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# Melatonin enhances NK cell function in aged mice by increasing T-bet expression via the JAK3-STAT5 signaling pathway

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**Abstract**

Natural killer (NK) cells are crucial innate immune cells that provide defense against viruses and tumors. However, aging is associated with alterations in NK cell composition and compromised cell functions. Melatonin, known for its anti-tumor effects, has been reported to improve NK cell function. However, the molecular mechanism underlying melatonin's effect on senescent NK cells remains unclear. In this study, we aimed to elucidate the mechanism by which melatonin enhances the function of senescent NK cells. Our findings revealed that melatonin significantly increased the number and function of NK cells in aging mice. The results suggest that melatonin enhances NK cell proliferation, degranulation, and IFN-γ secretion. Further investigations demonstrated that melatonin promotes NK cell maturation and activation, mainly via the JAK3/STAT5 signaling pathway, leading to increased expression of T-bet. These discoveries provide a theoretical basis for potential immunotherapy strategies based on melatonin-mediated modulation of NK cell function in aging individuals.

**Keywords** Melatonin, NK function, T-bet, Aging, JAK3-STAT5

## Introduction

Natural killer (NK) cells are a critical component of the innate immune system, playing a pivotal role in controlling cell cancerization and infection [31]. NK cells originate from hematopoietic stem cells in the bone marrow and can be found in various peripheral tissues, including lymphatic and non-lymphatic organs, such as the spleen, lymph nodes, liver, lung, and fat [7]. In response to infection or cancer cells, NK cells can be activated through activating receptors or down-regulation of MHC-I molecules. Upon activation, NK cells rapidly release cytotoxic molecules, pro-inflammatory cytokines, and chemokines to eliminate target cells and regulate immune responses [24]. The functions of NK cells are diverse, but their primary role is to exert cytotoxic effects on cells with abnormal MHC-I expression.

Aging is closely associated with significant changes in the immune system, affecting both innate and

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adaptive immunity [23]. NK cells, in particular, are adversely affected by aging, resulting in alterations in both their proportion and number. For instance, the $CD56^{bright}CD16^{-}$ NK cell subpopulation in the peripheral blood of aging individuals declines, while the $CD56^{dim}CD16^{+}$ NK cell subpopulation increases. Consequently, the ability of NK cells in elderly individuals to secrete cytokines and chemokines is reduced, leading to compromised immune function [2, 26]. The decrease in $CD56^{bright}$ NK cell subsets in aging individuals is attributed to reduced immature NK cell export from the bone marrow, indicating that aging affects NK cell subgroups within the bone marrow [11]. However, the precise molecular mechanisms underlying the decline in NK cell function with aging remain unclear, underscoring the need to identify therapeutic agents capable of activating senescent NK cells.

Melatonin, a substance known for its anti-tumor effects, also serves as an adjunctive treatment in tumor therapies such as chemotherapy and radiotherapy [20]. A critical role of melatonin is in modulating the anti-tumor immune response. As a time-varying hormone, melatonin is mainly synthesized and secreted by the pineal gland [9]. It acts as an endogenous free radical scavenger and indirect antioxidant, participating in the regulation of cellular redox reactions. Notably, the removal of the pineal gland is associated with immune cell loss, and various cytokines and growth factors, including IFN-γ, granulocyte-macrophage colony-stimulating factor, and granulocyte colony-stimulating factor, can stimulate melatonin release [17]. Additionally, immune cells such as $CD4^{+}$ Th cells and cytotoxic T lymphocytes (CTLs) express melatonin receptors, and melatonin can stimulate the release of IL-2 through melatonin receptor 1, ultimately leading to an increase in the number of NK cells [3, 14]. Moreover, melatonin enhances the antigen presentation ability of macrophages, leading to CTL activation and proliferation. It also promotes CTL proliferation and anti-tumor activity by stimulating anti-tumor factors such as IFN-γ, TNF-α, IL-6, while inhibiting IL-4 [19]. Nevertheless, the molecular mechanism by which melatonin improves NK cell function remains elusive.

In our study, we observed that melatonin improves the secretion of IFN-γ and degranulation in NK cells. Furthermore, melatonin treatment led to an increased number of NK cells in the spleen, bone marrow, and liver of aging mice, likely due to its ability to enhance NK cell proliferation. Melatonin was also found to promote the maturation and activation of NK cells in aging mice, which likely contributes to the improvement of their function. We further investigated the molecular mechanism of melatonin's effect on NK cell function, discovering that melatonin treatment increased the activation level of NK cells and up-regulated the Janus tyrosine Kinase 3 (JAK3)/Signal Transducer and Activator of Tranion (STAT5) signaling pathway, subsequently leading to increased the expression of T-bet which is an important transcription factor for the immune system, orchestrating NK cell immune responses. Therefore, it is speculated that melatonin enhances the function of NK cells in aging mice by up-regulating T-bet expression through the JAK3/STAT5 signaling pathway. Our research has revealed a novel function of melatonin in enhancing NK cell function and elucidated its molecular mechanism for regulating NK cell function. These findings provide a new theoretical basis for immunotherapy based on NK cells.

## Materials and methods

### Animals and Experimental Design

The animal research protocols conducted in this study were approved by the Animal Ethics Committee of The Sixth Affiliated Hospital, School of Medicine, South China University of Technology. Aging C57BL/6 mice (18 months old) were obtained from Guangdong Medical Animal Center (Foshan, China) and housed in temperature-controlled (22±1 °C) quarters with a 12/12 light-dark (LD) cycle and ad libitum access to water and food. The mice were divided into two groups: the melatonin treatment group and the control group. In the melatonin treatment group, aged mice were orally administered with 10 mg/kg of melatonin once every two days for one month.

### Flow Cytometry

Flow cytometry analysis was performed using a BD CANTO II (BD Biosciences), and cell sorting was conducted on a BD Aria II (BD Biosciences). Monoclonal antibodies specific for mouse CD3, NK1.1, NKp46, CD71, CD98, CD11b, CD27, IFN-γ, CD107a, Annexin V, ki67, JAK3, p-STAT5, T-bet, Eomes, STAT5, IL-2, and isotype controls were obtained from eBioscience (San Diego, CA) or Biolegend (San Diego, CA). For the analysis of NK cell surface markers, antibodies were diluted in PBS containing 2% FBS. For the analysis of intracellularly expressed proteins, cells were fixed and permeabilized with Phosflow Perm buffer III (BD) and subsequently stained with the appropriate antibodies.

### Detection of CD107a Expression and Intracellular Staining of IFN-γ

In vitro experiments involved intraperitoneal injection of 200 μg poly I: C in each mouse. After 18 h, NK cells ($1\times10^{6}$) were sorted from the spleen of wild-type mice using FACS. NK cells were then treated with melatonin (100μM) for 24 h and/or co-cultured with RMA-S tumor cells. GolgiStop™ reagent (BD Biosciences) was added to inhibit intracellular protein release. Subsequently, APC-conjugated anti-CD107a antibody, PE-conjugated

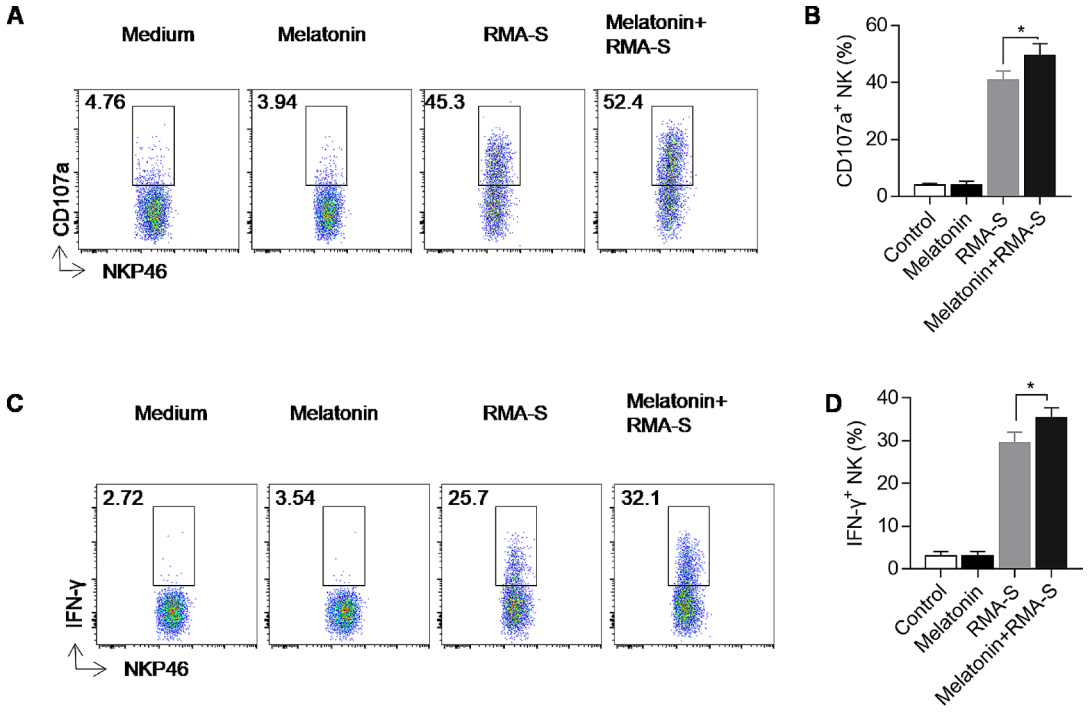


**Fig. 1** Melatonin treatment enhances the expression of CD107a and the secretion of IFN-γ in NK cells. (**A**) NK cells were isolated from mouse spleen, subjected to in vitro melatonin treatment, and subsequently stimulated with RMA-S cells to assess the NK cells' capability to secrete CD107a. (**B**) Statistical chart representing CD107a secretion by NK cells. (**C**) Isolating NK cells from mouse spleens, subjecting them to in vitro melatonin treatment, and then stimulating them with RMA-S cells to assess the NK cells' potential to secrete IFN-γ. (**D**) Statistical chart depicting IFN-γ secretion by NK Cells. The data is presented as means ± SD. $n = 6$. Statistical analysis was performed using Prism software with one-way ANOVA. A significance level of < 0.05 was deemed as statistically significant. *$P < 0.05$

anti-IFN-γ, or isotype controls were added. For in vivo experiments, aging mice were treated with melatonin (10 mg/kg) for 3 months. Each mouse was intraperitoneally injected with 200 μg poly I: C to activate NK cells. After 18 h, poly I: C-activated spleen cells ($2\times10^6$) were co-cultured with the same number of RMA-S cells. GolgiStop™ reagent (BD Biosciences) was added, followed by APC-conjugated anti-CD107a antibody, PE-conjugated anti-IFN-γ, or isotype controls. Medium only was used as a negative control. After 4 h, cells were harvested for the detection of intracellular IFN-γ and CD107a.

### Detection of Apoptosis and Proliferation of NK Cells by FACS

Splenocytes were stained with antibodies against Nkp46, Annexin V, and Ki67 (eBiosciences). Fluorescence intensity was measured by FACS according to the manufacturer's instructions (BD Biosciences).

### Inhibition of the expression of STAT5 in NK cells

Splenic NK cells were isolated from aged mice (1×10^6), pre-treated with BD750 (STAT5 antagonist, 1μM, MCE: HY-131140) for 2 h, followed by treatment with 100μM melatonin for 24 h, The expression of STAT5 and T-bet in NK cell was detected using flow cytometry.

### Block melatonin receptors on NK cells

Splenic NK cells were isolated from aged mice (1×10^6), pre-treated with S26131 (melatonin receptor 1 antagonist, 10μM, MCE: HY-122136) or 4-P-PDOT (melatonin receptor 2 antagonist, 10μM, MCE: HY-100609) for 1 h, followed by treatment with 100μM melatonin for 24 h, the expression of CD107a and IFN-γ was detected using flow cytometry.

### Statistical analysis

All experimental results were expressed as the mean ± standard deviation (SD) and were plotted using GraphPad Prism 7 software (IBM Corp., Armonk, NY, US). Unpaired Student's t-tests (two-tailed) were performed using GraphPad Prism 7, Tukey's test or Dunnett's post-tests were performed based on the ANOVA for multivariate data analysis using SPSS 22.0 (SPSS Inc., Chicago, IL, US). A P-value of less than 0.05 was considered statistically significant (* denotes significance compared to control: *$P<0.05$, **$P<0.01$, and ***$P<0.001$).

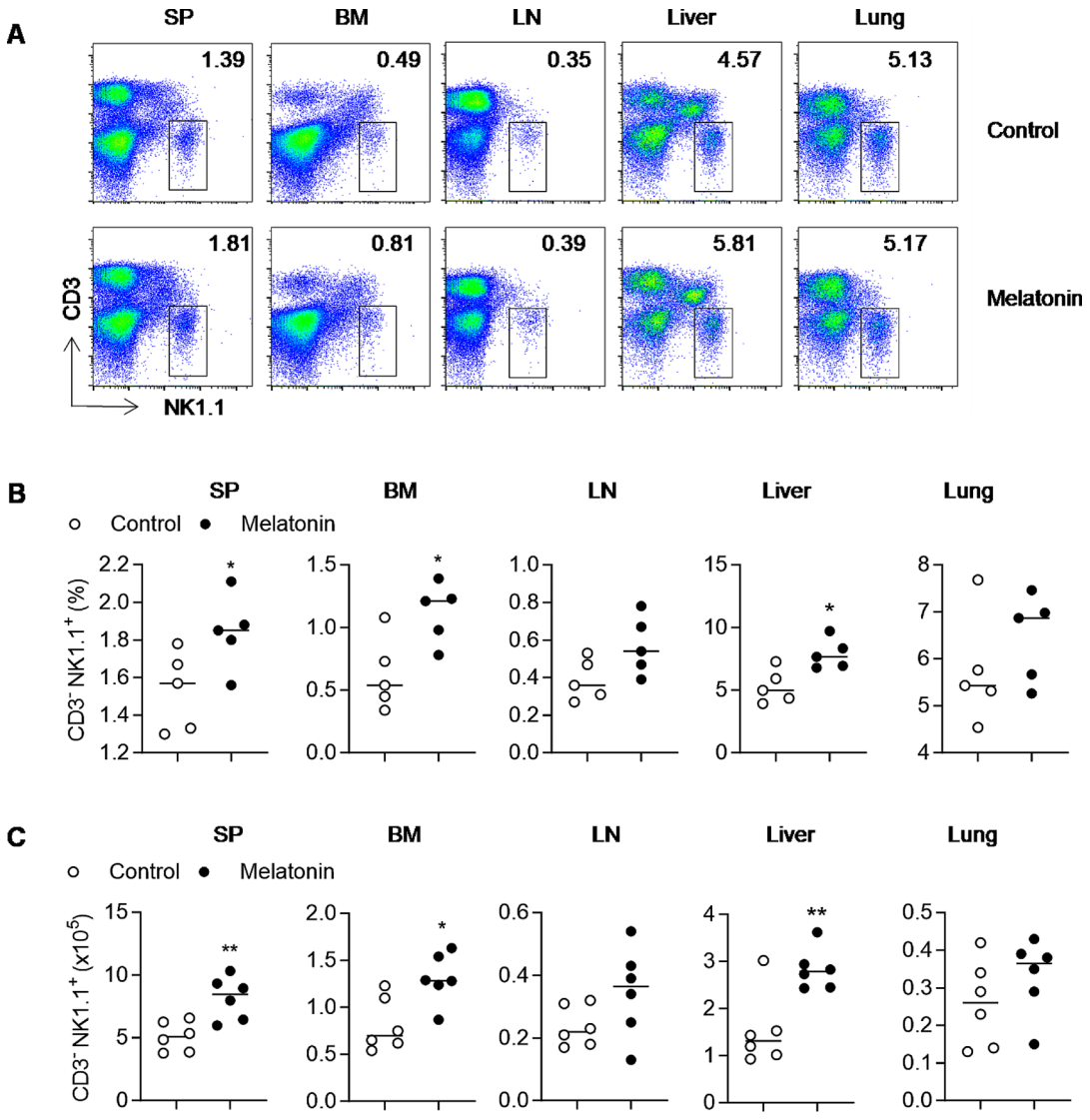


**Fig. 2** Melatonin treatment results in an increased quantity of NK cells in the spleen, bone marrow, and liver of aged mice. (**A**) Following melatonin administration, flow cytometry was employed to analyze the proportion of NK cells in the spleen, bone marrow, lymph nodes, liver, and lungs of the mice. (**B**) Statistical graph depicting the proportion of NK Cells in various mouse organs. (**C**) Statistical graph illustrating the absolute numbers of NK Cells in various mouse organs. The data is presented as means ± SD. $n = 6$. Unpaired two-tailed Student's t-tests were conducted utilizing Prism software. A p-value of less than 0.05 was deemed statistically significant. $^{*}P < 0.05$, $^{**}P < 0.01$

## Result

### The addition of melatonin improves the degranulation and IFN-γ secretion abilities of RMA-S co-cultured NK cells

Our previous studies have revealed that disrupting the circadian rhythm of mice impairs the immune surveillance capacity of NK cells, emphasizing the importance of maintaining a normal circadian rhythm for NK cell function [36]. Melatonin, a hormone secreted by the pineal gland, is known to regulate circadian rhythms. Tian et al. [27] demonstrated NK cell recovery activity in aged mice after melatonin supplementation. Currier et al. (2000) showed an increase in the absolute number of NK cells in the spleen and bone marrow of mice fed melatonin compared to control mice. In this study, we conducted in vitro experiments by adding melatonin to explore its effect on NK cell function. Our results demonstrate that the additional addition of melatonin increases the expression of CD107a in NK cells co-incubated with RMA-S cells (Fig. 1A, B). Furthermore, melatonin supplementation also enhances the ability of NK cells co-incubated with RMA-S cells to secrete IFN-γ (Fig. 1C, D). These findings lead us to speculate that melatonin treatment can improve the function of NK cells.

### In vivo Melatonin treatment increases the number of NK cells in the spleen, bone marrow, and liver of aged mice

Given the positive effects of melatonin on NK cell function observed in vitro, we were curious to investigate

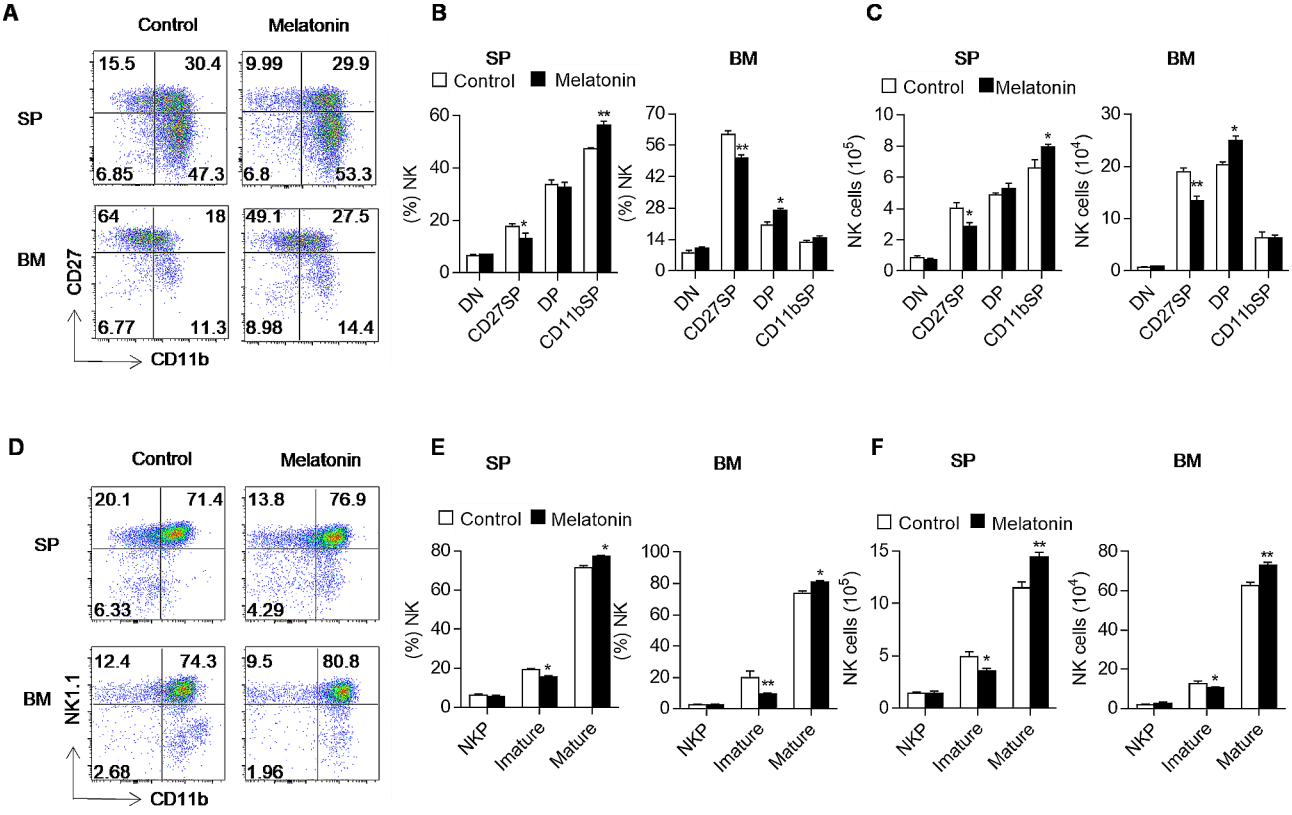


**Fig. 3** Melatonin treatment enhances the maturation of NK cells in the spleen and bone marrow of aged mice. (**A**) Following melatonin treatment, flow cytometry was employed to observe the progression of NK cell development, with CD27 and CD11b serving as markers for distinct developmental stages of NK cells. (**B**) Statistical graph illustrating the proportion of NK Cell subsets in the spleen and bone marrow of mice across various developmental stages. (**C**) Statistical graph depicting the absolute numbers of NK cell subsets in the spleen and bone marrow of mice across various developmental stages. (**D**) Following melatonin treatment, NK cell development was assessed using a flow cytometer, with NK1.1 and CD11b serving as markers for distinct developmental stages of NK cells. (**E**) Statistical graph representing the proportion of NK cell subsets in the spleen and bone marrow of mice across various developmental stages. (**F**) Statistics depicting the absolute numbers of NK cell subsets across different developmental stages in mouse spleen and bone marrow. The data is presented as means ± SD. $n = 6$. Unpaired two-tailed Student's t-tests were conducted using Prism software. A p-value of less than 0.05 was deemed statistically significant. $^{*}P < 0.05$, $^{**}P < 0.01$

whether melatonin treatment also impacts the number of NK cells in the immune organs of aged mice. To explore this, we administered melatonin to aging wildtype mice and isolated lymphocytes from the spleen, bone marrow, lymph nodes, liver, and lungs. By employing flow cytometry, we analyzed the proportion and number of NK cells in each lymphatic organ or tissue. Our results revealed a significant increase in the proportion and number of NK cells in the spleen, bone marrow, and liver of mice following melatonin treatment (Fig. 2A-C). This finding suggests that melatonin treatment contributes to maintaining a higher number of NK cells in the body, which may, in turn, enhance the immune function of elderly mice. However, when we treated young mice (8–12 weeks) with melatonin, we found that melatonin did not increase the number of NK cells in the spleen, bone marrow, and liver (Fig. S1A-C) nor did it enhance the function of NK cells (Fig. S1D-G).

### Melatonin treatment promotes the development and maturation of NK cells in aged mice

The proper development of NK cells is pivotal for determining their functionality. In light of this, we investigated the impact of melatonin treatment on the development of NK cells in the spleen and bone marrow of aged mice using flow cytometry. We utilized CD27 and CD11b markers to classify NK cells into four developmental stages: CD27 single positive (CD27SP, pre-stage), double positive (DP, immature), and CD11b single positive (CD11bSP, mature). Our results demonstrated a notable increase in mature NK cells, specifically in the CD11bSP subset, and a reduction in the CD27SP subset following melatonin treatment (Fig. 3A-C). Additionally, we employed another gating method using NK1.1 and CD11b markers to distinguish different developmental stages, including NK cell precursors (DN), immature NK cells (NK1.1SP), and mature NK cells (DP). Similar results were observed, with a significant increase in mature NK cells and a decrease in immature NK cells in

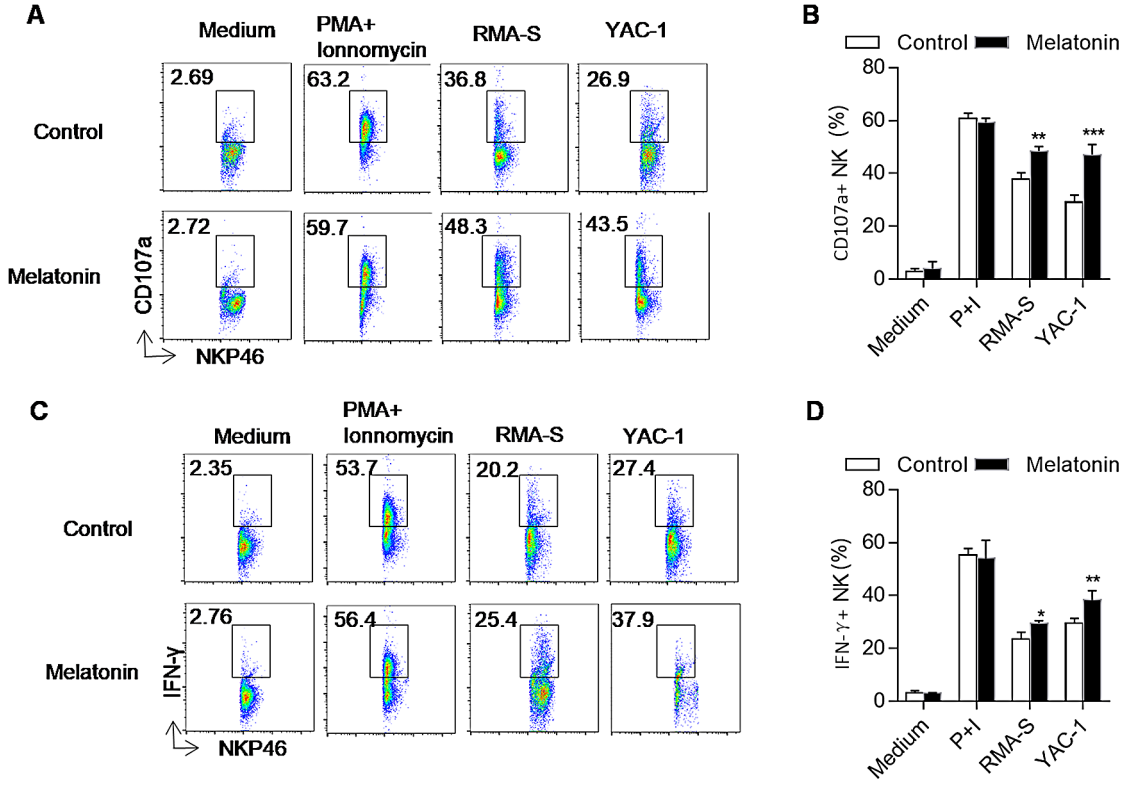


**Fig. 4** In vivo melatonin treatment enhances the capacity of NK cells in aged mice to exhibit CD107a expression and IFN-γ secretion. (**A**) Following melatonin treatment, spleen lymphocyte cells are collected. Subsequently, upon stimulation with RMA-S and YAC-1 cells, the secretion of CD107a is measured through flow cytometry. (**B**) Statistical chart representing CD107a secretion by NK cells. (**C**) Following melatonin treatment, spleen lymphocyte cells were collected. Subsequent to stimulation with RMA-S and YAC-1 cells, flow cytometry was employed to analyze the secretion of IFN-γ. (**D**) Statistical chart depicting IFN-γ secretion by NK Cells. The data is presented as means ± SD. $n = 6$. Unpaired two-tailed Student's t-tests were conducted using Prism software. A p-value of less than 0.05 was considered statistically significant. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$

both the spleen and bone marrow after melatonin treatment (Fig. 3D-F). These findings indicate that melatonin promotes the maturation of NK cells.

### Melatonin treatment improves NK cell degranulation and IFN-γ secretion in aging mice

To further validate the beneficial effects of melatonin on NK cell function in aged mice, we isolated splenocytes from aging mice treated with melatonin and stimulated them with RMA-S or YAC-1 tumor cells. Flow cytometry analysis was conducted to assess the expression of CD107a and IFN-γ in NK cells. The results showed an increase in CD107a expression in NK cells stimulated by RMA-S or YAC-1 after melatonin treatment, indicating an enhancement in NK cell degranulation (Fig. 4A, B). Additionally, melatonin treatment also augmented the ability of NK cells to secrete IFN-γ (Fig. 4C, D). These findings provide further evidence of melatonin's positive impact on NK cell function in aging mice.

### Melatonin treatment increases the proliferation of $CD27^{-}CD11b^{+}$ NK cells in the spleen of aged mice

To understand the underlying reason behind the increase in NK cell numbers after melatonin treatment in aged mice, we analyzed the proliferation and apoptosis of NK cells at various developmental stages in the spleen of melatonin-treated aging mice. Interestingly, we observed a significant increase in $Ki67^{+}$ cells among the total NK cell population, particularly in the $CD27^{-}CD11b^{+}$ subset (Fig. 5A, B). This finding suggests that melatonin treatment may increase the number of NK cells in aging mice by promoting the proliferation of $CD27^{-}CD11b^{+}$ mature NK cells. Furthermore, we evaluated NK cell apoptosis in melatonin-treated aged mice and found no significant changes in the total NK cell population or the number of Annexin $V^{+}$ cells in each developmental stage (Fig. 5C, D). These results suggest that melatonin's effects on NK cell numbers in aging mice are likely attributed to increased proliferation rather than reduced apoptosis.

### Melatonin treatment activates NK cells in aged mice

To gain insights into the mechanism underlying melatonin's positive influence on NK cell function in aged

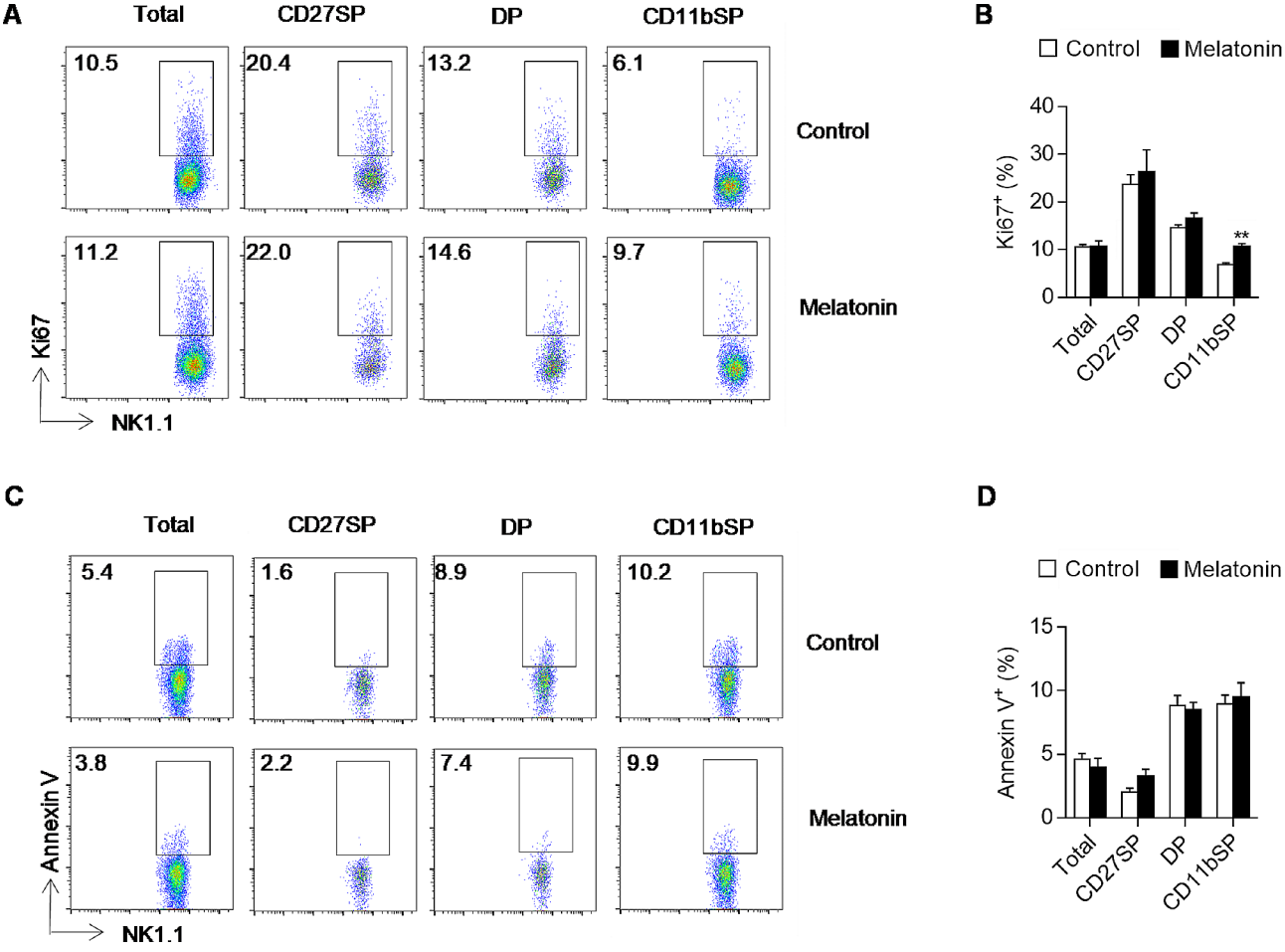


**Fig. 5** Melatonin stimulates the proliferation of CD11b single-positive NK cells in the spleen of aged mice. (**A**) Following melatonin treatment, flow cytometry was utilized to assess the expression of Ki67 in various developmental stages of NK cells. (**B**) Statistics of Ki67-positive cells in various subgroups of spleen NK Cells. (**C**) Following melatonin treatment, flow cytometry was employed to detect the expression of Annexin V in NK cell subgroups at different developmental stages. (**D**) Statistical Chart Depicting Annexin V-Positive Cells in Different Subgroups of Spleen NK Cells. The data is presented as means ± SD. $n = 6$. Unpaired two-tailed Student's t-tests were conducted using Prism software. A p-value of less than 0.05 was considered statistically significant. $^{*}P < 0.05$, $^{**}P < 0.01$

mice, we investigated the expression of activation markers CD71 and CD98 in melatonin-treated aged mice. CD71 and CD98 are nutrient receptors, and their expression levels reflect cell metabolism levels. Our results demonstrated significantly higher expression levels of CD71 on NK cells in melatonin-treated mice compared to aging mice. Specifically, the CD11bSP mature NK cell subset showed a notable increase in CD71 expression, while other developmental stages did not exhibit significant differences (Fig. 6A, B). Furthermore, we observed increased expression of CD98 in NK cells from melatonin-treated mice compared to aging mice (Fig. 6C, D). Notably, the expression of CD98 was significantly increased in most developmental stages, except for the early developmental stage of CD27SP. These findings indicate that melatonin may improve NK cell function by activating NK cells.

### Melatonin enhances T-bet expression through the JAK3/STAT5 signaling pathway to enhance NK cell function

To further elucidate the molecular mechanism underlying melatonin's positive effects on NK cell function, we focused on the signaling pathways involved in NK cell development and function, particularly the JAK3/STAT5 pathway. We assessed the changes in JAK3/STAT5 in NK cells of aged mice treated with melatonin and found a significant increase in JAK3 expression, particularly in the CD11bSP mature NK cell subset (Fig. 7A, B). Additionally, we observed a notable increase in the phosphorylation level of STAT5, a downstream molecule of JAK3, in the spleen NK cells of melatonin-treated mice, indicating the activation of STAT5 signaling (Fig. 7C, D). As T-bet is a crucial transcription factor involved in NK cell development and function, we investigated its expression in spleen NK cells of melatonin-treated mice. Remarkably, the expression of T-bet was significantly increased in

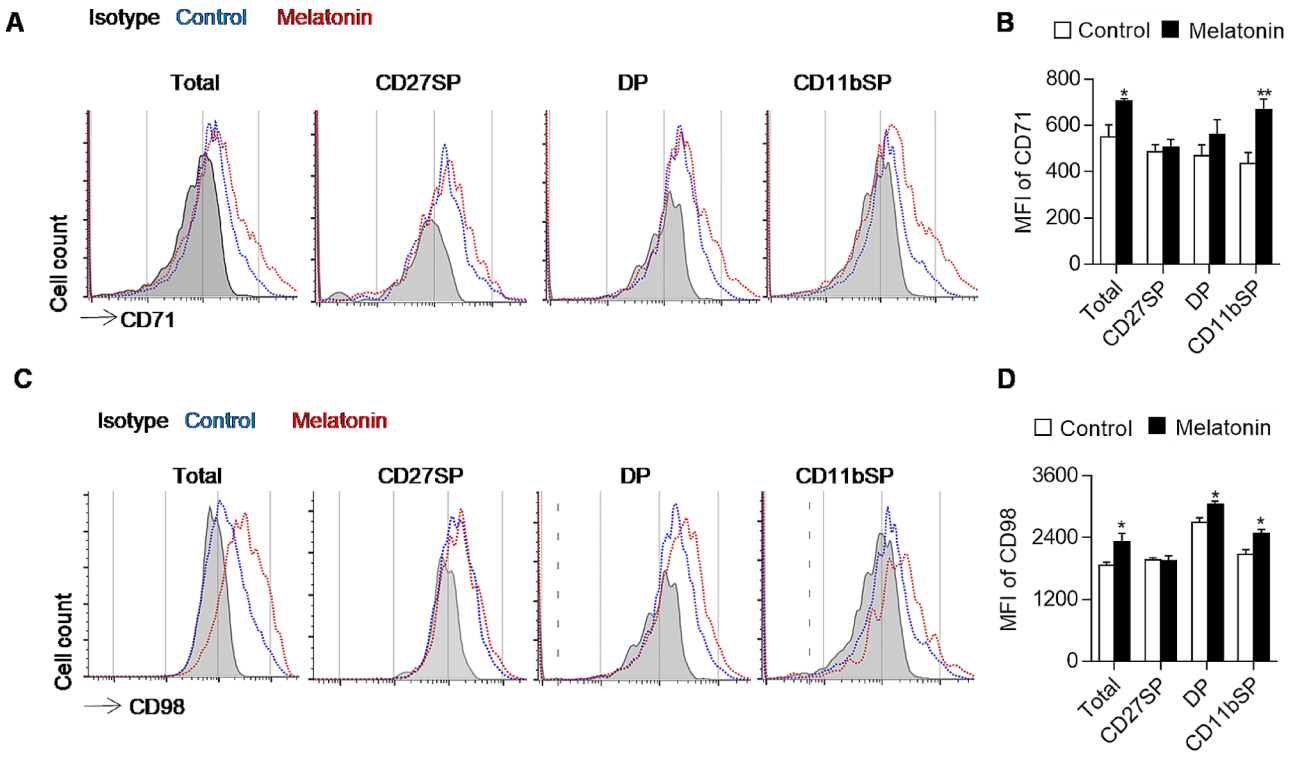


**Fig. 6** Melatonin enhances the expression of CD71 and CD98 in spleen NK cells of aged mice. (**A**) Following melatonin treatment, flow cytometry was employed to assess the expression of CD71 in NK cell subgroups at various developmental stages. (**B**) Statistical graph depicting CD71 fluorescence intensity in different subgroups of spleen NK Cells. (**C**) Following melatonin treatment, flow cytometry was employed to analyze the expression of CD98 in NK cell subgroups at various developmental stages. (**D**) Statistical graph illustrating CD98 fluorescence intensity in different subgroups of spleen NK Cells. The data is presented as means ± SD. $n = 6$. Unpaired two-tailed Student's t-tests were conducted using Prism software. A p-value of less than 0.05 was considered statistically significant. $^{*}P < 0.05$, $^{**}P < 0.01$

melatonin-treated mice, including in NK cells at various developmental stages (Fig. 7E-F). However, the expression of another important transcription factor regulating NK cell function, Eomes, did not change (Fig. 7G-H). We used the inhibitor BD750 to inhibit STAT5 expression in NK cells and examined the expression of T-bet. The results showed that after inhibiting STAT5, there was a significant decrease in T-bet expression in NK cells (Fig. 7I-L). From these findings, we speculate that melatonin enhances the expression of T-bet through the JAK3/STAT5 signaling pathway, ultimately augmenting the immune surveillance function of NK cells.

### Melatonin enhances T-bet expression through the JAK3/STAT5 signaling pathway to enhance NK cell function

We hypothesize that melatonin may enhance NK cell function either by directly activating intracellular signals through surface melatonin receptors on NK cells or indirectly by increasing IL-2 secretion from CD4+T cells. We treated aged mice NK cells with either the melatonin receptor 1 (MT1) antagonist S26131 or the melatonin receptor 1 (MT2) antagonist 4-P-PDOT, followed by melatonin treatment, and assessed NK cell expression of CD107a and IFN-γ.The results indicate that inhibiting melatonin receptors does not affect melatonin's effects on NK cells (Fig. 8A-D), suggesting that melatonin does not exert its effects directly through it's receptors expression on NK cells. We found that melatonin treatment significantly increases IL-2 expression in CD4+T cells of aged mice (Fig. 8E-F). Using a CD4+T cell sorting kit, we depleted CD4+T cells from mouse splenic mononuclear cells (PBMCs). Results showed that PBMCs depleted of CD4+T cells and treated with melatonin significantly reduced NK cell IFN-γ production (Fig. 8G-H), indicating that melatonin enhances NK cell function in aged mice by increasing IL-2 expression in CD4+T cells.

## Discussion

Melatonin, a hormone responsible for maintaining the body's circadian rhythm, gradually decreases with age due to reduced secretion by the pineal gland. This decline in melatonin content leads to reduced sleep quality and compromised immunity in aging mice (Li, Li, 2017; Reiter et al., [21, 30]. To investigate the impact of melatonin on NK cells in aging mice, we conducted experiments, which demonstrated that treating NK cells with melatonin in vivo improves their ability to degranulate and secrete IFN-γ. This finding highlights the significant

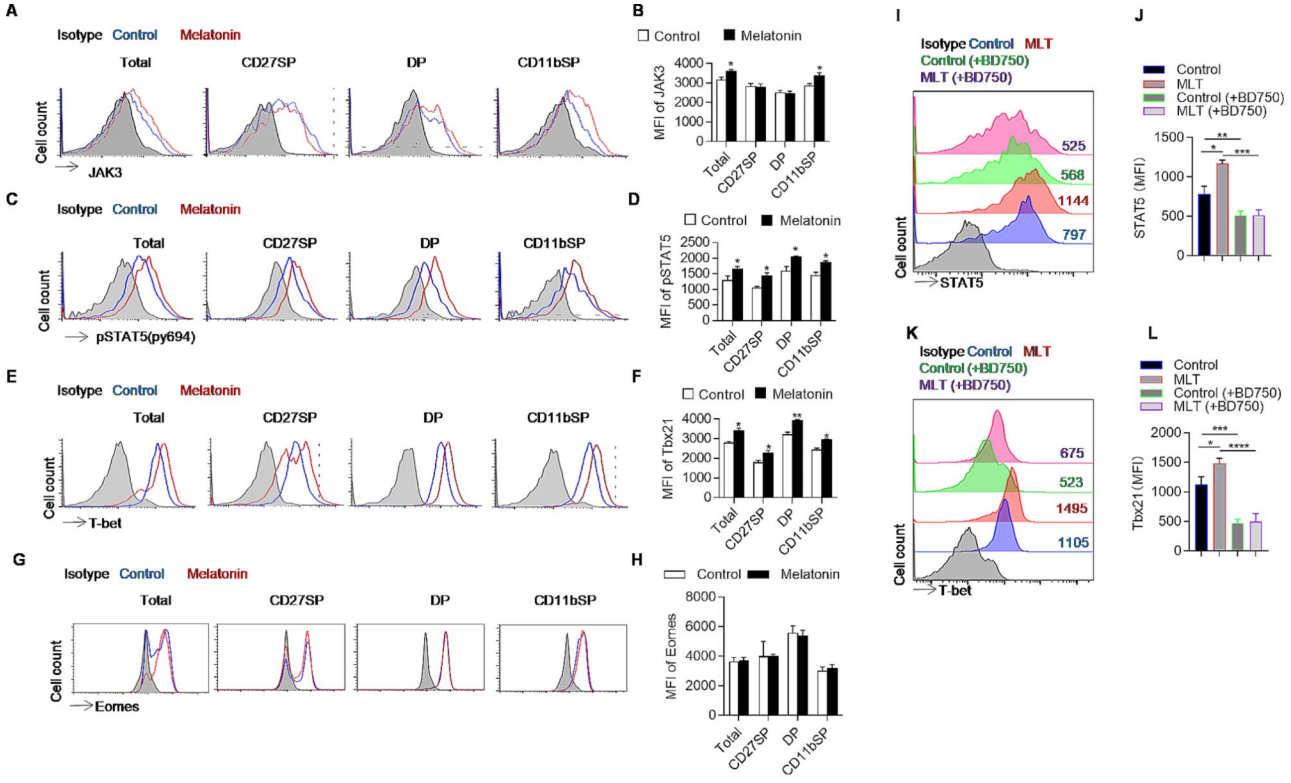


**Fig. 7** Melatonin enhances the expression of JAK3/p-STAT5 and T-bet in spleen NK cells of aged mice. (**A**) Following melatonin treatment, flow cytometry was employed to detect JAK3 expression in NK cell subgroups at various developmental stages. (**B**) Statistical Graph Depicting JAK3 fluorescence intensity in different subgroups of spleen NK Cells. (**C**) Following melatonin treatment, flow cytometry was utilized to detect the expression of p-STAT5 in NK cell subgroups at various developmental stages. (**D**) Statistical graph illustrating fluorescence intensity of p-STAT5 in different subgroups of splenic NK Cells. (**E**) Following melatonin treatment, flow cytometry was employed to detect the expression of T-bet in NK cell subgroups at various developmental stages. (**F**) Statistical graph depicting T-bet fluorescence intensity in different subgroups of spleen NK Cells. (**G**) Following melatonin treatment, flow cytometry was employed to detect the expression of Eomes in NK cell subgroups at various developmental stages. (**H**) Statistical graph depicting T-bet fluorescence intensity in different subgroups of spleen NK Cells. (**I**) Splenic NK cells were isolated from aged mice (1 × 10^6), pre-treated with BD750 (STAT5 antagonist, 1μM) for 2 h, followed by treatment with 100μM melatonin for 24 h, NK cell STAT5 expression was detected using flow cytometry. (**J**) The statistical graph of NK cell STAT5 expression. K. Splenic NK cells were isolated from aged mice (1 × 10^6), pre-treated with BD750 (STAT5 antagonist, 1μM) for 2 h, followed by treatment with 100μM melatonin for 24 h, NK cell T-bet expression was detected using flow cytometry. L. The statistical graph of NK cell T-bet expression. The data is presented as means ± SD. $n = 6$. Unpaired two-tailed Student's t-tests or one-way ANOVA. were conducted using Prism software. A p-value of less than 0.05 was considered statistically significant. *$P < 0.05$, **$P < 0.01$,***$P < 0.001$, ****$P < 0.0001$

role of melatonin in maintaining NK cell function. Animal experiments further revealed that melatonin treatment for 1–2 weeks significantly increased the number of NK cells in the spleen and bone marrow [8]. Additionally, in leukemia model mice, melatonin supplementation increased NK cell numbers and extended mouse survival time [8], suggesting the potential of melatonin in enhancing NK cell function in aging mice. However, melatonin treatment does not enhance NK cell function in young mice. This might be because NK cells in young mice are already functioning well, rendering melatonin's restorative effects unnecessary.

Subsequently, we explored the in vivo effects of melatonin on NK cell function in aging mice. The results indicated a significant increase in the number of NK cells in the spleen, bone marrow, and liver of aging mice supplemented with melatonin for an extended period, suggesting that melatonin may enhance the immune function of the NK cell population by increasing their numbers. Further investigation into the mechanisms underlying the increase in NK cell numbers revealed that melatonin enhanced NK cell proliferation, particularly in the mature CD11bSP stage, without affecting apoptosis. This suggests that melatonin may promote NK cell proliferation to increase their numbers. Similarly, melatonin has been found to reduce the proliferation of $CD4^+CD25^+$ Treg and exert an anti-tumor effect [22]. Additionally, melatonin enhances the secretion of IL-2, leading to increased $CD8^+$ killer T cell proliferation [18], and stimulates the number and activity of $CD8^+$ killer T cells, promoting bone marrow hematopoiesis [4]. The C57BL/6J (B6) is the most common inbred mouse strain used in biomedical research around the world. Yet, this strain is notoriously known for being deficient

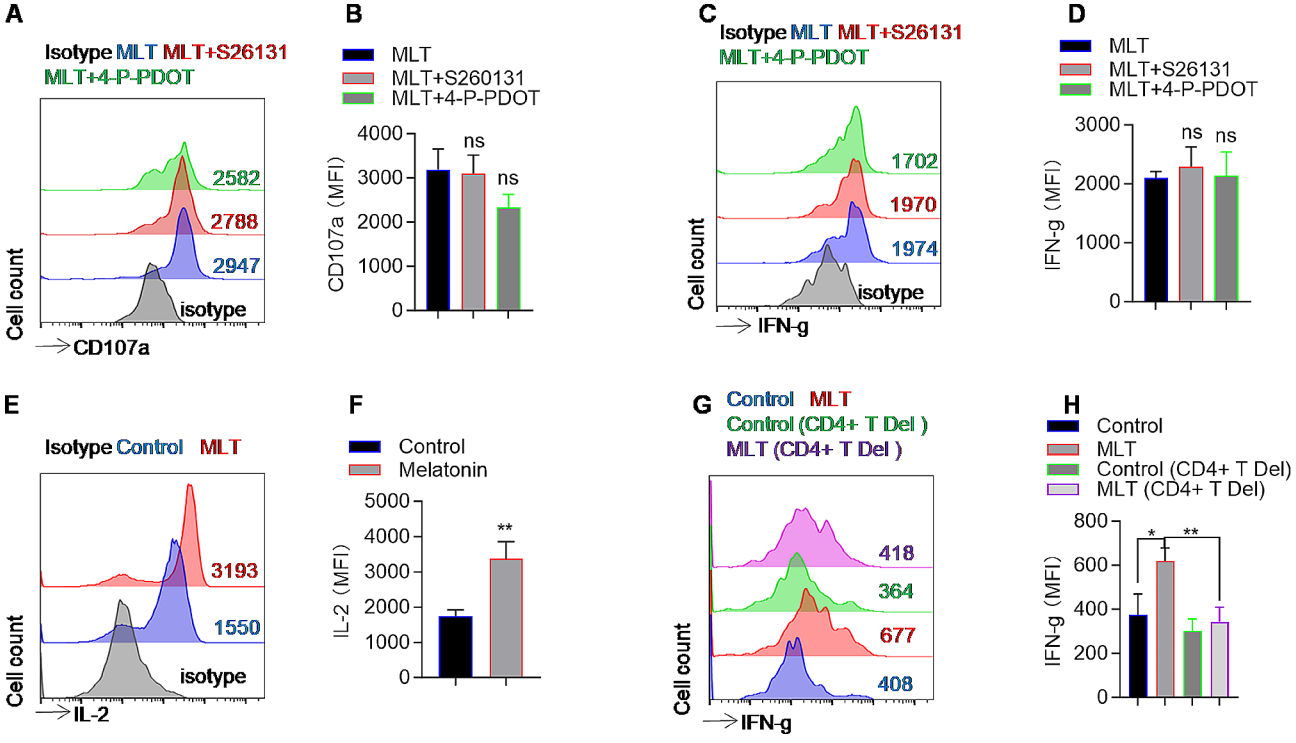


**Fig. 8** Melatonin promotes NK cell function by enhancing IL-2 secretion from CD4 + T cells rather than directly acting on melatonin receptors. (**A**) Splenic NK cells were isolated from aged mice (1 × 10^6), pre-treated with S26131 (MT1 antagonist, 10μM) or 4-P-PDOT (MT2 antagonist, 10μM) for 1 h, followed by treatment with 100μM melatonin for 24 h, NK cell CD107a expression was detected using flow cytometry. (**B**) The statistical graph of NK cell CD107a expression. (**C**) Splenic NK cells were isolated from aged mice (1 × 10^6), pre-treated with S26131 (MT1 antagonist, 10μM) or 4-P-PDOT (MT2 antagonist, 10μM) for 1 h, followed by treatment with 100μM melatonin for 24 h, NK cell IFN-γexpression was detected using flow cytometry. (**D**) The statistical graph of NK cell IFN-γ expression. (**E**) Flow cytometry was employed to assess the expression of IL-2 in CD4 + T cells from the spleens of aged mice treated with melatonin or control. (**F**) The statistical graph of IL-2 expression in CD4 + T cells. (**G**) We used a CD4 + T cell isolation kit to deplete CD4 + T cells from splenic PBMCs of aged mice, followed by treatment of the remaining PBMCs with 100 μM melatonin for 24 h, and assessed NK cell IFN-γ expression using flow cytometry. (**H**) The statistical graph of NK cell IFN-γ expression. The data is presented as means ± SD. $n = 6$. Unpaired two-tailed Student's t-tests or one-way ANOVA. were conducted using Prism software. A p-value of less than 0.05 was considered statistically significant. $^{*}P < 0.05$, $^{**}P < 0.01$

in the biosynthesis of melatonin. Melatonin deficiency in this strain results from non-functional alleles of the genes coding two key enzymes of the melatonin synthesis pathway: arylalkylamine-N-acetyltransferase (Aanat) and Nacetylserotonin-O-methyltransferase (Asmt). It has been proposed that melatonin-deficiency has a favorable impact on domestic mice in breeding colonies by eliminating the antigonadal action of melatonin [10]. Therefore, endogenous melatonin is absent in C57BL/6J (B6) mice, which will not interfere with the effects of exogenous melatonin.

Melatonin exerts pleiotropic effects in the immune system by regulating various cellular functions. It participates in protecting the electron transfer chain and preventing mitochondrial oxidative damage, thereby regulating redox balance [6]. Melatonin also inhibits aerobic glycolysis, survival signaling pathways, and cancer metastasis [15]. In the context of NK cell function, the JAK3/STAT5 signaling pathway plays a critical role in their development and function [29]. Disruption of the IL-15/STAT5 signaling pathway leads to loss of immune function in NK cells [33]. The PI3K-AKT-mTOR signaling pathway plays a crucial role in both the proliferation and effector functions of NK cells [13], Li D et al.,2021). Phosphorylated STAT3 upregulates the transcription of the activating receptor NKG2D on the surface of NK cells, thereby activating them Zhu S et al., [35]. Additionally, T-bet, a key transcription factor, influences NK cell development and maturation. NK cells lacking T-bet exhibit high expression of immature transcription factors [25, 34]. Many signaling pathways can induce T-bet expression in NK cells. T-bet expression in NK cells can be induced by the IFN-γR/STAT1 pathway [1]. T-bet expression in NK cells can be induced by the IL-12R/STAT4 pathway [28]. T-bet expression in NK cells is promoted by the mTOR signaling pathway [32]. T-bet and Eomes are T-box transcription factors that drive the differentiation and function of cytotoxic lymphocytes such as NK cells. Eomes promotes NK cell development and maturation. T-bet promotes terminal NK cell maturation by cooperating with Zeb2 [34]. Eomesodermin spatiotemporally orchestrates the early and late stages of NK

cell development by targeting KLF2 and T-bet, respectively [12].

Our research reveals that melatonin-treated aged mice exhibited activation of the JAK3/STAT5 signaling pathway and up-regulation of T-bet expression, which in turn enhanced NK cell function. This suggests that melatonin may improve NK cell function in aging mice by up-regulating T-bet expression. However, our research has some limitations, such as not using transgenic mice with endogenous melatonin synthesis for the experiments. In future studies, we plan to use transgenic mice with endogenous melatonin synthesis and STAT5 and T-bet gene knockout mice to validate their roles in NK cell maturation and function.

## Supplementary Information

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Supplementary Material 1

### Author contributions

C Liang designed experiments; C Liang, R Song, and J Zhang performed study; X Zeng, Z Guan, J Yao analysed the data. X Zeng supervised the study.

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### Data availability

The data used to support the findings of this study are available from the corresponding author upon request.

## Declarations

### Ethics approval

We did not use any human specimens in this research. The animal experiment protocol was approved by the Animal Ethics Committee of The Sixth Affliated Hospital, School of Medicine, South China University of Technology.

### Consent for publication

All authors have agreed to publish this manuscript.

### Competing interests

The authors declare no competing interests.

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