vesicle (47, 50, 52). We thus overexpressed wild-type LAMP-5
230 (LAMP-5-WT), a YKHM mutant (LAMP-5-Y276A), or control vector (EV) in MV4;11 and
231 THP-1 cells, followed by selective knockdown of endogenous *LAMP5* using an shRNA
232 targeting the 3'UTR region of *LAMP5*. Overexpression of LAMP-5-WT completely
233 prevented the cell growth inhibition upon knockdown of endogenous *LAMP5*, validating
234 *LAMP5* as the main target of the shRNA. In contrast, LAMP-5-Y276A was unable to
235 rescue the cell growth in both MV4;11 and THP-1 cell lines (**Figure 5A**). In pDCs, *LAMP5*
236 knockdown or overexpression of LAMP-5-Y276A induced IFN-1 activation upon TLR9-

237 stimulation, due to retention of TLR9 in the IRF-SE. To determine the effect of LAMP-5
238 on IFN-1 signaling in MLL-r leukemia, we turned to THP-1-ISG-SEAP cells that contain
239 an Interferon Stimulated Gene (ISG) inducible-SEAP reporter. Upon TLR activation by
240 PAM3CSK4, IFN-1 signaling activation was evident only in the LAMP-5-depleted cells but
241 not in the control LAMP-5 depleted condition (**Figure 5B**). To ensure this effect was not
242 limited to one cell line, we validated the increase in IFN-1 signaling in other MLL-r cell
243 lines by demonstrable increased expression of Interferon Regulatory Factor 7 (*IRF7*), one
244 of the downstream effectors of TLR activation, Interferon Alpha 2 (*IFNA2*) and Signal
245 transducer and activator of transcription 1 (*STAT1*) upon depletion of LAMP-5 (**Figure**
246 **5C**) (69). To assess the role of LAMP5-depletion mediated IFN-1 activation on cell growth,
247 we performed knockdown of *IRF7* along with *LAMP5* in MV4;11 cells. We found that loss
248 of *IRF7* alone had no significant effect on MLL-r leukemia cell growth but importantly, its
249 depletion prevented the growth inhibition observed upon LAMP-5 knockdown (**Figures**
250 **5D and 5E**) (**Supplemental Figure 6A-B**). Collectively, these results demonstrate that a
251 critical function of LAMP-5 in MLL-r leukemias is to promote the transfer of TLR/IL1R from
252 the IFN-1-activating signaling cascade to the pro-inflammatory signaling cascade.
253 Depletion of LAMP-5 thus leads not only to loss of NF-κB activation but also to activation
254 of IFN-1-signaling, the latter inducing cell-death.

255 **Surface LAMP-5 can be detected and targeted with Antibody Drug Conjugate** 256 **therapy**

257 Previous studies have shown that all LAMP family members can localize to the plasma
258 membrane (70–73). Furthermore, LAMP-5 was found to briefly localize in the plasma
259 membrane of cortical neurons in mice, and is highly predicted to reach the cell membrane

260 based on the human surfaceome(29, 47). We thus sought to confirm if LAMP-5 was
261 expressed on the surface of MLL-r leukemia cells. Using an antibody targeting the N-
262 terminus of LAMP-5, we were able to detect LAMP-5 on the surface of MLL-r leukemia
263 cell lines, while none was detected in the MLL-Germ leukemias (**Figure 5A-B**). To
264 validate the specificity of the antibody, we overexpressed LAMP-5 or control empty vector
265 (EV) in the LAMP-5-negative MLL-Germ leukemia cell line, Kasumi-1. We detected
266 surface LAMP-5 only in the cells that express high levels of LAMP-5 (**Figure 5C**). Using
267 primary AML patient samples, we further validated that surface LAMP-5 can discriminate
268 between MLL-r leukemia and MLL-Germ leukemias (**Figure 5D**). As proof-of-concept for
269 potential therapeutic use, we used a secondary antibody conjugated to the tubulin-toxin
270 Mertansine, targeting the surface-LAMP-5 antibody. We observed that a 72-hour
271 treatment with this antibody-sandwich comprised of the surface LAMP-5 antibody along
272 with the secondary ADC antibody is sufficient to reduce cell viability in MLL-r leukemia
273 cell lines MOLM-13, RS4;11 and THP-1, while no effect was seen in the LAMP-5-negative
274 Kasumi-1 cell line (**Figure 5E**). These results suggest that LAMP-5 could be exploited as
275 a MLL-r specific biomarker and could potentially be used as a target for immunotherapy.

276 **Discussion**

277 Our findings identify *LAMP5* as a novel core gene in MLL-r leukemias, directly up-
278 regulated by the MLL-FP. Additionally, we found that LAMP-5 is essential for MLL-r
279 leukemia cell survival. Further, we found that one of the critical functions of LAMP-5 is to
280 regulate innate-immune signaling in MLL-r leukemias, specifically directing the flux of
281 activity away from IRF-SE towards the PI-SE, leading to constant activation of NF-kB.

282 Recent discoveries have highlighted how the specific subcellular location and timing of
283 TLR activation affect signaling outcomes in normal immune cells(74). However, how
284 these mechanisms function in leukemia is still poorly understood. Recently, Combes et
285 al. showed that LAMP-5 is a negative regulator of IFN-1 signaling in pDCs by transporting
286 activated TLR9 from the IRF-SE to the PI-SE. Although dispensable for pDCs cell
287 survival, LAMP-5 depletion leads to unrestricted activation of IFN-1 signaling.
288 Furthermore, aberrant expression of LAMP-5 can lead to diminished activation of pDCs
289 in tumors and contribute to their immunomodulatory phenotype by decreasing the IFN-1
290 production capacity(52). Innate immune signaling and inflammation have been shown to
291 play a crucial role in acute leukemias(59, 60). MLL-r leukemias rely on activation of NF-
292 kB downstream of TLR/IL1R to maintain the MLL-FP gene signature and block cell
293 differentiation(62, 63). Furthermore, it has been shown that treatment with IFN- β or
294 activation of IFN-1 signaling is deleterious for MLL-r leukemias (75). In our study, we
295 describe a novel role for LAMP-5 in maintaining NF-kB activation and blocking IFN-1
296 signaling downstream of TLR/IL1R activation in MLL-r leukemias. We show that LAMP-5
297 acts as a molecular switch to maintain TLR/IL1R signaling in the pro-inflammatory
298 endosome leading to NF-kB activation, whereas LAMP-5 depletion leads to activation of
299 IFN-1 signaling and cell death. This suggests that both LAMP-5-mediated induction of
300 pro-inflammatory signaling and inhibition of IFN-1 signaling contribute to the pathogenesis
301 of MLL-r leukemias. We confirmed that activated IFN-1 signaling upon LAMP-5 depletion
302 induced cell death by depleting IRF7, which rescued cell growth and clonogenicity in
303 LAMP5-depleted cells. This suggests that increased IFN-1 signaling is at least partly
304 responsible for inducing cell death upon LAMP-5 depletion. Additionally, overexpression

305 of *LAMP5* in MLL-Germ leukemia leads to increased activation of NF- κ B, p38 and JNK,
306 and increased cell growth, which suggests that this signaling pathway might be
307 contributing to the therapy-resistant phenotype of MLL-r leukemias.

308 LAMP-5 is part of the lysosomal associated membrane protein family, of which two main
309 members LAMP-1 and LAMP-2 are expressed in the endo-lysosomal vesicles
310 ubiquitously in all tissues (76). In contrast, LAMP-3 and CD68 (LAMP-4, microsialin) show
311 a more restricted expression in human tissues (72, 77). Regardless of their prominent
312 localization in the endo-lysosomal compartment of cells, all LAMP members have been
313 shown to localize in the plasma membrane either at steady state or as a result of immune
314 activation(70–73). In humans, *LAMP5* expression is generally restricted to the brain,
315 ovaries and blood. In blood, *LAMP5* is exclusively expressed in nonactivated pDCs (50,
316 51). Interestingly, in non-activated pDCs, LAMP-5 is found in the ERGIC compartment
317 and it requires the activation of TLR9 to leave the compartment and join the endo-
318 lysosomal vesicles(50, 52). We found that the aberrant increased expression of LAMP-5
319 in MLL-r leukemia leads to its accumulation in the plasma membrane, as evidenced by a
320 novel LAMP-5 antibody targeting the N-terminus of the protein. The detection of LAMP-5
321 on the surface of MLL leukemias provides the opportunity to potentially use it as a target
322 for immunotherapy in this treatment-refractory malignancy. These results provide a
323 rationale to develop immunotherapies targeting LAMP-5. Furthermore, LAMP5 is highly
324 expressed in other cancers such as multiple myeloma (MM) and blastic plasmacytoid
325 dendritic cell neoplasm (BPDCN)(78, 79). Therefore LAMP5 immunotherapies could
326 benefit other blood diseases. Finally, total loss of Lamp5 in mice had no major effects on

327 health or lifespan with minor behavioral effects, suggesting that there could be a wide
328 therapeutic window for LAMP-5-directed therapies in humans (48, 49).

329 Similar to our observations, Wang et al. recently showed that LAMP-5 is essential for the
330 survival of MLL-r leukemias *in vitro* and *in vivo* using shRNA knockdown. However, they
331 propose that LAMP-5 is a negative regulator of autophagy leading to MLL-FP
332 stabilization. They show that LAMP-5 and ATG5 colocalize in MLL-r leukemia cells and
333 that blockade of autophagy is sufficient to rescue the increased levels of apoptosis after
334 LAMP-5 knockdown(68). We were unable to detect any significant change in the levels
335 of the MLL-FP upon LAMP5 knockdown. Since TLR-mediated innate immune signaling
336 can regulate autophagy, it is possible that the function of LAMP-5 in regulating autophagy
337 as described by Wang et al is downstream of its impact on endosome-lysosome
338 trafficking(80–84). On the other hand, it is also possible that these effects are not directly
339 linked and that LAMP-5 might exert its growth-promoting effects in MLL-r leukemia by
340 multiple mechanisms. It is notable however that the role of autophagy in leukemia is
341 controversial(85). In murine MLL leukemia models, heterozygous loss of *Atg5* leads to
342 increased leukemia cell proliferation *in vitro* and more aggressive leukemia *in vivo*, while
343 homozygous loss is lethal to these cells(86). Additionally, while some studies suggest
344 that *Atg5*-dependent autophagy may contribute to the development of MLL-AF9 driven
345 leukemia but dispensable for propagation and chemosensitivity, others suggest that *Atg5*-
346 dependent autophagy is dispensable altogether(87–89). Overall, our results show that
347 LAMP-5 localizes both on the surface and in LAMP-1⁺ endosomes in leukemia, leading
348 to constitutive activation of pro-inflammatory signaling, and dampening of interferon-
349 signaling, and that it can be used as a target for immunotherapy.

350 **Materials and Methods:**

351 **Cell lines and primary patient cells**

352 Human leukemia cell lines were maintained in Iscove's modified Dulbecco medium
353 (IMDM) or Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10%
354 fetal bovine serum (FBS), 1% penicillin and 1% streptomycin.

355 MLL-AF9 Tet-off human CD34⁺ cells were a kind gift from Dr. James Mulloy, and were
356 generated as follows: umbilical cord blood (UCB) was obtained from the Translational
357 Trial Development and Support Laboratory of CCHMC. CD34⁺ cells were isolated from
358 UCB using the EasySep CD34⁺ isolation kit (StemCell Technologies). CD34⁺ cells were
359 pre-stimulated in IMDM/20%FBS containing 100ng/mL SCF, TPO, Flt3-L, and IL-6 and
360 20ng/mL IL-3 for 2 days before transduction. Transduction was carried out using
361 spinoculation onto Retronectin (Takara Bio) coated plates along with 4ug/mL polybrene.
362 Cells were transduced with both the pSIN-TREtight-dsRED-MLL/AF9 lentivirus and the
363 MSCV-GFP-IRES-tTA34 retrovirus. After transduction, cells were maintained in
364 IMDM/20% FBS with 10ng/mL of each cytokine. A pure population of dsRED⁺GFP⁺ cells
365 were selected over several weeks of culture.

366 Fully de-identified primary patient cells were obtained from the Cincinnati Children's
367 Hospital Medical Center Biorepository. Patient identities have been protected. By the time
368 the mice displayed signs of leukemia, the only human cells that remained in the mice
369 were leukemic cells (MLL-rearrangement was confirmed with FISH).

370 Human CD34⁺ cells and cells from patients with MLL-r AML were cultured in IMDM
371 supplemented with 20% FBS and 10 ng/ml human cytokines including SCF, FLT3-Ligand,

372 Thrombopoietin, IL-3 and IL-6. Cell lines were periodically validated by STR genotyping
373 through Genetica Cell Line Testing (LabCorp). Cells were tested and were negative for
374 mycoplasma contamination. None of the cell lines utilized in this study are recognized by
375 the ICLAC as being commonly misidentified.

376 **Animal experiments**

377 All animals used for this study were 6-12 weeks old. All animal experiments were carried
378 out in accordance with the guidelines of the Institutional Animal Care and Use Committee
379 (IACUC). For xenograft experiments with MV4;11 and MLL-r primary patient cells,
380 immunocompromised NOD-*Rag1*^{null} *IL2r γ* ^{null} (NRG) (Jackson Laboratories, stock no.
381 007799) recipient mice were conditioned with 30 mg/kg busulfan and transplanted with 2-
382 7.5×10^5 cells 24 hours later. In xenograft experiments bone marrow samples were
383 collected four weeks after transplantation as well as when signs of leukemia were present;
384 aspirates were analyzed via flow cytometry for the presence of human CD45⁺ cells and
385 the presence of shRNA-transduced Venus⁺ cells

386 **Retroviral and lentiviral transductions**

387 Retroviral and lentiviral supernatants were generated by transfection of HEK293T cells
388 using the FuGENE 6 reagent (Promega) according to the manufacturer's
389 recommendations.

390 The lentiviral shRNA pLKO.1-Puro plasmids TRCN0000129410 (shLAMP5-1),
391 TRCN0000129378 (shLAMP5-2) and TRCN0000014861 (shIRF7) were purchased from
392 Millipore Sigma. Human cells were incubated with a single dose of lentiviral supernatant
393 overnight. Cells transduced with constructs granting puromycin resistance were selected

394 in 0.5-5 ug/ml of Puromycin for 72 hours. Cells transduced with constructs containing a
395 fluorescent marker (Venus) were isolated on between days 4-5 after transduction by
396 sorting using MoFlo XDP, FACSAria (BD Biosciences) or a SONY SH800S (Sony
397 Biotechnology)

398 The retroviral IKBKB-S177E-S181E (IKBKB-EE) plasmid was a gift from Anjana Rao
399 (Addgene plasmid # 11105; <http://n2t.net/addgene:11105>; RRID:Addgene_11105). Flag-
400 LAMP5-WT and Flag-LAMP5-Y276A plasmids were as described previously(47) FLAG-
401 LAMP5-WT and FLAG-LAMP5-Y276A were cloned into the MSCV-IRES-Puro vector.

402 **Flow cytometry**

403 For apoptosis assays, cells were incubated with allophycocyanin (APC)-conjugated
404 Annexin V (BD Bioscience) for 15 minutes at RT in 1X Annexin V Binding Buffer (BD
405 Bioscience) followed by staining with 7-aminoactinomycin (7-AAD) (eBioscience). For
406 surface LAMP-5 detection, cells were incubated with 3 µg anti-human LAMP-5
407 therapeutic antibody (TAB-0643CL, Creative Biolabs, NY, USA) overnight and then
408 stained with anti-mouse IgG1-PE (eBioscience). Data were acquired on a FACS Canto I
409 and results were analyzed using FlowJo Version 10 (FlowJo).

410 **Colony-forming unit assays**

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

414

415

416 **RT- and Quantitative RT-PCR**

417 Total RNA was extracted from human Puromycin-selected or sorted Venus⁺ cells using
418 the RNeasy Mini kit (Qiagen). RNA was reversed transcribed into cDNA using iScript
419 Advanced cDNA Synthesis kit (Bio-Rad Laboratories). For Quantitative RT-PCR 5-10ng
420 of cDNA was analyzed using iTaq Universal SYBR Green Supermix and iTaq Universal
421 Probes Supermix (Bio-Rad) in a StepOnePlus Real-Time PCR machine (Applied
422 Biosystems). Taqman probes were purchased from Applied BioSystems. Primers and
423 probes used for Quantitative RT-PCR were: *LAMP5* (5'-
424 TACGACTCCTCGGAGAAAACC-3' and 5'-TGACACTCATAGGACTTCCCAG-3') *IRF7*
425 (5'-CCCACGCTATACCATCTACCT-3' and 5'-GATGTTCGTCATAGAGGCTGTTG-3'),
426 *LAMP5*(Hs00202136_m1), *STAT1* (Hs01013996_m1), *IFNA2* (Hs00265051_s1), *ACTIN*
427 (Hs99999903-m1)

428 **Western blotting**

429 The primary antibodies used were anti-LAMP-5 (Thermo Fisher Scientific Cat# 14-9778-
430 80, RRID:AB_2573029), anti-AF9 (Bethyl Cat# A300-597A, RRID:AB_495520), anti-
431 MLL1 (Cell Signaling Technology Cat# 14689, RRID:AB_2688009), anti-phospho-JNK
432 (Cell Signaling Technology Cat# 4668, RRID:AB_823588), Phospho-IKK (Cell Signaling
433 Technology Cat# 2697, RRID:AB_2079382), Phospho-p38 MAPK (Cell Signaling
434 Technology Cat# 4511, RRID:AB_2139682), Phospho-NF-kB p65 (Cell Signaling
435 Technology Cat# 4025, RRID:AB_10827881), anti-Actin (Cell Signalling Technology,
436 13E5, RRID: AB_2223172), and anti- β -tubulin (Cell Signaling Technology, 9F3, RRID:
437 AB_823664). Whole cell lysates were isolated using RIPA buffer (Sigma) and the amount
438 of protein was determined using the BCA Protein Assay Kit (Thermo Scientific). 30 μ g of

439 protein was separated by SDS-PAGE on a 4-20% gradient gel (Bio-Rad). After transfer
440 to PVDF membranes, blots were blocked with Odyssey® Blocking Buffer TBS (LI-COR)
441 for one hour and incubated with primary antibodies overnight. After washing, blots were
442 incubated with appropriate secondary IRDye 680RD goat anti-mouse (LI-COR) and
443 IRDye 800CW goat anti-rabbit (LI-COR) antibodies at a dilution of 1:10,000 for one hour.
444 Images were obtained using the Odyssey CLx Infrared Imaging System (LI-COR).

445 **ChIP-seq Analysis**

446 ChIP-seq data were downloaded from the Gene Expression Omnibus using the listed
447 accession numbers (GSE84116 for MLL-Af4 transformed CB CD34⁺, GSE95511 for ML-
448 2 data, GSE79899 for MV4;11 and THP-1 data, GSE38403 for RS4;11 data, and
449 GSE38338 for SEM data) and visualized with the UCSC Genome Browser, assembly
450 hg19. For consistency, data for RS4;11 and SEM were converted from their originally
451 mapped hg18 to hg19 using the liftOver tool from the UCSC Genome Browser Utilities.

452 **Cell Viability**

453 MOLM-13, RS4;11, THP-1 and Kasumi-1 cells were plated at 10,000 cells per well in a
454 96-well plate. Cells were incubated with LAMP-5 therapeutic antibody (TAB-0643CL
455 Creative Biolabs) and Anti-Mouse IgG Fc-DM1 Antibody with Non-Cleavable Linker (AM-
456 103D1-50, Moradec LLC) at 5ng/uL and 1ng/uL final concentrations respectively. To
457 measure cell viability CellTiter-Glo® 2.0 Cell Viability Assay (Promega Cat# G9242 WI,
458 USA) was used following manufacture protocol.

459

460

461 **SEAP reporters**

462 THP-1-Blue NF- κ B/ISG cells (InvivoGen) carrying a stable integrated NF- κ B-inducible or
463 ISG-inducible secreted embryonic alkaline phosphatase (SEAP) reporter construct were
464 transduced with shLAMP5-2-Venus and sorted for Venus expression after 48 hours. Cells
465 were then plated at a concentration of 2×10^4 cells/well and stimulated with Pam3CSK4
466 (10ng/mL) or LPS (100ng/mL) for 24 hours. Cells were centrifuged and 20uL supernatant
467 was incubated with 180uL QUANTI-Blue reagent at 37 degrees for 30 min - 2 hours. The
468 levels of NF- κ B-induced or ISG-induced SEAP was measured in a microplate reader at
469 620 nm.

470 **Immunofluorescence**

471 Cells seeded on alcian blue-treated coverslips were fixed with 3.5% PFA and
472 permeabilized with 0.05% saponin. Cells were then stained overnight with primary
473 antibodies: anti-LAMP-5 (Thermo Fisher Scientific Cat# 14-9778-80,
474 RRID:AB_2573029), anti-MYD88 (R and D Systems Cat# AF2928, RRID:AB_2297977),
475 anti-LAMP-1 BV421 (BioLegend Cat# 328626, RRID:AB_11203537) anti-LAMP-2 AF647
476 (Thermo Fisher Scientific Cat# A15464, RRID:AB_2534477). Immunofluorescence and
477 confocal microscopy was performed with a Zeiss LSM580 63x objective and
478 accompanying imaging softwares

479 **Statistics**

480 The statistical methodology used and sample sizes are described in the individual Figure
481 legends. *t* tests were two tailed unless otherwise stated. Results are presented as mean
482 \pm SEM unless otherwise stated. A two-sided time-stratified Cochran-Mantel-Haenszel

483 was used for the Kaplan-Meier Survival analysis. ROC curves were used to determine
484 the diagnostic utility of LAMP5 mRNA. The sensitivity and specificity were identified at the
485 optimal cutoff point that was chosen at which the Youden's index was maximal. A
486 significance level cutoff of 0.05 was used unless otherwise stated. Statistical analysis was
487 performed using GraphPad Prism.

488 **Authors Contributions:**

489 G.G.M., L.H.L., and A.R.K. contributed to study conception and design. G.G.M., J.C., and
490 D.L. acquired data. G.G.M., J.C., M.W., M.B., D.L., P.P., E.G., and L.H.L. analyzed and
491 interpreted data. G.G.M., J.C., L.H.L. and A.R.K. wrote and revised the manuscript. M.W.,
492 D.L., P.P., E.G., reviewed the manuscript. M.W., D.L., and J.C., provided administrative,
493 technical or material support.

494 **Acknowledgments:**

495 We would like to thank Daniel Starzcynowski, PhD, for his intellectual input. We would
496 like to acknowledge the assistance of the Research Flow Cytometry Core in the Division
497 of Rheumatology at Cincinnati Children's Hospital Medical Center. All flow cytometric data
498 were acquired using equipment maintained by the Research Flow Cytometry Core in the
499 Division of Rheumatology at Cincinnati Children's Hospital Medical Center.

500 M.W. was supported by an NIH grant (R50 CA211404). L.H.L. is a St. Baldrick's
501 Foundation Scholar and is supported by grants from CancerFree KIDS and the NIH (L40
502 HL143713-01) A.R.K. was supported by a Hyundai Hope on Wheels grant.

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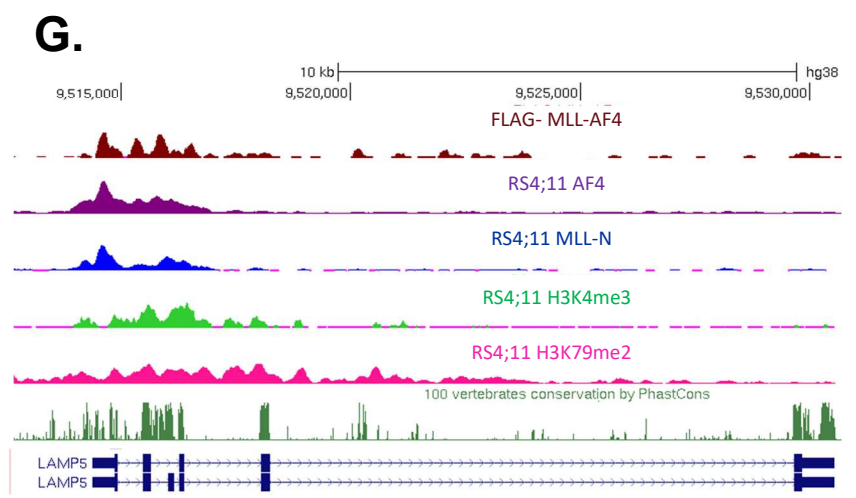
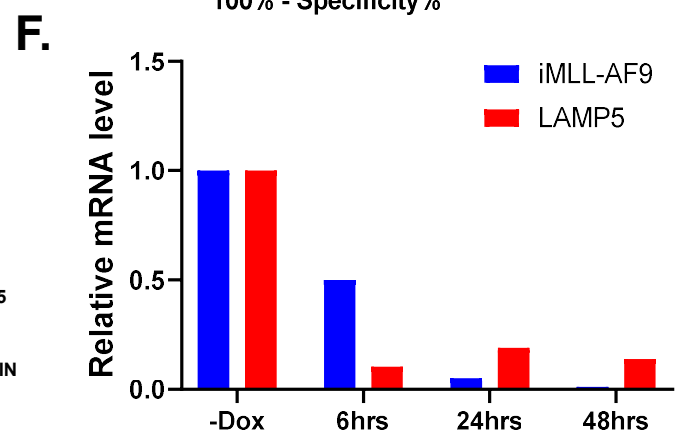
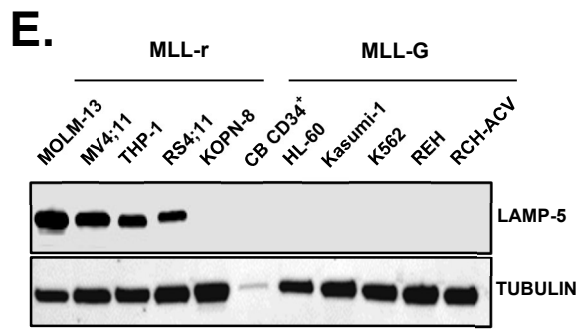
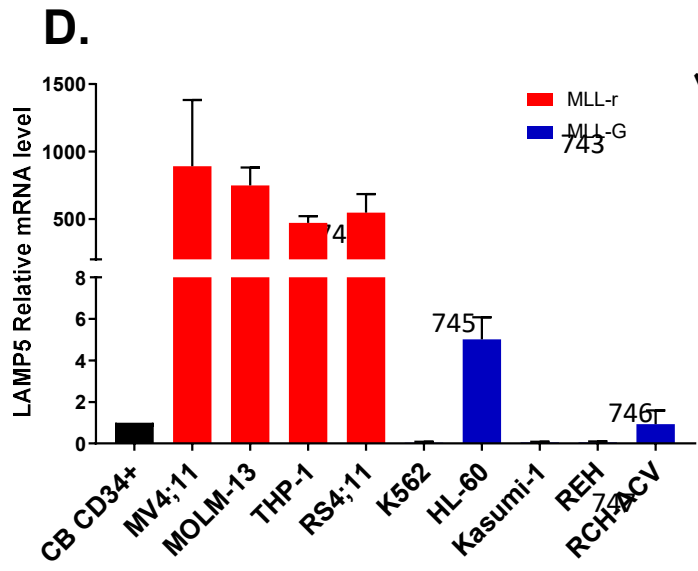
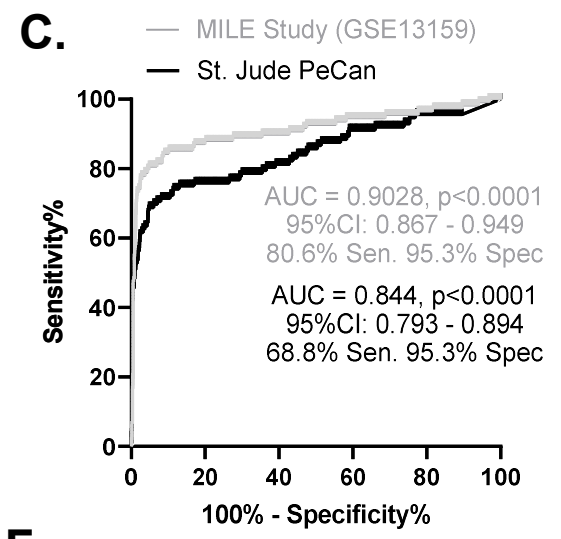
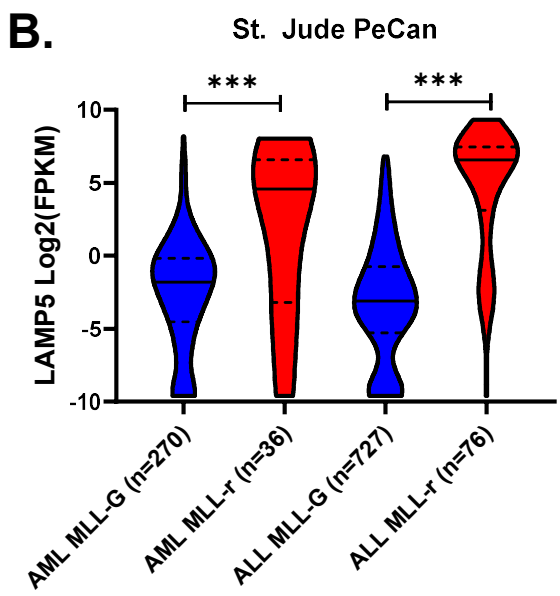
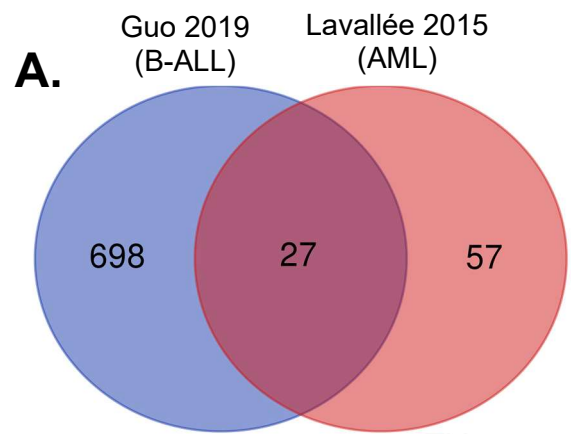
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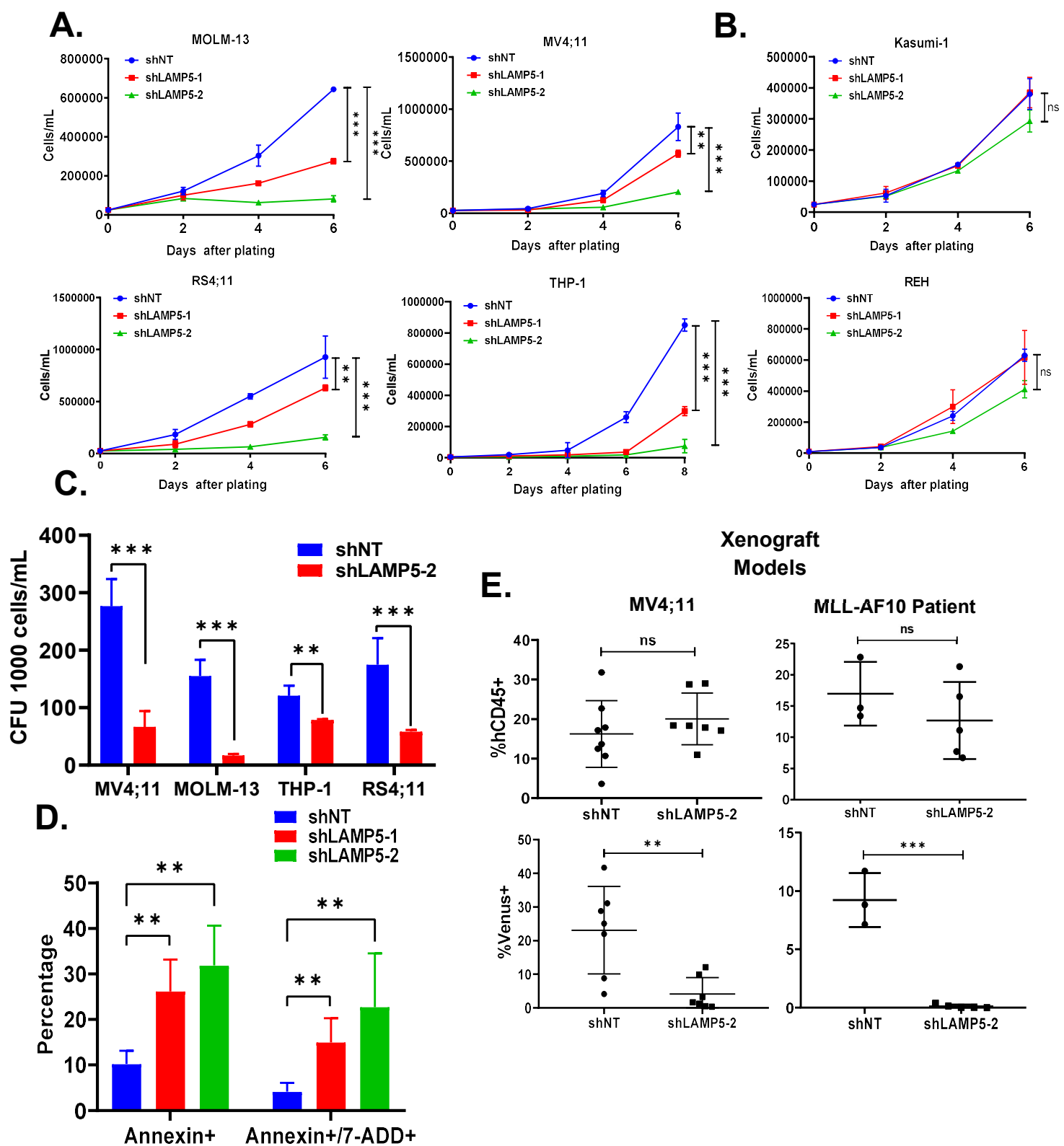
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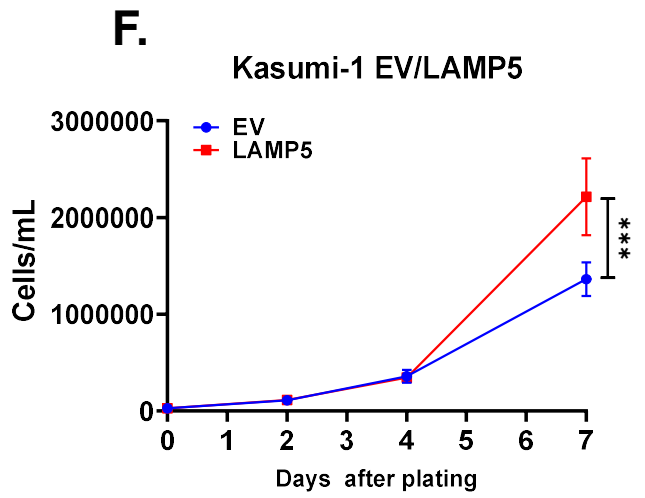
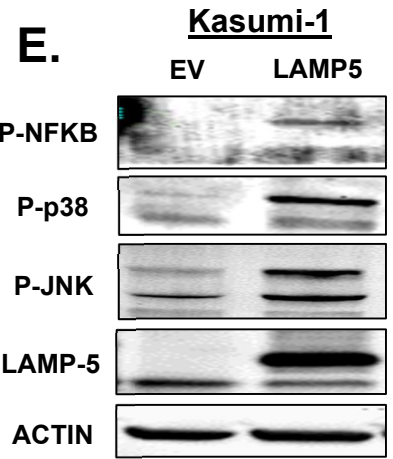
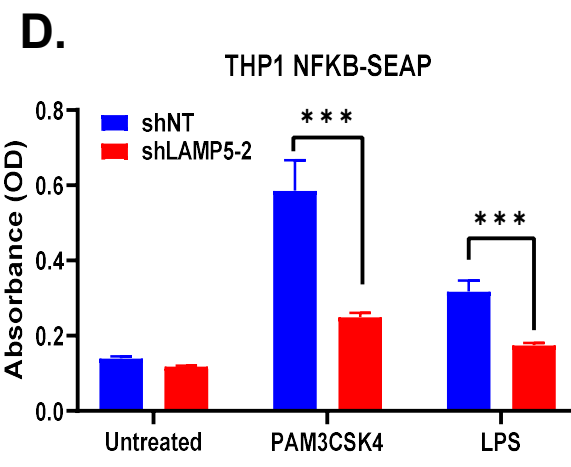
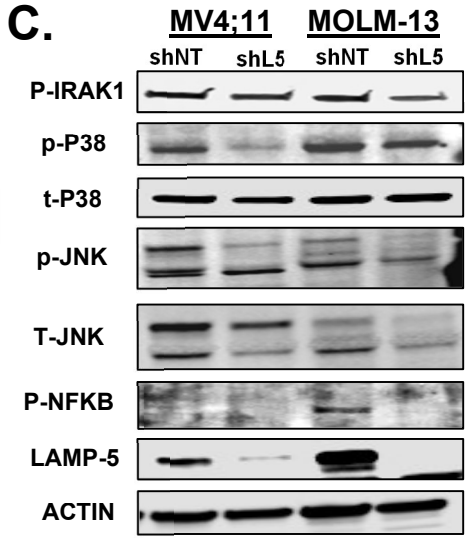
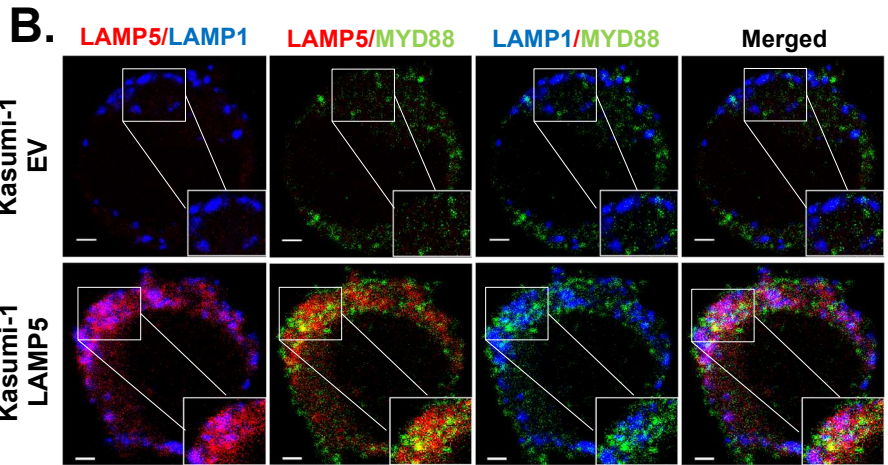
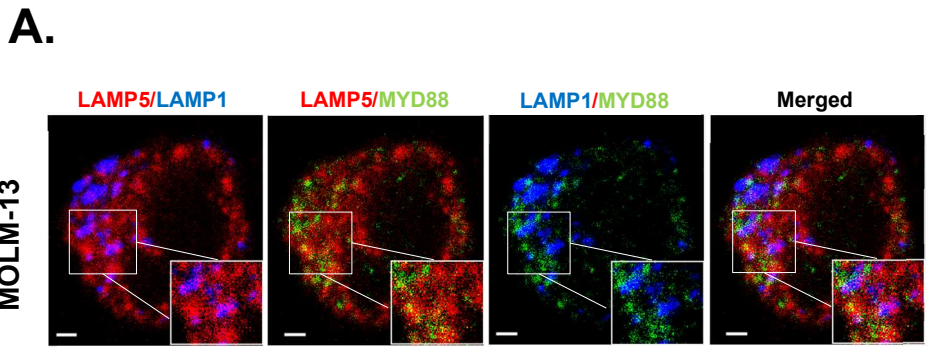


749 **Figure 1: *LAMP5* is highly expressed in MLL-r leukemias and is a direct target of the MLL-fusion**
750 **protein (MLL-FP). (A)** Intersection of published gene expression signatures composed of genes
751 overexpressed in MLL-rearranged AML and ALL when compared to MLL-Germine leukemias. **(B)** Log2
752 FPKM expression of *LAMP5* in AML and ALL pediatric patients with MLL-rearrangement (AML MLL-r,
753 n=36 and ALL MLL-r, n=76) compared to MLL-Germine (AML MLL-G, n=270) (ALL MLL-G, n=727)
754 patients. Data obtained from the St. Jude PeCan Portal and presented as median value with quartiles
755 (*t*-test, *p*< 0.0001) **(C)** Receiving Operating Curve (ROC) analysis showing capacity of *LAMP5* to
756 discriminate acute leukemia patients with MLL-Germ or MLL-r leukemias. Data obtained from
757 GSE13159 and St. Jude Pecan Portal **(D)** Relative expression of *LAMP5* in MLL-r leukemia (MV4;11,
758 MOLM-13, KOPN-8, THP-1 and RS4;11) and MLL-Germ leukemia (K562, HL-60, Kasumi-1, REH,
759 RCH-ACV) cell lines. The graph represents relative expression of *LAMP5* normalized to *B-ACTIN*. Data
760 is from 3 biological replicates. *LAMP5* expression in cord blood cells was set as 1.0. Bars show mean
761 ± SEM. **(E)** Western blot analysis of the *LAMP5* levels in MLL-r leukemia and MLL-Germ leukemia
762 human cell lines. CD34⁺ cord blood cells were used as control. TUBULIN was used as a loading control.
763 **(F)** Graph represents the relative expression of *LAMP5* and *MLL-AF9* upon incubation with
764 Doxycycline. Relative expression of *LAMP5* and *MLL-AF9* normalized to *B-ACTIN*. **(G)** ChIP-seq
765 profiles of human ALL cell lines expressing MLL-AF4 show binding of the MLL N-terminus, AF4 C-
766 terminus and significant H3K4me3 and H3K79me2 mark in the *LAMP5* promoter and gene body. ChIP-
767 seq data were obtained for the CB CD34⁺ transformed with MLL-Af4 and RS4;11 from GSE84116 and
768 GSE38403 respectively.



771 **Figure 2: LAMP5 expression is required for MLL-r leukemia survival *in vitro* and *in vivo*. (A-B)**
772 *In vitro* growth of MLL-r and MLL-G leukemia cell lines **(A)** (MOLM-13, MV4;11, RS4;11 and THP-1)
773 and **(B)** (Kasumi-1 and REH) respectively upon shRNA knockdown of *LAMP5*. Data are from 3
774 independent experiments, *t* test, (**,p<0.01, ***, p< 0.001). **(C)** Colony forming units (CFU) of MV4;11
775 and MOLM-13 cells upon *LAMP5* shRNA knockdown. Data are from 3 biological replicates,
776 represented as mean and SEM (**,p<0.01, ***, p< 0.001). **(D)** Percentage of Annexin⁺ or Annexin⁺/7-
777 AAD⁺ cells after transduction with shNT, shLAMP5-1 and shLAMP5-2. Data are from 3 biological
778 replicates, represented as mean and SEM of at least 3 experiments. *t* test, p< 0.01 **(E)** Plots show
779 percentage of human CD45⁺ (upper) and Venus⁺ cells in the CD45⁺ fraction (lower) in MV4;11 (left)
780 and MLL-AF10 primary patient sample (right). Data are from 8 biological replicates for MV4;11 and 5
781 biological replicates for the MLL-AF10 primary patient, represented as mean and SEM. *t* test, p< 0.01

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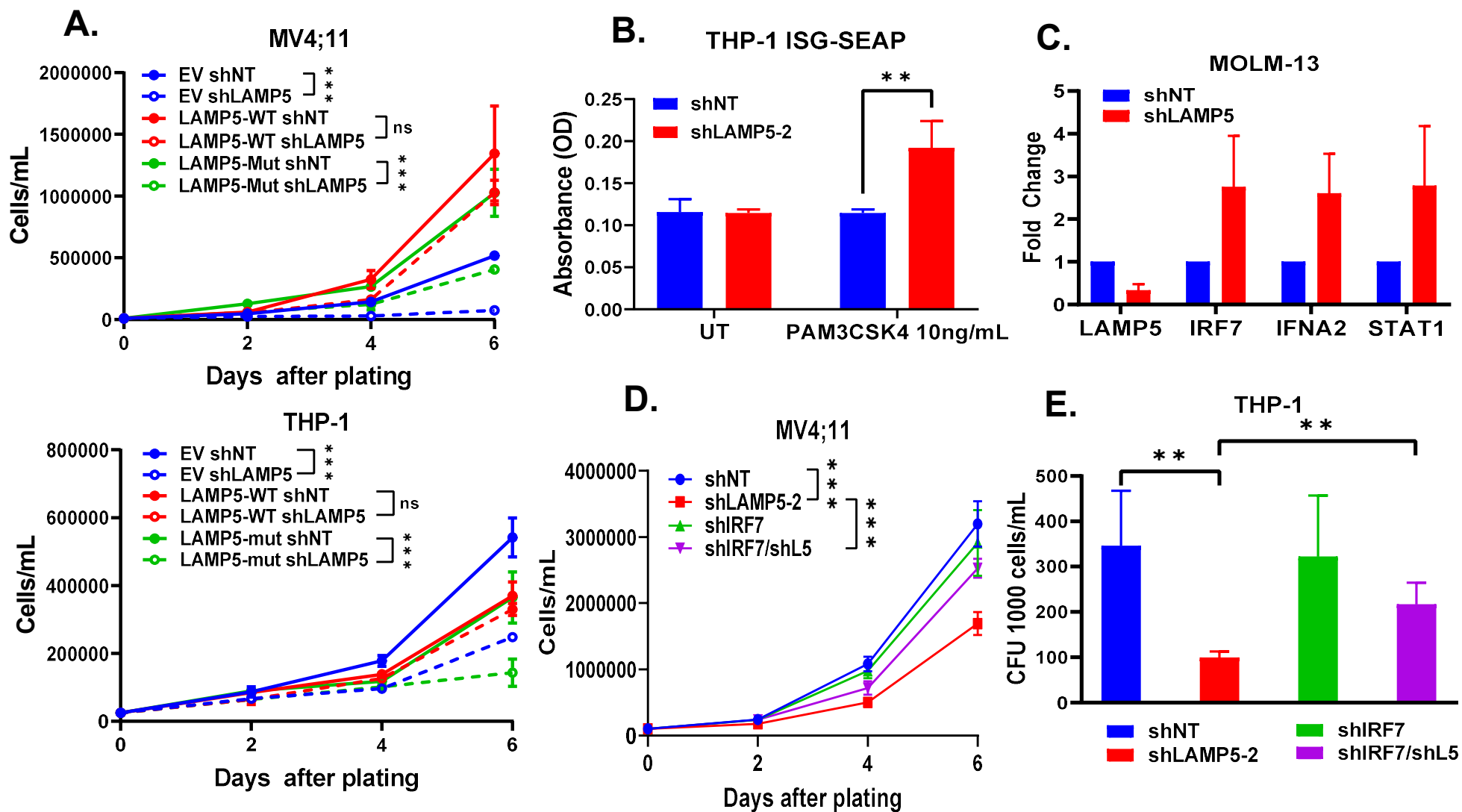


806 **Figure 3: LAMP-5 is required for activation of TLR/IL1R signaling (A)** Representative confocal
807 microscopy images showing MV4;11 cells stained with LAMP-5, LAMP-1 and MYD88. Scale bar = 1µm
808 **(B)** Confocal microscopy image showing Kasumi-1 cells overexpressing EV or *LAMP5* stained with
809 LAMP-5, LAMP-2 and MYD88. Scale bar = 1µm **(C)** Western blot analysis showing that LAMP-5
810 depletion (shL5) led to a decrease of p-IRAK1, p-p38, p-JNK, and p-NFKB, known downstream targets
811 of TLR signaling. **(D)** THP-1-Blue-NFKB reporter cell line was treated with PAM3CSK2 10ng/mL or LPS
812 100ng/mL in the presence or absence of LAMP5. Data are from 3 independent experiments t-test, ***,
813 $p < 0.001$. **(E)** Western blot analysis showing Kasumi-1 cells with overexpression of EV or *LAMP5*
814 showing increase activation of p-NFKB, p-p38 and p-JNK. **(F)** *In vitro* cell growth of Kasumi-1 cells
815 overexpressing EV or LAMP5. Data are from 3 individual experiments, t-test, ***, $p < 0.001$.

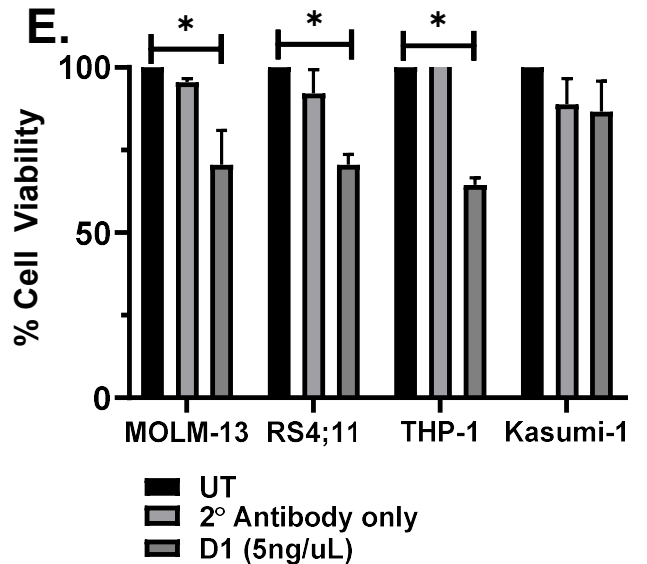
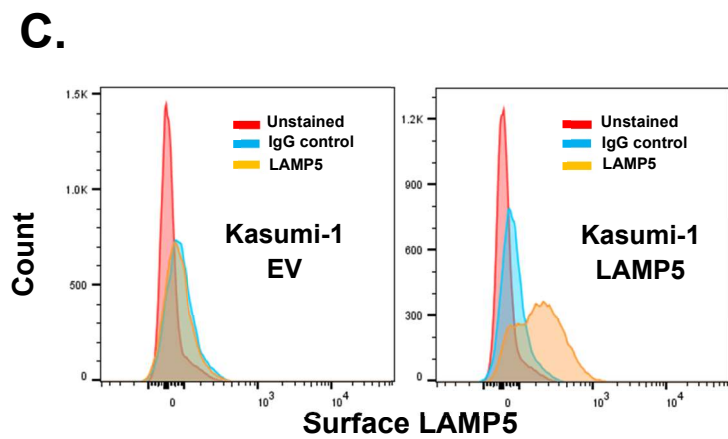
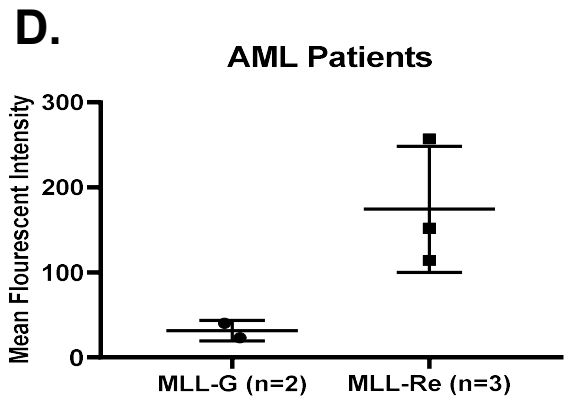
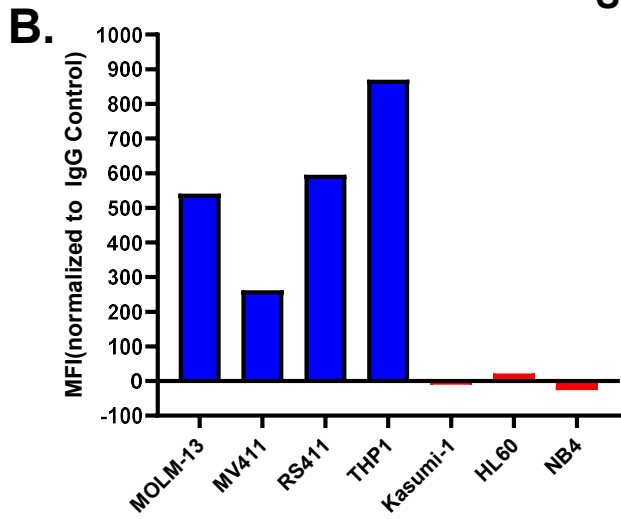
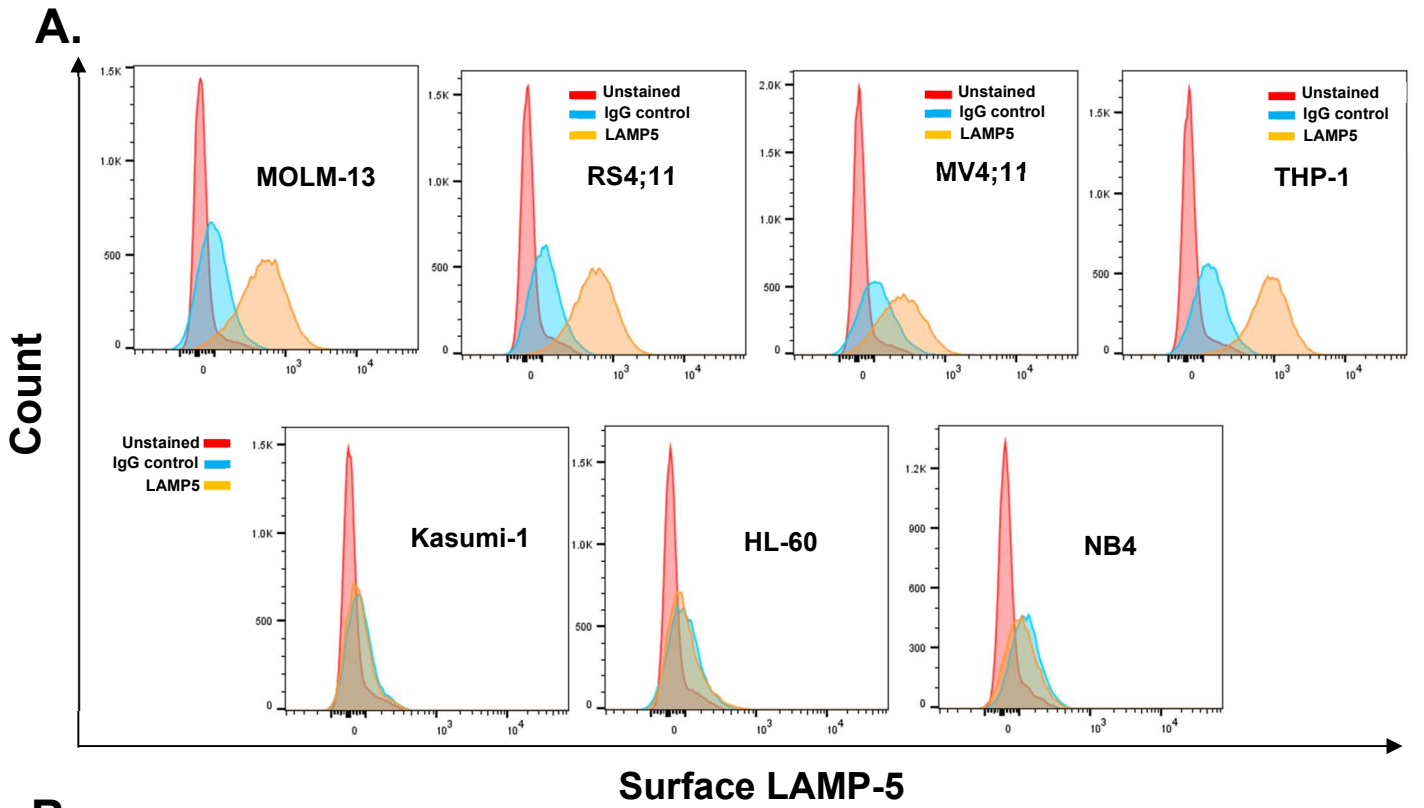
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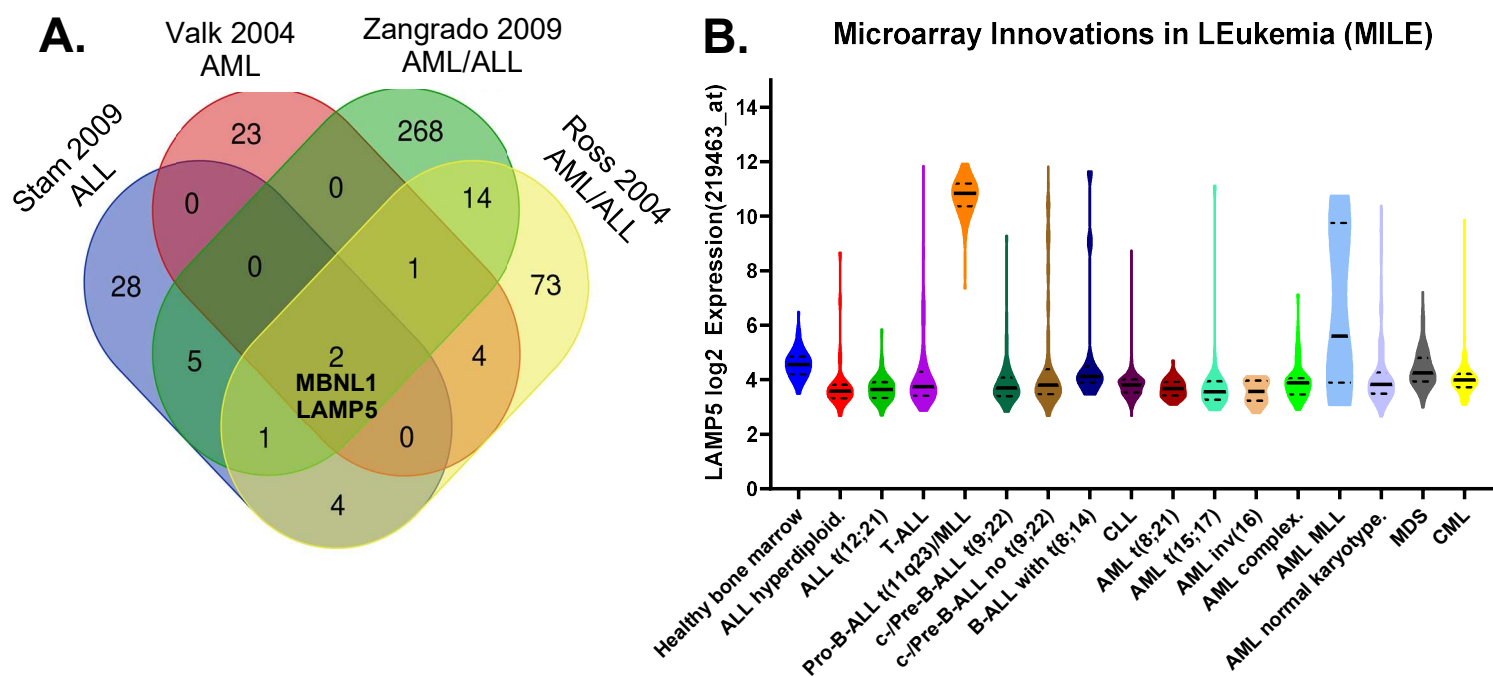


820 **Figure 4: LAMP5 is a negative regulator of IFN-1 signaling in MLL-r leukemias (A)** In-vitro growth
821 of MV4;11 and THP-1 cells overexpressing EV, LAMP-5 WT, or LAMP-5-mut upon shRNA knockdown
822 of LAMP-5. Data are from 3 independent experiments. t-test ^{***}, p< 0.001. **(B)** THP-1 ISG blue reporter
823 cell line was treated with 10ng/mL PAM3CSK2 in the presence or absence of LAMP-5. Data are from
824 3 independent experiments. Bars show mean ± SEM. t-test ^{**}, p<0.01. **(C)** Relative expression of
825 *LAMP5*, *IRF7*, *IFNA2* and *STAT1* upon knockdown of LAMP-5 in MOLM-13 cells. The graph represents
826 relative expression of *LAMP5*, *IRF7*, *IFNA2*, and *STAT1* normalized to β -*ACTIN*. Data are from 3
827 biological replicates. Bars show mean ± SEM. **(D)** *In vitro* growth of MV4;11 after LAMP5, or IRF7 or
828 LAMP5 and IRF7 shRNA knockdown. Data are from 3 independent experiments, represented as mean
829 and ±SD. ^{***}, p< 0.001. **(E)** Colony forming units (CFU) of THP-1 cells upon shRNA knockdown of
830 LAMP-5, IRF7, or LAMP-5 and IRF7 together. Data are from 3 independent experiment, represented
831 as mean and ±SD, t-test ^{**}, p<0.01.

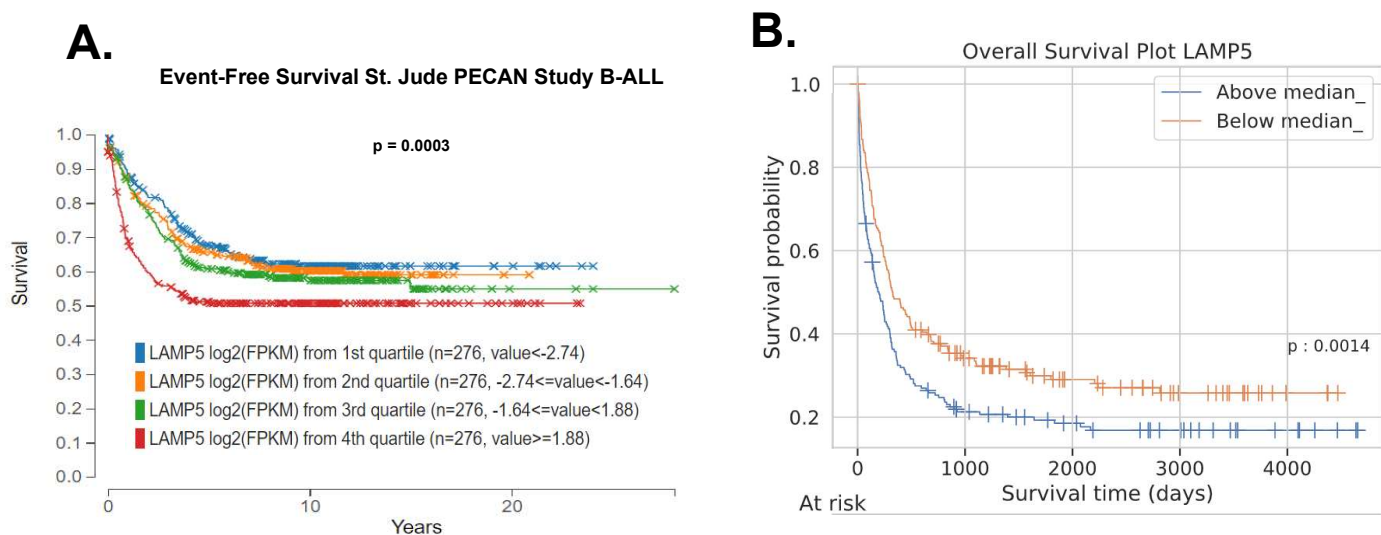


860 Figure 5: Surface LAMP-5 can be detected and targeted with Antibody Drug Conjugate therapy

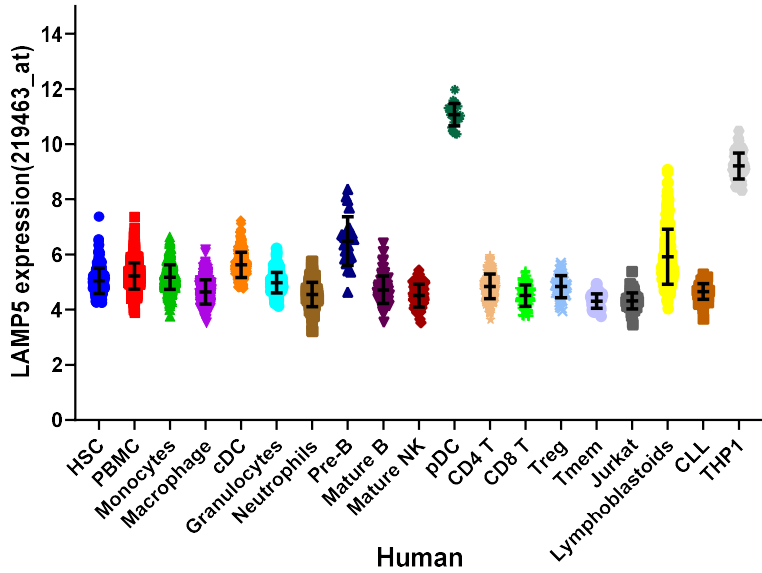
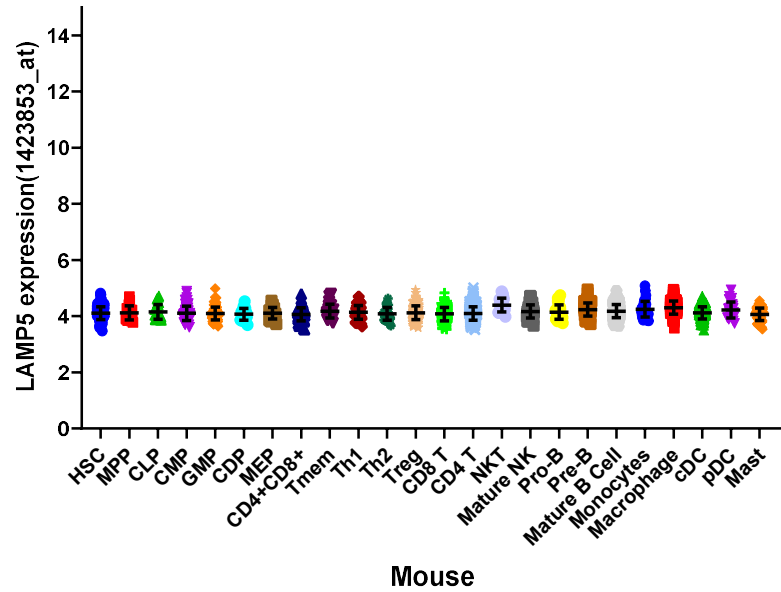
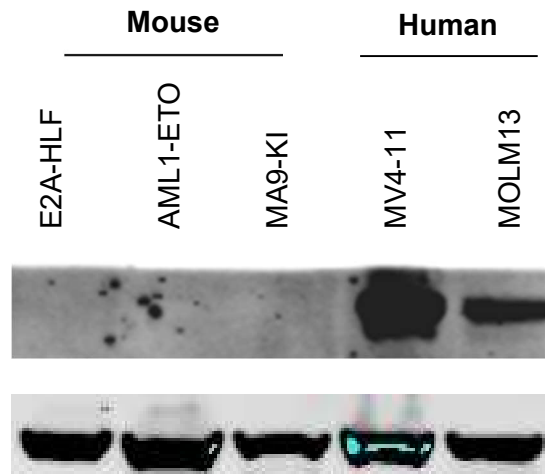
861 **(A)** Representative histogram plots showing LAMP-5 surface expression in MLL-r leukemia (MOLM-
862 13, RS4;11, MV4;11, and THP-1) and MLL-Germ leukemia (Kasumi-1, HL-60 and NB4) cell lines **(B)**
863 Graph showing mean fluorescence intensity (MFI) of LAMP-5 surface staining in MLL-r leukemias vs
864 MLL-Germ leukemias. **(C)** Representative histogram of LAMP-5 staining in Kasumi-1 expressing EV or
865 LAMP5, confirming the specificity of the antibody. **(D)** MFI of LAMP-5 surface expression in MLL-r and
866 MLL-Germ AML patients. **(E)** MOLM-13, RS4;11, THP-1 and Kasumi-1 cells were incubated with
867 surface LAMP-5 antibody clone D1 and α MFc-NC-DM1 ADC antibody for 72h. Bar graph represents
868 cell viability from 3 biological replicates presented as Mean and \pm SEM



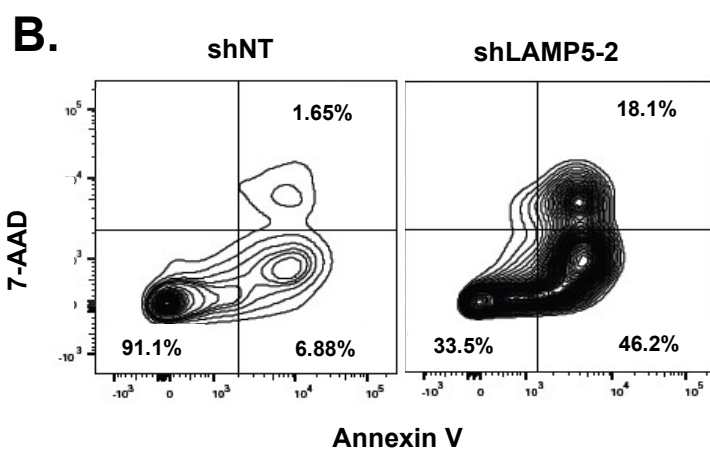
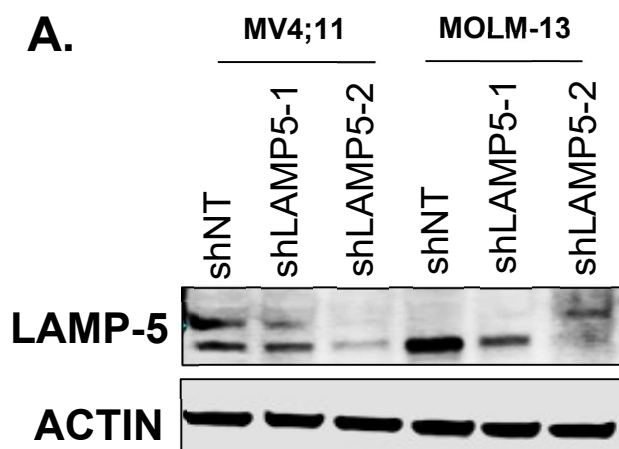
Supplemental Figure 1: (A) Intersection of published gene expression signatures composed of genes overexpressed in MLL-rearranged AML and ALL when compared to other MLL-germline leukemias. **(B)** Log₂ microarray expression of *LAMP5* in multiple molecular subtypes of leukemia from the Microarray Innovations in Leukemia (MILE) Study. Data shown as median values and quartiles. Normalized data from Bloodspot.eu



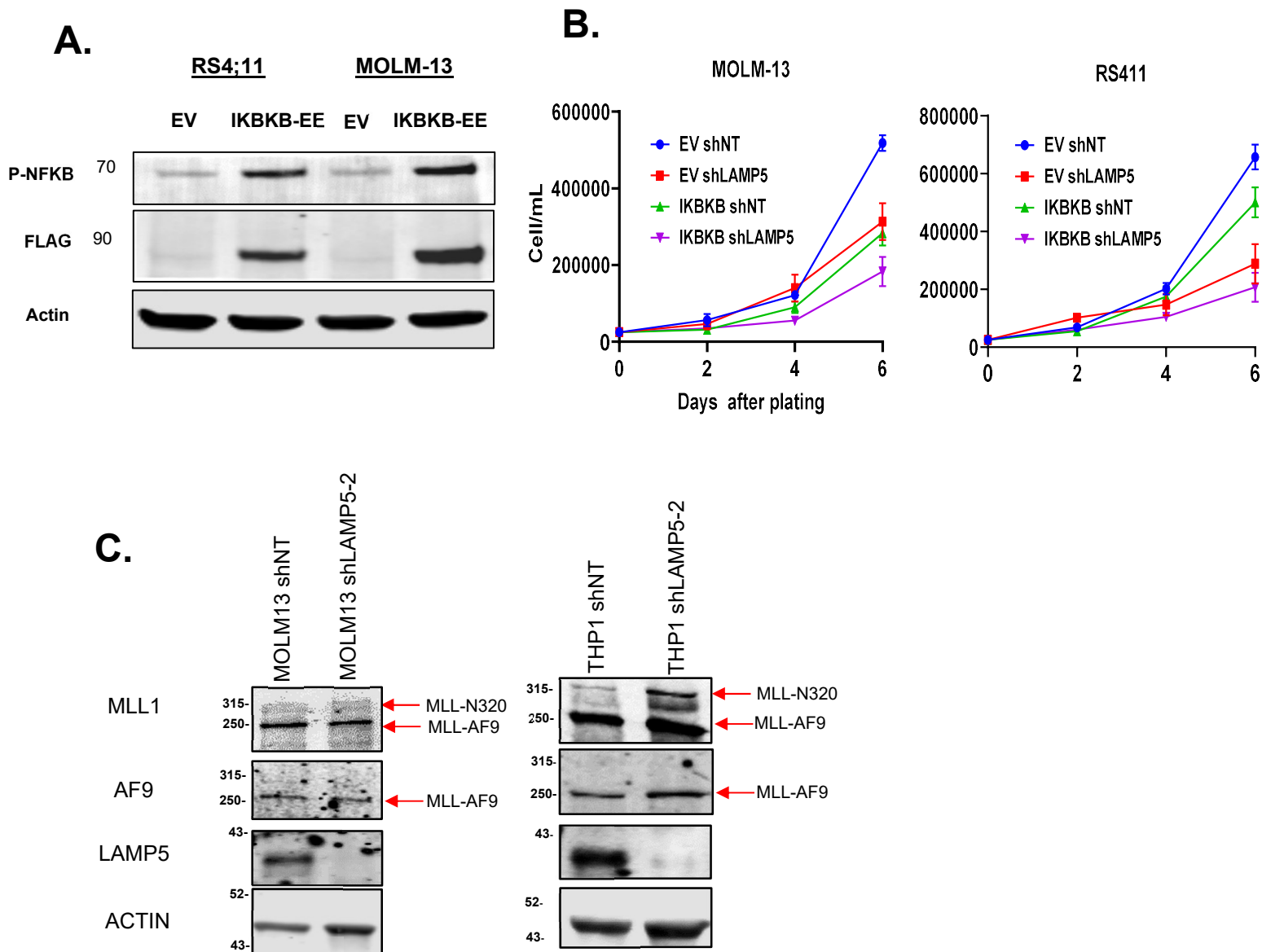
Supplemental Figure 2: (A) Kaplan-Meier Event-free survival curve of B-ALL patients based on *LAMP5* Log₂(FPKM) expression (n=1,104 two-sided time-stratified Cochran–Mantel–Haenszel test p=0.003). Data obtained from the St. Jude PeCan Portal. **(B)** Kaplan-Meier Overall survival curve of AML patients based on *LAMP5* (TPM) expression (n=374 two-sided time-stratified Cochran–Mantel–Haenszel test p=0.0014). Data obtained from the Leucegene Data Portal.

A.**B.****C.**

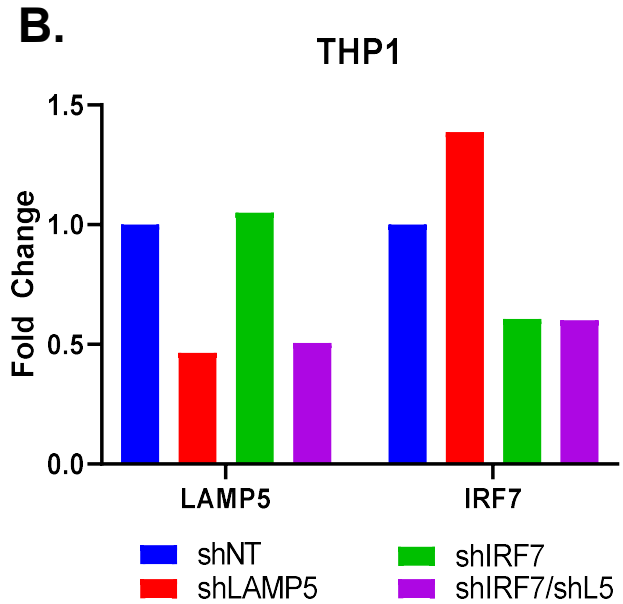
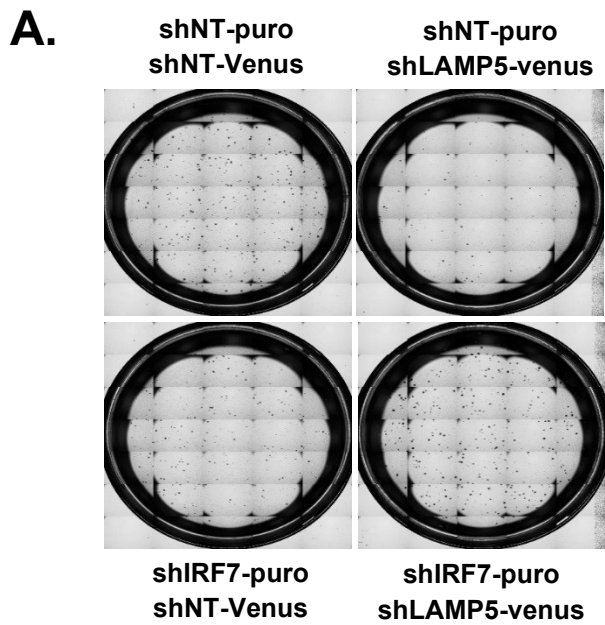
Supplemental Figure 3: (A) Normalized Microarray expression data of LAMP5 in human blood and leukemia (THP-1 as MLL-AF9 control). Data from Immuno-Navigator portal, presented as Mean with SD. (B) Normalized Microarray expression data of *Lamp5* in mouse blood. Data from Immuno-Navigator portal, presented as Mean with SD. (C) Western blot analysis of LAMP-5 expression in Mouse Lin(-) cells transformed with E2A-HLF, AML1-ETO, MLL-AF9. MV4-11 and MOLM-13 cells were used as control.



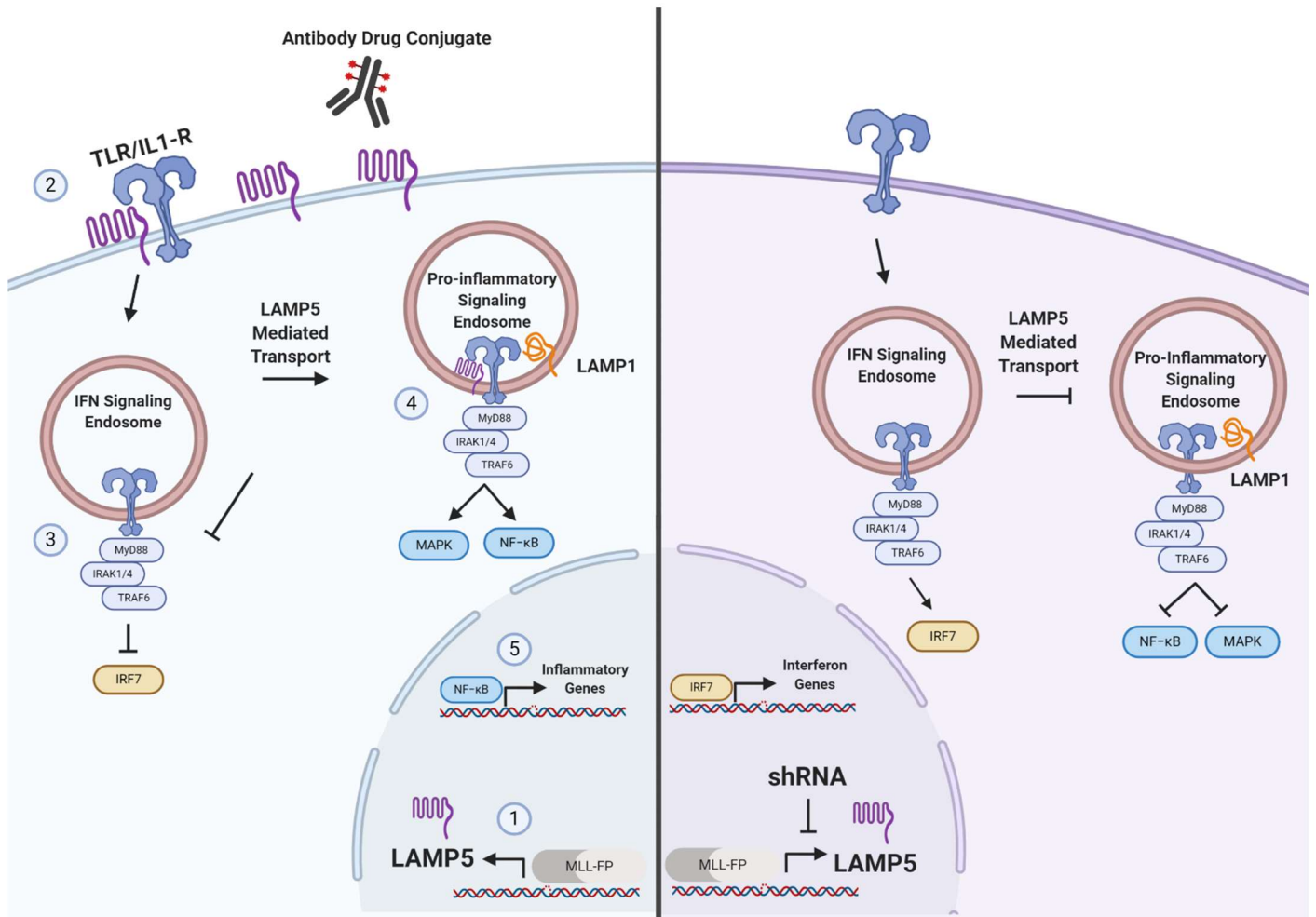
Supplemental Figure 4: (A) Western blot analysis of LAMP-5 expression in MV4;11 and MOLM-13 cells after transduction of shLAMP5-1 and shLAMP5-2. **(B)** Representative contour plot of MOLM-13 cells after transduction with shNT and shLAMP5-2.



Supplemental Figure 5: (A) Western blot analysis showing overexpression of IKKB-EE (FLAG) and activation of p-NFKB. **(B)** In-vitro cell growth of MOLM-13 and RS4;11 overexpressing EV or IKKB-EE upon LAMP5 downregulation. Data is from 3 technical replicates, representative of at least 3 experiments. **(C)** Western blot analysis showing the expression of the Germline MLL1 and MLL-AF9, C-term AF9, LAMP5 and actin after LAMP5 knockdown in MOLM13 and THP1 cell lines.



Supplemental Figure 6: (A) Representative images of THP-1 cells transduced with shNT/shIRF7/shLAMP5/shCombo(shIRF7/shLAMP5) after one week in methylcellulose. (F) Relative expression of *LAMP5* and *IRF7* after knockdown of LAMP-5, IRF7, or LAMP-5 and IRF7 together.



Supplemental Figure 7: Graphical Abstract. **Left panel:** ①The MLL-FP induces expression of LAMP5. ② LAMP5 gets internalized from the cell surface to the IFN signaling Endosome (IFN-SE), ③. ④LAMP5 is quickly shuttled to the LAMP1+ pro-inflammatory signaling endosome (PI-SE), activating NF-κB signaling. ⑤NF-κB activates pro-inflammatory signaling **Right panel:** Depletion of LAMP5 leads to blockage of transport and retention of TLR in the PI-SE, with activation of the IFN-SE and induction of Interferon related genes. Left panel depicts surface-LAMP5 can be targeted in MLL-r leukemias with immunotherapies.