

Cholinergic signals regulate acute myeloid leukemia and chemotherapy response

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Abstract

The bone marrow niche, where MSCs and the autonomic nervous system play regulatory roles, supports AML self-renewal, survival and chemoresistance. The autonomic nervous system utilizes noradrenaline and acetylcholine as postganglionic neurotransmitters for adrenergic and cholinergic regulation, respectively. While adrenergic regulation of hematopoiesis has been characterized, cholinergic AML regulation has remained elusive. Here, we identify autonomic cholinergic signaling as a regulator of AML development, maintenance, and chemotherapy response. Leukemia cells increase neural plasticity and cholinergic activity, promoting leukemogenesis via activation of nicotinic acetylcholine receptors and PI3K signaling in AML. In the niche, cholinergic signals increase CXCL12 via $\alpha 7$ nicotinic acetylcholine receptor activation in MSCs. This divergent, niche-dependent cholinergic regulation promotes leukemia stem cell quiescence and chemoresistance. These findings suggest that leukemia exploits cholinergic regulation, which exerts a dual, context-dependent role in leukemic cells and their niche. Targeting cholinergic niche signalling may therefore guide future strategies to improve treatment outcomes in AML.

Main Text

In acute myeloid leukemia (AML), leukemia stem cells (LSCs) resemble hematopoietic stem cells (HSCs) in their quiescence and can escape anti-proliferative chemotherapy, giving rise to relapse^{1–6}. LSCs can evade chemotherapy by hijacking energy sources and antioxidant protection mechanisms from HSC-niche forming mesenchymal stromal cells (MSCs)^{7,8}, which additionally support AML translational demands at relapse⁹. Therefore, understanding the regulatory mechanisms of the bone marrow (BM) niche are key to facilitate LSC eradication¹⁰. The BM is densely innervated by sympathetic nerve fibers¹¹. The autonomic nervous system comprises two branches that frequently fulfil opposite homeostatic actions: the sympathetic (SNS) and the parasympathetic nervous systems (PNS). Noradrenaline is the most common postganglionic neurotransmitter of the SNS and regulates hematopoietic stem and progenitor cell (HSPC) proliferation, differentiation and migration directly^{12,13} or indirectly, through the modulation of endothelial cells and sub-endothelial MSCs, which play a key role in the HSC niche^{8,14}. Acetylcholine (ACh) is the main postganglionic neurotransmitter of the PNS and a subset of sympathetic fibers innervating the skeleton. However, cholinergic regulation of normal and malignant hematopoiesis is under-investigated. Cholinergic signals cooperate with noradrenergic signals to regulate daily migration of HSCs and leukocytes^{14,15}. ACh produced by different cells, including neurons, osteolineage cells and recirculating B cells, restrains HSC proliferation^{16–18}. Myeloablative treatments used for cancer treatment and as conditioning regimens for HSC transplantation induce ACh secretion, which plays a key role preserving the quiescence of HSCs near the bone surface (endosteal BM) during hematopoietic regeneration^{16,18}. ACh preferentially regulates HSCs via MSCs, where $\alpha 7$ nicotinic ACh receptor ($\alpha 7$ nAChR) activation leads to increased CXCL12 expression¹⁶. The end result is HSC¹⁹ and LSC²⁰ lodgement and quiescence in a protective microenvironment²¹. While a previous study reported reduced

sympathetic noradrenergic nerve fibers in the BM of AML mice²², the role of the cholinergic signaling in AML development, maintenance and response to chemotherapy has not been characterized, prompting the current study.

AML promotes cholinergic signaling

To gain insight into the possible role of ACh in AML, we analyzed serum samples and found doubled ACh concentration in newly diagnosed AML patients, compared with healthy donors (Fig. 1a, Table S1). Importantly, high ACh concentration was associated with poor overall survival in AML (Fig. 1b), regardless of the AML stratification risk (Extended Data Fig. 1a). A mild correlation was detected between age and serum ACh concentration (Extended Data Fig. 1b), which was found to be a strong predictor of AML risk in multivariate analysis (Table S2). These results suggest that high ACh might be a risk factor in human AML.

Serum ACh concentration was similarly increased in AML mice carrying the MLL-AF9 oncogene fusion protein (Fig. 1c), which were used as a research model²³. AML frequently develops in the BM. To investigate the role of ACh secreted by BM nerve terminals¹⁶ we compared the density of cholinergic and noradrenergic nerve fibers in the BM of inducible (i) MLL-AF9 mice²⁴ upon AML development. In line with a previous study²², noradrenergic nerve fiber density was markedly reduced (Fig. 1d-g); in contrast, we found preservation of cholinergic nerve fibers (Fig. 1h-k). Consistently, the mRNA expression of the cholinergic markers glial-derived-neurotrophic-factor (GDNF)-family receptor 2 (*GFRA2*) and its ligand *neurturin* was unchanged in the BM of AML mice (Extended Data Fig. 1c-d). Therefore, contrasting the reduction of noradrenergic nerve fibers, cholinergic nerve fibers are maintained in AML BM.

Increased human serum ACh and selective preservation of mouse cholinergic BM fibers in AML raised the question whether AML instructs cholinergic phenotypes. We previously showed that interleukin (IL)-6 induces a cholinergic “switch” of skeletal sympathetic adrenergic neurons during postnatal development, and in response to physical activity in adults¹⁷. As AML cells overproduce IL-6^{10,25–27}, we co-cultured them with sympathetic neurons isolated from the superior cervical ganglion, which contains neurons with combined noradrenergic and cholinergic properties^{28,29} (Extended Data Fig. 1e). Remarkably, conditioned medium collected from AML blasts induced in neurons the mRNA expression of the cholinergic marker *GFRA2* (compared with the adrenergic marker *TH*); this effect was reproduced by co-cultures of SCG neurons with AML blasts and inhibited by IL6-blocking antibody (Fig. 1l-m and Extended Data Fig. 1f-g). The results were confirmed by immunofluorescence of GFR α 2 and TH proteins (Fig. 1n-r), suggesting that AML promotes cholinergic neural phenotypes.

The vagus nerve promotes LSC maintenance and self-renewal

Whilst the SNS innervates the BM, ACh is the main postganglionic neurotransmitter of the PNS, which regulates the activity of peripheral organs (including secondary lymphoid organs like the spleen, where AML cells bidirectionally traffic to/from BM) mainly through the vagus nerve. We measured c-Fos expression, which is a well-established marker of neuronal activation, in the dorsal motor nucleus of the vagus and found it increased in AML mice (Fig. 2a–c), suggesting enhanced PNS activity.

To determine the functional contribution of vagal signaling, we performed bilateral subdiaphragmatic vagotomy with pyloroplasty in naïve C57BL/6 mice (Extended Data Fig. 2a). Successful denervation was confirmed by retrograde tracing with Fluoro-Gold, which showed in vagotomized animals a virtual loss of retrograde labelling of the dorsal motor nucleus of the vagus (Extended Data Fig. 2b–c). Three weeks after surgery, mice were transplanted with MLL–AF9–GFP-transduced HSPCs (see Extended Data Fig. 2a). Five weeks later, vagotomized animals showed reduced serum ACh concentration (Fig. 2d), concomitantly with reduced BM AML cells (Fig. 2e), while BM cell viability was unaffected (Extended Data Fig. 2d). A similar reduction of primitive (GFP⁺Lin[−]) BM AML cells and a further reduction of leukemic granulocyte–macrophage progenitors (L-GMPs, which represent LSCs in this AML model²³) suggested that vagal AML regulation starts at the top of the hierarchy (Fig. 2f–g). Consistently, vagotomy decreased AML colony-forming capacity *in vitro* and reduced leukemia-initiating cell frequency seven-fold, whilst their normal BM counterparts in vagotomized naïve mice were unaffected (Fig. 2h–j and Extended Data Fig. 2e–h). Similarly, leukemia burden and L-GMP numbers were reduced in the spleen of vagotomized AML mice (Extended Data Fig. 2i–l). These results suggest a selective vagal regulation of LSCs. Indeed, L-GMPs from vagotomized animals exhibited elevated expression of the differentiation marker GR-1 and increased BrdU retention (Fig. 2k–l), suggesting that vagal activity promotes a stem-like phenotype in LSCs. This was consistent with reduced frequency of c-kit⁺ AML cells in vagotomized mice (Fig. 2m).

To interrogate the underlying mechanism, we performed bulk RNA sequencing on L-GMPs from vagotomized and sham-operated AML mice (Table S3). Gene set enrichment analysis (GSEA) indicated that vagal signaling activates stemness-associated transcriptional programs, including phosphoinositide 3-kinase (PI3K) signaling and epigenetic regulatory pathways (Fig. 2n). Consistent with a vagal epigenetic LSC regulation, ATAC–seq profiling showed that genes with increased chromatin accessibility at transcription start sites in sham-operated L-GMPs were enriched for pathways related to synaptic signaling, ion channel and neurotransmitter activity (Fig. 2o). These results suggest that AML-induced parasympathetic activity promotes LSC maintenance via the vagus nerve.

Cholinergic signals directly promote L-GMP stemness and proliferation via PI3K

To determine whether ACh directly regulates L-GMPs, we cultured these cells in presence/absence of ACh and assessed colony formation in methylcellulose. ACh significantly increased colony formation, an effect that persisted upon replating them without ACh addition (Fig. 3a). Since the vagus nerve primarily

regulates myeloid cells in the spleen via $\alpha 7$ nAChR³⁰, we tested the nAChR inhibitor hexamethonium. The ACh-induced clonogenic activity in L-GMPs was abolished by hexamethonium (Fig. 3b), suggesting that it is transduced by nicotinic cholinergic receptors. In contrast, the clonogenic capacity of normal HSPCs was unaffected (Fig. 3c), indicating that ACh selectively enhances LSC self-renewal. A similar increase in colony formation was observed in primary human LSCs following ACh treatment, and this effect similarly persisted upon replating (Fig. 3d). Consistently, ACh treatment induced L-GMP proliferation (Fig. 3e-g). ACh-treated L-GMPs also retained a more primitive immunophenotype, with lower GR-1 and higher c-Kit expression (Fig. 3h-i).

To investigate the underlying mechanism, we performed RNA sequencing of L-GMPs upon ACh treatment (Table S3). GSEA revealed ACh-induced stemness-associated and PI3K signaling programs in L-GMPs (Fig. 3j). Notably, downregulated genes in vagotomized mice ("vagotomy-downregulated") were significantly upregulated in ACh-treated L-GMPs (Fig. 3k). To further investigate PI3K signaling, we assessed p-AKT levels by Western Blot. ACh increased p-AKT expression (Fig. 3l), and this effect was dependent on nAChR signaling (Fig. 3m), consistent with the transcriptional changes observed following vagotomy or pharmacological nAChR blockade (see Fig. 2n-o). ACh-induced PI3K activation was confirmed in MV4-11 AML cells, which exhibited increased growth upon ACh treatment (Extended Data Fig. 3a). Therefore, cells were pretreated with Bimiralisib (dual PI3K/mTOR inhibitor), Idelalisib (PI3K δ inhibitor) or AZD8835 (PI3K α /PI3K δ inhibitor) before ACh exposure. ACh-induced AKT phosphorylation was reduced by AZD883, restoring signaling to baseline levels (Extended Data Fig. 3b). Consistently, pharmacological inhibition of PI3K with AZD8835 abrogated the ACh-induced MV4-11 cell proliferation (Extended Data Fig. 3c). Together, these data identify ACh as a direct regulator of L-GMP growth and self-renewal through PI3K.

Nicotinic cholinergic signals promote AML leukemogenesis via nicotinic receptors

The nAChR agonist nicotine is a major bioactive compound of tobacco, and smoking history has been associated with delayed hematopoietic recovery in leukemia patients receiving allogeneic HSC transplantation¹⁶. In experimental models, nicotine induces quiescence of normal HSCs¹⁶. Therefore, nicotine was administered to AML mice as a gain-of-function (GOF) model (Extended Data Fig. 4a). Nicotine accelerated leukemogenesis and increased leukemic burden in peripheral blood and BM (Fig. 4a-c). During incipient leukemogenesis in chimeric mice carrying AML cells and WT hematopoietic cells (see Extended Data Fig. 4a), nicotine treatment inhibited cell cycle progression of WT HSPCs, appearing to reduce HSPCs in the endosteal BM, and likely conferring a competitive advantage to AML cells (Fig. 4d-k).

Therefore, AML mice were treated with the peripheral nAChR inhibitor hexamethonium, as a complementary loss-of-function (LOF) model. Hexamethonium reduced BM cellularity (Fig. 4l). FACS analysis, colony formation assays, and ELDA analysis confirmed a significant reduction in phenotypic

and functional L-GMPs in hexamethonium-treated mice (Fig. 4m-p). Consequently, hexamethonium delayed leukemogenesis (Fig. 4q). In contrast, *in vivo* blockade of muscarinic ACh receptors (for comparison) did not affect L-GMPs (Extended Data Fig. 4b-c). Bulk RNA sequencing of purified L-GMPs further supported the enrichment of stemness and PI3K-associated transcriptional programs conferred by nAChR activation (Fig. 4r and Table S3). Strikingly, genes differentially expressed in vagotomized mice (“vagotomy-downregulated”) also showed altered expression in L-GMPs from hexamethonium-treated AML mice (Fig. 4s). Together, these results suggest that nicotinic cholinergic signals maintain LSCs and promote leukemogenesis in AML via nAChR.

Divergent AML cell-autonomous and niche-mediated cholinergic regulation

Since LSCs are supported by MSCs in the BM niche^{7,9}, we next compared the effect of nAChR modulators on L-GMPs cultured alone, or in co-culture with self-renewing mesenchymal spheres (mesenspheres)⁸, as a source of MSCs (Extended Data Fig. 4d). In contrast to ACh-enhanced survival and proliferation of L-GMPs in monoculture (see Fig. 3a-i), in co-culture with MSCs ACh increased L-GMP quiescence, which was prevented by α -bungarotoxin (Fig. 5a-b), which preferentially inhibits $\alpha 7$ nAChR. Additionally, α -bungarotoxin increased apoptosis of iMLL-AF9 LSC-derived AML blasts in co-culture with BMSCs, but not in monocultured blasts (Extended Data Fig. 4e-k). These observations suggest divergent effects of cholinergic regulation on AML LSCs and their niches.

$\alpha 7$ nAChR in BMSCs promotes LSC quiescence via CXCL12

To confirm these findings *in vivo* using cKO mice, we examined AML development in mice lacking $\alpha 7$ nAChR in BMSCs targeted by Leptin receptor³¹ (*LepR*; *LepR-Cre*; *ChRNA7^{f/f}* mice, Extended Data Fig. 5a). Targeted loss of $\alpha 7$ nAChR by *LepR-Cre* reduced BM CXCL12 concentration (Fig. 5c) and rendered endosteal BM LSCs more proliferative (Fig. 5d). Consequently, *LepR-Cre*; *ChRNA7^{f/f}* mice developed AML faster than their control littermates (Fig. 5e), leading to increased leukemia burden in the spleen (Fig. 5f). LSC quiescence exit in *LepR-Cre*; *ChRNA7^{f/f}* mice rendered LSCs more sensitive to anti-proliferative chemotherapy, manifested as decreased spleen weight in cKO mice treated with Ara-C (Fig. 5g and Extended Data Fig. 5b). However, LSCs escaping chemotherapy remained more proliferative in *LepR-Cre*; *ChRNA7^{f/f}* (Fig. 5h-i), cancelling out differences in relapse-free survival and leukemic burden at relapse (Extended Data Fig. 5c-d). For confirmation in an independent model, CXCL12 was deleted in BMSCs targeted by the regulatory elements of *Nestin* promoter (*Nes-Cre^{ERT2}* mice), which largely overlap with BMSCs targeted by *LepR-Cre*^{32,33} (Extended Data Fig. 5e). CXCL12 depletion in nestin⁺ cells similarly induced endosteal BM LSC quiescence exit, but LSCs escaping chemotherapy continued to proliferate more (Fig. 5j) and post-Ara-C AML kinetics was unchanged (Extended Data Fig. 5f), phenocopying the results with *LepR-Cre*. Therefore, these results suggest that LSCs hijack niche

pathways that regulate HSC quiescence¹⁶, and that $\alpha 7$ nAChR in BM MSCs promotes LSC quiescence via CXCL12.

Sympathetic cholinergic fibers promote LSC quiescence

Sympathetic cholinergic fibers promote normal HSC quiescence in endosteal BM niches and are deficient in mice deficient in the neurotrophic factor receptor $GFR\alpha 2^{16,34}$. Therefore, we used $GFR\alpha 2^{-/-}$ mice to investigate the sympathetic cholinergic neural regulation of AML (Extended Data Fig. 5g). Lack of cholinergic nerve fibers in $GFR\alpha 2^{-/-}$ mice transplanted with AML cells accelerated AML development (Fig. 5k). After AraC treatment, increased LSC proliferation in cholinergic-neural-deficient mice led to augmented leukemia burden (Fig. 5l-m), phenocopying $\alpha 7$ nAChR or CXCL12 deletion in MSCs (see Fig. 5f-j). Resembling the latter, cholinergic neural deficiency triggered LSC quiescence exit, which sensitized LSCs to chemotherapy, but also accelerated the expansion of LSCs that escaped chemotherapy, leading to similar AML regrowth (Fig. 5n). Together, these results suggest that sympathetic cholinergic fibers promote LSC quiescence in the endosteal BM niche via $\alpha 7$ nAChR in BM MSCs.

BM niche sympathetic cholinergic signaling blockade sensitizes AML to induction chemotherapy

Since cholinergic signals mainly regulate in LSCs the G0-G1 transition, where AML cells are not sensitive to Ara-C, we tested a more intense chemotherapy targeting cells throughout their cell cycle. Induction chemotherapy combining Ara-C with anthracycline, which is commonly used as intensive clinical first-line AML treatment, was mimicked in mice (Extended Data Fig. 6a). Lack of cholinergic neurons triggered endosteal LSC quiescence exit (Fig. 5o-p). Induction therapy was much more efficient in $GFR\alpha 2^{-/-}$ mice, since AML relapse was delayed 9-fold in cholinergic-neural-deficient mice (Fig. 5q). To complement the $GFR\alpha 2^{-/-}$ LOF model with a GOF model, AML mice were treated with a cholinesterase inhibitor (neostigmine bromide, which prolongs the effect of endogenous ACh) in combination with induction therapy (Extended Data Fig. 6b). Contrasting cell cycle progression in cholinergic-neural-deficient mice, enhanced cholinergic signaling rendered endosteal LSCs more quiescent (Fig. 5r-s). These results suggest that toning down cholinergic neural activity might sensitize LSCs to induction therapy in AML.

nAChR blockade sensitizes AML to induction chemotherapy

We investigated whether pharmacological inhibition of nAChRs affects the sensitivity of AML to induction chemotherapy. AML mice were treated with induction therapy alone or combined with the peripheral nAChR inhibitor hexamethonium bromide (Extended Data Fig. 6c). nAChR inhibition decreased the quiescence of endosteal BM LSCs (Fig. 6a). Consequently, combination therapy was synergistic,

eliminating twice as many L-GMPs as induction therapy alone (Fig. 6b). As a result, disease-free survival time was doubled (Fig. 6c). At relapse, combination therapy reduced leukemic burden in the endosteal BM and peripheral blood (Fig. 6d-e).

To evaluate the possible impact of nAChR blockade on LSCs evading chemotherapy, colony-forming-units in culture (CFU-Cs) were assayed using BM cells from AML mice treated with induction therapy alone or combined with hexamethonium bromide (see Extended Data Fig. 6c). The frequency of CFU-Cs was 2.5-fold reduced in replating assays (Fig. 6f), confirming a reduction of leukemia-initiating cells after combination therapy. However, the average colony size was significantly increased (Fig. 6g-i), consistent with the proliferative effect of nAChR blockade. Consequently, secondary recipient mice transplanted with residual BM cells after combination therapy showed accelerated AML development (Fig. 6j). These results highlight a double-edged sword effect of cholinergic blockade in AML: while it induces quiescence exit of LSCs and renders them sensitive to induction chemotherapy, those LSCs escaping combination therapy are more proliferative and may accelerate relapse in these highly chemo-resistant AML models. Therefore, carefully finetuning the dose and time of treatment might be key to maximize LSC elimination and avoid counteracting expansion.

Discussion

The autonomic nervous system is a master regulator evolutionarily selected to maintain organismal homeostasis and to efficiently respond to stress. Cumulative evidence suggests that cancer cells must subvert autonomic neural regulation to drive oncogenesis³⁵. The autonomic nervous system comprises two branches, sympathetic and parasympathetic, which preferentially utilize the postganglionic neurotransmitters noradrenaline and ACh, respectively. While a previous study reported reduced sympathetic noradrenergic nerve fibers in AML BM²², the role of cholinergic signals in AML development, maintenance and response to chemotherapy has remained elusive.

This study demonstrates that AML actively induces cholinergic signals, which promote LSC maintenance and chemotherapy resistance via nAChRs. Inhibition of cholinergic signaling disrupts LSC quiescence, driving dormant LSCs into cycle and sensitizing them to induction chemotherapy, thereby revealing a therapeutic vulnerability.

The SNS and PNS frequently utilize their postganglionic neurotransmitters noradrenaline and ACh to exert distinct/opposite effects in innervated organs, regulate inflammation and maintain homeostasis³⁶. Among ACh receptors, $\alpha 7$ nAChR stands out as a key regulator of immune cell activation^{30,37}. In the hematopoietic system, circadian oscillations of cholinergic and noradrenergic signals orchestrate the daily migration of HSCs and leucocytes^{14,15}. However, the autonomic neural regulation of AML had not been characterized in detail so far. Our results confirm the reduced noradrenergic activity previously reported in AML BM²², and suggest that this is not the mere consequence of broad neuropathic damage, but rather represents an active induction of cholinergic signaling. This interpretation is supported by 1) increased cholinergic marker expression in sympathetic neurons exposed to AML cells (at least partly

dependent on IL-6); 2) increased neuronal activity marker expression in the dorsal motor nucleus of the vagus, which is the main nerve delivering parasympathetic signals to peripheral organs including the spleen, where AML cells migrate to/from BM; 3) increased ACh concentration in the serum of AML patients and mice; and 4) preserved cholinergic fibers in AML BM, contrasting the marked reduction of noradrenergic fibers. These results suggest that AML cells exploit autonomic neural adrenergic/cholinergic plasticity³⁸ to instruct cholinergic phenotypes. This effect resembles the developmental “cholinergic switch” of noradrenergic neurons³⁹, which is IL-6-dependent and has bone-anabolic effects both in postnatal development and in response to physical activity in adults¹⁷. This cholinergic induction was similarly observed after pro-inflammatory myeloablation used for cancer treatment and as conditioning regimens for HSC transplantation¹⁶.

The reason for cholinergic induction by AML appears to be a pro-leukemogenic action of ACh. AML is sustained by LSCs, which are resistant to conventional therapy and drive relapse^{4–6}. Targeting LSCs remains a major clinical challenge, as their self-renewal programs—conserved mechanisms that maintain stem cell pools—are reinforced by oncogenic networks and microenvironmental signals. High LSC frequency and stemness-associated gene signatures predict resistance and poor outcomes in AML^{40–43}, and disrupting LSC self-renewal shows therapeutic potential in preclinical studies^{3,23,44–46}. Here, we demonstrate that parasympathetic signaling directly promotes LSC self-renewal, most likely through epigenetic regulation. The parasympathetic nervous system regulates the activity of peripheral organs mainly through the vagus nerve. Vagotomy or pharmacological nAChR blockade suppresses LSC frequency and leukemogenicity, while sparing normal hematopoiesis. Therefore, these results reveal selective pro-leukemogenic effects of autonomic cholinergic signals.

Pro-oncogenic effects of ACh in solid tumors have been previously attributed primarily to nAChR–dependent activation of PI3K/AKT and MAPK signaling cascades⁴⁷, which drive cellular proliferation, angiogenesis, and therapy resistance. Extending these findings to AML, we show that ACh promotes PI3K/mTOR-dependent transcriptional reprogramming, proliferation, and stemness in murine and human LSCs. Bulk RNA sequencing reveals that ACh-induced transcriptional programs overlap with those enriched in AML LSCs *in vivo*, suggesting that cholinergic signaling reinforces canonical LSC maintenance pathways.

The PI3K/AKT/mTOR axis—a central regulator of growth, metabolism, and stem cell fate—is constitutively activated in 50–80% of AML cases⁴⁸. PI3K signaling sustains LSC survival and metabolic reprogramming while preventing differentiation⁴⁹, and its hyperactivation correlates with therapy resistance and poor prognosis. Inhibition of PI3K or its downstream effectors AKT and mTOR reduces LSC frequency and sensitizes AML blasts to standard therapies in preclinical models⁴⁸. Class I PI3K isoforms have been implicated across leukemia^{49,50}, establishing PI3K as a key driver of leukemogenesis and a targetable vulnerability in AML. Therapeutically, these findings suggest that targeting cholinergic signaling may represent a novel strategy to target LSCs, since nAChR antagonists

such as hexamethonium effectively reduced LSC function and PI3K activity *in vivo*, mirroring the effects of vagotomy.

These leukemogenic effects of ACh align well with our finding that serum ACh levels are elevated in AML patients and may serve as an independent adverse prognostic marker for overall survival. This paves the way for future studies that evaluate serum ACh concentration as a candidate biomarker of LSC activity, complementing genomic risk stratification and potentially guiding patient-tailored therapy, and/or as a surrogate of stemness-associated transcriptional states that confer treatment resistance.

LSCs resemble HSCs in their quiescence, which allows them to escape anti-proliferative chemotherapy¹⁻⁶. Furthermore, LSCs can evade chemotherapy by hijacking energy sources and antioxidant protection mechanisms from HSC-niche forming BMSCs^{7,8}, and rely on them to translationally build blocks at relapse⁹. Moreover, ACh-induced $\alpha 7$ nAChR activation increases CXCL12 expression in BMSCs, preserving HSC quiescence and maintenance during proliferative hematopoietic regeneration^{16,18}. Therefore, we asked whether LSCs might exploit cholinergic-niche-dependent quiescence for chemotherapy protection and dissected LSC-autonomous and niche-dependent effects of ACh on LSC quiescence and chemosensitivity. Intriguingly, contrasting ACh-enhanced survival and proliferation of LSCs in monoculture, in co-culture with MSCs ACh rendered LSCs more quiescent. These results suggested divergent effects of cholinergic regulation on AML LSCs and their niches, which were confirmed *in vivo*, allowing us to conclude that $\alpha 7$ nAChR in BMSCs promotes LSC quiescence via CXCL12. This conclusion is supported by the following evidence: 1) $\alpha 7$ nAChR deletion in MSCs (*LepR-Cre*) reduced BM CXCL12 concentration and rendered LSCs more proliferative; 2) this result was confirmed and phenocopied by targeted CXCL12 deletion in MSCs (*Nes-Cre^{ERT2}* mice, which largely overlap with BMSCs targeted by *LepR-Cre*^{32,33}); 3) niche-dependent cholinergic regulation of LSC quiescence was similarly phenocopied in *GFR $\alpha 2$ ^{-/-}* mice lacking the sympathetic cholinergic fibers that promote HSC quiescence in endosteal BM niches^{16,34}.

Cholinergic-niche-induced LSC quiescence prompted us to test whether disruption of this regulation enhances chemotherapy sensitivity. Supporting this possibility, $\alpha 7$ nAChR deletion in MSCs (*LepR-Cre*) rendered LSCs more sensitive to anti-proliferative chemotherapy, and AML relapse after induction therapy was markedly delayed in cholinergic-neural-deficient mice. Therefore, we tested the peripheral nAChR inhibitor hexamethonium bromide, which does not cross the blood-brain barrier (to spare the central nervous system), in combination with induction chemotherapy. While combination therapy synergized in eliminating more LSCs, surviving LSCs were more proliferative and accelerated relapse in this notoriously highly chemo-resistant AML mouse model. Together, these results highlight key impacts of cholinergic signals in AML cells and their niche. They also suggest that carefully finetuning the timed administration of cholinergic modulators might impair the expansion and maintenance of LSCs while sensitizing them to chemotherapy.

Declarations

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Competing interests

The authors declare no competing interests.

Data and materials availability: The data that support the findings of this study are included in the paper or available from the corresponding author upon reasonable request.

Supplementary Materials

Materials and Methods

Extended Data Figs. 1 to 6

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Figures

Figure 1

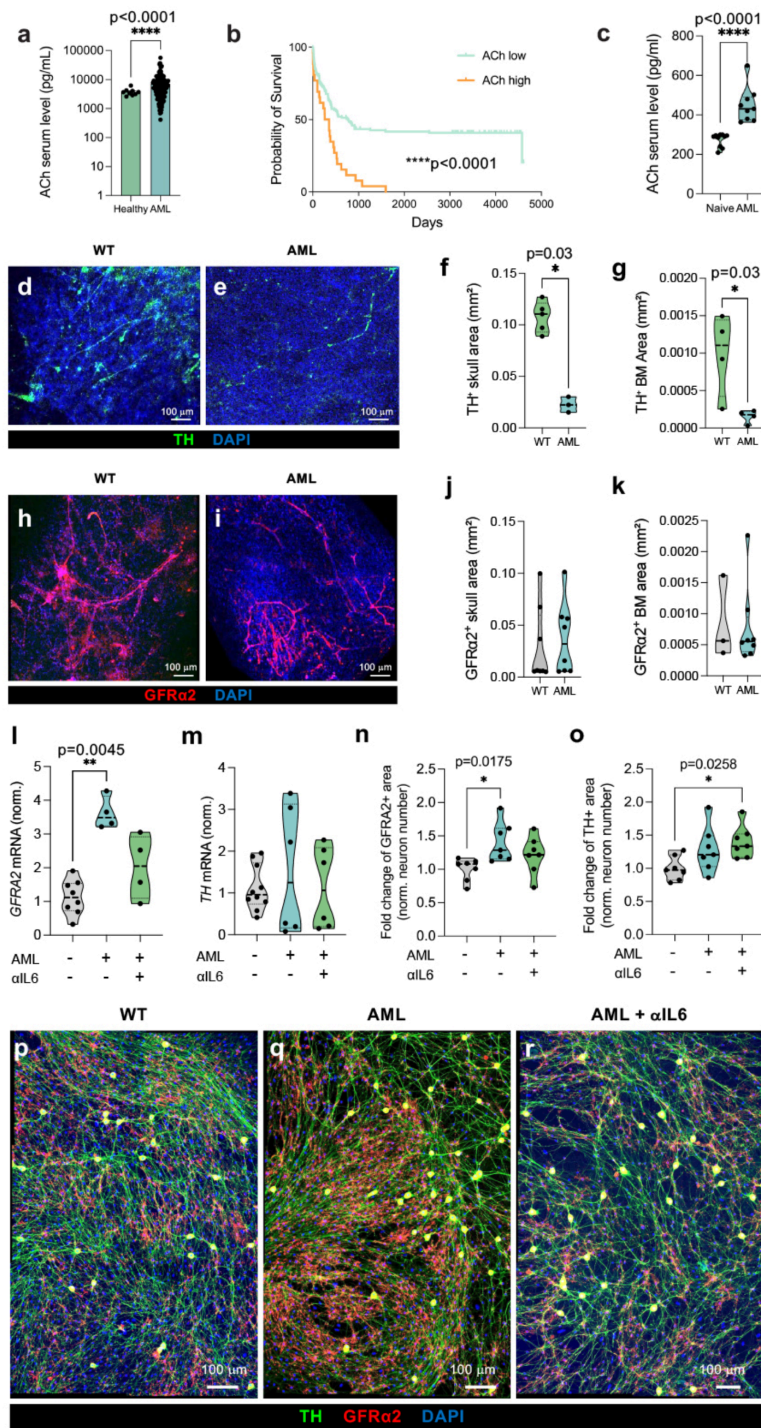


Figure 1

AML promotes cholinergic signalling.

a, Increased ACh serum concentration in newly diagnosed AML patient ($n = 177$) and age-matched healthy donors ($n = 9$).

b, Lower overall survival in ACh-high vs. ACh-low AML patients (cut-off = 10,255 pg/ml).

c, Increased ACh serum concentration in AML mice compared with naïve (WT) mice ($n=9-10$).

d-g, Representative immunofluorescence (d-e) of skull TH⁺ noradrenergic nerve fibers in (d) WT and (E) AML mice and (f-g) their reduction, quantified in (f) skull and (g) femoral BM ($n = 3-5$).

h-k, Representative immunofluorescence (H-I) of skull GFR α 2⁺ (a previously validated marker)¹⁷ cholinergic nerve fibers in (H) WT and (I) AML mice and (j-k) their maintenance, quantified in (j) skull and (k) femoral BM ($n = 3-8$).

l, Increased GFR α 2 mRNA expression in sympathetic neurons treated with conditioned medium from AML-neuron co-cultures was reverted by IL-6 blockade ($n = 4-12$).

m, Unchanged TH mRNA expression in sympathetic neurons treated with conditioned medium from AML-neuron co-cultures with/without IL-6-blocking antibody ($n = 4-12$).

n-r, Quantification (n-o) and representative immunofluorescence (p-r) of (n) GFR α 2 and (o) TH proteins in sympathetic neurons co-cultured in transwell with AML blasts with/without anti-IL6 ($n = 7$).

a, c, f, g, j, k, l-o, Data are means $\pm$ SEM. Each dot represents a biological replicate. * $P < 0.05$; ** $P < 0.01$; **** $P < 0.0001$. a, Welch's t-test. b, log-rank test. c, Mann Whitney t-test.

f-g, j-k, Unpaired two-tailed t test. l-o, Kruskal-Wallis test and Dunn's multiple comparisons after outlier exclusion by ROUT.

a DMV Naive **b** DMV AML

c $p=0.006$
c-fos⁺ cells/ μm^2
AML - +

d $p=0.0077$
ACh (pg/mL)
Vagotomy - +

e $p=0.0439$
GFP+ (% of viable cells)
Vagotomy - +

f $p=0.0302$
GFP+Lin- (% of viable cells)
Vagotomy - +

g $p=0.0611$
L-GMP (% of viable cells)
Vagotomy - +

h $p=0.0246$
of GFP+ colonies (5000 WBM cells)
Vagotomy - +

i $p=0.0077$
log fraction nonresponding
dose (number of cells)
Sham
Vagotomy

j

	Injected Cells	Responders
Sham	50'000	5/5
	5'000	5/5
	500	1/5
Vagotomy	50'000	5/5
	5'000	2/5
	500	0/5

Chi-square test

k $p=0.0305$
Gr-1 MFI on L-GMPs
Vagotomy - +

l $p=0.0131$
BrdU+ (% of L-GMPs)
Vagotomy - +

m $p=0.0452$
cKit+ (% of viable cells)
Vagotomy - +

n

MTOR SIGNALING
C-MYC PATHWAY
GAL_LEUKEMIC_STEM_CELL_UP
PI3K-AKT SIGNALING
REACTOME_PI3K_AKT_ACTIVATION_WNT_SIGNALING (BETA-CATENIN)
GENTLES_LEUKEMIC_STEM_CELL_UP
MACROAUTOPHAGY
PI3K-AKT-MTOR-SIGNALING PATHWAY CA, CAMP AND LIPID SIGNALING
POS. REG. OF CELL PROLIFERATION
CONSTITUTIVE SIGNALING BY ABERRANT PI3K IN CANCER
ROADMAP_HISTONE_H3K79ME2_H1_POS. REG. OF G1-S TRANSITION OF MITOTIC CELL CYCLE

Sham vs. Vagotomy

NES

Upregulated (NES)
1.6 1.2 0.8 0.4 0.0

P-value (-Log10)
0 1 2 3 4

o

GATED CHANNEL ACT.
LIGAND-GATED ION CHANNEL ACT.
LIGAND-GATED CHANNEL ACT.
TRANSMITTER-GATED ION CHANNEL ACT.
EXICITORY LIGAND-GATED ION CHANNEL ACT.
TRANSMITTER-GATED CHANNEL ACT.
NEUROTRANSMITTER REC. ACT. (POSTSYNAPTIC)
ION CHANNEL ACT.
NEUROTRANSMITTER REC. ACT.
CHANNEL ACT.
PASSIVE TRANSMEMBRANE TRANSPORTER ACT.
IONOTROPIC GLUTAMATE RECEPTOR ACT.
LIGAND-GATED ANION CHANNEL ACT.
GLUTAMATE REC. ACT.

Sham vs. Vagotomy

P-value
0.01 0.02 0.03

Count

Parasympathetic cholinergic signals promote leukemogenesis.

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- d, Decreased ACh serum concentration in vagotomized mice, compared with sham-operated ($n = 10$).
 - e, Reduced frequency of GFP⁺ leukemic BM cells in vagotomized mice ($n = 5-6$).
 - f, Reduced frequency of BM lin⁻GFP⁺ leukemic cells in vagotomized mice ($n = 5-6$).
 - g, Reduced frequency of BM L-GMPs in vagotomized mice ($n = 5-6$).
 - h, Reduced CFU-Cs per 5,000 BM cells in vagotomized mice ($n = 5-6$).
 - i-j, Extreme Limiting Dilution Assay (ELDA, I) and (J) estimated stem cell frequency ($n=5$).
 - k, Increased Gr-1 expression in L-GMPs in vagotomized mice ($n = 5-6$).
 - l, Increased BrdU retention in L-GMPs indicating reduced LSC proliferation in vagotomized mice ($n = 5-6$).
 - m, Reduced frequency of ckit⁺ cells in vagotomized mice ($n = 5-6$).
 - n, Gene set enrichment analysis (GSEA) on RNAseq data in BM L-GMPs from vagotomized or sham-operated mice ($n = 3-4$).
 - o, GSEA on ATAC-seq data in BM L-GMPs from vagotomized or sham-operated mice ($n = 3-4$).
- c-h, k-m, Data are means $\pm$ SEM. Each dot represents a biological replicate. $*P < 0.05$; $**P < 0.01$. c-h, k-m, Student's t-test with Welch's correction. J, χ^2 test.

Figure 3

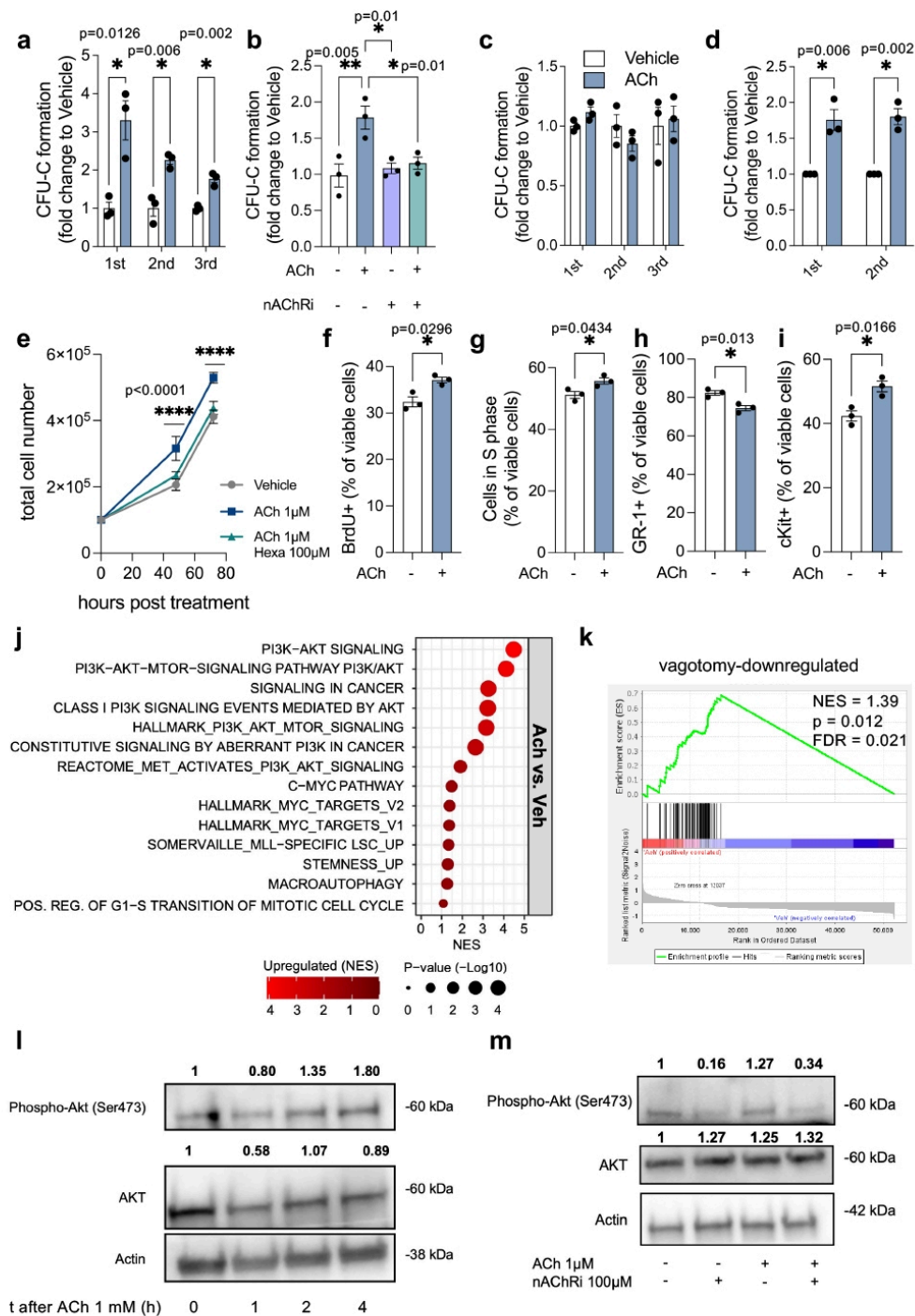


Figure 3

Cholinergic signals directly promote L-GMP stemness and proliferation.

a, Increased colony-forming activity and re-plating efficiency of ACh-treated L-GMPs (n = 3).

- b, Increased colony formation of ACh-treated L-GMPs is reversed by the nAChR inhibitor (i) hexamethonium ($n = 3$).
- c, Unaltered colony and re-plating capacity of ACh-treated normal Lin-Sca1+ckit+ HSPCs ($n = 3$).
- d, Increased colony-forming activity and re-plating efficiency of ACh-treated primary human LSCs ($n=3$ AML patients).
- e, Increased L-GMPs growth by ACh is reversed by the nAChRi hexamethonium ($n = 3$).
- f, Increased L-GMP proliferation (BrdU incorporation) after ACh treatment ($n=3$).
- g, Increased frequency of L-GMPs in S phase after ACh treatment ($n=3$).
- h, i, Reduced frequency of (h) GR-1+ (differentiation marker) and (i) increased c-kit+ (primitive marker) in L-GMPs after ACh treatment ($n=3$).
- j, GSEA analysis of RNA sequencing data derived from ACh-treated and vehicle-treated L-GMPs ($n = 5-6$).
- k, GSEA plot “vagotomy-downregulated”.
- l, m, Western plot for phospho-Akt and Akt in L-GMPs treated with ACh for 1, 2, and 4h (l) or 4h in presence or absence of hexamethonium (m).
- a-i, Each dot is a biological replicate. a, c, d, f-i, Student’s t-test with Welch’s correction. b, One-way ANOVA with Dunnett’s multiple comparisons. e, Two-way ANOVA with Bonferroni multiple comparisons.

Figure 4

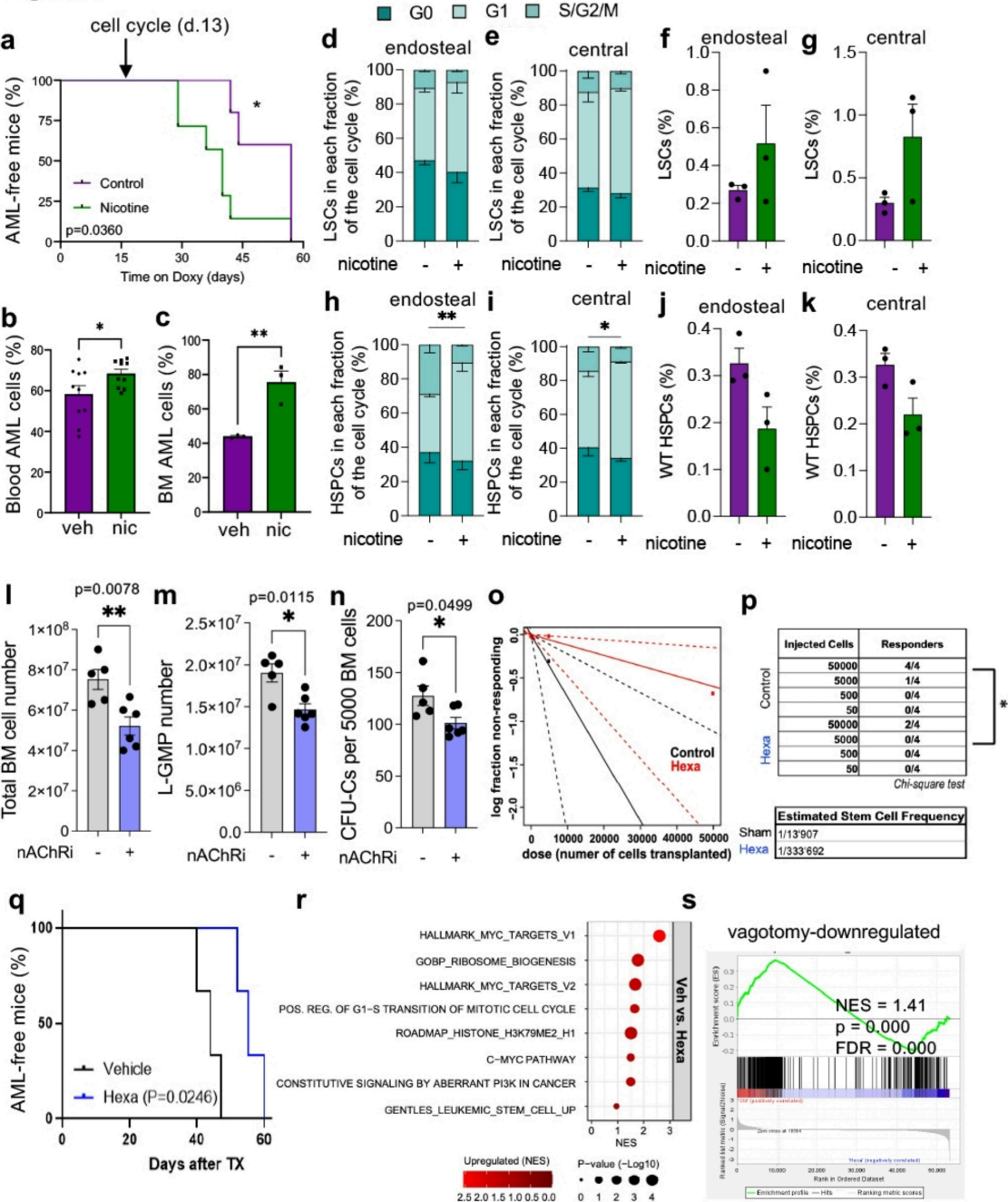


Figure 4

Nicotinic cholinergic signals promote leukemogenesis via $\alpha 7$ nAChR.

a, Accelerated AML development in mice treated with nicotine (green) compared with vehicle (purple) ($n = 5-6$).

b-c, Increased frequency of AML cells in (b) blood and (c) BM after nicotine treatment ($n = 3-10$).

d-e, Frequency of (d) endosteal or (e) central BM LSCs in each phase of the cell cycle ($n = 3$) during incipient AML in mice.

f-g, Trend towards increased frequency of (f) endosteal and (g) central BM LSCs after nicotine treatment ($n=3$).

h-i, Increased frequency of quiescent (h) endosteal or (i) central BM WT HSPCs after nicotine treatment ($n = 3$).

j-k, Trend towards reduced frequency of BM (j) endosteal and (k) central BM WT HSPCs after nicotine treatment ($n= 3$).

l-n, Reduced BM nucleated cells (l), L-GMPs (m) and CFU-Cs (n) in AML mice treated with the nAChRi hexamethonium bromide ($n = 5-6$).

o-p, ELDA (o) and (p) estimated stem cell frequency showing reduced LSCs in mice treated with nAChRi ($n=4$).

q, Delayed leukemogenesis in mice treated with hexamethonium bromide (hexa, $n = 3$).

r, GSEA on RNA-seq data in BM L-GMPs from vehicle- or hexamethonium-treated AML mice ($n = 3-4$).

s, GSEA plot “vagotomy-downregulated”.

b-c, f-g, j-n, Data are means $\pm$ SEM. Each dot is a biological replicate. $*P < 0.05$, $**P < 0.01$. a,q, Log-rank (Mantel-Cox) test. b-c, f-g, j-k, Student's t test. d-e, h-i, Two-way ANOVA and Šídák's multiple comparisons. l-n, Student's t -test with Welch's correction. p, χ^2 test.

Figure 5

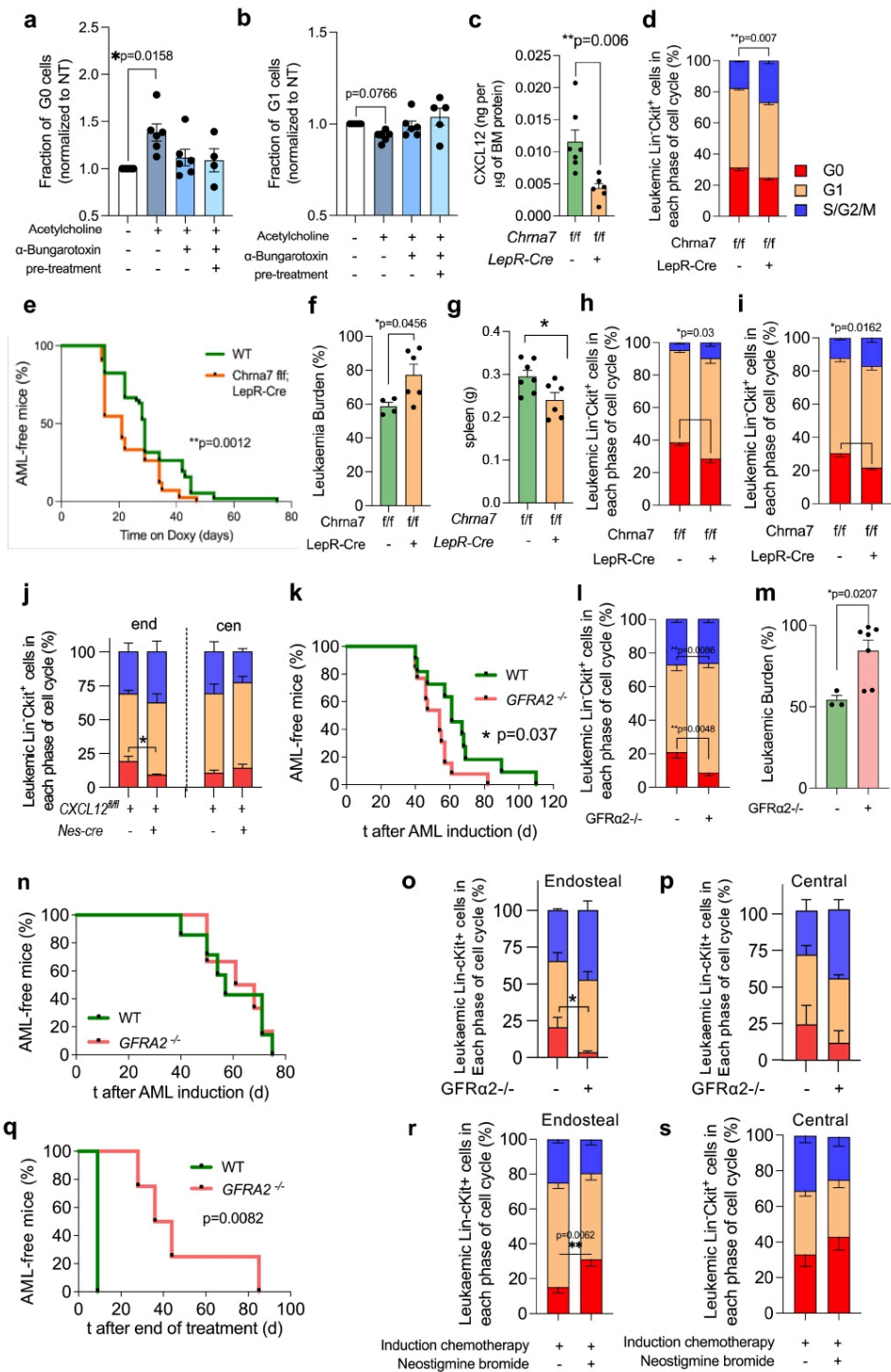


Figure 5

Cholinergic signals differentially regulate LSCs and their BM niche.

a-b, Fraction of L-GMPs sorted from AML mice in (a) G0 or (b) G1 phases of the cell cycle 4d after co-culture with mesenspheres in presence/absence of ACh and α -bungarotoxin ($n = 4-6$). Increased L-GMP quiescence by ACh was reversed by nAChR inhibition.

c, Reduced BM CXCL12 concentration in AML mice lacking $\alpha 7$ nAChR in MSCs (*LepR-Cre;Chrna7^{fl/fl}* mice) ($n = 6-7$).

d, Reduced frequency of quiescent leukemic BM Lin⁻ckit⁺ cells in mice lacking $\alpha 7$ nAChR in MSCs 4w after AML induction ($n = 4-6$).

e-f, Accelerated AML kinetics (E) and spleen leukaemia burden (F) in *LepR-Cre;Chrna7^{fl/fl}* mice (orange) compared with control group (green)($n = 42-57$).

g, Reduced spleen leukemia burden after Ara-C treatment of mice lacking $\alpha 7$ nAChR in MSCs ($n = 3-8$).

h-i, Reduced frequency of quiescent leukemic BM Lin⁻ckit⁺ cells in mice lacking $\alpha 7$ nAChR in MSCs after Ara-C treatment ($n = 6-7$).

j, Reduced frequency of quiescent leukemic Lin⁻ckit⁺ cells in the endosteal BM of mice lacking CXCL12 in MSCs (*Nes-Cre^{ERT2};CXCL12^{fl/fl}* mice) 24h after single AraC treatment (100mg/kg/day)($n = 6-7$).

k, Accelerated leukemogenesis in cholinergic-neural-deficient (*GFR α 2^{-/-}*, red) mice, compared with control group (green) or ($n = 11-13$).

l-m, Reduced frequency of quiescent leukemic BM Lin⁻ckit⁺ cells (l) and increased BM AML burden (m) in cholinergic-neural-deficient mice 24h after 5 doses of Ara-C (100mg/kg/day) ($n = 3-7$).

n, AML-free control (green) or *GFR α 2^{-/-}* (red) mice after 5 doses of Ara-C (100mg/kg/day) ($n = 6-7$).

o-p, Reduced frequency of quiescent endosteal (not central) BM Lin⁻ckit⁺ cells leukemic cells from cholinergic-neural-deficient mice 24h after induction chemotherapy (5 doses of AraC at 100mg/kg/day, together with 3 doses of doxorubicin at 3mg/kg/day for the first 3 days) ($n = 3-5$).

r-s, Increased frequency of quiescent endosteal (not central) BM Lin⁻ckit⁺ cells leukemic cells when induction chemotherapy is combined in WT mice with a cholinesterase inhibitor (neostigmine bromide, 80 μ g/kg/day), to enhance endogenous cholinergic signaling ($n = 7$).

q, AML relapse-free survival of control (green) or *GFR α 2^{-/-}* (red) mice after induction chemotherapy ($n = 4$).

a-c, f-g, m, Data are means $\pm$ SEM. Each dot is a biological replicate. * $P < 0.05$, ** $P < 0.01$. a-b, One-way ANOVA and Tukey's comparisons. Outliers were identified using the ROUT

method. e,k,n,q, Log-rank (Mantel-Cox) test. c,f,g,m, Unpaired two-tailed t test with Welch's correction. d,h-j,l,o,p,r,s Two-way ANOVA and Šídák's comparisons.

Figure 6

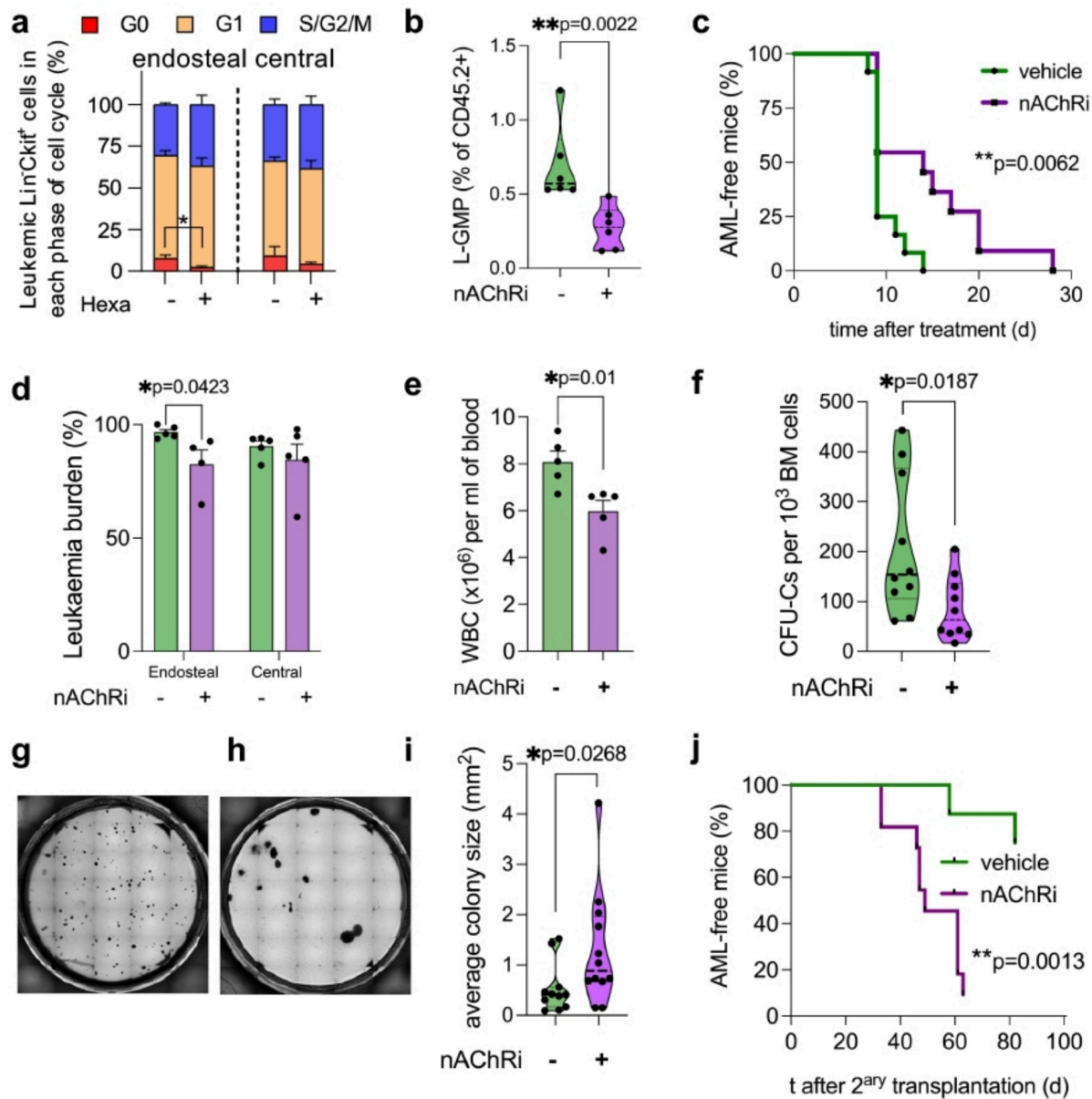


Figure 6

Pharmacological blockade of nAChRs sensitises AML to chemotherapy.

a, Reduced frequency of quiescent endosteal (not central) BM Lin⁺ckit⁺ cells leukemic cells 4d after induction therapy alone or combined with the nAChR inhibitor hexamethonium bromide (n = 5-6).

b, Reduced L-GMP burden in the BM 4d after nAChR inhibitor treatment combined with induction AML therapy (5 doses of AraC at 100mg/kg/day, together with 3 doses of doxorubicin at 3mg/kg/day for the first 3 days) (n=6).

c, Delayed AML relapse kinetics after combined nAChR inhibitor and AML induction therapy (n=11-12).

d, Reduced leukemic burden in the endosteal (not central) BM 4d after combined nAChR inhibitor and AML induction therapy (n=4-5).

e, Reduced leukocyte count in blood 4d after combined nAChR inhibitor and AML induction therapy (n=5).

f-h, Reduced replating CFU-C ability (f) (n=10-11) and increased colony size after combined nAChR inhibitor and AML induction therapy (h), compared with induction therapy alone (g).

i, Average size of colonies from f-h (n=11-12).

j, AML relapse-free mice after secondary transplantation of BM cells from mice treated with induction chemotherapy or combination therapy (n=8-11).

Each dot is a mouse. Data are means $\pm$ SEM. $*P < 0.05$, $**P < 0.01$. a, Two-way ANOVA and Šídák's multiple comparisons test. c,j, Log-rank (Mantel-Cox) test. b,e-f,i, Mann-Whitney test. Outliers were identified using the ROUT method. d, One-way ANOVA with Tukey's multiple comparisons.

Supplementary Files

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