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Research Article

Vagal-mAChR4 signaling promotes Friend virus complex (FV)-induced acute erythroleukemia

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ABSTRACT

Erythroleukemia belongs to acute myeloid leukemia (AML) type 6 (M6), and treatment remains difficult due to the poor prognosis of the disease. Friend virus (FV) is a complex of two viruses: Friend murine leukemia virus (F-MuLV) strain along with a defective spleen focus-forming virus (SFFV), which can induce acute erythroleukemia in mice. We have previously reported that activation of vagal α7 nicotinic acetylcholine receptor (nAChR) signaling promotes HIV-1 transcription. Whether vagal muscarinic signaling mediates FV-induced erythroleukemia and the underlying mechanisms remain unclear. In this study, sham and vagotomized mice were intraperitoneally injected with FV. FV infection caused anemia in sham mice, and vagotomy reversed this change. FV infection increased erythroblasts ProE, EryA, and EryB cells in the spleen, and these changes were blocked by vagotomy. In bone marrow, FV infection reduced EryC cells in sham mice, an effect that was counteracted by vagotomy. FV infection increased choline acetyltransferase (ChAT) expression in splenic $CD4^+$ and $CD8^+$ T cells, and this change was reversed by vagotomy. Furthermore, the increase of EryA and EryB cells in spleen of FV-infected wild-type mice was reversed after deletion of *ChAT* in $CD4^+$ T cells. In bone marrow, FV infection reduced EryB and EryC cells in sham mice, whereas lack of *ChAT* in $CD4^+$ T cells did not affect this change. Activation of muscarinic acetylcholine receptor 4 (mAChR4) by clozapine N-oxide (CNO) significantly increased EryB in the spleen but decreased the EryC cell population in the bone marrow of FV-infected mice. Thus, vagal-mAChR4 signaling in the spleen and bone marrow synergistically promotes the pathogenesis of acute erythroleukemia. We uncover an unrecognized mechanism of neuromodulation in erythroleukemia.

1. Introduction

Erythroleukemia belongs to type 6 (M6) of acute myeloid leukemia (AML), which is more common in the elderly and has no obvious cytological features (Michiels et al., 1997; Park et al., 2002). The World Health Organization (WHO) classifies erythroleukemia according to its pathogenic characteristics into two categories: M6a, in which erythrocyte precursor cells account for more than 50% of the number of enucleated red blood cells in the bone marrow, and myeloblasts account for more than 20%; M6b, immature tumor proliferation occurs only in erythrocytes (Harris, 2001; Wickrema and Crispino, 2007). Although the incidence of erythroleukemia in acute myeloid leukemia is low, the treatment is still difficult due to the poor prognosis of the disease, which has great research value. Earlier studies found that transcription factors such as *Spi1*, *Gata1/2*, *Nfe2*, and *Klf1* play important roles in red blood cell proliferation and differentiation (Wickrema and Crispino, 2007).

Friend virus (FV), which comprises replication-competent Friend murine leukemia virus (F-MuLV) plus replication-defective spleen focus-forming virus (SFFV), induces a multistage erythroleukemia (Eckner, 1975; Jones et al., 1988; Ney and D'Andrea, 2000; Hasenkrug and Dittmer, 2007). As a helper virus, F-MuLV can provide an envelope for SFFV, helping the latter to amplify in the host. Replication-defective SFFV

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appears to be the major determinant of erythroleukemia induction in both newborn and adult mice (MacDonald et al., 1980). The SFFV glycoprotein encoded by oncogene *gp55* activates the erythropoietin receptor, resulting in the abnormal proliferation of infected erythroblasts. Within 48 h after infected by FV, erythroblasts begin to proliferate and to migrate from bone marrow to spleen. By 9 days, erythropoietic foci cover the spleen, which enlarges during the next few weeks from 0.09 g to 2–4 g (Hoatlin and Kabat, 1995).

Vagus efferents originate from the dorsal motor nucleus of the vagus (DMV) and have extensive innervation, especially in the spleen and bone marrow. The vagus nerve innervates the spleen and releases norepinephrine (NE), which acts on beta 2 adrenergic receptors (ADRs) expressed by choline acetyltransferase (ChAT)-expressing T cells. These cells produce acetylcholine to activate the α7 nicotinic acetylcholine receptor (nAChR), which mediates the cholinergic anti-inflammatory response by reducing the production of pro-inflammatory cytokines through inhibiting the NF-κB transcriptional pathway or activating the tyrosine kinase JAK2 (Guarini et al., 2003; de Jonge et al., 2005; Pavlov and Tracey, 2006). We have demonstrated that disruption of vagal-α7 nAChR signaling may impair splenic inflammatory responses (Zhao et al., 2017) and bone marrow α7 nAChR^{+}Sca 1^{+} cell proliferation egress (Chen et al., 2020). In addition to nicotinic acetylcholine receptors, the vagus nerve can also modulate muscarinic acetylcholine receptors (M1–M5). Muscarinic acetylcholine receptor 4 (mAChR4) regulates erythroid burst-forming unit (BFU-E) self-renewal, and mAChR4 downregulation corrects myelodysplastic syndromes, aging, and hemolytic anemia (Trivedi et al., 2019). However, whether and how the vagus nerve regulates erythroleukemia development is unclear.

Due to the anti-inflammatory response of the vagus nerve, its regulatory role in the immune system and erythropoiesis remains of great interest. We have reported that activation of α7 nAChR promotes HIV-1 transcription in an ROS/p-p38 MAPK/LMNB1/NFATC4 signaling dependent manner (Wen et al., 2022). In this study, we performed vagotomy in FV-infected mice and found that FV replication was not significantly affected after vagotomy, however, the acute erythroleukemia and anemia after infection were corrected. Furthermore, we found that ChAT in CD4^{+} T cells and mAChR4 could mediate acute erythroleukemia, confirming the pathogenic role of the vagus nerve-mAChR4 signaling in FV-induced erythroleukemia. Our study was the first to discover the role of vagus-mAChR signaling in virus-induced erythrocyte dysplasia, which may be another piece of evidence that the vagus nerve affects blood cell development.

2. Materials and methods

2.1. Animal

BALB/c and C57BL/6J mice were purchased from Beijing Weitong Lihua Laboratory Animal Technology Co., Ltd. C57BL/6J-*CD4*^{Cre} *ChAT*$^{flox/flox}$ mice (*ChAT*[flox] strain# 016920) were purchased from Jax Lab and housed in an SPF facility. For viral infection, 6-week-old female C57BL/6J mice were intraperitoneally inoculated with 5×10^8 copies of FV virus. For mAChR4 activation studies, C57BL/6J mice were dosed with clozapine N-oxide (CNO) (10 mg/kg/day) 24 h before FV (5×10^8 copies) infection and then daily until sacrifice. The experimental protocol was approved by the Institutional Animal Care and Use Committee of the Pasteur Institute, Shanghai.

2.2. Vagotomy

Mice were anesthetized with 0.6 mL/20 g body weight of 1.2% avertin and fixed under the stereoscope with medical tape. After wiping the pharynx with iodophor and medical alcohol, a small incision of about 1 cm is made longitudinally. The surrounding muscle and mucosal tissue were carefully excised, and the vagus nerve was severed with fine scissors. After severing the right vagus nerve, the pharyngeal wounds of the mice were closed with surgical sutures. The sham group only underwent surgery without severing the vagus nerve. The mice recovered for seven days and the corresponding experiments were carried out.

2.3. Reagents

Reagents involved in this study include: RNA reverse transcription kit (TOYOBO company), TaqMan real-time fluorescence quantitative kit (CWBiao, Suzhou, China), CNO (Selleck Corporation, Shanghai, China), Milameline hydrochloride (MH, Selleck Corporation, Shanghai, China), VU0238429 (VU, Selleck Corporation), anti-mAChR4 antibody (18C7.2, Abcam, UK), and Intracellular Fixation and Permeabilization Buffer (eBioscience, Thermo Fisher Scientific, US).

2.4. Cell culture

Murine erythroleukemia (MEL) cell lines (Tongpai Biotechnology Co., Ltd, Shanghai, China) are erythroid progenitor cells derived from the spleens of susceptible mice, which were culture in DMEM medium (Gibco, Thermo Fisher Scientific, US) with 10% FBS (Gibco, Thermo Fisher Scientific), 100× penicillin (Gibco, Thermo Fisher Scientific) and 100× streptomycin (Gibco, Thermo Fisher Scientific) at 37 °C, 5% CO_2, infected with the Friend virus complex 1. These cells can initiate proliferation when infected with FV. To study the effect of mAChR5 agonist on erythropoiesis genes, MEL cells were incubated with 50 μmol/L MH, 2 μmol/L VU or 8 nmol/L CNO for 24 h before FV infection (5×10^8 copies).

2.5. Virus identification, amplification and storage

The virus adopted in this study was donated by Prof. Fu, Linchun from Guangzhou University of Traditional Chinese Medicine (GeneBank accession number: OQ718806) (Zhou et al., 2008). We have previously demonstrated that this strain can infect adult C57BL/6 mice (Song et al., 2022). The genomic sequences of this virus were listed in Supplementary data. Sequence alignment was performed (https://www.vectorbuilder.cn/tool/sequence-alignment.html) to analyze the similarity between this strain and two F-MuLV strains FB29 (NC_001362) and FB-57 (X02794). In addition, to verify the presence of SFFV in this strain, three pairs of primers were designed using the SFFV reference sequence on NCBI (NC_001500.1), which is formed by gp1, gp2/3, and gp4 fragments, respectively (Supplementary Figs. S1A and 1B). Using viral RNA as a template and three pairs of primers, RT-PCR was performed, and the obtained products were verified by electrophoresis (Supplementary Fig. S1C) and Sanger sequencing (Supplementary Fig. S1D). Blast was used to compare the sequencing results with the SFFV reference sequence.

For virus amplification, 1×10^9 copies of virus were intraperitoneally (i.p.) injected into BALB/c mice. Mice were sacrificed on 14 days post-infection with spleen removed. 10% splenic suspension was made in PBS. After centrifugation at 2000×*g* for 20 min, the supernatant was collected, filtered through a 45 μm filter, and stored in −80 °C for future use.

2.6. Assay for viral replication

At necropsy, spleen, blood or bone marrow (BM) were harvested, and total cellular RNAs were extracted with TRIzol reagent (Life Technologies) and then reversely transcribed to cDNA with ReverTra Ace qPCR ET Master Mix with genome DNA Remover Kit (TOYOBO). Viral replication was quantified by measuring the expression of viral *gag* gene with PCR quantification using the GoldStar TaqMan Mixture (CWBiao, Suzhou, China). PCR was performed using the Thunderbird SYBR qPCR Mix (TOYOBO) on the ABI 7900HT Real-time PCR system (Applied Biosystems), with an initial denaturation step for 10 min at 95 °C, amplification with 40 cycles of denaturation (95 °C, 15 s), annealing and

extension (60 °C, 1 min). The primers for *gag*, forward, 5′-CTCTTTCTCCGAGGACCCAG-3′, reverse, 5′-GTCATTGGGCAGCTGAGTTG-3′, and the probe: 5′-(FAM) ACAGCTTTGATCGAGTCCGTTCTCCT (TAMRA)-3′.

2.7. RT-PCR

RT-PCR reactions were prepared using GenStar SYBR qPCR Mix and performed on an ABI 7900HT Real-Time PCR instrument. Data are shown as relative values normalized by GAPDH. The relevant primer information was listed in Supplementary Table S1.

2.8. Cell proliferation assay

A total of 1×10^9 MEL cells was incubated with 10 μmol/L 5,6-carboxyfluorescein diacetate succinimidyl ester (CFSE) (Target Molecule Corp., Shanghai, China) at 37 °C for 10 min. The labeling reaction was stopped for 1 min by adding an equal volume of heat inactivated fetal bovine serum. The CFSE labeled cells were washed twice with PBS and recounted, and the cell concentration was adjusted to 6×10^4 cells/mL in IMDM containing 10% FBS.

2.9. Western blot

Cells were harvested by centrifugation at 300 ×*g* for 10 min, then resuspended in 200 μL of lysis buffer. The samples were mixed with 5× protein loading buffer and heated at 100 °C for 10 min, then loaded into an SDS-PAGE gel and run at 120 V for 70 min to separate the different proteins. After SDS-PAGE electrophoresis, PVDF membranes were treated with methanol for 5 min. Perform protein transfer at 250 mA for 75 min using a fully wet transfer apparatus. After protein transfer, PVDF membranes were blocked with 5% skim milk, incubated at room temperature for 30 min, and washed with 1× TBST buffer for 5 min, 3 times. It was then incubated with the primary antibody [mouse *anti*-mAChR4 (18C7.2, Abcam); 1:1000] overnight at 4 °C. After antibody incubation, wash with 1× TBST buffer for 5 min (3 times) before adding secondary antibody [donkey anti-mouse IgG (H + L)-HRP (715-035-151, Jackson, USA); 1:10,000]. After incubation for 2 h at room temperature, the membrane was then washed for three times with 1× TBST buffer for 5 min each. The PVDF membrane was immersed in ECL buffer and imaged with a chemiluminescence imager (Tanon, Shanghai, China).

2.10. Flow cytometry

The phenotypes of proerythroblast (ProE), erythroblast A (EryA), erythroblast B (EryB), and erythroblast C (EryC) were determined by flow cytometry by immunostaining with specific antibodies. The strategy of gating erythroblasts at different stages was used as described previously (Koulnis et al., 2011). Four stages of erythroblasts were defined as: immature, large "ProE" erythroblasts: $CD71^{high}Ter119^{intermediate}$; less mature, large "EryA" erythroblasts: $CD71^{high}Ter119^{high}FSC^{high}$; smaller, more mature "EryB" erythroblasts: $CD71^{high}Ter119^{high}FSC^{low}$; mature "EryC" erythroblast subset: $CD71^{low}Ter119^{high}FSC^{low}$. The specific monoclonal antibodies against the antigens or isotype-matched IgG controls used were: Fc blocker: CD16/CD32 (FCR4G8, eBioscience, Thermo Fisher Scientific, US), CD71 (C2F2, BD Pharmingen, US), Ter119 (Ter119, BD Pharmingen), DAPI (Sigma-Aldrich, US). For intracellular staining, cells were treated with intracellular fixation and permeabilization Buffer (eBioscience, Thermo Fisher Scientific), and anti-ChAT antibody (ab181023, abcam). The stained cells were detected using a Fortessa flow cytometer (BD Pharmingen) and analyzed with FlowJo 10.1 software (BD Biosciences, US). The frequency (%) of total cells was presented in all the experiments.

2.11. Statistical analysis

Graphpad Prism 7.0 (GraphPad Software) was used for statistical analysis. For multiple comparison, one-way ANOVAs with Tukey justification were performed.

3. Results

3.1. The vagus nerve promotes anemia induced by acute FV infection

To verify the viral strain adopted in the study, sequence alignment was performed and the results showed that the virus shared 89.36% and 88.76% similarity to the complete genome of F-MuLV strains FB29 and FB-57, respectively (Supplementary Table S2). We also confirmed that the gp2/3 fragment of SFFV was existed (Supplementary Figs. S1C and 1D), indicating that the virus used in this experiment is the FV complex of F-MuLV and SFFV.

To explore the role of the vagus nerve in FV-induced disorders, the right vagus nerve in C57BL/6J mice was severed by vagotomy (VGX). Mice were intraperitoneally infected with FV one week later. And five days after infection, viral loads in mouse spleen, blood, and BM were measured by qPCR (Fig. 1A). Mice lost weight for the first 4 days and then recovered 7 days after vagotomy (Fig. 1B). There was no significant difference of viral loads in blood, spleen, and BM between FV-infected sham and vagotomy mice (Fig. 1C). Five days after infection, sham mice developed anemia, manifested by decreased red blood cell counts, hematocrit, and hemoglobin. These parameters were reversed by vagotomy (Fig. 1D). These findings suggest that the vagus nerve can contribute to FV-induced acute anemia.

3.2. Vagus nerve differentially regulates the proliferation and differentiation of progenitor erythrocytes in the spleen and bone marrow during FV acute infection

To investigate the role of vagus nerve in regulating progenitor erythroblast proliferation and differentiation during FV infection, we examined the frequency of erythroblasts at different stages in the BM or spleen by flow cytometry (Fig. 2A). FV infection increased splenic ProE, EryA, and EryB cells, and these changes were blocked by vagotomy (Fig. 2B). The mRNA levels of *Epor*, *Nfe2*, *Klf1*, and *Gata1* (key genes for erythropoiesis) in spleen were elevated after FV infection and decreased by vagotomy (Fig. 2C). In bone marrow, FV infection reduced EryC cells in sham mice, an effect that was counteracted by vagotomy (Fig. 2D). These findings support that vagal signaling contributes to FV-induced abnormal erythropoiesis by promoting erythroblast proliferation and differentiation in the spleen and inhibiting their maturation in the BM.

3.3. Vagotomy decreases the proportion of ChAT-expressing $CD4^+$ and $CD8^+$ T cells induced by FV infection in spleen

Lymphocytes in the spleen, especially T lymphocytes, can produce acetylcholine. To investigate the relationship between FV-associated erythroleukemia and acetylcholine production by immune cells in the spleen, we counted the ChAT-expressing $CD4^+$ and $CD8^+$ T cells in the spleen by flow cytometry. FV infection increased the proportion of ChAT-expressing $CD4^+$ and $CD8^+$ T cells, whereas vagotomy abolished this change (Fig. 3A). This finding suggests that the occurrence of vagus-involved erythroleukemia may be related to the formation of acetylcholine in $CD4^+$ and $CD8^+$ T cells from spleen.

3.4. ChAT mediates the progression of FV-induced erythroleukemia

To investigate whether ChAT expressed by $CD4^+$ T cells affects FV-induced erythroleukemia, we challenged $ChAT^{flox/flox}$ and $CD4^{Cre}ChAT^{flox/flox}$ mice with FV virus. Viral copy numbers were reduced in FV-infected $CD4^{Cre}ChAT^{flox/flox}$ mice compared with FV-infected

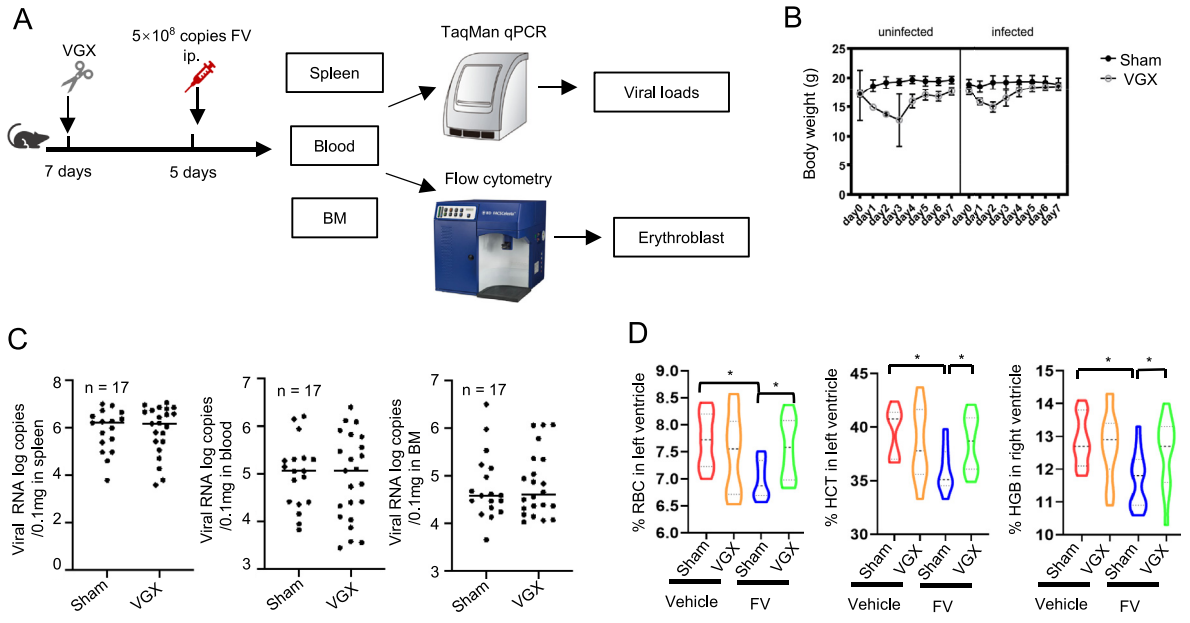

Fig. 1. Vagotomized (VGX) reverses the anemia caused by FV infection *in vivo*. **A** Flow diagram of experiment. Vagus nerve on the right side was cut off in the vagotomy group. The sham group was operated but keeping nerve intact. Mice recovered for seven days, then infected by FV (5×10^8 copies). Mice were sacrificed on five days post-infection. **B** The weight change of mice after VGX. **C** The viral loads in spleen, blood and bone marrow (BM) were detected by qPCR (n = 17). **D** The mature red blood cell (RBC), hematocrit (HCT) and hemoglobin (HGB) level in the left ventricle and right ventricle were detected (n = 10). Data are mean ± standard deviation, and the experimental results were analyzed by ANOVA. $^*P < 0.05$.

ChAT$^{flox/flox}$ mice (Fig. 3B). Flow cytometric analysis of erythroblasts in BM and spleen was performed. We found that FV infection increased ProE, EryA and EryB cells in the spleen. And the increase of EryA and EryB cells was reversed by deletion of ChAT in CD4$^+$ T cells (Fig. 3C). In BM, FV infection reduced EryB and EryC cells in sham mice, and the absence of ChAT in CD4$^+$ T cells did not affect this change (Fig. 3D). These data suggest that the effect of splenic vagal innervation on proliferation and differentiation of EryA and EryB cell is dependent on ChAT expression in CD4$^+$ T cells.

3.5. FV induces proliferation of MEL cells expressing muscarinic acetylcholine receptors, and activation of mAChR5 does not affect the expression of erythropoietic genes

After we verified that MEL (a mouse erytheoleukemia cell line) could be infected by FV (Fig. 4A), we used CellTrace™ CFSE to trace FV-infected MEL. By flow cytometry analysis, we found that FV infection drove these cells to proliferate (Fig. 4B and C). To study whether MEL express acetylcholine receptors, we performed RT-PCR targeting *Chrna1–a10*, *Chrnb1–b4*, *Chrnd*, *Chrne*, *Chrng*, and *Chrm1–m5*. We found that acetylcholine receptors, especially *Chrna9*, *Chrnb1–b3*, *Chrm4–m5* were expressed on the MEL (Fig. 4C). Considering that muscarinic acetylcholine receptor antagonists could correct anemias in mouse models of myelodysplastic syndromes (MDS), aging, and hemolysis *in vivo* (Trivedi et al., 2019), we first studied whether activation of mAChR5 (coded by *Chrm5*) affects erythropoiesis genes (*Nfe2* and *Gata1*) in FV-infected MEL treated with VU [a positive allosteric modulator of mAChR5 (Bridges et al., 2009)] combining with or without MH (a non-selective muscarinic receptor agonist). We found that FV infection upregulated *Nfe2* and *Gata1*, but activation of mAChR5 by MH, VU or their combination did not affect this change (Fig. 4E and F).

3.6. mAChR4 promotes FV-induced splenic erythroblast proliferation in vivo

We examined whether activation of mAChR4 affects proliferation of erythroblasts *in vivo* during FV acute infection, using CNO (a selective mAChR4 agonist, Zorn et al., 1994). We found that CNO treatment significantly promoted the viral load of FV in spleen (Fig. 5A). Splenic EryB cell population in FV-infected mice was significantly increased after CNO treatment, while other cell populations remained unchanged (Fig. 5B). Concurrently, CNO treatment significantly elevated the mRNA level of erythropoiesis genes (*Epor*, *Nfe2*, and *Gata1*) in FV-infected mice (Fig. 5C). In BM, CNO treatment significantly reduced EryC cell population in FV-infected mice (Fig. 5D). These findings suggest that activation of mAChR4 increases EryB in the spleen and inhibits EryC in the bone marrow, thus disrupting erythrocyte differentiation and maturation.

3.7. FV infection increases mAChR4 expression and activation of this receptor boosts Spi1 expression in MEL cells

FV infection in MEL increased mAChR4 expression and CNO could boost this effect confirmed by Western blotting (Fig. 6A) and flow cytometric analysis (Fig. 6B and C). FV infection did not affect expression of *Spi1* and *Fli1* in MEL, but activation of mAChR4 by CNO could increase mRNA level of *Spi1* in FV-infected cells (Fig. 6D and E). Considering that *Spi1* is a putative oncogene in virally induced murine erythroleukemia

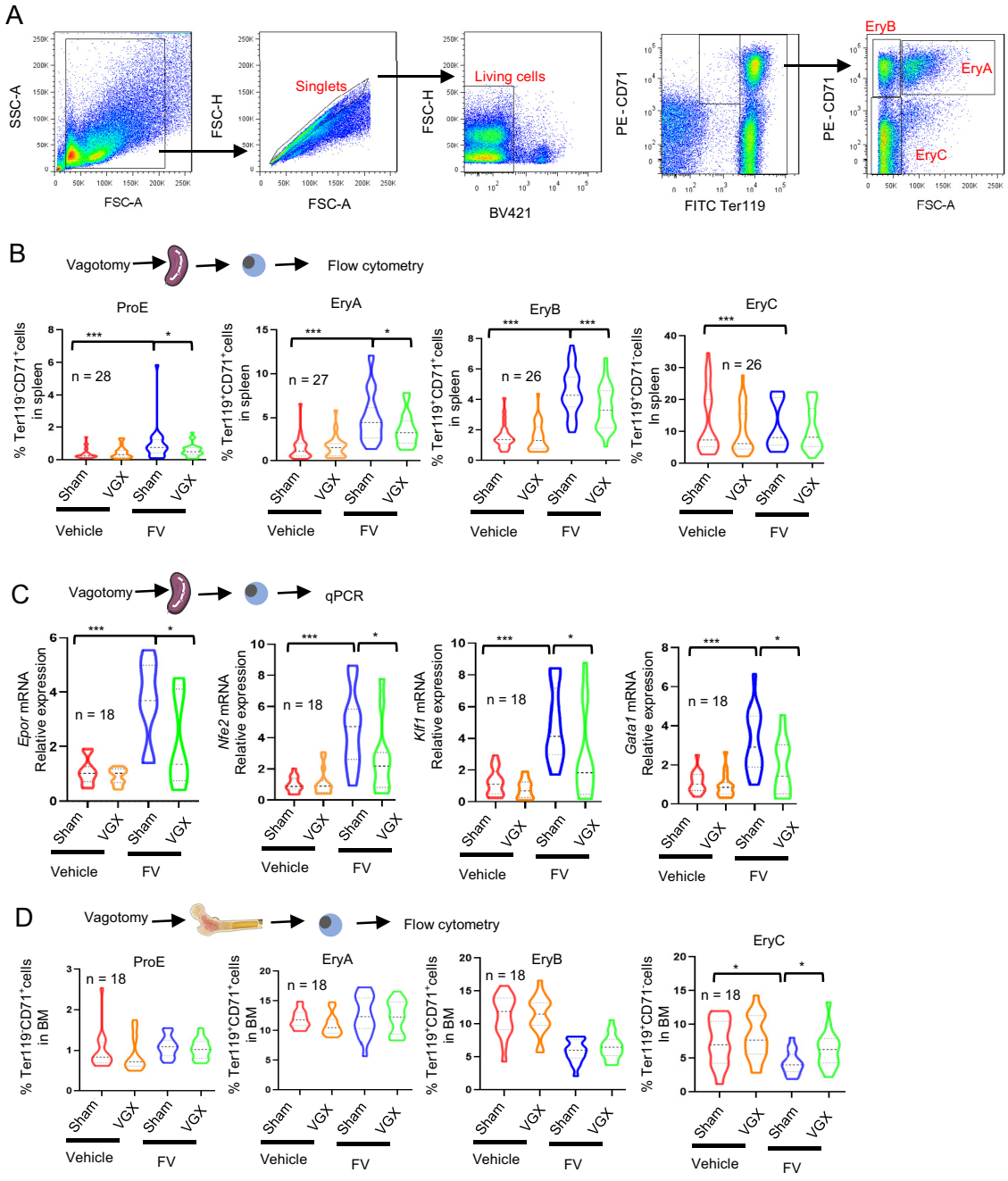

Fig. 2. Vagus nerve regulates the proliferation and differentiation of erythroblasts in the spleen and bone marrow (BM) during FV acute infection. **A** Gating strategy of different-stage erythroblasts. The frequencies of progenitor erythrocytes (ProE), erythroblast type A (EryA), erythroblast type B (EryB), and erythroblast type C (EryC) in spleen and BM were detected by flow cytometry. **B** The frequencies of erythroblasts at each stage in spleen were detected by flow cytometry at five days post-infection. **C** Effect of vagotomy on RNA levels of erythroid differentiation-related genes in the spleen. Real-time quantitative PCR was used to detect the expression level of erythrocyte differentiation-related genes *Epor*, *Nfe2*, *Klf1* and *Gata1* in the spleen normalized by *GAPDH*. **D** The frequencies of erythroblasts at each stage in BM were detected by flow cytometry. Data are mean ± standard deviation, and the experimental results were analyzed by ANOVA. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. n = 18 in each group.

(Moreau-Gachelin et al., 1988), our data indicates mAChR4-SPI1 signaling may regulate the development of acute erythroleukemia.

4. Discussion

Friend virus (FV) is a complex virus including a replication-defective spleen focus-forming virus (SFFV) and a replication-competent helper virus (F-MuLV). SFFV plays a major role in inducing erythroleukemia; however, it needs F-MuLV for replication. Generally, C57BL mice are resistant to FV replication and to FV-induced disease. After the young adults C57BL mice were infected N type or B-type tropic FV with SFFV for 21 days, little or no virus is recovered and no splenomegaly were seen (Axelrad, 1989). However, other research found that 8- to 10-week-old C57BL/6J mice can also be infected by NB-tropic FV (Van der Gaag and Axelrad, 1990). Thus, there is no absolute restriction to SFFV replication early after infection

in young adult C57BL/6 mice. The immune response might play an important role in resistance of C57BL/6 mice against FV (Del Guercio et al., 1982; Zelinskyy et al., 2004; Tsuji-Kawahara et al., 2013). C57BL/6 (B6) (Fv-2rr) mice have been found to differ from B6.S (Fv-2ss) mice not only in their response to this virus but also in the proliferative state of their erythropoietic progenitor cells BFU-E: in B6 mice the majority of BFU-E are normally quiescent, while in B6·S mice approximately 50% are actively synthesizing DNA at any time (Mak et al., 1979; Axelrad et al., 1982). We have previously reported that FV infected adult 6-week-old C57BL/6 mice and triggered mast cell activation and degranulation (Song et al., 2022). Considering the differences of immune response between C57BL/6 and BALB/c, to be consistent with our previous studies, in this research we firstly used C57BL/6 mice to investigate whether vagus nerve can impact FV replication. We found though the FV replication was not impacted, the frequency of erythroblasts had a great change after vagotomy. The virus strain adopted in this study shares about 90% similarity with FB29 and FV57 (Zhou et al., 2008). Our sequencing data also confirmed that this strain contained SFFV fragments (gp2/3). Therefore, it is feasible to use this FV strain to test our hypothesis that vagal-mAChR4 signaling affects FV-induced acute erythroleukemia. Moreover, FV-infected mice did not develop splenomegaly, but a large number of erythroblasts and erythropoiesis genes were increased in the spleen. Therefore, the use of FV-induced mouse models of acute anemia and erythroleukemia is sufficient for our experimental needs, and FV can induce erythroleukemia-like disease in C57 mice in this study, while such phenomena are not observed in other reports.

The vagus nerve is the tenth pair of cranial nerves in the body, the main component of the parasympathetic nerve in the autonomic nervous system. Vagus nerve is involved in regulation of innate immune responses and inflammatory responses during microbial invasion and tissue damage (Tracey K. J., 2002; Baccala et al., 2009; Tracey Kevin J, 2009; Andersson and Tracey, 2012). We proposed the pulmonary parasympathetic inflammatory reflex theory (Yang et al., 2014) and found that vagal α7 nAChR signaling cooperates with the spleen to regulate lung infection, inflammation, and injury (Zhao et al., 2017). Vagus-α7 nAChR signaling modulates lung inflammation during influenza virus (Gao et al., 2021). Recently, we found that activation of the α7 nAChR can trigger the ROS/p-p38 MAPK/LMNB1/NFATC4 signaling pathway to enhance HIV-1 transcription (Wen et al., 2022). These findings provide a solid foundation for our study of vagal modulation on FV infection and its role in the pathogenesis of acute erythroleukemia.

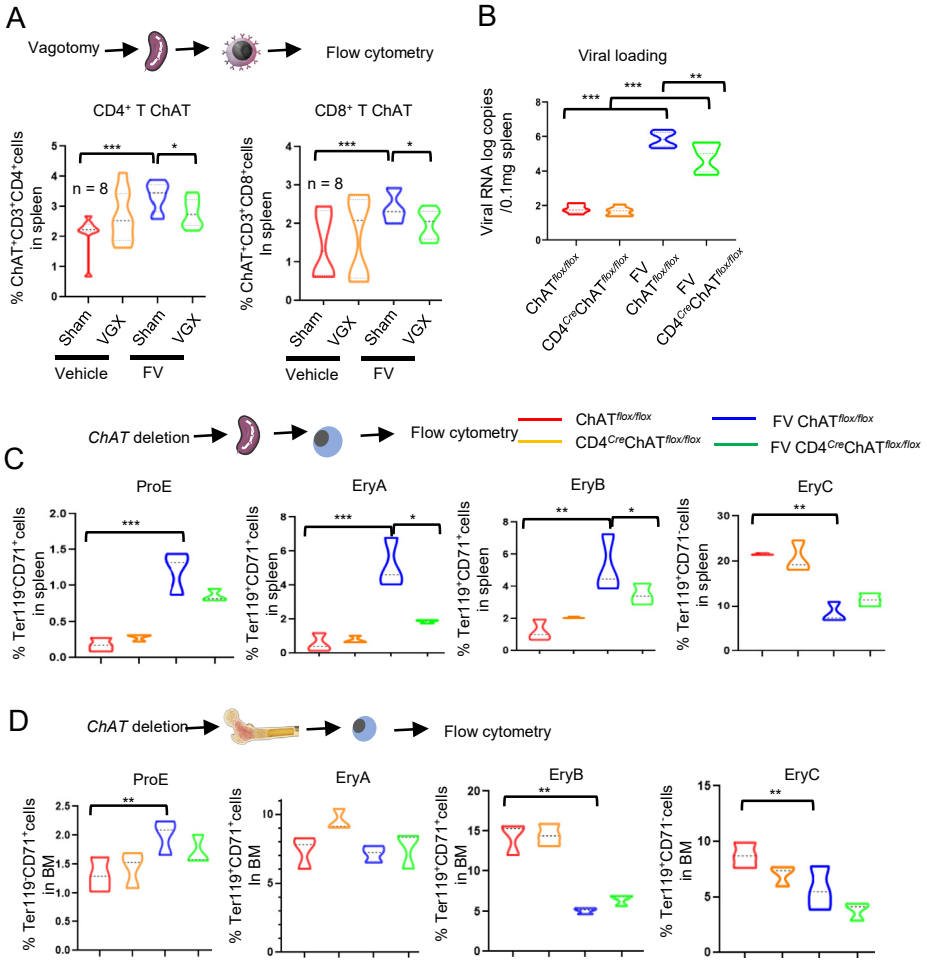

Fig. 3. Vagus nerve coupling with acetylcholinesterase (ChAT) in $CD4^+$ T cells promotes the erythroleukemia induced by FV. **A** The right vagus nerve was severed in the vagotomy (VGX) group. The sham group received an incision but kept the vagus nerve intact. Mice recovered for 7 days and were then infected with FV (5×10^8 copies). ChAT production levels in $CD4^+$ and $CD8^+$ T cells in the spleen were detected by flow cytometry (n = 8 in each group) at five days post-infection. **B** $ChAT^{flox/flox}$ *and* $CD4^{Cre}ChAT^{flox/flox}$ mice were infected with FV (5×10^8 copies), respectively. Mice were euthanized five days after infection. Viral loads in spleens were detected by qPCR (in each group). The frequency of erythroblasts in each stage of spleen (**C**) and bone marrow (BM, **D**) was measured by flow cytometry (n = 5). The data are the mean ± standard deviation, and the results were analyzed by ANOVA and representative of two independent experiments. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

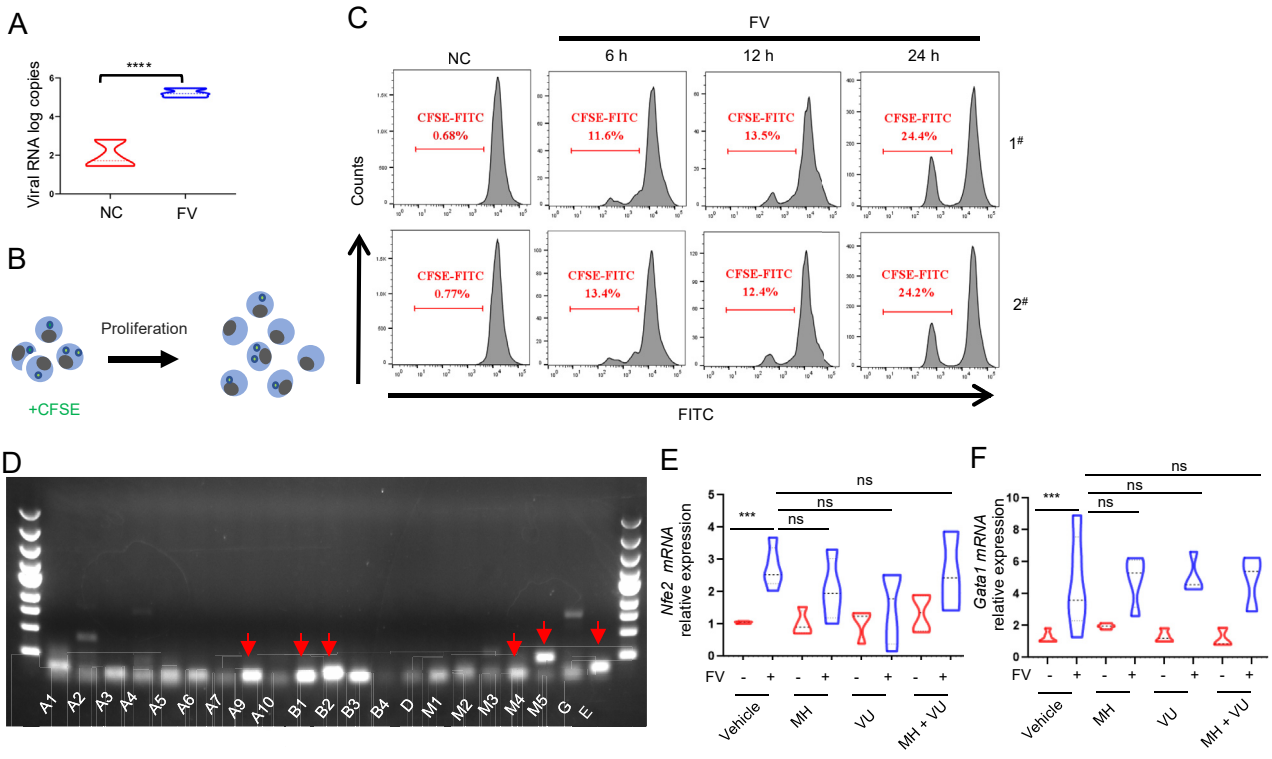

Fig. 4. FV induces proliferation of MEL cells and these cells express muscarinic acetylcholine receptors, whereas activation of mAChR5 does not affect erythropoietic genes. **A** Change of viral load in FV- MEL cells. 2×10^5 MEL cells were infected with FV (5×10^8 copies) for 48 h. The viral loading of cells was detected by RT-PCR. $^{****}P < 0.0001$. **B, C** The proliferation of MEL was detected by CFSE tracing. MEL cells were labeled by CFSE, then infected with FV for 6, 12 or 24 h and flow cytometry was performed. Two independent experiments (1# and 2#) were conducted. **D** The expressions of various acetylcholine receptors in MEL were detected by RT-PCR and electrophoresis. The high expression of acetylcholine receptors was indicated by red arrowhead. **E, F** The effect of mAChR5 agonist on erythropoiesis genes. 2×10^5 MEL cells were incubated respectively with MH, VU or both for 24 h, then infected with FV (5×10^8 copies) for 48 h. The expressions of *Nfe2* and *Gata1* were detected by RT-PCR and normalized by *GAPDH*. MH, Milameline hydrochloride, 50 μmol/L; VU, VU0238429, 2 μmol/L. Two independent experiments were conducted. n = 4–5 in each group. Data are mean ± standard deviation, and the experimental results were analyzed by ANOVA. $^{***}P < 0.001$.

Studies have shown that nAChR plays an important role in HIV-associated neurocognitive disorders (HAND) (Capo-Velez et al., 2018). Activation of α7 nAChR leads to significant neuroprotection against HIV-1 Tat-induced HAND (Zhao X. et al., 2021). HIV gp120 protein can upregulate the expression of α7 nAChR on the surface of monocyte-derived macrophages in human blood through the CXCR4 pathway (Gundavarapu et al., 2013). So far, it is unclear whether the vagus nerve modulates FV infection. In this study, we found that acetylcholine receptors, especially *Chrna9*, *Chrnb1–3*, *Chrm4–5*, were expressed on mouse erythroleukemia cells. Since only mAChR4 (encoded by *Chrm4*) and mAChR5 (encoded by *Chrm5*) agonists are commercially available, we investigated the role of these two receptors in FV infection. FV infection increased MEL proliferation, but activation of mAChR5 did not affect FV infection-promoted erythropoiesis genes (Fig. 4E and F). However, activation of mAChR4 can promote FV replication in the spleens, and increase the expression of erythropoiesis genes (Fig. 5A and B). *In vitro*, FV infection promotes mAChR4 expression in MEL, and activation of mAChR4 by CNO boosts the expression of erythropoiesis gene *Spi1* (Fig. 6A–C). Deletion of *ChAT* in $CD4^+$ T cells reduced FV replication in the spleens and correspondingly decreased proliferation of erythroblasts in the spleens (Fig. 3C). These data are different from our *in vivo* finding that vagotomy did not affect the viral loads in the spleen, blood, and bone marrow (Fig. 1C). One possible explanation is that the vagus nerve, distinct from ChAT, may have an unknown mechanism regulating FV replication.

Although the role of the vagus nerve in the replication of the FV during acute infection was not found, we found that after the right vagus nerve was cut, the level of virus-induced pro-erythrocytic proliferation in the spleen was reduced, the symptoms of host anemia were relieved, and the signaling factors of erythrocyte differentiation (*Gata1*, *Nfe2*, *Klf1*) were significantly reduced (Figs. 1D, 2B and 2C). This suggests that the vagus nerve plays a contributing role in the pathogenesis of erythroleukemia.

Before the vagus nerve enters the spleen, it needs to be passed through the celiac ganglia, and the signals generated by the vagus nerve are received by the sympathetic splenic nerve (Ding et al., 2019). NE released from sympathetic nerve endings stimulates spleen $CD4^+ChAT^+$ T cells to produce acetylcholine (Rosas-Ballina et al., 2011), which promotes erythroblast proliferation and differentiation. In this study, we found that FV infection increased ChAT expression in splenic $CD4^+$ and $CD8^+$ T cells, which could be blocked by vagotomy (Fig. 3A). This finding suggests that the vagus nerve promotes the expression of ChAT expression in $CD4^+$ T cells. More importantly, both vagotomy and loss of ChAT in $CD4^+$ T cells reduced splenic EryA and EryB numbers (Fig. 2B, C, 3B), supporting that vagus nerve-ChAT expressing $CD4^+$ T cells mediate FV infection-induced abnormal splenic erythropoiesis.

Erythropoiesis occurs mostly in bone marrow and ends in blood stream. Maturation from erythroid-committed precursors is called terminal erythropoiesis and occurs in the bone marrow within

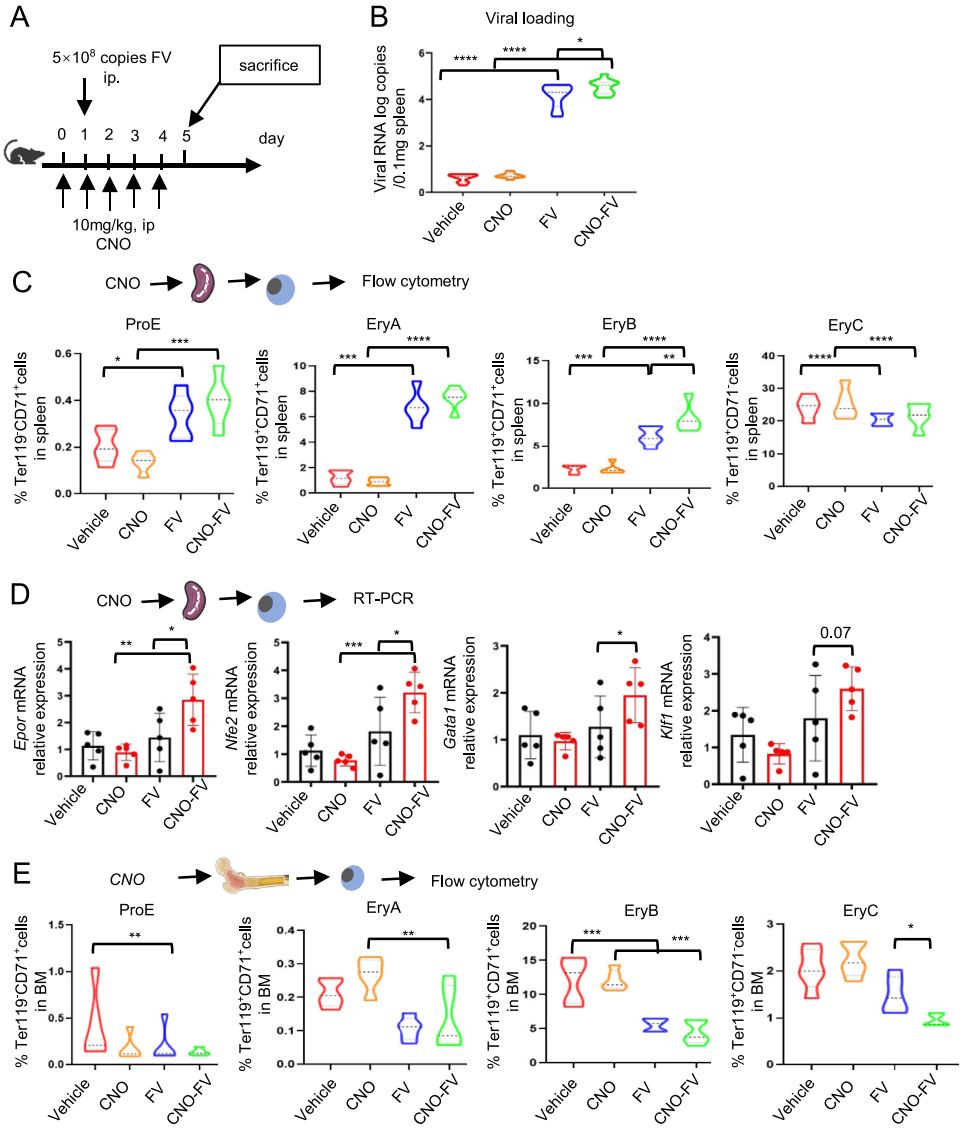

Fig. 5. Effects of mAChR4 agonist (CNO) on viral load, differentiation and proliferation of erythroblasts in FV-infected mice. **A** Mice were injected via i.p. with either vehicle or CNO (10 mg/kg) 24 h before infection, then administrated every day till sacrificed. Mice were euthanized 5 days after infection. **B** The viral loads in spleen were detected by qPCR. **C, D** The effect of mAChR4 agonist on frequencies of erythroblasts and expression of erythropoiesis genes in spleen. The frequencies of erythroblasts at each stage in spleen were detected by flow cytometry. Real-time quantitative PCR was used to detect the mRNA level of erythrocyte differentiation-related genes *Epor*, *Nfe2*, *Gata1,* and *Klf1*, which normalized by *GAPDH.* **E** The frequencies of erythroblasts at each stage in bone marrow (BM) were detected by flow cytometry. Data are mean ± standard deviation, and the results were analyzed by ANOVA and representatives of two independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, and $^{****}P < 0.0001$. n = 4–5 in each group.

erythroblastic islands (Moras et al., 2017). Acetylcholinesterase (AChE) hydrolyzes acetylcholine at cholinergic synapses. In the erythrocytes of *Ache*$^{-/-}$ mice, the amount of hemoglobin, especially α-globin, was found to be significantly reduced, suggesting that AChE plays a role in erythroblast maturation (Xu et al., 2019). In our study, FV infection decreased EryC and impaired erythrocyte maturation in bone marrow. This process can be reversed by vagotomy, but is facilitated by the activation of mAChR4 by its agonist CNO, confirming the blockade of vagal-mAChR4 signaling in erythrocyte maturation in bone marrow (Figs. 2D and 5E). Considering that the vagus nerve can directly innervate the bone marrow (Chen et al., 2020) and does not require CD4^{+} T cells to produce ACh, the absence of ChAT in CD4^{+} T cells does not affect the maturation of EryC in the bone marrow.

F-MuLV is the replication-competent "helper" virus, which is necessary for replication and packaging of SFFV, and SFFV is the replication-defective component. SFFV plays a role in the early stage of FV disease and a crucial role in the late stage of the disease *in vivo* (Jones et al., 1988). The pre-leukemic phase of the disease results from the abnormal activation of the erythropoietin (Epo) receptor by the gp55 *env* gene product of SFFV (Pereira et al., 2000). NF-E2 activity is essential for erythroid differentiation of murine erythroleukemia cells (Nagai et al., 1998). Erythroid Kruppel-like factor (EKLF/KLF1) is an erythroid specific, C(2)H(2) zinc finger transcription factor that is essential for the proper chromatin structure and expression of the adult beta-globin gene (Quadrini and Bieker, 2006). Peak GATA-1 and KLF1 mRNA levels preceded the globin gene peak, but the highest NF-E2 levels coincided with maximal globin levels, suggesting a role of NF-E2 in the maintenance, rather than the initiation of globin gene expression (Hodges et al., 1999). We found that activation of mAChR4 by CNO significantly increased erythropoiesis genes (*Epor*, *Nfe2*, and

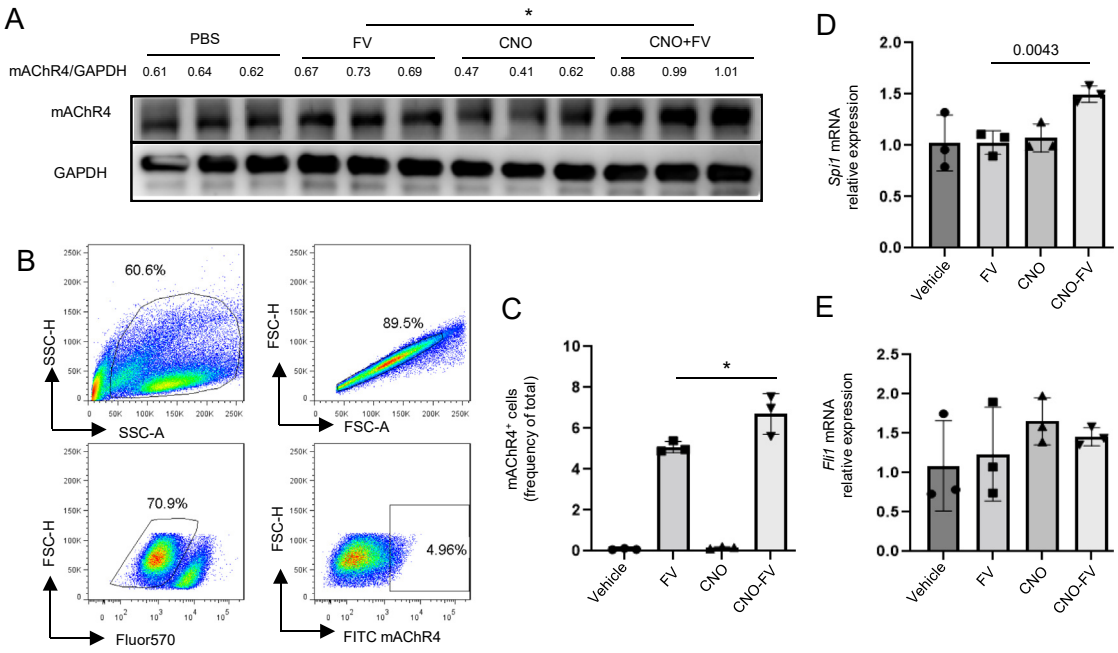

Fig. 6. FV infection increases mAChR4 expression and activation of mAChR4 enhances *Spi1* expression in FV-infected MEL cells. 2×10^5 MEL cells were incubated respectively with CNO for 24 h, then infected with FV infection (5×10^8 copies) for 48 h. **A** Western blotting of mAChR4 in PBS, FV, CNO, and CNO + FV treated MEL cells at 48 h. *$P < 0.05$. **B, C** Flow cytometric analysis of mAChR4 expression. *$P < 0.05$. **D, E** *Spi1* and *Fli1* expression in PBS, FV, CNO, and CNO + FV treated MEL cells at 48 h detected by RT-PCR. The cell concentration 2×10^5/well, 200 μL viral aliquot, CNO, 10 nmol/L n = 3 in each group. Data are mean ± standard deviation, and the experimental results were analyzed by unpaired *t*-test. $P < 0.05$ was considered significant difference.

Gata1) in FV-infected mouse spleens (Fig. 5D), which supports our hypothesis that activation of mAChR4 promotes FV-induced acute erythroleukemia.

Spi-1 gene activation is a general feature in the malignant proerythroblastic transformation which occurs in mice infected with Friend and Rauscher viruses (Moreau-Gachelin et al., 1990). Constitutive expression of *Spi-1* is related to the block of the differentiation of erythroid precursors (Moreau-Gachelin et al., 1996). High expression of *Spi-1* in erythroid cells may be the cause of the block in differentiation that is characteristic of SFFV-transformed erythroid cells (Ruscetti, 1995). Thus, high expression of *Spi1* in CNO-treated FV-infected MEL (Fig. 6D) might be an important factor contributing to acute erythroleukemia.

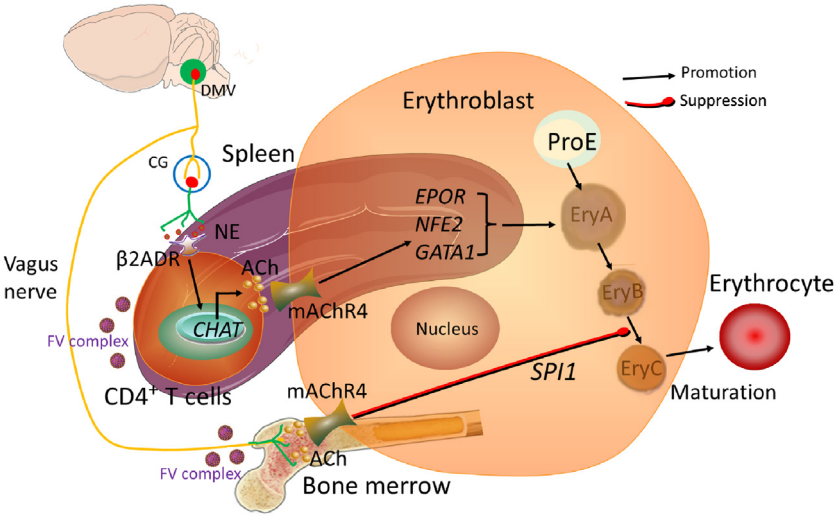

Fig. 7. The hypothetical model: vagal-muscarinic acetylcholine receptor 4 (mAChR4) signaling promotes FV-induced acute erythroleukemia. Vagus nerve innervation in the spleen drives acetylcholine (ACh) production in choline acetyltransferase (ChAT)-expressed $CD4^+$ T cells during FV acute infection. The ACh activates mAChR4 in erythroblasts and boosts their proliferation and differentiation. In bone marrow, vagus nerve endings directly release ACh, which activates mAChR4 and suppresses maturation of EryC. Vagal-mAChR4 signaling in spleen and bone marrow synergistically contributes to the pathogenesis of acute erythroleukemia. DMV, dorsal motor nucleus of vagus nerve; CG, cardiac ganglion; NE, noradrenaline; β2ADR, recombinant adrenergic receptor beta 2; ChAT, choline acetyl-transferase; ProE, progenitor erythroblast; EryA, erythroblast A; EryB, erythroblast B; EryC, erythroblast C.

Taken together, vagus nerve innervation in the spleen drives ACh production in ChAT-expressed $CD4^+$ T cells during FV acute infection. The ACh activates mAChR4 in erythroblasts and boosts their proliferation and differentiation. In bone marrow, vagus nerve endings directly release ACh, which activates mAChR4 and suppresses maturation of EryC. Vagal-mAChR4 signaling in spleen and bone marrow synergistically contributes to the pathogenesis of acute erythroleukemia (Fig. 7). We have revealed an unrecognized mechanism of neuroimmune regulation of erythroleukemia. However, in this study, we only examined the influence of vagus nerve on short-time FV infection, which is needed to be done for a longer period of infection. Although we observed the anemia after 5 dpi, other indicators of erythroleukemia, such as splenomegaly, did not appeared but seems to need to be measured. Moreover, and the molecular mechanism needs further study.

5. Conclusions

In conclusion, vagotomy reversed the increase in ProE, EryA, and EryB cells in the spleen and the decrease in EryC cells in the bone marrow caused by FV infection; loss of *ChAT* in $CD4^+$ T cells reversed the increase in EryA and EryB cells in FV-infected spleen; and activation of mAChR4 increased splenic EryB but decreased bone marrow EryC cell populations from FV-infected mice. Vagus-mAChR4 signaling increases erythroblast proliferation and blocks its maturation, thereby promoting the development of acute erythroleukemia.

Data availability

The GeneBank accession number of the virus strain: OQ718806. The original contributions presented in the study are included in the article/Supplementary data, and further inquiries can be directed to the corresponding authors.

Ethics statement

The study protocol was approved by the Animal Ethics and Welfare Committee of the Institut Pasteur of Shanghai, Chinese Academy of Sciences, and adhered to the guidelines for the Rules for the Implementation of Laboratory Animal Medicine (1998) from the Ministry of Health, China.

Author contributions

Shuting Song: conceptualization, methodology, investigation, formal analysis, software, visualization and writing (original draft). Zhekai Lin: methodology, investigation, formal analysis, and writing (original draft). Caiqi Zhao: investigation, animals, discussions, and resources. Jing Wen: investigation, animals, discussions, and resources. Jie Chen: investigation, animals, discussions, and resources. Shitao Xie: investigation, animals, discussions, and resources. Huaxin Qi: Investigation, animals, discussions, and resources. Jianhua Wang: Resources. Xiao Su: conceptualization, project administration, funding acquisition, and writing (review and editing).

Conflict of interest

The authors declare that they have no competing interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.virs.2023.05.005178-186.

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