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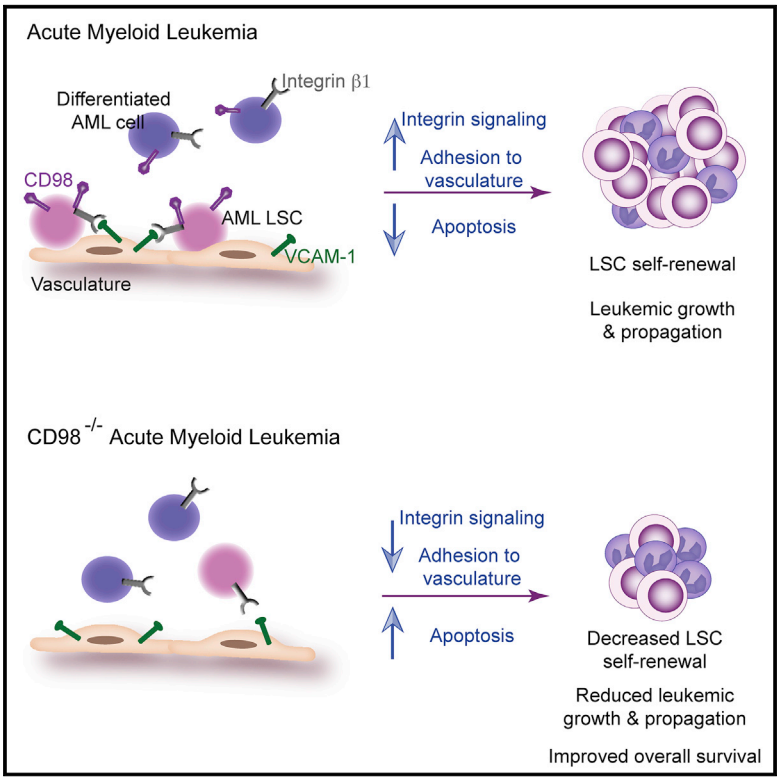
CD98-Mediated Adhesive Signaling Enables the Establishment and Propagation of Acute Myelogenous Leukemia

Graphical Abstract



Highlights

- Development and analysis of conditional CD98$^{-/-}$ model in the hematopoietic system
- CD98 loss impairs propagation of established AML in mouse models of disease
- Antibody-mediated CD98 blockade impairs primary human AML growth
- CD98-mediated adhesion to vasculature promotes leukemia stem cell maintenance

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In Brief

Bajaj et al. demonstrate the importance of CD98-mediated adhesion for survival of acute myelogenous leukemia (AML) and show that genetic deletion of CD98 in mice or use of a therapeutic CD98 antibody in patient-derived xenografts blocks AML growth.

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CD98-Mediated Adhesive Signaling Enables the Establishment and Propagation of Acute Myelogenous Leukemia

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SUMMARY

Acute myelogenous leukemia (AML) is an aggressive disease associated with drug resistance and relapse. To improve therapeutic strategies, it is critical to better understand the mechanisms that underlie AML progression. Here we show that the integrin binding glycoprotein CD98 plays a central role in AML. CD98 promotes AML propagation and lethality by driving engagement of leukemia cells with their microenvironment and maintaining leukemic stem cells. Further, delivery of a humanized anti-CD98 antibody blocks growth of patient-derived AML, highlighting the importance of this pathway in human disease. These findings indicate that microenvironmental interactions are key regulators of AML and that disrupting these signals with targeted inhibitors such as CD98 antibodies may be a valuable therapeutic approach for adults and children with this disease.

INTRODUCTION

The microenvironment that surrounds cancer cells can play a critical regulatory role in malignant growth and expansion. Early studies in breast cancer using both 3D cultures and in vivo approaches identified a role for stromal elements such as fibroblasts, vascular cells, and the extracellular matrix in sustaining tumor growth and dissemination, in part by activating transforming growth factor β, SDF1-α/CXCR4 signaling pathways, and through the release of metalloproteinases (Bissell and Radisky,

Significance

Little is known about how adhesive signals from the microenvironment control leukemia growth. In this study we show that CD98, which controls both adhesion and amino acid transport, plays a critical role in AML. CD98 deletion in a mouse model of AML impaired tumor initiation and propagation and increased survival. In vivo imaging revealed that CD98$^{-/-}$ leukemia cells had fewer stable interactions with the endothelium, and restoring CD98's adhesive function could rescue CD98$^{-/-}$ defects. Finally, CD98 blocking antibodies effectively impaired the growth of human AML cells in vitro and in xenografts. These findings identify CD98 as a mediator of adhesive signaling in AML, and provide key genetic evidence for the clinical utility of targeting CD98 in pediatric and adult AML.

2001; Orimo et al., 2005; Petersen et al., 1992). Further, epithelial interactions with extracellular matrix components were subsequently shown to be necessary for effective dissemination and metastasis of breast cancers (Desgrosellier et al., 2009; Desgrosellier and Cheresh, 2010), and to contribute to chemoresistance (Nakasone et al., 2012). Recent studies have challenged the dogma that the microenvironment is always supportive of oncogenesis by showing that targeted depletion of stromal cells can enhance pancreatic cancer growth (Lee et al., 2014; Ozdemir et al., 2014; Rhim et al., 2014). Although leukemic cells experience less anchorage than solid tumor cells, and are often thought to not be as spatially restricted, they do grow and reside within the bone marrow surrounded by a large network of microenvironmental cells. However, the specific molecular cues that drive the engagement of leukemia cells with the microenvironment, and their role in sustaining and promoting oncogenesis, remain poorly understood.

To address these key questions we have focused on CD98, a molecule that amplifies adhesive signals induced by a variety of extracellular matrix components through interactions with integrins (Fenczik et al., 1997; Feral et al., 2005), and plays an important regulatory role in assembly of a fibronectin matrix (Feral et al., 2007). Because of its role in mediating signals from multiple integrins, blocking CD98 can impair a broad spectrum of adhesive signals and be a powerful approach to disrupting interactions of cancer cells with their microenvironment. Structurally, CD98 is a transmembrane protein complex that consists of a single-pass heavy-chain (CD98hc encoded by *SLC3A2*) disulfide linked to a multipass light chain (consisting of amino acid transporters such as LAT1). The heavy chain of CD98 promotes adhesive signals in part by binding to multiple β1 and β3 integrins (Cantor et al., 2008; Zent et al., 2000) and increasing cell spreading, migration, survival, and growth. The association of CD98 heavy chain with any one of six light chains also regulates essential amino acid transport, which can contribute to cell survival (Imai et al., 2010; Rosilio et al., 2014). In the immune system, modulating CD98 function through blocking antibodies has implicated CD98 in B and T cell proliferation and activation (Haynes et al., 1981). In addition, conditional deletion of CD98 has been shown to inhibit clonal proliferation of T cells in response to antigens and prevent the establishment of autoimmune disease (Cantor et al., 2011), as well as abrogate the ability of B cells to respond to mitogens, leading to defects in plasma cell formation (Cantor et al., 2009).

Because of its role in clonal expansion, CD98 has also been studied in cancer. CD98 is highly expressed in many solid tumors, and its expression associated with poor prognosis (reviewed in Cantor and Ginsberg, 2012). The functional relevance of CD98 has primarily been tested in context of solid cancer cell lines. For example, ectopic expression of CD98 can transform epithelial cell lines such as CHO (Henderson et al., 2004) and NIH3T3 (Hara et al., 1999) and promote their anchorage-independent growth and tumor formation in immunocompromised mice, while inhibitory antibodies against CD98 can block the proliferation of bladder cancer cell lines (Yagita et al., 1986). Further, the inhibition of a CD98 light chain (LAT1) can affect proliferation of breast and lung cancer cell lines by impairing amino acid transport (Imai et al., 2010; Shennan and Thomson, 2008). The development of a conditional *Slc3a2* (referred to as *Cd98hc*) allele (Feral et al., 2007) has allowed genetic analysis of the role of CD98. *Cd98hc* deletion can prevent the formation of embryonic stem cell teratomas (Feral et al., 2005) and squamous cell carcinomas (Estrach et al., 2014), and heterozygous loss of *Cd98hc* reduces intestinal adenoma formation (Nguyen et al., 2011). However, the role of CD98 in hematologic malignancies and the relative contribution of adhesive signaling and amino acid transport functions to cancer have not been examined. To address this, we tested whether CD98 controls the establishment and propagation of primary leukemia cells within their native microenvironment by using both a genetic approach and the delivery of a newly developed therapeutic in the context of de novo acute myelogenous leukemia (AML).

RESULTS

CD98 in Normal Hematopoiesis

Given that CD98 is expressed in all hematopoietic lineages (Figures S1A and S1B), we tested its function by conditionally deleting *Cd98hc* using the *Rosa26-CreER*^{T2} model. Cre-mediated recombination leads to the excision of exons 1 and 2, which encode the transmembrane and cytoplasmic regions of the CD98 heavy chain, and results in complete loss of gene expression (Feral et al., 2007). The *Cd98hc*$^{fl/fl}$;*Rosa26-CreER*$^{+/+}$ mice (denoted hereafter as *Cd98hc*$^{fl/fl}$) allowed temporal control over CD98 expression and enabled efficient loss of CD98 after tamoxifen delivery (Figure S1C).

Conditional deletion of *Cd98hc* (Figures S1C and S1D; denoted as CD98$^{+/+}$ or CD98$^{-/-}$ post treatment) did not adversely affect bone marrow cellularity (Figure S1E) or hematopoietic stem cell (HSC) numbers (cKit^{+}Lin^{-}Sca1^{+}CD150^{+}CD48^{-} or KLSCD34$^{+/-}$Flk2^{-}; Figure 1A). Some changes, such as an increase in KLSCD150^{+}CD48^{-} cells (Figure 1A), a reduction in multipotent myeloid progenitors (Figures 1B and S1F), and an increase in differentiated B and T cells (Figure 1C), were noted and could be due to alterations in differentiation and/or proliferation. To identify the cell intrinsic impact of eliminating CD98 expression, we transplanted *Cd98hc*$^{fl/fl}$ or *Cd98hc*$^{+/+}$ HSCs into wild-type recipients. Deletion following stable reconstitution 2 months after transplant had no effect on apoptosis (Figure S1G) and led to a minor 1.5-fold decrease in chimerism (Figures 2A–2C). However, transplantation of HSCs lacking CD98 showed a functional dependence on this signal in the context of rapid proliferative need (Figure S1H): thus, while CD98$^{+/+}$ chimerism increased from 38% to 57%, CD98$^{-/-}$ chimerism dropped from 17% to 10% (Figure 2D). These results suggest that while CD98 loss may not affect the numbers of established HSCs and progenitors, it can affect their proliferative and regenerative capacity.

CD98 in De Novo AML Initiation and Maintenance

We tested the importance of CD98 in leukemogenesis using a mixed-lineage leukemia fusion protein (MLL)-driven model of de novo AML, a highly drug-resistant disease in adults and children. Because MLL-driven AML often presents with *NRAS* mutations, we used MLL-AF9 and NRAS^{G12V} oncogenes to establish myeloid leukemia with *Cd98hc*$^{fl/fl}$ cells (Figures 3A and S2A–S2C). CD98 loss led to a marked increase in survival (55%) relative to controls (0%) (Figures 3A, S2D, and S2E), indicating that CD98 is important for MLL-leukemia initiation. To determine

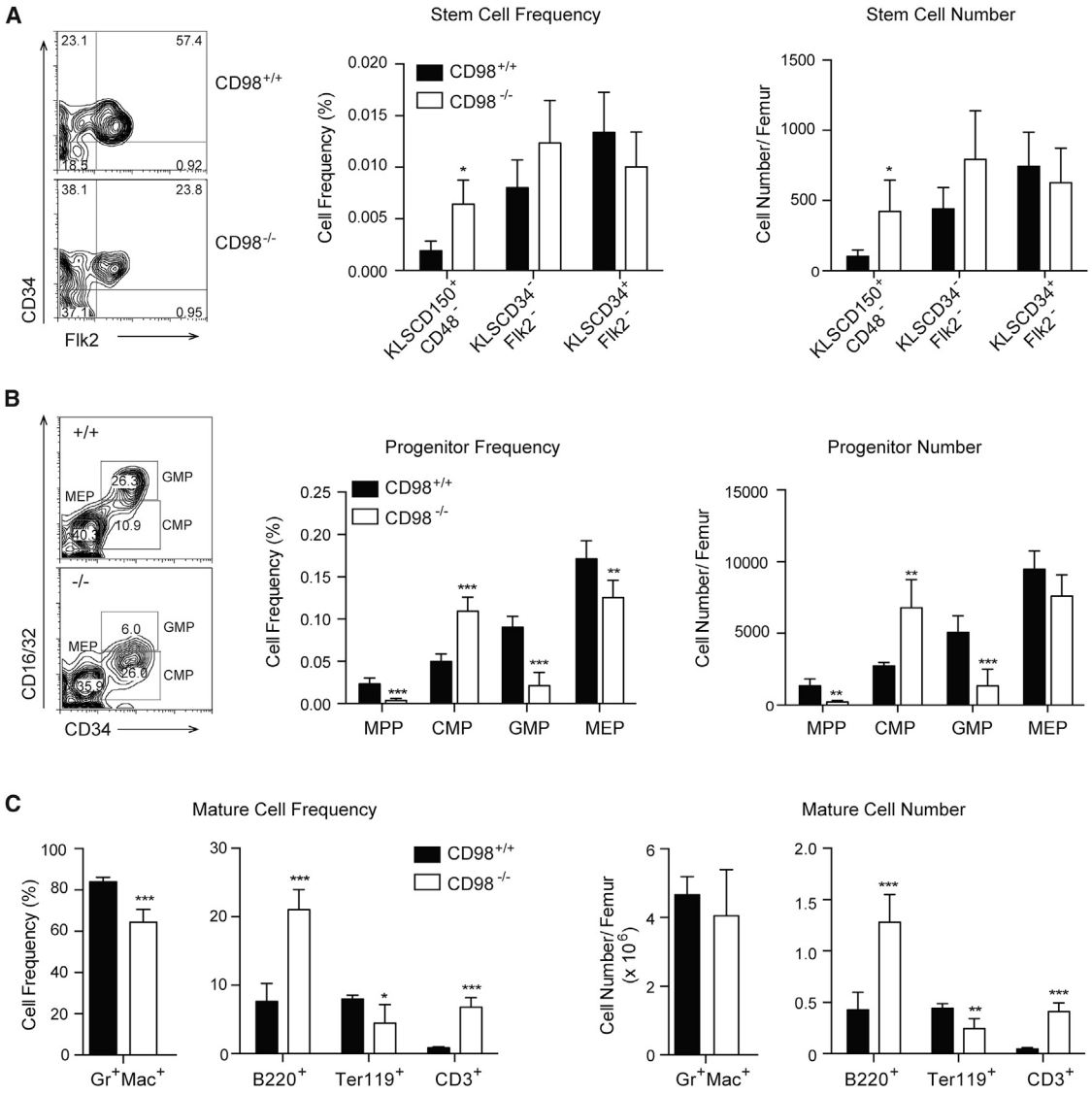

Figure 1. Generation and Analysis of Conditional CD98-Deficient Mice

(A) *Cd98hc*$^{+/+}$;*Rosa26-CreER* (denoted as CD98$^{+/+}$) and *Cd98hc*$^{fl/fl}$;*Rosa26-CreER* (denoted as CD98$^{-/-}$) mice were treated with tamoxifen and analyzed 3 days later (n = 5 for each cohort). Representative fluorescence-activated cell sorting (FACS) plots (gated on KLS cells), average stem cell frequencies, and absolute numbers of stem and progenitor cells in CD98$^{+/+}$ and CD98$^{-/-}$ mice are shown.

(B) Representative FACS plots show myeloid and erythroid progenitor frequency in CD98$^{+/+}$ and CD98$^{-/-}$ mice (gated on Lin$^-$IL7R$^-$cKit$^+$Sca$^-$ cells). The average frequencies and numbers of progenitors (MPP: KLSCD34$^+$Flk2$^+$; GMP: Lin$^-$IL7Ra$^-$Kit$^+$Sca1$^-$CD34$^+$CD16/32$^+$; CMP: Lin$^-$IL7Ra$^-$Kit$^+$Sca1$^-$CD34$^+$CD16/32$^-$; MEP: Lin$^-$IL7Ra$^-$Kit$^+$Sca1$^-$CD34$^-$CD16/32$^-$) are shown.

(C) Average frequencies and numbers of differentiated cells in CD98$^{+/+}$ and CD98$^{-/-}$ mice.

Error bars represent ±SEM. *p < 0.05, **p < 0.01, ***p < 0.001, unpaired t test. See also Figure S1.

whether continued AML propagation requires CD98, we assessed the capacity of sorted cKit$^+$ leukemia cells to propagate disease (Figure 3B). Tamoxifen-induced CD98 loss led to a dramatic reduction in the number of leukemic colonies formed in vitro (Figure 3C) and significantly increased the median survival of mice transplanted with cKit$^+$ AML cells from 21.5 days to 33 days (Figure 3D). These data indicate that abrogating CD98 expression reduced morbidity from established disease. Serial transplantation of CD98$^{-/-}$ cKit$^+$ leukemia cells not only impaired self-renewal but also led to a striking improvement in survival (0% for CD98$^{+/+}$ versus 46% for CD98$^{-/-}$) (Figures 3D and S2F). Finally, because patients generally present with full-blown disease, we modeled a more clinically relevant setting by eliminating CD98 expression after AML establishment (Figure 3E). Loss of CD98 in this context led to a significant increase in survival (0% for CD98$^{+/+}$ versus 66% for CD98$^{-/-}$, Figure 3E). CD98 appeared to be broadly required by non-MLL AML as well, with its loss improving survival dramatically in disease driven by AML-ETO9a and NRAS^{G12V} (Figure 3F). These data demonstrated a requirement for CD98 in the initiation, self-renewal,

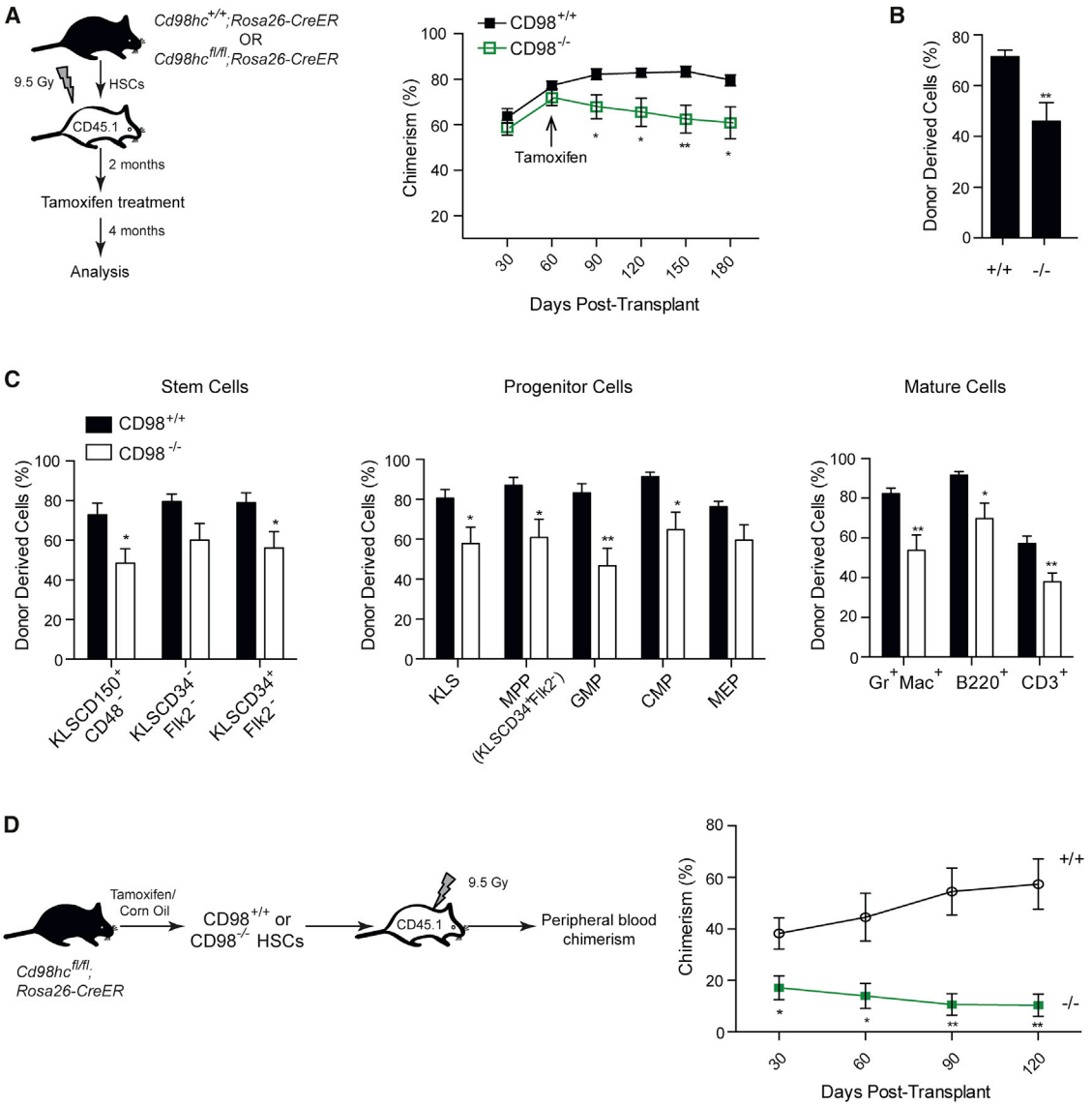

Figure 2. Effect of CD98 Loss on HSC Function

(A) KLSCD34^{-}Flk2^{-} cells from *Cd98hc*$^{+/+}$;*Rosa26-CreER* (denoted as CD98$^{+/+}$) or *Cd98hc*$^{fl/fl}$;*Rosa26-CreER* (denoted as CD98$^{-/-}$) mice were transplanted and tamoxifen delivered after 2 months (schematic on the left). HSCs were subsequently analyzed at 4 months after transplant (n = 9 per cohort). Average donor chimerism in the peripheral blood prior to and after tamoxifen treatment is shown (right).

(B) Average donor chimerism in the bone marrow of transplanted mice 4 months after tamoxifen delivery.

(C) Average frequency of HSCs, progenitors, and differentiated cells in the bone marrow of mice transplanted with CD98$^{+/+}$ and CD98$^{-/-}$ HSCs (n = 9 for each cohort).

(D) HSCs from tamoxifen or corn oil treated *Cd98hc*$^{fl/fl}$;*Rosa26-CreER* mice were transplanted (schematic on the left), and peripheral blood chimerism analyzed (right, n = 5–6 for per cohort).

Error bars represent ±SEM. *p < 0.05, **p < 0.01, Mann-Whitney U test.

and propagation of de novo AML, and provided clear genetic evidence that targeting CD98 may be of therapeutic value.

CD98 Loss Impairs Survival and Depletes Leukemia Stem Cells

To determine the cellular basis of CD98's effect, we analyzed the impact of CD98 loss on leukemia proliferation, apoptosis, and differentiation. Loss of CD98 led to a modest decline in proliferation (Figures 4A and 4B) but triggered a dramatic rise in apoptosis, suggesting that CD98 is critical for leukemia cell survival (Figures 4C and 4D). Gene expression analysis of candidate apoptosis-related genes showed that the loss of CD98 led to decreased expression of anti-apoptotic genes such as *Bcl2L1* and *Akt1* and increased expression of pro-apoptosis genes such as *Casp1* (Figure 4E). To determine whether these play a functional role downstream of CD98, we tested whether their re-expression could rescue the colony-forming ability of CD98$^{-/-}$ AML cells. While *Bcl2L* alone was able to partially

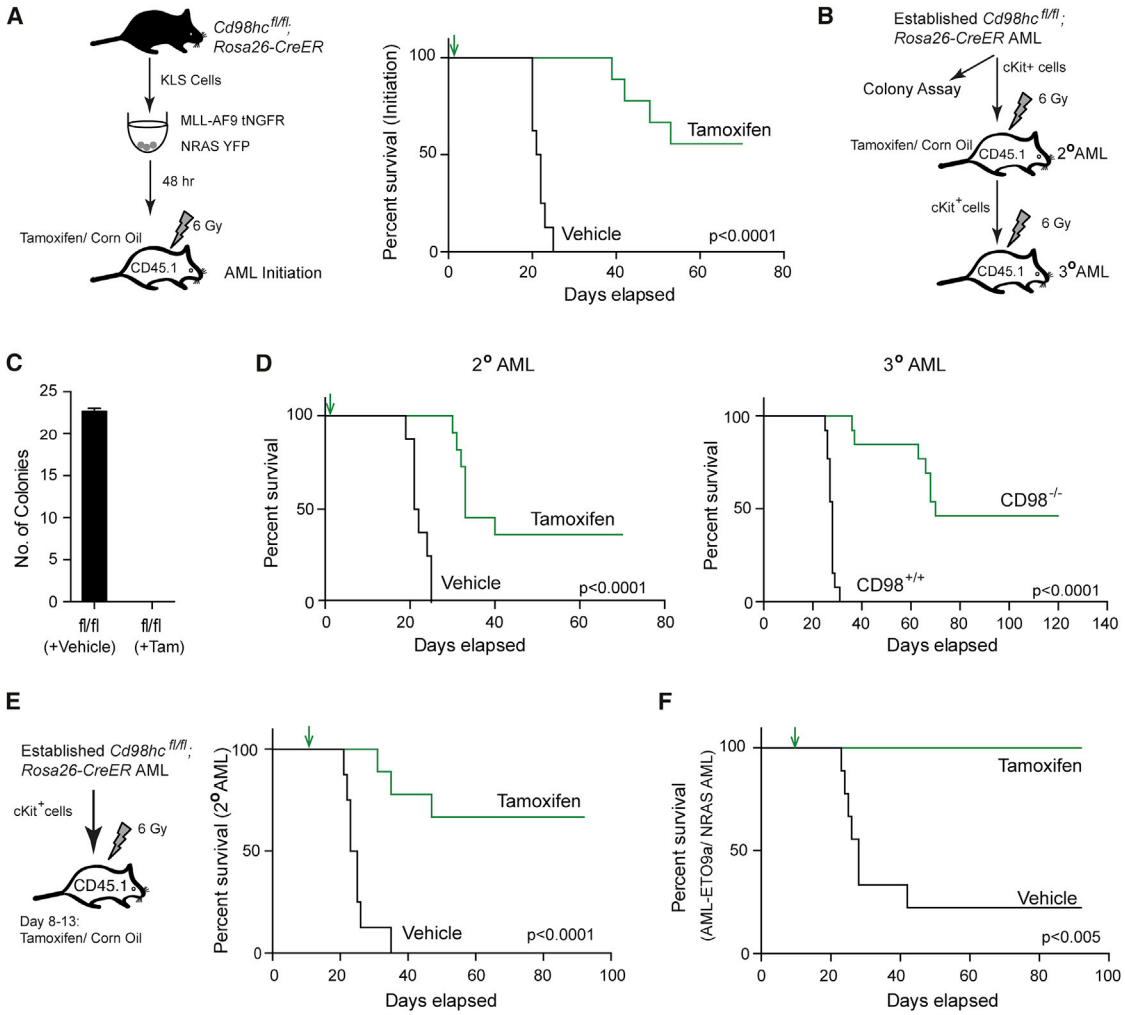

Figure 3. CD98 Loss Impairs AML Growth and Propagation

(A) KLS cells from *Cd98hc*$^{fl/fl}$;*Rosa26-CreER* mice were retrovirally transduced with MLL-AF9-IRES-tNGFR and NRAS^{G12V}-IRES-YFP and transplanted to establish disease (schematic on the left). The start of treatment is indicated by an arrow on the survival curve (right; n = 8–9 for each cohort, data combined from two independent experiments).

(B–D) cKit$^+$ cells from established *Cd98hc*$^{fl/fl}$;*Rosa26-CreER* leukemia (schematic in B) were isolated and cultured in vitro in the presence of tamoxifen or vehicle, and colony formation assessed (C). Cells were transplanted, 2° recipients treated with tamoxifen or vehicle, and survival monitored (n = 8 for vehicle and n = 11 for tamoxifen treatment, data combined from two independent experiments). The cKit$^+$ CD98$^{+/+}$ or CD98$^{-/-}$ leukemia cells from 2° mice were also transplanted into 3° recipients to monitor in vivo self-renewal capacity (D). The start of treatment is indicated by an arrow on the survival curve (n = 13 for each cohort, data combined from two independent experiments).

(E) cKit$^+$ *Cd98hc*$^{fl/fl}$;*Rosa26-CreER* AML cells were transplanted, recipients treated with tamoxifen or vehicle from day 8 to day 13 (schematic on the left), and survival monitored (right; n = 8 for control and n = 9 for tamoxifen treatment, data combined from two independent experiments). The start of treatment is indicated by an arrow on the survival curve.

(F) KLS cells from *Cd98hc*$^{fl/fl}$;*Rosa26-CreER* mice were isolated, infected with AML-ETO9a and NRAS oncogenes, and transplanted. Mice were subsequently treated with tamoxifen or corn oil, starting 8 days after transplant. The start of treatment is indicated by an arrow on the survival curve (n = 9 for vehicle and n = 8 for tamoxifen treatment, data combined from two independent experiments).

Survival curves depict log-rank test p values. Error bars represent ±SEM. See also Figure S2.

rescue the colony formation of CD98$^{-/-}$ cells to ~18% of control, the addition of activated Myr-AKT increased colony-forming ability to 50% (Figure S3A).

We also analyzed leukemia stem cells (LSCs), marked in this model as cKit$^+$ or Gr1$^{-/low}$cKit$^+$ (Shi et al., 2013; Somervaille and Cleary, 2006; Zuber et al., 2011). CD98 loss clearly affected the frequency of LSCs, consistent with enriched CD98 expression in this population (Figures 4F and S3B). Leukemias arising from CD98$^{-/-}$ cells showed a marked depletion of both cKit$^+$ (Figure 4G) and Gr1^{lo}cKit$^+$ stem cells (Figures S3C–S3G). Consistent with this, CD98$^{-/-}$ AML stem cells expressed significantly lower levels of MLL-leukemia stem cell-associated genes (Somervaille et al., 2009) (Figure 4H). These data collectively indicate that CD98 is important for promoting the survival of leukemia cells and for maintaining LSCs in MLL-driven AML.

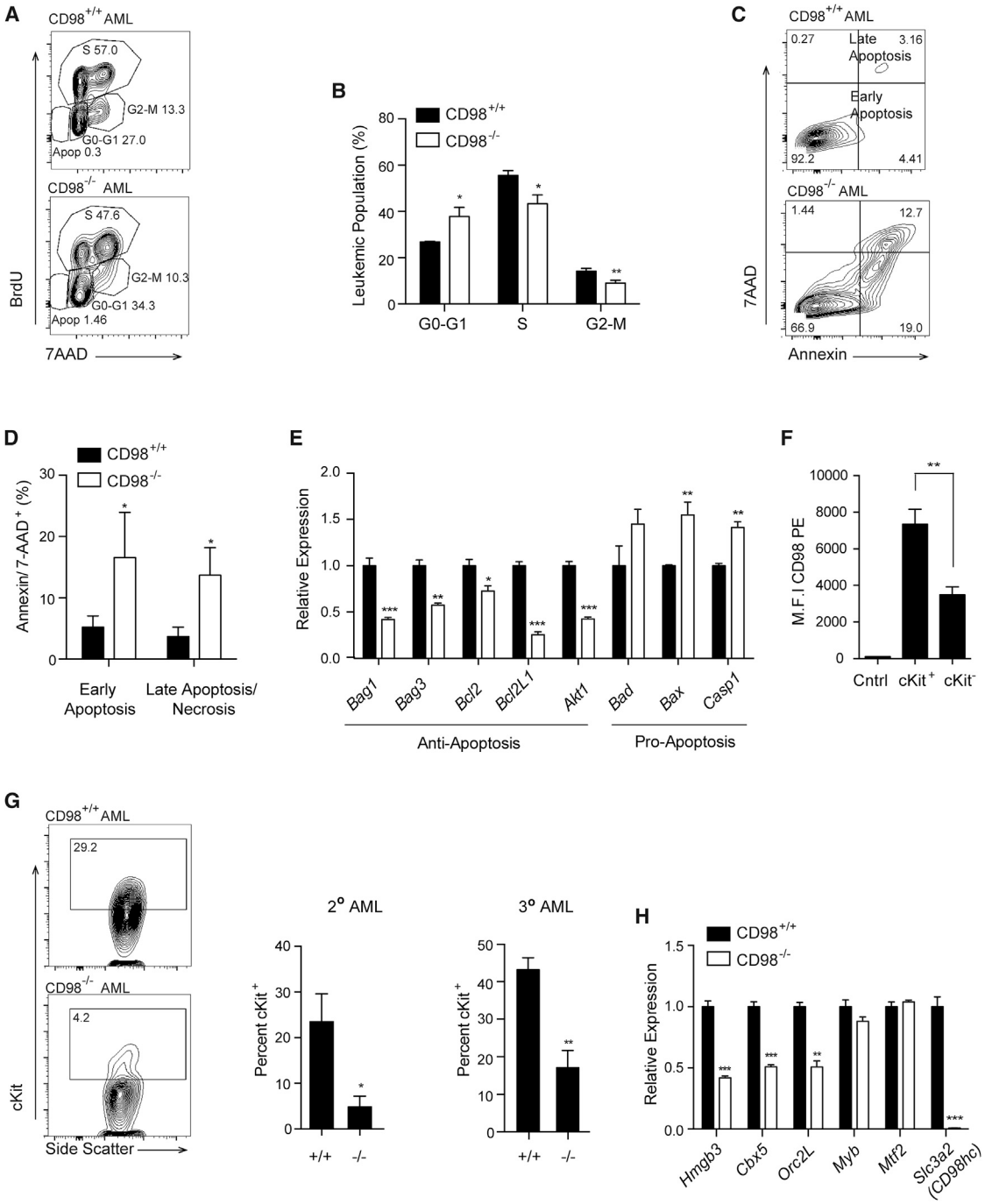

Figure 4. Loss of CD98 Triggers Apoptosis and Depletion of AML Stem Cells

(A and B) cKit$^+$ *Cd98hc$^{fl/fl}$;Rosa26-CreER* AML cells were transplanted and treated with tamoxifen or corn oil. Eighteen days after transplant, mice were injected with bromodeoxyuridine (BrdU) and cells analyzed for incorporation 22 hr later. Representative FACS plots show BrdU and 7AAD staining of MLL-AF9$^+$NRAS$^+$ leukemia cells from CD98$^{+/+}$ and CD98$^{-/-}$ mice (A). Average frequency of cells in distinct phases of the cell cycle (n = 2–3 per cohort) is shown (B).

(C and D) Representative FACS plots (C) and graph (D) show analysis of early and late apoptosis in MLL-AF9$^+$NRAS$^+$ leukemia cells 18–21 days post transplant (n = 3 per cohort).

(E) Expression of apoptosis-associated genes in leukemia cells from representative wild-type and CD98$^{-/-}$ leukemia. Data shown are from triplicates of one sample per cohort. Similar results were obtained from three independent samples per cohort.

(F) Mean fluorescence intensity of CD98 expression in control unstained, cKit$^+$ and cKit$^-$ MLL-AF9$^+$NRAS$^+$ leukemia cells (n = 4).

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CD98-Mediated Integrin Signaling Is Required for Leukemia Growth

Since CD98 can regulate both integrin-mediated signaling and amino acid transport, we tested the importance of each function for leukemia progression by reconstituting the two functions individually in CD98$^{-/-}$ cells using chimeric human CD98-CD69 proteins (Figures 5A and S4A). These chimeric proteins separated CD98 functions by replacing either the integrin binding cytoplasmic and transmembrane domain or the extracellular amino acid transport domain of CD98 with parts of CD69, another type II transmembrane protein (Fenczik et al., 2001; Feral et al., 2005). Loss of colony-forming ability could be rescued almost completely by the full-length human CD98 heavy chain (as a control) as well as partially by the chimera that restored integrin binding and signaling (Figure 5B), indicating that downstream activation of integrin signaling is critical for leukemia growth. In contrast, the chimera that only restored amino acid transport failed to rescue the defect. Interestingly, rescue was most efficient when the chimera that preserves integrin binding was co-delivered with the chimera that restores amino acid transport. These data suggest that the integrin binding function of CD98 is critically required for leukemic growth and that the amino acid transport function can synergize with integrin binding to fully restore the normal function of LSCs. We should note that CD98 loss affected cells grown on semi-soft substrates such as methylcellulose or in vivo, but not when plated in liquid culture (Figures 3C, 3D, and 5B; data not shown). This is consistent with previous work showing that CD98-mediated signaling is required for proliferation and survival on soft substrates but not stiff substrates (Estrach et al., 2014), possibly because there is enough tension on contact with stiff substrates (such as plastic) that CD98 is not needed to amplify integrin signaling.

As integrin-dependent adhesion mediates leukemia cell interactions with their microenvironment, we tested whether eliminating CD98 expression affected the dynamic in vivo associations of leukemic cells with their niche by real-time imaging. We first tested whether wild-type leukemia cells were capable of forming long-term interactions with blood vessels over time and found that about 25% of cells in the marrow space remained in close contact with vessels for 25 min or longer. The loss of CD98 reduced long-term interactions by 2-fold (Figures 5C and 5D; Movies S1 and S2), indicating that CD98 enables stable interactions of leukemia cells with their niche in vivo.

To define whether the interactions of leukemia cells with endothelium are physiologically important for maintaining stem cell properties, we used human umbilical vein endothelial cells (HUVECs) as a surrogate. Consistent with in vivo imaging analysis, the ability of cKit^{+} leukemia cells to adhere to HUVECs was reduced by ~40% with CD98 loss (Figures 5E and 5F). To interfere with adhesion and not other signals regulated by CD98, we used an anti-VCAM-1 (vascular cell adhesion molecule 1) antibody to block integrin-mediated adhesion. Endothelial cells express the integrin ligand VCAM-1 (Swerlick et al., 1992), and AML cells express the integrin $\alpha4/\beta1$ (or VLA-4) (Matsunaga et al., 2003), providing a rationale for testing whether adhesion of cKit^{+} leukemia cells could be blocked by VCAM-1 inhibition, and thus mimic the effect of CD98 loss on adhesion. While blocking VCAM-1 reduced the adherence of wild-type cKit^{+} cells by ~2-fold, it had no impact on the attachment of CD98-deficient cKit^{+} cells, confirming that CD98 is required for VCAM-1/VLA-4 mediated interaction of leukemic cells with blood vessels (Figures 5G, S4B, and S4C). Importantly, blocking adhesion led to a 9-fold loss of cKit^{+} leukemia cells (Figure 5H), predominantly due to apoptosis (Figure 5I). These data indicate that CD98-integrin-mediated interactions with endothelial cells are needed for survival and maintenance of LSCs.

CD98 Is Required for Human AML Propagation

Analysis of human normal cells and LSCs revealed that while CD98 was expressed at the cell surface on normal CD34^{+} stem and progenitor cells, its expression was 3-fold higher in AML CD34^{+} cells (Figure 6A). CD98 expression was also 1.8-fold higher in the CD34^{+} AML stem cells compared with CD34^{-} cells, consistent with enriched expression observed in murine LSCs (Figures 4F and S3B). Functionally, small hairpin RNA (shRNA) delivery in primary patient-derived AML cells as well as cell lines led to a consistent, albeit inefficient, reduction of CD98 expression, and affected both adhesion to endothelial cell lines (Figures S5A and S5B), and colony formation by 2.7- to 15-fold (Figures 6B, 6C, and S5C–S5E). Importantly, CD98 inhibition affected colony formation of human AMLs driven by both MLL translocations (Figure 6B) or by other mutations (Figures 6C and S5D; Table S1). Most importantly, CD98 knockdown led to reduced leukemia burden across several primary patient-derived xenografts (Figures S5F and S5G).

As CD98 is a cell-surface molecule, we tested the impact of antibody-mediated targeting in the context of primary AML in vitro and in vivo. To this end we used a humanized CD98 antibody, IGN523 (Hayes et al., 2014). Treatment with IGN523 antibody (denoted as CD98 mAb) significantly affected the adhesion of AML cells to endothelial cells (Figure S5H). Further, six primary patient samples showed a ~2- to 7-fold decrease in colony-forming ability when treated with the antibody (Figures 6D, 6E, S5I, and S5J). This suggests that inhibition of CD98 via a deliverable could be as effective as genetic silencing of CD98 in blocking the growth of human AML.

Finally, we tested the impact of CD98 inhibition on in vivo propagation of human AML. Immunodeficient mice were transplanted with patient-derived leukemia cells and treated with CD98 monoclonal antibody (mAb) (18-2A, non-humanized clone) or control immunoglobulin G (IgG) starting 1 day after transplant. This resulted in a complete depletion of leukemia engraftment in antibody-treated mice 4 weeks after transplantation (Figures 7A–7C and Table S2), indicating that CD98 expression and function

(G) Representative FACS plots of cKit expression in CD98$^{+/+}$ and CD98$^{-/-}$ leukemia cells. Average frequency of cKit^{+} leukemia stem cells in secondary and tertiary MLL leukemia is shown (n = 3–5 per cohort for secondary leukemia and n = 3 per cohort for tertiary leukemia).
(H) Expression of MLL-leukemia stem cell genes in sorted cKit^{+} cells from representative wild-type and CD98$^{-/-}$ leukemia. Data shown are from triplicates of one sample per cohort. Similar results were obtained from three independent experiments.
Error bars represent ±SEM. *p < 0.05, **p < 0.01, ***p < 0.001, unpaired t test. See also Figure S3.

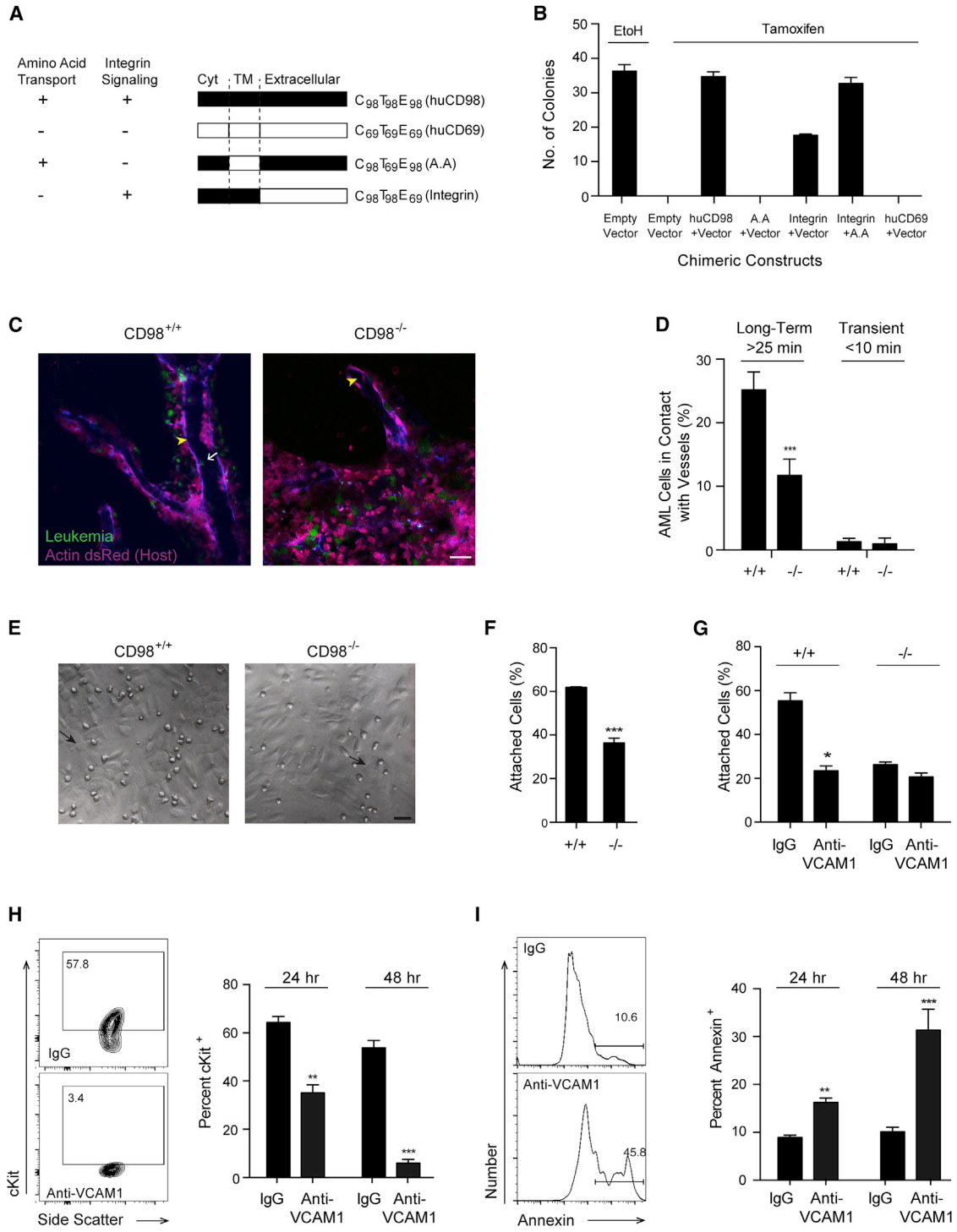

Figure 5. AML Is Dependent on CD98-Mediated Integrin Signaling

(A) Human CD98-CD69 chimeric constructs designed to specifically reconstitute either the amino acid transport or the integrin signaling function of CD98.

(B) Average number of colonies formed by *Cd98hc*$^{fl/fl}$ cKit^{+} cells transduced with human CD98 chimeric proteins and grown in the presence of ethanol (vehicle) or tamoxifen (to delete *Cd98hc*) (n = 3).

(C) Representative images of calvarial bone marrow (magenta) of mice transplanted with CD98$^{+/+}$ and CD98$^{-/-}$ AML cells (green). Arrowheads indicate blood vessels (marked by VE-cadherin in blue), and arrow points to a leukemia cell adhering to vessel. Scale bar, 25 μm.

(D) Average frequency of CD98$^{+/+}$ and CD98$^{-/-}$ leukemia cells in contact with blood vessels (distance <0.5 μm) for 25 min or longer (stable/long-term) or for less than 10 min (transient/short-term) (n = 12 movies for each group, from 4 to 5 mice per cohort; data were compiled from two independent experiments).

(E) Representative pictomicrographs show CD98$^{+/+}$ and CD98$^{-/-}$ leukemia cells (indicated by arrows) adhering to HUVECs. Scale bar, 25 μm.

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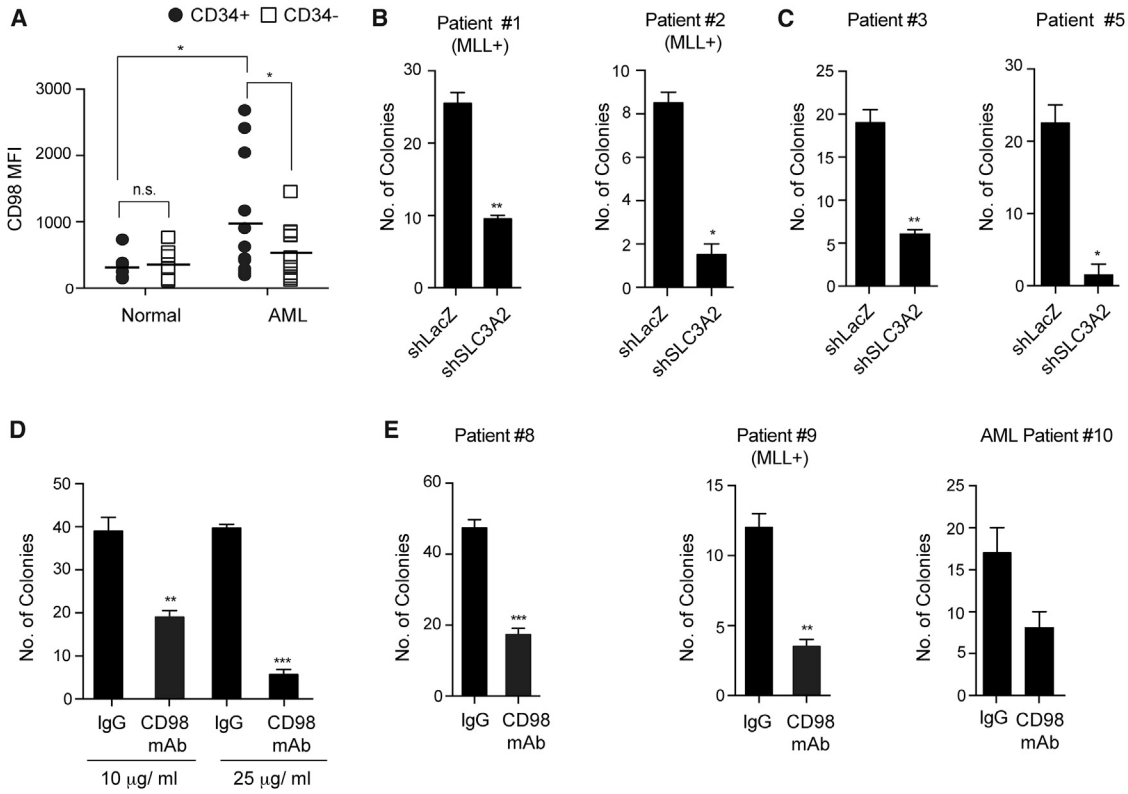

Figure 6. CD98 Inhibition Impairs Human AML Growth In Vitro

(A) Mean fluorescence intensity of CD98 in $CD34^+$ and $CD34^-$ cells from normal donors (n = 7) and AML patients (n = 12).

(B and C) Human MLL^+ AML (B) and non-MLL AML (C) samples were transduced with lentiviral shRNAs targeting LacZ (control) or human SLC3A2 (CD98hc). Infected cells were sorted and plated in methylcellulose. Average numbers of colonies formed are shown (n = 2–3 for each group).

(D) Primary human AML cells (patient #6) were seeded in methylcellulose in the presence of varying concentrations of control IgG or CD98 mAb. Average colony formation at each concentration was scored to determine optimal dose (n = 3 for each condition).

(E) Three independent AML patient samples were grown in methylcellulose in the presence or absence of CD98 mAb (25 μg/mL). Average numbers of colonies formed are shown (n = 2–3 for each group).

Error bars represent ±SEM. *p < 0.05, **p < 0.01, ***p < 0.001; n.s., not significant, unpaired t test. See also Figure S5 and Table S1.

is required for human AML establishment in vivo. To determine whether the antibody can have an impact on established disease, we treated mice with detectable chimerism in the peripheral blood; mice were distributed into two cohorts with more of the high-chimerism mice assigned to the CD98 antibody treatment group to test the ability of CD98 inhibition to control high-burden disease (Figures 7D–7F). While chimerism increased in control-treated animals, CD98 antibody delivery dramatically reduced the disease burden in the peripheral blood by ~33%–100% (average 66% reduction, Figure 7G). In addition, CD98 antibody delivery reduced the disease burden by ~12%–98% in the bone marrow of treated mice (average 67% reduction, Figure 7G). Importantly, CD98 antibody delivery had no detectable effect on the ability of normal $CD34^+$ cells to form colonies in vitro (Figure S6A) or to engraft in vivo (Figures S6B and S6C), paralleling data from the CD98-deficient mouse models. The fact that the antibody may have a more marked effect on human leukemia cells identifies a potential therapeutic window, and indicates that the CD98 mAb may be effective in the clinic.

DISCUSSION

Our work here focuses on defining the molecular signals that mediate microenvironmental influences on leukemia growth. To this end, we generated a conditional knockout model of CD98, a surface molecule critical for integrating adhesive signals

(F) Average frequency of $CD98^{+/+}$ and $CD98^{-/-}$ $cKit^+$ cells adhering to HUVECs (n = 3 from a representative experiment; similar results were obtained from two independent experiments).

(G) Impact of anti-VCAM-1 antibody on adhesion of $CD98^{+/+}$ and $CD98^{-/-}$ $cKit^+$ cells to HUVECs (n = 3).

(H) Representative FACS plots show cKit expression in wild-type leukemia cells after co-culture with HUVECs in the presence or absence of anti-VCAM-1. Average frequency of $cKit^+$ cells at the indicated time points is quantified (n = 3 for each condition).

(I) Flow cytometric plots showing frequency of $Annexin^+$ cells in $cKit^+$ AML. Average frequency of $Annexin^+$ cells at the indicated time points is quantified (n = 3 for each condition).

Error bars represent ±SEM. *p < 0.05, **p < 0.01, ***p < 0.001, unpaired t test. See also Figure S4; Movies S1 and S2.

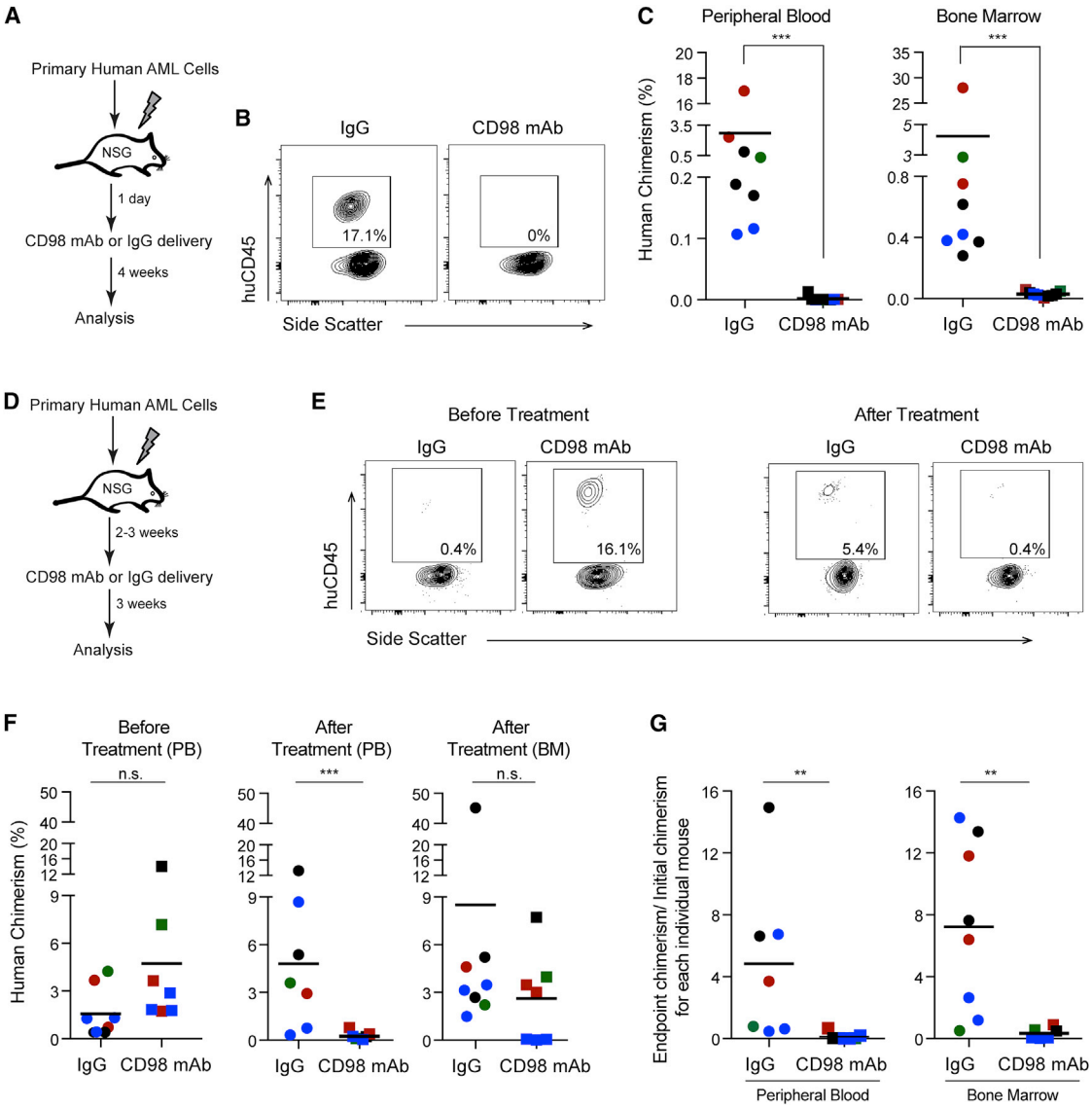

Figure 7. Loss of CD98 Impairs Human AML Growth In Vivo

(A–C) NSG mice transplanted with cells from four primary AML patient samples were treated with either control IgG or CD98 mAb (15 mg/kg) once a week, starting 1 day after transplant (schematic in A). Representative FACS plots (B) show human cell chimerism in the peripheral blood. Chimerism in individual xenograft recipients at 4 weeks in peripheral blood and bone marrow (C). Xenografts include two mice per cohort for AML sample #12 (red); one mouse per cohort for #13 (green); two to three mice per cohort for #16 (blue); and three mice per cohort for #17 (black).

(D–G) NSG mice were transplanted with cells from four primary AML patient samples: two to three mice per cohort for #7 (black); two mice per cohort for #14 (red); one mouse per cohort for #15 (green); and three mice per cohort for #18 (blue). Disease was allowed to establish until there was detectable chimerism in peripheral blood (schematic in D). Mice were then treated with either control IgG or CD98 mAb (15 mg/kg) once a week for 3 weeks. Representative FACS plots (E) show human cell chimerism in peripheral blood before and after treatment. (F) Leukemia cell chimerism in peripheral blood (PB) or bone marrow (BM) of individual xenograft recipients. (G) Fold change in leukemia cell chimerism in peripheral blood or bone marrow following delivery of control or CD98 mAb, shown relative to peripheral blood chimerism measured at treatment initiation.

**p < 0.01, ***p < 0.001; n.s., not significant, Mann-Whitney U test. See also Figure S6 and Table S2.

from the environment. While CD98 loss did not significantly affect the normal hematopoietic cell pool, it had a dramatic impact on the development and propagation of MLL-driven de novo AML. The impact of eliminating CD98 expression could be attributed, at least in part, to defects in localization and adhesion with endothelial cells, elements critical for sustaining LSC survival (see the model in Figure 8). We corroborated our genetic data by testing an inhibitory humanized CD98 antibody, which effectively blocked the growth of primary AML samples in vitro and in patient-derived xenografts. Collectively these data provide strong genetic evidence for the requirement of CD98 in leukemia development, and suggest that use of therapeutic anti-CD98 antibodies in AML could be beneficial for both adult and pediatric AML.

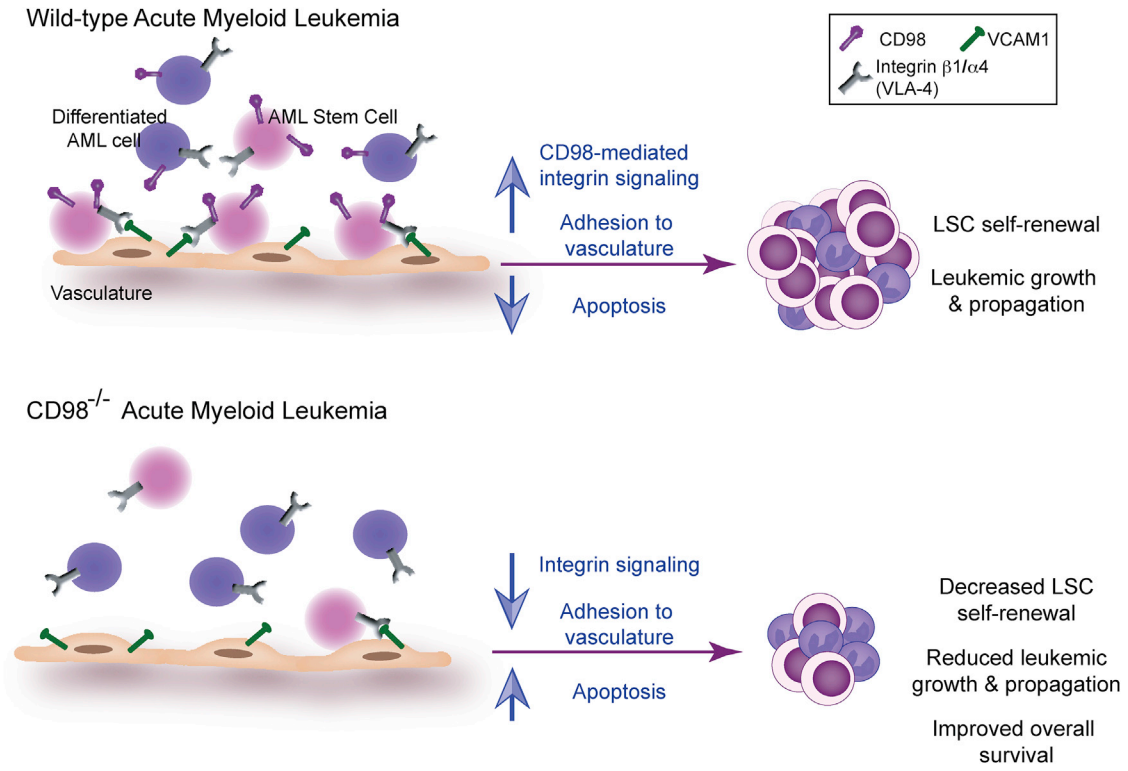

Figure 8. Functional Role of CD98 in AML

Model depicting the role of CD98 in wild-type AML and the effect of its deletion on disease progression.

Analysis of CD98$^{-/-}$ mice provided definitive genetic insight into the role of CD98 in normal hematopoiesis. While CD98 deficiency had only a minor impact on the normal adult hematopoietic system in vivo, the CD98$^{-/-}$ HSCs exhibited a markedly reduced ability to form colonies and reconstitute hematopoiesis in a transplant setting. This suggests that while CD98 is not critical for maintaining an established pool of hematopoietic cells, it is needed to drive extensive HSC expansion. These findings are consistent with previous data showing that while CD98 is not required for homeostatic proliferation and maintenance of B and T cells, it is required for their rapid antigen-driven clonal expansion (Cantor et al., 2009, 2011). Thus, the fact that inhibition of CD98 did not affect the established hematopoietic compartment suggests that the therapeutic use of CD98 antibody in context of leukemia should be further explored.

The inability of standard chemotherapies to trigger a durable remission in AML has led to concerted efforts to identify regulatory events that allow continued propagation of disease, and may serve as effective targets for therapy. These efforts have predominantly centered around intrinsic signals such as β-catenin (Wang et al., 2010), phosphatidylinositol 3-kinase (Doepfner et al., 2007; Xu et al., 2003), nuclear factor κB (Guzman et al., 2001), mitogen-activated protein kinase (Milella et al., 2001), and Msi2 (Kwon et al., 2015; Park et al., 2015). However, the specific molecules that integrate signal reception from the microenvironment remain relatively less explored. In this context, our data identify CD98 as a key molecular player in mediating the influence of the bone marrow niche on AML initiation and progression. The finding that CD98 works in part by enabling MLL-AF9 leukemia cells to establish long-term interactions with blood vessels in vivo reveals a remarkable parallel with the fact that endothelial cells have been implicated in the survival and proliferation of normal stem cells (Ding et al., 2012; Kunisaki et al., 2013). This also complements prior work on interactions of MLL-AF9 transduced progenitors with osteoblasts (Lane et al., 2011) and provides a distinct view of the role, and molecular basis, of the interactions of LSCs with endothelial cells. Consistent with adhesion being a key mechanism of action for sustaining leukemia cells, we find that active blockade of LSC adhesion to endothelial cells can deplete stem cells and thus impair cancer progression. As CD98 heavy chain can bind to the cytoplasmic tails of both integrin β1 and β3, its loss likely results in decreased VCAM-1/VLA-4 (integrin α4β1) signaling. The identification of CD98 as a key molecular driver of adhesive events important in leukemogenesis contributes to an emerging view that adhesive signals, such as those mediated by CXCR4 (Sison et al., 2013; Tavor et al., 2004), CD44 (Jin et al., 2006), Tspan3 (Kwon et al., 2015), and FAKs (Despeaux et al., 2012; Tyner et al., 2008), play a crucial role in controlling distinct aspects of cancer (Desgrosellier and Cheresh, 2010; Konopleva and Jordan, 2011; Seguin et al., 2015). This work also indicates that other proteins that bind and amplify integrin signals, such as Talin and Kindlin (Calderwood et al., 2013), should be studied as potential regulators of myeloid leukemia initiation and maintenance.

Our study with CD98, which binds to integrin β1, provides an important genetic complement to prior work showing that

inhibition of VLA-4-mediated adhesive interactions with fibronectin can prevent chemoresistance in AML (Jacamo et al., 2014; Matsunaga et al., 2003). The fact that integrin β3, which is also upstream of CD98, can reduce *MLL*-driven leukemia (Miller et al., 2013), suggests that activity of several adhesive molecules is integrated through CD98 to collectively mediate and control initiation and progression of AML in vivo. Although the integrin binding function is critical for the observed effect of CD98 on AML growth, we should note that more complete rescue of $CD98^{-/-}$ defects is only observed following restoration of the amino acid transport as well as integrin signaling functions. The possibility that the combined inhibition of integrin binding and amino acid transport activity of CD98 are necessary to fully resolve human disease progression in vivo, suggests that targeting CD98 may be more powerful than solely targeting upstream adhesive signals.

Because CD98 is particularly well suited for antibody-mediated therapy, an anti-CD98 antibody (IGN523) has recently been developed (Hayes et al., 2014). Both genetic deletion and mAb-mediated blockade of CD98 suggest that CD98 inhibition impairs adhesion and survival of leukemia cells. We should note that previous work (Hayes et al., 2014) indicates that IGN523, which reduces CD98 expression, can also increase lysosomal membrane permeability, reduce amino acid transport, and trigger antibody-dependent cellular cytotoxicity (ADCC). Thus, the anti-CD98 mAb could act through multiple mechanisms that include defects in cell adhesion to the microenvironment, increased apoptosis, and ADCC. While the CD98 mAb has been shown to delay growth of AML cell lines and patient-derived lung cancer in xenografts, whether CD98 is needed for growth of primary patient samples at physiological sites such as the bone marrow and peripheral blood has remained unknown. The CD98 conditional knockout mouse models reported here provide important genetic evidence for a role of CD98 in AML; further, the finding that blocking CD98 with either shRNAs or a humanized antibody strongly affects the growth of human leukemia both in vitro and at physiologic sites in patient-derived xenografts strongly supports the use of CD98-directed therapy in myeloid leukemia. Because the models used here were predominantly driven by MLL rearrangements—translocations found in more than two-thirds of all infant leukemias, and associated with a particularly poor prognosis (Krivtsov and Armstrong, 2007)—our data also suggest that CD98 inhibition should be considered for targeting both adult and pediatric leukemia.

EXPERIMENTAL PROCEDURES

See Supplemental Experimental Procedures for more extensive methods.

Generation of Experimental Mice

The conditional knockout mice (*Cd98hc*$^{fl/fl}$, also *Slc3a2*-loxP) (Feral et al., 2007) were mated with *Rosa26-CreER*^{T2} mice (Strain: B6;*129-Gt(ROSA)26Sor*$^{tm1(cre/Esr1)Tyj}$) mice to generate the *Cd98hc*$^{fl/fl}$;*Rosa26-CreER* mice. B6-CD45.1 (Strain: B6.SJL-*Ptprc*^{a}*Pepc*^{b}/BoyJ) mice were used as transplant recipients. All mice were 6–16 weeks of age. Mice were bred and maintained in the animal care facilities at the University of California San Diego. All animal experiments were performed according to protocols approved by the UC San Diego Institutional Animal Care and Use Committee.

In Vivo Transplantation Assays

For bone marrow transplants, 500 LT-HSCs (KLSCD150^{+}CD48^{-} or KLSFlk2^{-}CD34^{-}) isolated from bone marrow of mice expressing CD45.2 were transplanted into lethally irradiated (9.5 Gy) congenic recipient mice (expressing CD45.1) along with 2 × 10^5 Sca1-depleted bone marrow rescue cells. Peripheral blood of recipient mice was collected every 4 weeks for 4–6 months after transplant. Where indicated, adult mice were administered tamoxifen (Sigma) in corn oil (20 mg/mL) daily by intraperitoneal injection (150 μg per gram of body weight) for 5 consecutive days.

Generation and Analysis of Leukemia Models

Bone marrow KLS cells were sorted from *Cd98hc*$^{fl/fl}$;*Rosa26-CreER*$^{+/+}$ mice and cultured overnight in RPMI medium (Life Technologies) supplemented with 20% fetal bovine serum (FBS), 50 μM 2-mercaptoethanol, 100 ng/mL stem cell factor (SCF) (R&D Systems), and 10 ng/mL interleukin-3 (IL-3) and 10 ng/mL IL-6 (R&D Systems). Cells were retrovirally infected with MSCV-MLL-AF9-IRES-tNGFR and MSCV-NRAS^{G12V}-IRES-YFP. Cells were harvested 48 hr after infection and retro-orbitally transplanted into cohorts of sublethally irradiated (6 Gy) B6-CD45.1 mice. For secondary transplants, cells from primary transplanted mice were sorted for cKit^{+} MLL-AF9-IRES-tNGFR^{+}/MSCV-NRAS-IRES-YFP^{+} cells, and 2,000 to 4,000 cells were transplanted per mouse. Where indicated, adult mice were administered tamoxifen (Sigma) in corn oil (20 mg/mL) daily by intraperitoneal injection (150 μg per gram of body weight) for 5 consecutive days. Recipients were maintained on antibiotic water (sulfamethoxazole and trimethoprim) and evaluated daily for signs of morbidity, weight loss, failure to groom, and splenomegaly. Premorbid animals were euthanized and relevant tissues harvested and analyzed by flow cytometry. For homing assays, lethally irradiated recipients were transplanted with 10^6 CD98$^{+/+}$ or CD98$^{-/-}$ MLL-AF9-IRES-tNGFR^{+}/MSCV-NRAS-IRES-YFP^{+} cells, and bone marrow analyzed 16 hr after transplant. Apoptosis assays were performed by staining cells with annexin V and 7AAD (eBioscience). Analysis of in vivo bromodeoxyuridine (BrdU) incorporation was performed using the APC BrdU Flow Kit (BD Pharmingen) after a single intraperitoneal injection of BrdU (2 mg at 10 mg/mL).

Human Leukemia Samples and Cell Lines

Eighteen patient samples were obtained from Singapore General Hospital, the Duke Adult Bone Marrow Transplant Clinic, the Fred Hutchinson Cancer Research Center, and UC San Diego Moores Cancer Center from institutional review board-approved protocols with written informed consent in accordance with the Declaration of Helsinki. Leukemia cells were cultured in Iscove's modified Dulbecco's medium with 10% FBS, 100 IU/mL penicillin, 100 μg/mL streptomycin, and 55 μM 2-mercaptoethanol, and supplemented with 100 ng/mL SCF and TPO (R&D Systems). The human AML cell lines THP1 and M-V-411 (ATCC) were maintained in RPMI with 10% FBS, 100 IU/mL penicillin, and 100 μg/mL streptomycin. For colony-forming assays with shRNAs, primary leukemia cells were transduced with lentiviral shRNA and RFP^{+} cells were sorted 48 hr post-infection and plated in complete methylcellulose medium (H4434 StemCell Technologies). Colony numbers were counted 10–14 days after plating. All experiments were conducted with two independent hairpin shRNAs targeting SLC3A2 that more effectively knock down expression in AML cells. For in vivo experiments, NSG mice (Strain: NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Wjl}/SzJ) were sublethally irradiated (2.5 Gy) and retro-orbitally transplanted with primary human AML cells.

In Vitro Adhesion Assays

For in vitro adhesion assays, 5000 cKit^{+} AML cells from CD98$^{+/+}$ or CD98$^{-/-}$ leukemia were plated on confluent HUVEC cells (Life Technologies) activated with 10 ng/mL human tumor necrosis factor α (eBioscience) in RPMI with 20% FBS, 100 IU/mL penicillin, 100 μg/mL streptomycin, and 55 μM 2-mercaptoethanol in a 96-well plate, and incubated at 37°C for 2 hr. To determine the effect of loss of adhesion on cKit expression and apoptosis, we added 30 μg/mL anti-VCAM-1 blocking antibody (R&D Systems) or control IgG (Sigma) along with the leukemia cells where indicated. The wells were washed six or seven times to remove unattached cells and images were taken on an inverted microscope (Leica). Cells were trypsinized and analyzed on FACSAria III (Becton Dickinson) to obtain an absolute count of adherent leukemia cells per well.

In Vivo Imaging

Imaging was done as described previously (Fox et al., 2016; Koechlein et al., 2016; Kwon et al., 2015; Zimdahl et al., 2014). In brief, Actin-dsRed NOD SCID mice (Strain: NOD.Cg-*Prkdc*^{scid} Tg(CAG-DsRed*MST)1Nagy/KupwJ) were transplanted with CD98$^{+/+}$ and CD98$^{-/-}$ MLL-leukemia cells and imaged 7–10 days post transplant. VE-Cadherin conjugated to Alexa Fluor 647 (eBiosciences) was administered at a concentration of 10 μg diluted in 100 μL, 15 min prior to imaging. Images were acquired by the Leica LAS AF 2.7.3 software with a TCS SP5 upright DM600 CFS Leica confocal system using an HCX APO L 20×/1.00 W Leica Plan Apochromat objective. Images were continuously captured in 1,024 × 1,024 format (approximately 7 s per scan) for up to 1 hr. Images were analyzed using the Leica AF 2.7.3 software.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, six figures, two tables, and two movies and can be found with this article online at http://dx.doi.org/10.1016/j.ccell.2016.10.003.

AUTHOR CONTRIBUTIONS

J.B. designed and performed experiments on human and murine leukemia progression, provided analysis of mAb impact, imaging, and mechanistic studies, and helped write the paper. T.K. designed and performed experiments on normal hematopoiesis and leukemia progression in mouse models. N.K.L., H.Y.K., J.N.A., and J.M.C. provided experimental data and help. D.R., C.C., V.G.O., E.H.B., and E.D.B. provided primary leukemia patient samples and experimental advice. E.H.v.d.H. provided the CD98 antibody and experimental advice. M.H.G. and T.R. conceived the project, planned and guided the research, and wrote the paper.

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