Therapeutic Benefit of Melatonin in Choroidal Neovascularization During Aging Through the Regulation of Senescent Macrophage/Microglia Polarization

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Received: February 21, 2023
Accepted: July 27, 2023
Published: August 14, 2023

Citation: Cui K, Tang X, Hu A, et al. Therapeutic benefit of melatonin in choroidal neovascularization during aging through the regulation of senescent macrophage/microglia polarization. *Invest Ophthalmol Vis Sci.* 2023;64(11):19. https://doi.org/10.1167/iovs.64.11.19

Purpose. This study aimed to investigate the age-dependent anti-angiogenic capability of melatonin in choroidal neovascularization (CNV) and to explore the underlying molecular mechanisms.

Methods. In the present study, a laser-induced CNV model was established in both young (three months of age) and old (18 months of age) mice, and the size of CNV lesions and vascular leakage was detected by morphological and imaging examination. Next, Western blot and immunostaining were used to observe the levels of M2 markers, senescence-related markers, and molecules involved in IL-10/STAT3 pathway. Additionally, colivelin was used to study the effect of IL-10/STAT3 pathway activation on the expression of M2 markers and senescence-related markers by Western blot and immunostaining. Finally, the effects of colivelin on melatonin-induced reduction of CNV size and vascular leakage in mice at different ages were assessed using morphological and imaging examination.

Results. Our results revealed that aging promoted M2 macrophage/microglia polarization, and aggravated CNV and vascular leakage. Melatonin significantly inhibited the M2 polarization of senescent macrophage/microglia and reduced the CNV area and vascular leakage. Moreover, melatonin markedly suppressed IL-10/STAT3 pathway activation in the macrophage/microglia of old mice, and STAT3 activator colivelin reversed the suppressive effect of melatonin on M2 polarization of senescent macrophage/microglia and laser-induced CNV in old mice.

Conclusions. Our data demonstrated that melatonin significantly prevented the M2 polarization of senescent macrophage/microglia by inhibiting the IL-10/STAT3 pathway, and eventually attenuated senescence-associated CNV. These findings suggested that melatonin could serve as a promising therapeutic agent to treat CNV and other age-related ocular diseases.

Keywords: melatonin, choroidal neovascularization, polarization, senescence, IL-10/STAT3 signaling pathway

Age-related macular degeneration (AMD), a progressive disease of the aging eye, is the leading cause of irreversible blindness among the elderly in industrialized countries.[1] With a global increase in the aging population, the number of AMD patients is predicted to reach 288 million by 2040.[2] Phenotypically, AMD presents in two forms: dry (nonneovascular) and wet (neovascular).[3] Neovascular AMD is characterized by the formation of choroidal neovascularization (CNV), which is abnormal vessel growth from the choroid through Bruch's membrane toward the neuroretina, causing subretinal hemorrhage, photoreceptor damage, and eventually severe central visual loss.[4] Currently, the first-line treatment for CNV is administration of anti-vascular endothelial growth factor (VEGF) agents, which have some obvious limitations such as the burden of frequent intravitreal injections and resistance to treatment.[5] Therefore an in-depth study on the pathogenesis of CNV and search for novel drugs are still necessary.

Numerous studies have demonstrated that innate immunity, especially macrophage/microglia, play a pivotal role in the regulation of CNV in AMD.[6,7] Macrophages are highly plastic and functionally diverse as they can be polarized into a classically activated (M1) type that resists angiogenesis or an alternatively activated (M2) type that promotes angiogenesis.[8] M1-type macrophages, which can be induced by lipopolysaccharide or interferon-γ, mainly increase the

levels of interleukin-1β (IL-1β), IL-6, CD16/32 and CD80.[9] On the other hand, M2-type macrophages are characterized by increased secretion of arginase-1(Arg-1), resistin-like-α (Fizz-1), transforming growth factor-β, chitinase 3-like 3 (Ym-1), CD163 and CD206, which can be stimulated by IL-4 or IL-10.[10] Accumulating evidence suggests that senescent macrophage/microglia, which might actually polarize toward the M2 phenotype, play a key role in abnormal angiogenesis.[11,12] However, the detailed mechanisms that regulate macrophage/microglia phenotypes and functions, particularly in aging-associated eye diseases, remain largely unclear.

IL-10 is a pleiotropic immunoregulatory cytokine that is indispensable in maintaining immune homeostasis and is expressed in various immune cell types, such as T cells, B cells, and macrophages.[13,14] IL-10 binds to a heterodimeric transmembrane receptor complex consisting of the ligand-binding subunit IL-10R1 and the accessory subunit IL-10R2, resulting in the recruitment and activation of Janus kinase 1 and tyrosine kinase 2, which phosphorylates signal transducer and activator of transcription 3 (STAT3).[15,16] In certain conditions, phosphorylated STAT3 dimerizes and translocates to the nucleus, where it regulates transcription of target genes, subsequently activating signaling pathways that promote M2 polarization of macrophages.[17,18] A recent study found that IL10-driven STAT3 signaling promoted M2 polarization in senescent macrophages and played a critical role in pathological angiogenesis.[19]

Melatonin (N-acetyl-5-methoxytryptamine), an endogenous indoleamine, is not exclusively synthesized and secreted in the pineal gland,[20] because it is also locally produced in several tissues and organs, particularly the retina,[21,22] lens,[23] skin,[24] bone marrow,[25] and gastrointestinal tract.[26] Melatonin has a wide spectrum of biological functions, including adjustment of sleep and circadian rhythm, as well as antiaging, antioxidant, and antitumor activities.[27,28] As a potent free radical scavenger and a protector of mitochondria function, melatonin exhibits powerful antiaging properties and represents a potent tool to counteract age-related diseases.[29] In most cases, melatonin has been reported to affect macrophage phenotypes by inhibiting M1 polarization while promoting M2 macrophage properties.[30] However, previous studies revealed that the production of M1 markers (IL-6, IL-1β, and TNF-α) in murine macrophages cannot be affected by melatonin in some situations.[31,32] Additionally, Cheng et al.[33] found that exosomes from melatonin treated hepatocellular carcinoma cells decreased the expression of IL-10 and STAT3 activation in tumor-associated macrophages, indicating that melatonin inhibited the function of M2 macrophages in tumors. These studies suggested that regulation of macrophage/microglia polarization by melatonin is very complicated and likely varies depending on disease types.[30] Our previous study found that melatonin attenuated CNV and vascular leakage in a laser-induced CNV mouse model by switching macrophage/microglia polarization.[34] However, age, which is an important independent risk factor for AMD, was not taken into account in the previous study.[35] Therefore elucidating the mechanism by which aging influences the progression of CNV and demonstrating the anti-aging effect of melatonin on the development of CNV in blinding eye diseases remains imperative.

In this study, we used mice at different ages to explore the effect of melatonin behind age-dependent regulation of macrophage/microglia polarization and angiogenic function, and we sought to elucidate the underlying molecular mechanism. Our findings proved that melatonin significantly inhibited the polarization of senescent macrophage/microglia toward the M2 phenotype, and eventually attenuated senescence-associated CNV and vascular leakage, primarily by restraining the IL-10/STAT3 pathway.

Material and Methods

Reagents and Chemicals

Melatonin was purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Fluorescein sodium was acquired from Alcon Laboratories, Inc. (Fort Worth, TX, USA). Colivelin was obtained from R&D Systems (Minneapolis, MN, USA). The antibodies for Western blot and immunofluorescence in this study are listed in Supplementary Table S1.

Animals

The young (three months of age) and old (18 months of age) male C57BL/6J mice were acquired from the Animal Laboratory of Zhongshan Ophthalmic Center (Guangzhou, China). All experimental procedures were performed following the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by the Institutional Animal Care and Use Committee of Zhongshan Ophthalmic Center.

Laser-Induced CNV Mouse Model

The laser-induced CNV mouse model was established according to previously described methods.[34,36] The young and old mice were anesthetized with 1% pentobarbital sodium (50 mg/kg) by intraperitoneal injection, and the pupils were dilated with compound tropicamide eye drops. Laser photocoagulation (810 nm wavelength, 120 mW power, 75 µm spot size, 75 ms duration) was performed using an OcuLight Infrared Laser System (IRIDEX Corporation, Mountain View, CA, USA) under a slit lamp microscope. A total of four laser spots were induced at the 3-, 6-, 9-, and 12-o'clock positions, which were approximately two to three disc diameters from the optical disc, avoiding the main blood vessels. The production of cavitation bubbles indicated the disruption of Bruch's membrane, confirming that the laser-induced CNV model was successfully constructed.

Drug Administration and Experimental Design

Melatonin was initially dissolved in absolute ethanol, diluted in normal saline solution, and then intraperitoneally injected at a dose of 20 mg/kg/d for seven days. All mice were randomly separated into four groups: (a) CNV group of young mice: young mice with laser photocoagulation; (b) CNV + melatonin group of young mice: young mice with laser photocoagulation and melatonin administration; (c) CNV group of old mice: old mice with laser photocoagulation; (d) CNV + melatonin group of old mice: old mice with laser photocoagulation and melatonin administration. Furthermore, during the pharmacological experiments on STAT3 activator, two additional groups were added: (e) CNV + melatonin + colivelin group of young mice: young mice with laser photocoagulation, and intraperitoneal injection of melatonin and colivelin; (f) CNV + melatonin + colivelin group of old mice: old mice with laser photocoagulation, and intraperitoneal injection of melatonin and colivelin. Colivelin was initially dissolved in 20% ethanol, diluted in normal saline solution, and then intraperitoneally injected at a dose of 1 mg/kg/d for seven days. Melatonin and colivelin were

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intraperitoneally injected immediately after establishing the laser-induced CNV mouse model.

Histopathology

The eyeballs were removed at seven days after laser photocoagulation, fixed in 4% paraformaldehyde, and embedded in paraffin. Tissue sections (3 µm thick) were cut through the optic nerve and stained with hematoxylin and eosin (HE) for histological analysis. The paraffin sections, which passed through the center of each lesion with the largest cross-sectional area, were used to assess the size of CNV lesions. Images were captured with an optical microscope and analyzed using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

Choroidal Flat Mounts and Immunostaining

The mice eyes were enucleated and fixed in 4% paraformaldehyde for two hours, and the RPE–choroid–sclera complex eyecups were blocked and permeabilized overnight. Then the eyecups were incubated with primary antibodies (Supplementary Table S1) for 24 hours at 4 °C. After washing, the tissues were incubated for two hours with corresponding secondary antibodies. After staining, the eyecups were made into flat mounts by cutting radial incisions, and the images were captured using a Zeiss LSM 880 confocal microscope (Zeiss, Oberkochen, Germany).

Optical Coherence Tomography

One week after photocoagulation, mice were anesthetized, and their pupils were dilated. Optical coherence tomography (OCT) images were captured using the image-guided OCT system (Micron IV; Phoenix Research Laboratories, Pleasanton, CA, USA), which means that a live fundus image was used to guide OCT scanning in the CNV lesions of each eye. OCT images were analyzed using ImageJ software to determine the size of CNV lesions.

Fundus Fluorescein Angiography

Fluorescence leakage of CNV lesions was observed by Fundus Fluorescein Angiography (FFA) on day 7 after laser photocoagulation. After anesthesia and pupil dilation, 0.2 mL of 2% fluorescein sodium was injected intraperitoneally in mice. FFA images were obtained with a retina imaging system (Micron IV; Phoenix Research Laboratories) during the early phase (one to three minutes after fluorescein injection) and late phase (eight to 10 minutes after fluorescein injection). Two experienced specialists independently evaluated the CNV leakage grades, using the following criteria for FFA images: Grade I indicated the absence of hyperfluorescence, Grade II indicated the presence of hyperfluorescence without leakage, Grade III indicated hyperfluorescence in the early or mid-transit images and late leakage, and Grade IV indicated bright hyperfluorescence in the transit images and late leakage beyond the treated areas.[37]

Western Blot Analysis

The dissected samples of RPE–choroid–sclera complexes for Western blot analysis were processed as previously described.[34] In brief, the samples were separated by SDS-PAGE and then transferred to PVDF membranes. After blocking with 5% non-fat milk, the membranes were incubated with primary antibodies (Supplementary Table S1), followed by HRP-conjugated secondary antibodies. The blots were subsequently visualized by an enhanced chemiluminescence system.

Quantitative Real-Time PCR

Total RNA from RPE–choroid–sclera complexes was extracted with TRIzol and cDNA was synthesized using the cDNA Synthesis Kit. Quantitative real-time PCR was performed with SYBR Green Master Mix (Bio-Rad Life Science, Hercules, CA, USA). Gene expression was normalized to β-actin and analyzed using the $2^{-\Delta\Delta Cq}$ method. The sequences of primers are listed in Supplementary Table S2.

Statistical Analysis

All experiments were repeated at least three times. Data were shown as mean ± standard deviation (SD). Statistical analyses were performed using one-way ANOVA. $P < 0.05$ was considered statistically significant. Statistical analyses were performed with GraphPad Prism (v8.0) (GraphPad Software Inc., San Diego, CA, USA).

Results

Melatonin Significantly Reduced the Size of CNV Lesions in Old Mice

To investigate the effect of melatonin on CNV in mice at different ages, the laser-induced CNV model in young (three months of age) and old (18 months of age) mice was established. HE staining (Fig. 1A) showed that the thickness and length of CNV lesions were increased in old mice compared to young mice, they but were reduced to a similar level after intraperitoneal injection of melatonin in the two age groups (Figs. 1B, 1C), indicating that the inhibitory effect of melatonin on CNV was more pronounced in old mice. Additionally, CNV size was identified by the location of CD31-positive cells in choroidal flat mounts (Fig. 1D), which could intuitively show the formation of new vessels. Similar to the results of HE staining, the immunostaining of choroidal flat mounts showed that melatonin remarkably reduced the area of CNV in the old mice compared with the young mice (Fig. 1E), suggesting a significant inhibitory effect of melatonin on age-related CNV.

Next, we further assessed the antiangiogenic potential of melatonin on senescence-associated CNV with imaging examination. Direct comparison of the three-month-old and 18-month-old mouse OCT data (Fig. 1F), which is the thickness and length of CNV lesions, demonstrated that the older mice developed markedly larger CNV lesions compared to the younger mice. OCT analysis showed that intraperitoneal injection of melatonin led to markedly decreased CNV lesion thickness and length in aged mice (Figs. 1G, 1H), which was consistent with the results of morphological examinations above.

Melatonin Dramatically Attenuated Vascular Leakage of CNV Lesions in Old Mice

CNV is the aberrant growth of new blood vessels consisting of simple endothelial cells, which are fragile and hyperpermeable, and are prone to rupture and bleeding under the action of various pathological factors, resulting in vascular leakage.[36,38] The area and severity of fluorescein leakage from CNV in mice at different ages was assessed

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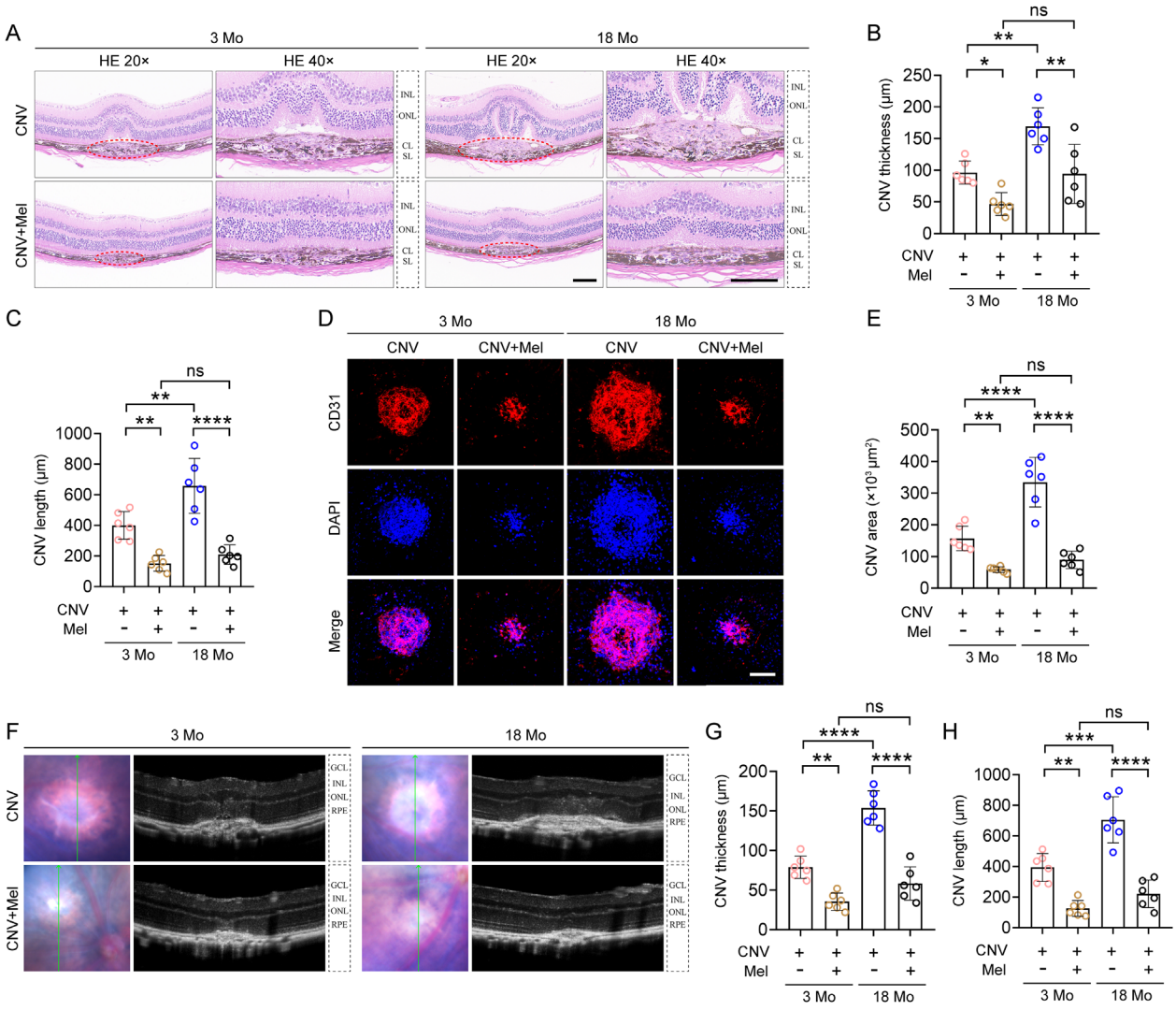

Figure 1. The effect of melatonin on the size of CNV lesions in mice at different ages. **(A)** Representative HE staining images of the CNV group and CNV + melatonin group in young and old mice are shown. INL, inner nuclear layer; ONL, outer nuclear layer; CL, choroid layer; SL, sclera layer. The CNV lesions are circled with *red dashed lines*. Magnified views are shown on the *right panel*. *Scale bar*: 100 μm. **(B, C)** Quantification of the thickness and length of CNV lesions in each group is shown in the bar graphs. **(D)** Representative immunofluorescent images of CNV lesions stained with CD31 (*red*) and DAPI (*blue*) in choroidal flat mounts are shown. *Scale bar*: 100 μm. **(E)** The area of CNV lesions in each group was quantified and is displayed in the bar graph. **(F)** Representative fundus photographs (*left*) and OCT images (*right*) from the CNV group and CNV + melatonin group in young and old mice are shown. GCL, ganglion cell layer; RPE, retinal pigment epithelium. **(G, H)** Quantification of the thickness and length of CNV lesions in each group is shown in the bar graphs. The data are mean ± SD, one-way ANOVA, $n = 6$. ns, not significant, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, and $^{****}P < 0.0001$.

using FFA (Fig. 2A). Comparison between the different ages revealed a significant increase in vascular leakage in the old mice relative to the young mice, and the severity of vascular leakage from CNV lesions decreased significantly with melatonin administration in old mice (Fig. 2B), indicating that the repressive effect of melatonin on laser-induced vascular leakage and pathological CNV was more significant in aged mice.

Melatonin Effectively Prevented Macrophage/Microglia Polarization Toward the M2 Phenotype in CNV of Old Mice

Our previous study found that melatonin prevented M2 polarization of macrophage/microglia in the development of CNV,[34] but aging, which is an important independent risk factor for AMD, was not taken into account in our experiments. Thus we investigated differences in the levels of M2 polarization markers in CNV of young and old mice. Western blot (Fig. 3A) showed that the levels of M2 polarization markers, such as Arg-1, YM-1, and Fizz-1, were markedly higher in old mice than in young mice. Nevertheless, melatonin treatment led to decreased protein expression of M2 markers in both age groups, and this effect was more pronounced in aged mice (Figs. 3B–D). In the immunostaining of choroidal flat mounts (Fig. 3E), CD206 (an M2 marker) was highly expressed in Iba1-positive macrophage/microglia, confirming the crucial role of M2 macrophage/microglia polarization in promoting CNV in the aged eye. Consistent with Western blot results, quantification of fluorescence intensity (Fig. 3F) found that CD206 expression was diminished after

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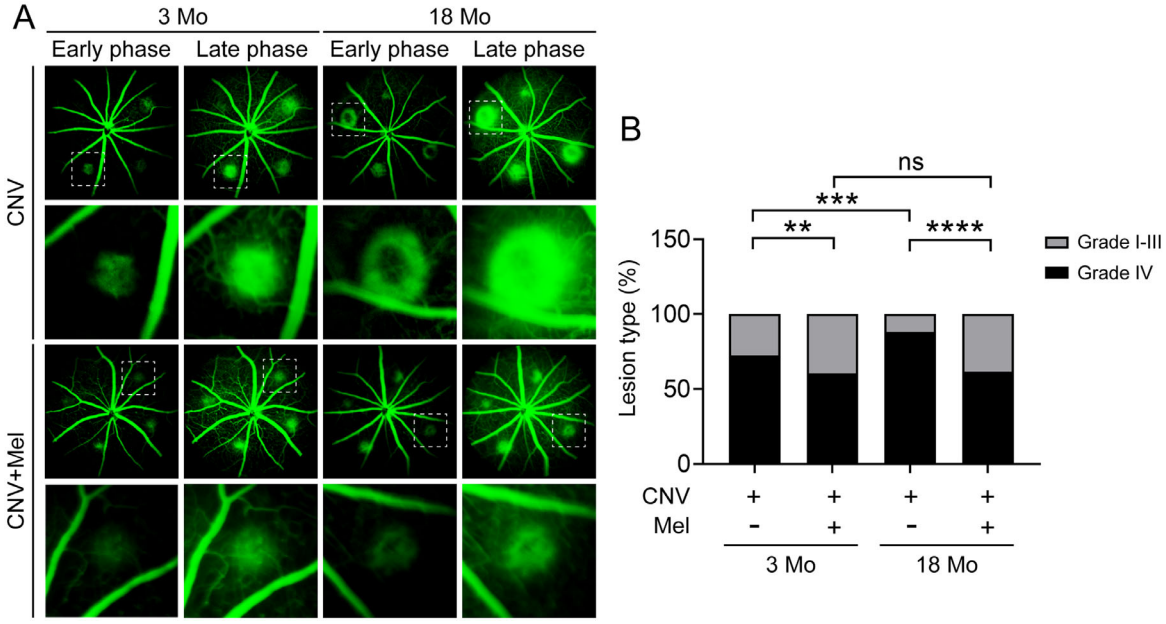

Figure 2. The effect of melatonin on vascular leakage of CNV lesions in mice at different ages. **(A)** Representative FFA images of different groups were acquired at two different times (the early phase and late phase) after intraperitoneal delivery of fluorescein. Magnified views in *white dotted boxes* are shown in the respective lower panel. **(B)** Fluorescein leakage in CNV lesions was graded on late images. The grading criteria of FFA images were as follows: Grade I indicated the absence of hyperfluorescence, Grade II indicated the presence of hyperfluorescence without leakage, Grade III indicated hyperfluorescence in the early or mid-transit images and late leakage, and Grade IV indicated bright hyperfluorescence in the transit images and late leakage beyond the treated areas. The data are mean ± SD, one-way ANOVA, $n = 6$. ns, not significant, $^{**}P < 0.01$, $^{***}P < 0.001$, and $^{****}P < 0.0001$.

intraperitoneal injection of melatonin in both young and old mice, but more so in old mice. Overall, we demonstrated a link between M2 polarization in macrophage and senescence and suggested that melatonin effectively prevented macrophage/microglia polarization toward the M2 phenotype in old mice.

To further investigate the effect of melatonin on M1 macrophage/microglia in the early stage of CNV, M1 markers in each group on day 3 after laser injury were examined by PCR (Supplementary Figs. S1A–E). The results showed that the expression of M1 markers (TNF-α, IL-6, IL-1β, and *CCL-2*) was reduced in old (18 months of age) mice as compared to young (three months of age) mice. However, melatonin did not significantly affect M1 markers levels, regardless of age. Additionally, *CCL-3* mRNA expression remained relatively unchanged among the four groups. Therefore our findings proved that melatonin did not significantly inhibit M1 type polarization in the early stage of CNV. Next, we assessed the effect of melatonin on the size of CNV lesions on day 3 after laser injury in mice at different ages. The immunostaining of choroidal flat mounts (Supplementary Figs. S1F, S1G) found that melatonin did not affect CNV area in two age groups, indicating that melatonin did not inhibit the size of CNV in the early stage of laser injury. Furthermore, we also found that there was no significant difference in CNV area at three days after laser injury in old mice compared with young mice, which may be due to incomplete CNV formation on day 3 after laser injury.[36]

Melatonin Inhibited Macrophage/Microglia Senescence in Old Mice

Considering the vital role of senescence in the pathogenesis of CNV,[39] we focused on the antiaging effect of melatonin by analyzing the levels of senescence markers in mice at different ages. Western blot (Fig. 4A) showed significant increases of the senescence-associated biomarkers p16, p21, and p53 in old mice. Intraperitoneal injections with melatonin dramatically downregulated the levels of senescence markers in old mice, whereas there was no significant difference in senescence markers expression in young mice before and after melatonin administration, because it was maintained at low levels (Figs. 4B–D). Immunofluorescence (Fig. 4E) showed colocalization of staining for p16 and Iba1 in choroidal flat mounts, indicating increased senescence in macrophage/microglia in old mice. Melatonin markedly reduced the expression of p16 in old mice but not young mice (Fig. 4F). Taken altogether, these data demonstrated that melatonin inhibited macrophage/microglia senescence in old mice, confirming the effective antiaging role of melatonin in senescence-associated CNV.

IL-10/STAT3 Signaling Pathway Might be Involved in the Inhibitory Effect of Melatonin on Senescence-Associated CNV

It has been reported that IL-10/STAT3 signaling pathway is an important determinant of M2 polarization in senescent macrophages and that it promotes angiogenesis in blinding eye diseases.[19] However, whether IL-10/STAT3 pathway is involved in the inhibitory effect of melatonin on senescence-associated CNV remains unclear. To further study the mechanism for melatonin's protective effects against CNV in mice at different ages, colivelin, a pharmacological activator of STAT3, was used in our experiments. Western blot (Fig. 5A) revealed that p-STAT3 expression was clearly elevated in old mice compared to young mice, and mela-

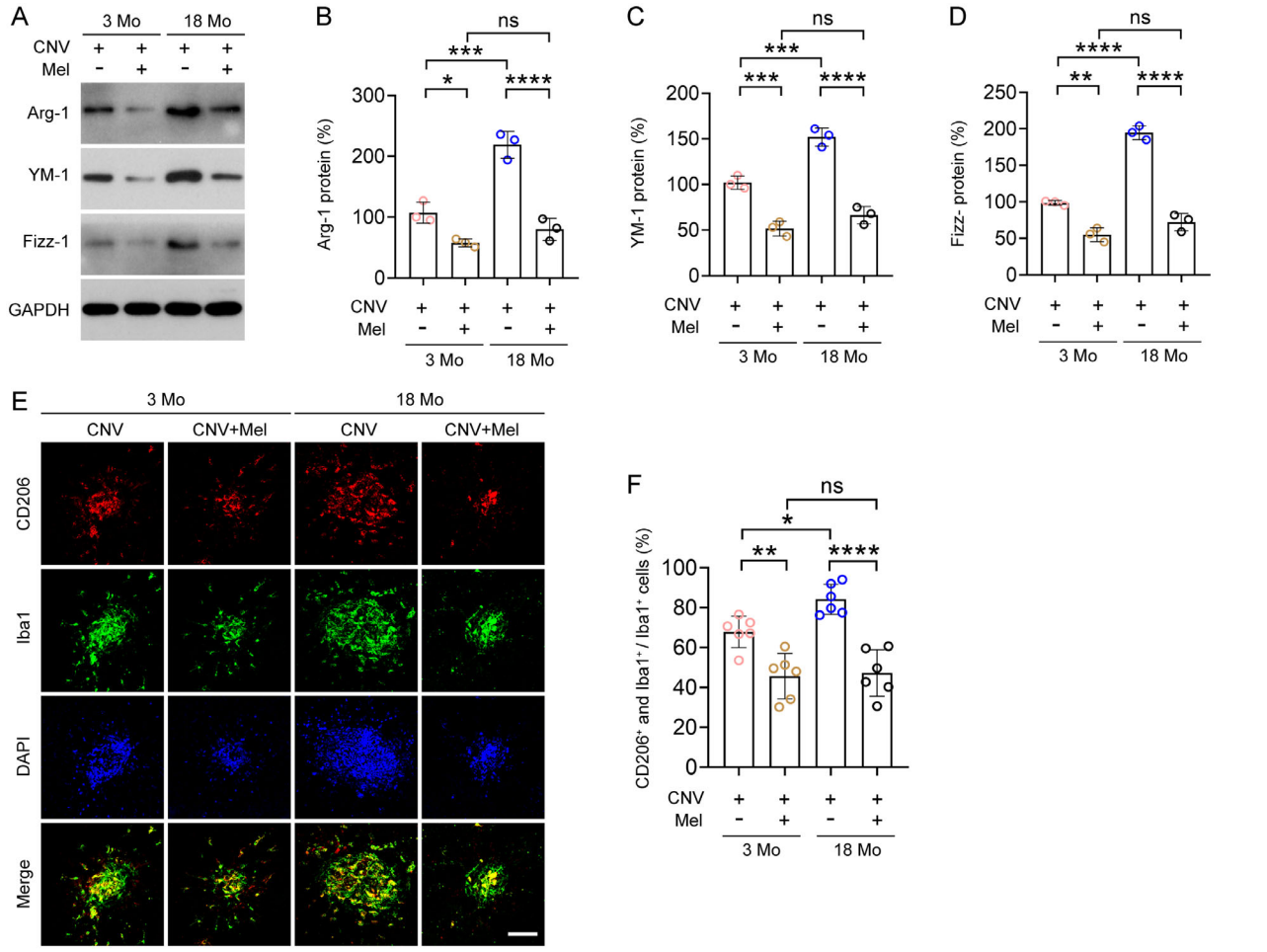

Figure 3. The effect of melatonin on macrophage/microglia polarization in mice at different ages. **(A)** The protein levels of M2 polarization markers (Arg-1, YM-1 and Fizz-1) in the RPE-choroid-sclera tissue containing CNV lesions were examined by Western blot. GAPDH served as the loading control. **(B–D)** Quantification of the Western blot is shown in the bar graphs. The data are mean ± SD, one-way ANOVA, $n = 3$. **(E)** The choroidal flat mounts were immunostained with CD206 (*red*), Iba1 (*green*), and DAPI (*blue*). Representative immunofluorescent images from each group in mice at different ages are shown. *Scale bar*: 100 µm. **(F)** The fluorescence intensity of CD206 was quantified and is displayed in the bar graph. The data are mean ± SD, one-way ANOVA, $n = 6$. ns, not significant, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, and $^{****}P < 0.0001$.

tonin administration resulted in diminished p-STAT3 levels in both age groups, which was more evident in old mice. Colivelin partly reversed the suppressive capacity of melatonin on STAT3 activation in old mice, but this effect was not observed in young mice (Fig. 5B). In addition, we also noted that the levels of IL-10R1 and IL-10R2 remained relatively unchanged among six groups (Figs. 5C, 5D). Quantitative analysis of gene expression by PCR (Fig. 5E) demonstrated an age-associated increase in IL-*10* mRNA expression in RPE-choroid-sclera tissue containing CNV lesions of old mice. However, a marked reduction in IL-10 mRNA was observed after melatonin administration in old mice, which could be partly reversed by colivelin. Meanwhile, the magnitude of change in IL-10 expression was significantly smaller in young mice. Additionally, immunofluorescence staining (Fig. 5F) revealed that p-STAT3 was mainly expressed in Iba1-positive macrophage/microglia of CNV lesions, and the changes in p-STAT3 expression were similar to the Western blot results (Fig. 5G). Collectively, the above-mentioned results suggested that IL-10/STAT3 pathway might be involved in the inhibitory effect of melatonin on senescence-associated CNV.

Colivelin Could Effectively Reverse the Inhibitory Effect of Melatonin on M2 Macrophage/Microglia Polarization in Old Mice

To further determine whether the IL-10/STAT3 signaling pathway was involved in the effect of melatonin on macrophage/microglia polarization, both young and old mice were intraperitoneally injected with melatonin alone or in combination with STAT3 activator colivelin for 7 days after laser photocoagulation. As shown in Fig. 6A, coadministration of colivelin and melatonin elicited a robust increase in levels of M2 markers (Arg-1, YM-1 and Fizz-1) in old mice observed on Western blot as compared to melatonin alone. On the other hand, only a slight increase of M2 markers expression was found in young mice (Figs. 6B–D). Immunofluorescence (Fig. 6E) also showed that CD206 expression was enhanced after cotreatment of colivelin with melatonin in both age groups of animals, but this effect was more prominent in old mice (Fig. 6F). Overall, these findings suggested that IL-10/STAT3 signaling pathway activation could effectively reverse the inhibitory effect of melatonin on M2 macrophage/microglia polarization in old mice.

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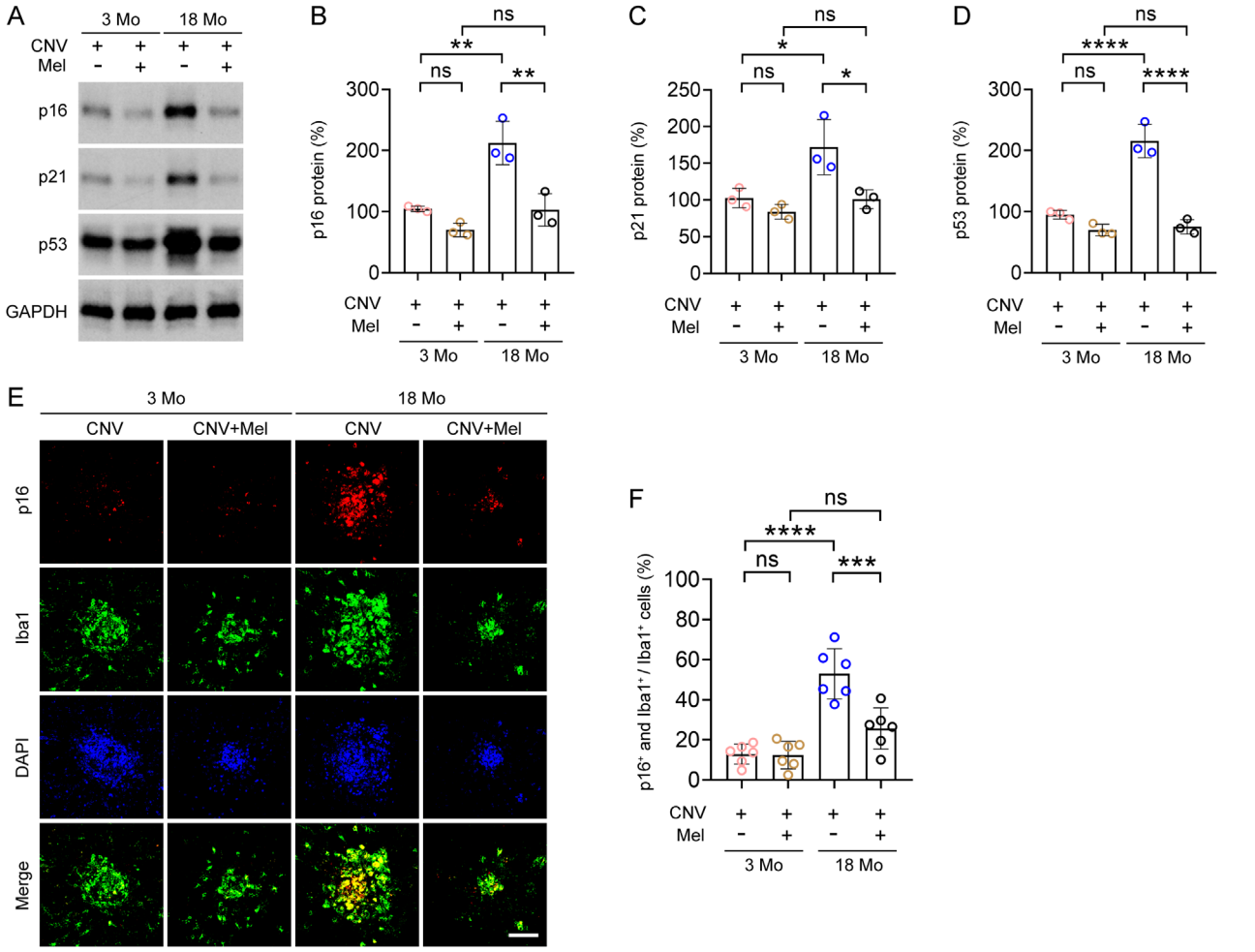

Figure 4. The effect of melatonin on macrophage/microglia senescence in mice at different ages. **(A)** The protein levels of senescence markers (p16, p21 and p53) in the RPE-choroid-sclera tissue containing CNV lesions were examined by Western blot. GAPDH served as the loading control. **(B–D)** Quantification of the Western blot is shown in the bar graphs. The data are mean ± SD, one-way ANOVA, $n = 3$. **(E)** The choroidal flat mounts were immunostained with p16 (*red*), Iba1 (*green*), and DAPI (*blue*). Representative immunofluorescent images from each group in mice at different ages are shown. *Scale bar*: 100 µm. **(F)** The fluorescence intensity of p16 was quantified and is displayed in the bar graph. The data are mean ± SD, one-way ANOVA, $n = 6$. ns, not significant, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, and $^{****}P < 0.0001$.

Colivelin Could Reverse the Antiaging Effect of Melatonin on Macrophage/Microglia of Old Mice

Next, we continued to explore whether the IL-10/STAT3 signaling pathway was involved in the antiaging effect of melatonin on macrophage/microglia. Western blot analysis (Fig. 7A) found that combination treatment of colivelin and melatonin dramatically up-regulated the levels of p16, p21, and p53, well-established biomarkers of cellular senescence, in old mice. In contrast, the levels of senescence markers remained almost constant after co-administration of colivelin and melatonin in young mice (Figs. 7B–D). In addition, the immunostaining of choroidal flat mounts (Fig. 7E) confirmed that combination therapy resulted in an increase of p16 expression primarily in old mice, but not in young mice (Fig. 7F). Taken together, these data suggested that IL-10/STAT3 pathway activation could effectively reverse the antiaging effect of melatonin on macrophage/microglia of old mice.

Colivelin Could Significantly Reverse the Inhibitory Effect of Melatonin Against Laser-Induced CNV and Associated Vascular Leakage in Old Mice

Having established the foundation that IL-10/STAT3 signaling pathway is a crucial regulator of polarization and senescence of macrophage/microglia, we sought to determine whether STAT3 activation could reverse melatonin-induced attenuation of CNV phenotype in mice at different ages. Seven days after CNV induction, HE staining (Fig. 8A) revealed that the old mice treated with colivelin and melatonin showed significantly increased CNV thickness and length compared with melatonin treatment alone, whereas the changes in young mice were slight (Figs. 8B, 8C). Moreover, CD31 immunostaining of choroidal flat mounts (Fig. 8D) revealed that colivelin and melatonin co-treatment led to larger CNV area in old mice (Fig. 8E). Furthermore, OCT (Fig. 8F) demonstrated that colivelin combined with

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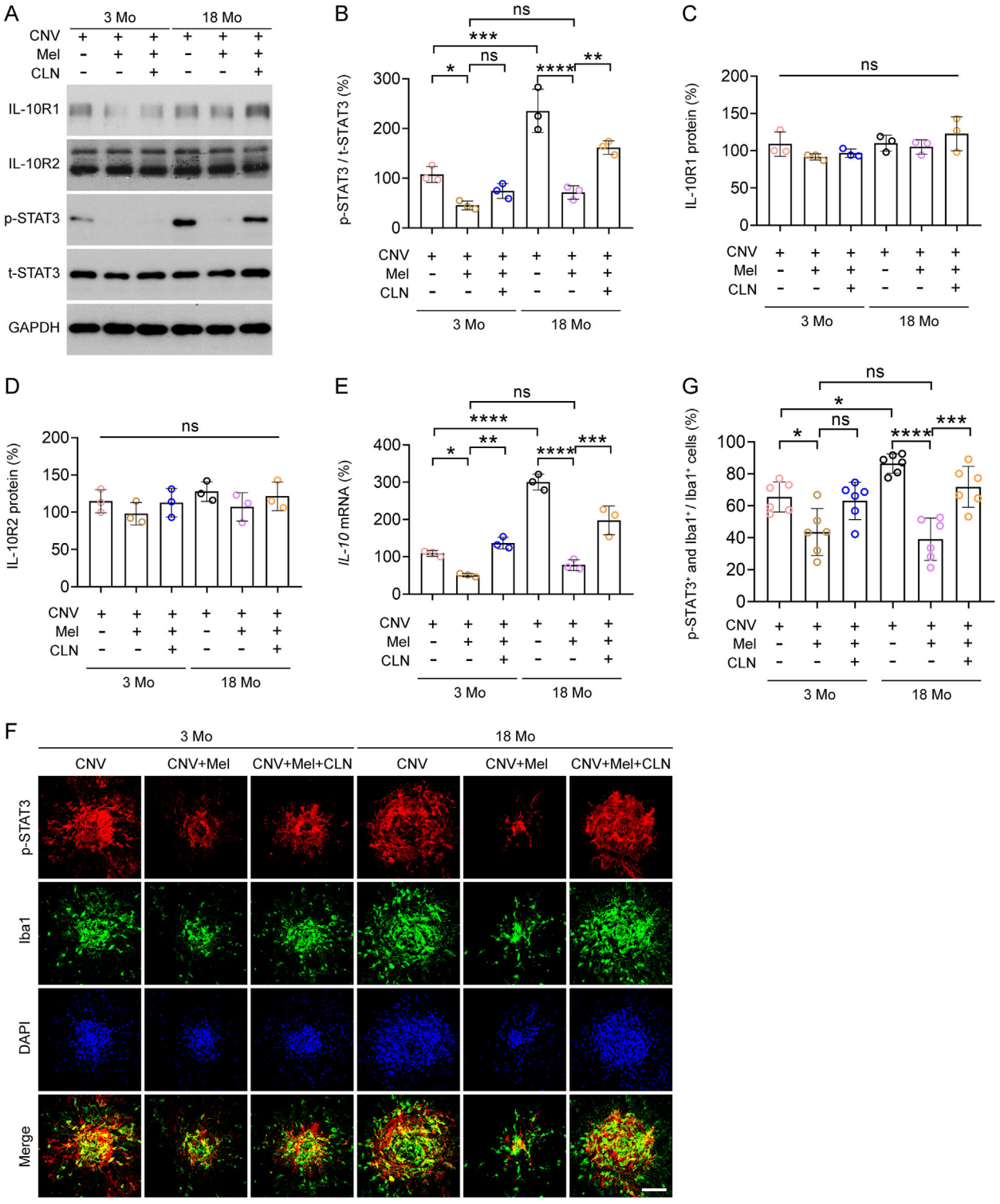

Figure 5. The effect of the IL-10/STAT3 signaling pathway on the antiaging ability of melatonin. **(A)** The protein levels of IL-10R1, IL-10R2, p-STAT3, and t-STAT3 in the RPE-choroid-sclera tissue containing CNV lesions were examined by Western blot. GAPDH served as the loading control. **(B–D)** Quantification of the Western blot is shown in the bar graphs. The data are mean ± SD, one-way ANOVA, $n = 3$. **(E)** The mRNA level of IL-10 in the RPE-choroid-sclera tissue containing CNV lesions was examined by PCR. The data are mean ± SD, one-way ANOVA, $n = 3$. **(F)** The choroidal flat mounts were immunostained with p-STAT3 (*red*), Iba1 (*green*), and DAPI (*blue*). Representative immunofluorescent images from each group of mice at different ages are shown. *Scale bar*: 100 µm. **(G)** The fluorescence intensity of p-STAT3 was quantified and is displayed in the bar graph. The data are mean ± SD, one-way ANOVA, $n = 6$. ns, not significant, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, and $^{****}P < 0.0001$.

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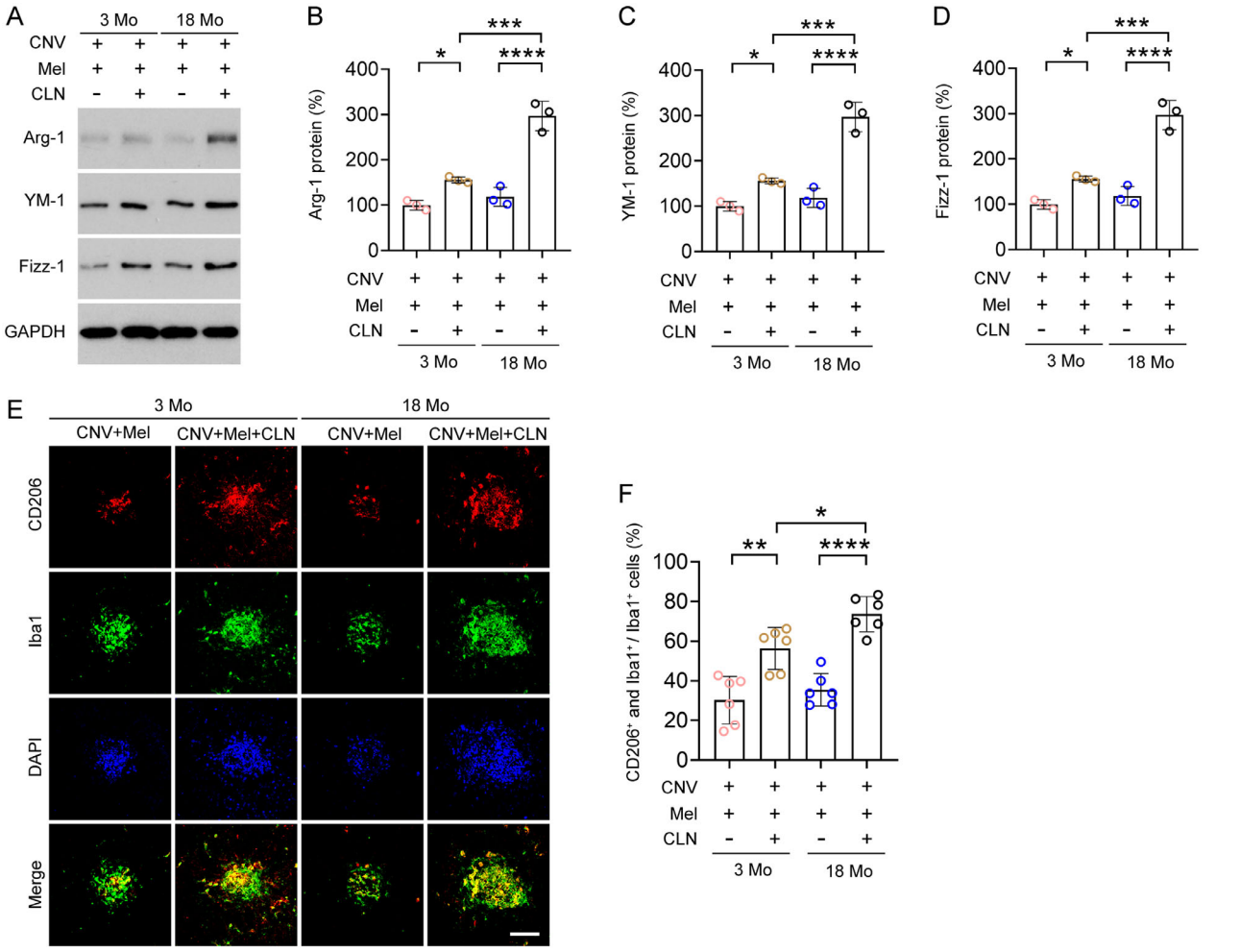

Figure 6. The effect of colivelin on macrophage/microglia polarization regulated by melatonin in mice at different ages. **(A)** The protein levels of M2 polarization markers (Arg-1, YM-1 and Fizz-1) in the RPE-choroid-sclera tissue containing CNV lesions were examined by Western blot. GAPDH served as the loading control. **(B–D)** Quantification of the Western blot is shown in the bar graphs. The data are mean ± SD, one-way ANOVA, $n = 3$. **(E)** The choroidal flat mounts were immunostained with CD206 (*red*), Iba1 (*green*), and DAPI (*blue*). Representative immunofluorescent images from each group in mice at different ages are shown. *Scale bar*: 100 µm. **(F, G)** The fluorescence intensity of CD206 was quantified and is displayed in the bar graph. The data are mean ± SD, one-way ANOVA, $n = 6$. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, and $^{****}P < 0.0001$.

melatonin increased the thickness and length of CNV lesion in two age groups, which was more pronounced in old mice (Figs. 8G, 8H).

Finally, we evaluated the effect of STAT3 activator colivelin on the inhibition of vascular leakage in CNV lesions by melatonin. FFA (Fig. 9A) showed that, after co-treatment of colivelin with melatonin, leakage intensity of CNV was markedly augmented in old mice, in both the early and late phase (Fig. 9B). In summary, these results demonstrated that activation of the IL-10/STAT3 pathway could effectively reverse the inhibitory effect of melatonin against laser-induced CNV and vascular leakage in old mice.

Discussion

It is well known that melatonin is a kind of multifunctional neuroendocrine hormone and has prominent antiaging, antioxidant, and antiangiogenic properties, which may represent a powerful tool to counteract various ophthalmic diseases.[29,40–42] In this study, we found that (a) melatonin significantly reduced the size of CNV and vascular leakage in old mice; (b) melatonin effectively prevented M2 polarization of macrophage/microglia in CNV of old mice; (c) melatonin inhibited macrophage/microglia senescence in old mice; (d) the IL-10/STAT3 pathway was involved in the inhibitory effect of melatonin on senescence-associated CNV. Taken together, we have elucidated the role of melatonin in inhibiting M2 polarization in senescent macrophage/microglia and its antiaging function of regulating angiogenesis in blinding eye diseases such as AMD.

Although age is considered the most important contributing risk factor for AMD, how age impacts the disease development of AMD and how to suppress this process are still not well understood.[43,44] In fact, the accumulation of senescent cells in human eyes with AMD is increasingly recognized.[45] Emerging studies indicate that senescence may directly and indirectly lead to the dysfunction and degeneration of various ocular tissues and cells, which greatly weaken their ability to maintain local homeostasis, resulting in progressive macular damage.[46–48] Melatonin, a

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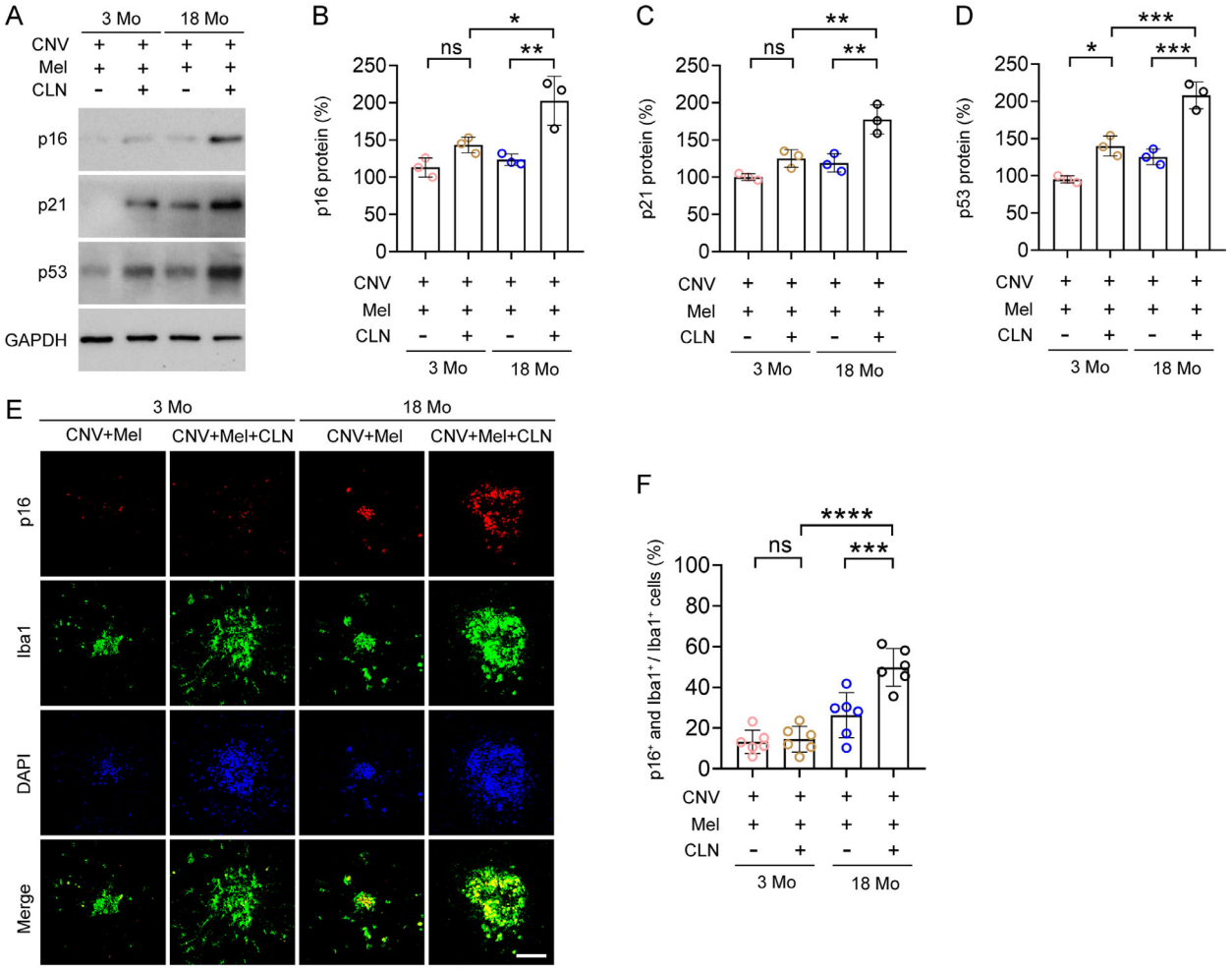

Figure 7. The effect of colivelin on macrophage/microglia senescence regulated by melatonin in mice at different ages. **(A)** The protein levels of senescence markers (p16, p21 and p53) in the RPE-choroid-sclera tissue containing CNV lesions were examined by Western blot. GAPDH served as the loading control. **(B–D)** Quantification of the Western blot is shown in the bar graphs. The data are mean ± SD, one-way ANOVA, $n = 3$. **(E)** The choroidal flat mounts were immunostained with p16 (*red*), Iba1 (*green*), and DAPI (*blue*). Representative immunofluorescent images from each group in mice at different ages are shown. *Scale bar*: 100 µm. **(F)** The fluorescence intensity of p16 was quantified and is displayed in the bar graph. The data are mean ± SD, one-way ANOVA, $n = 6$. ns, not significant, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, and $^{****}P < 0.0001$.

free radical scavenger with antioxidant and immunomodulatory activity and an ability to maintain mitochondrial homeostasis, contains potent anti-aging properties.[49] It has been suggested that degenerative diseases and their underlying age-dependent pathology are excellent targets for the anti-aging intervention strategies involving melatonin treatment.[50,51] Caballero et al.[52,53] and Nogues et al.[54] found that melatonin significantly diminished oxidative stress and the levels of senescent markers and enhanced apoptosis-related protein levels, confirming the effectiveness of melatonin in improving age-related impairments. In this study, our results demonstrated that CNV size and associated vascular leakage was significantly augmented in the laser-induced CNV model of old mice. However, melatonin significantly inhibited macrophage/microglia senescence and reduced the CNV size and vascular leakage in old mice, indicating the effective antiaging role of melatonin in senescence-associated CNV.

The recognition of multiple polarization states led to the discovery that M2 macrophages, rather than M1 macrophages, promoted angiogenesis in aging-associated diseases such as AMD.[11,19] Previous studies suggested that macrophages from old mice polarized more readily to an M2 type and promoted abnormal angiogenesis as seen in age-related diseases such as CNV in AMD.[11,55] Similarly, our present data indicated that the M2 markers levels were significantly higher in old mice and were accompanied by a significant increase in CNV lesions size and vascular leakage, confirming the crucial role of M2 macrophage/microglia polarization in aggravating CNV in the aged eye.

Melatonin has been suggested to play a key role in controlling the polarization and function of macrophage/microglia.[30,56] Zhang and colleagues[57] demonstrated that melatonin reduced M1 polarization markers but increased M2 markers in female rats with spinal cord injury, suggesting that melatonin induced an M1 to M2 phenotype switch to facilitate functional recovery. However, Cheng and colleagues[33] revealed that exosomes derived from hepatocellular carcinoma cells treated with melatonin decreased IL-10 expression and STAT3 activation in tumor-associated

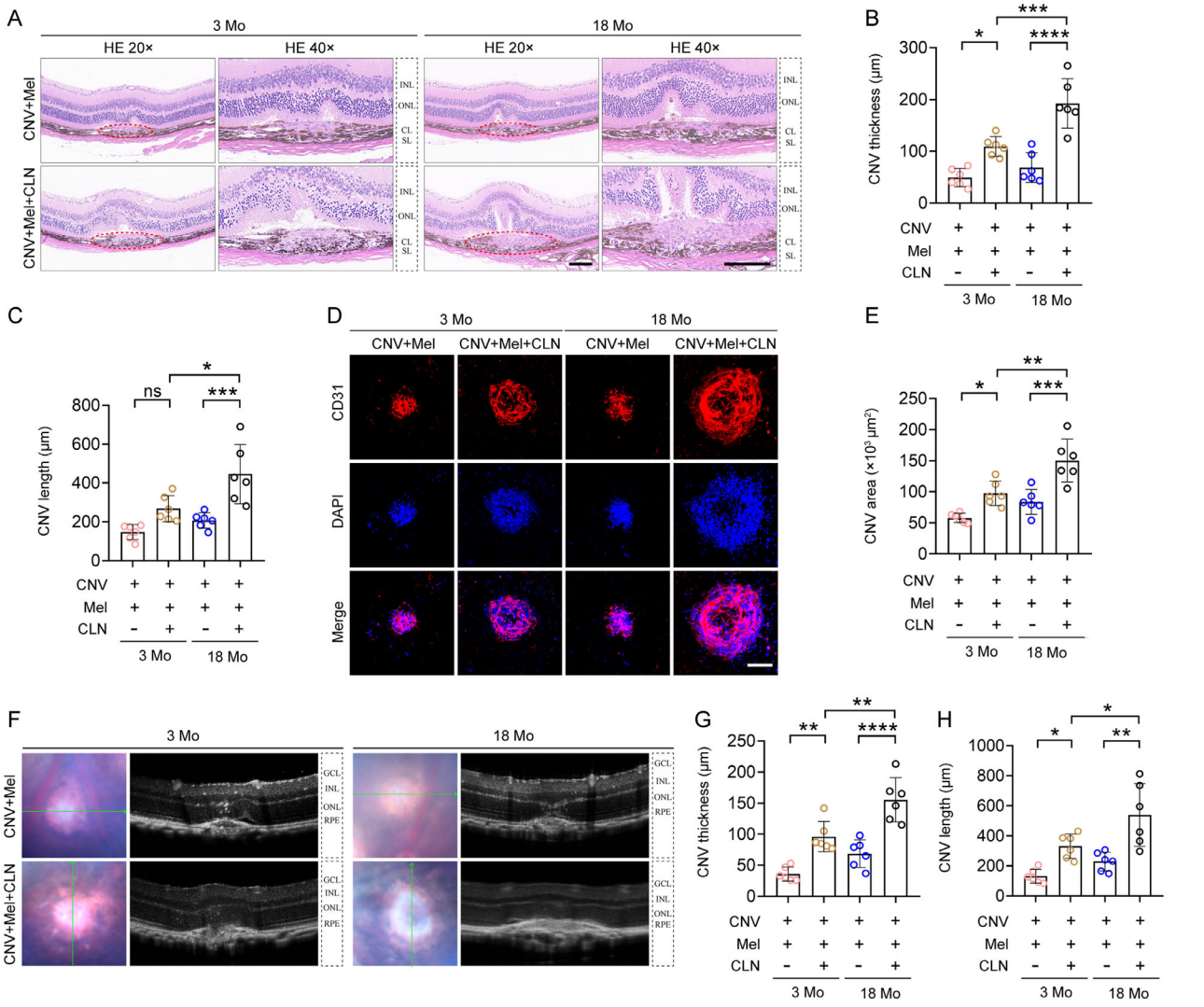

Figure 8. The effect of colivelin on melatonin-induced reduction of CNV size in mice at different ages. **(A)** Representative HE staining images of the CNV + melatonin group and CNV + melatonin + colivelin group in young and old mice are shown. INL, inner nuclear layer; ONL, outer nuclear layer; CL, choroid layer; SL, sclera layer. The CNV lesions are circled with *red dashed lines*. Magnified views are shown on the right panel. *Scale bar*: 100 μm. **(B, C)** Quantification of the thickness and length of CNV lesions in each group is shown in the bar graphs. **(D)** Representative immunofluorescent images of CNV lesions stained with CD31 (*red*) and DAPI (*blue*) in choroidal flat mounts are shown. *Scale bar*: 100 μm. **(E)** The area of CNV lesions in each group was quantified and is displayed in the bar graph. **(F)** Representative fundus photographs (*left*) and OCT images (*right*) from the CNV + melatonin group and CNV + melatonin + colivelin group in young and old mice are shown. GCL, ganglion cell layer; RPE, retinal pigment epithelium. **(G, H)** Quantification of the thickness and length of CNV lesions in each group is shown in the bar graphs. The data are mean ± SD, one-way ANOVA, $n = 6$. ns, not significant, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, and $^{****}P < 0.0001$.

macrophages, which are the major tumor-infiltrating macrophages in tumors that express M2-like phenotype. These studies showed that the regulation of macrophage/microglia polarization status by melatonin may differ based on the disease and that the molecular mechanisms involved are complex.[30,58] In our study, we discovered that melatonin effectively suppressed the polarization of senescent macrophage/microglia to an M2 phenotype in the CNV model of old mice compared with young mice, suggesting the beneficial effect of melatonin on age-dependent modulation of macrophage/microglia polarization.

A deeper understanding of the mechanism underlying the age-dependent antiangiogenic capability of melatonin is certainly necessary and urgent and may lead to new therapeutic strategies for age-related diseases such as AMD. It should be noted that some studies reported that melatonin treatment effectively reduced the level of IL-10,[59,60] whereas others demonstrated that melatonin up-regulated IL-10 expression.[61,62] A similar phenomenon was also observed in the regulation of STAT3 by melatonin. Carbajo-Pescador and colleagues[63] proved that melatonin exerts an antiangiogenic activity in HepG2 liver cancer cells by repressing the transcriptional activation of VEGF via blockade of HIF-1α and STAT3 signaling. However, Yang et al.[64] showed that melatonin pretreatment could improve postischemic cardiac function, reduce mitochondrial oxidative damage, and attenuate ischemia/reperfusion injury by activating the JAK2/STAT3 pathway. These results indicated that mela-

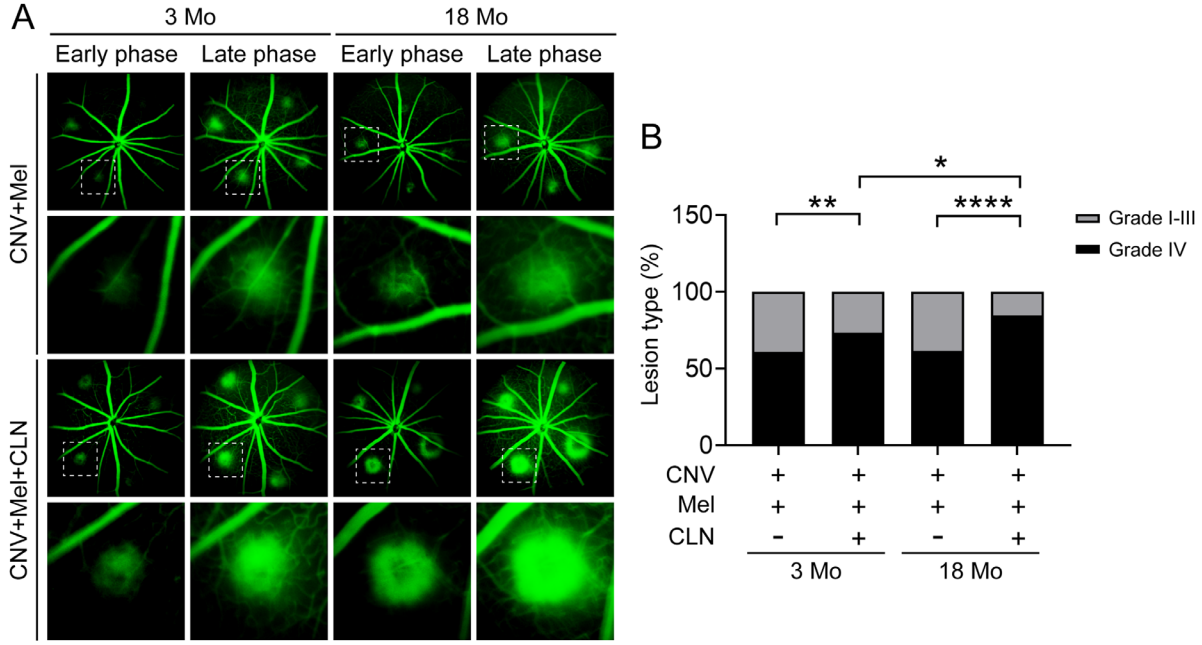

Figure 9. The effect of colivelin on melatonin-induced attenuation of CNV leakage in mice at different ages. **(A)** Representative FFA images of different groups were acquired at two different times (the early phase and late phase) after intraperitoneal delivery of fluorescein. Magnified views in *white dotted boxes* are shown in the respective lower panel. **(B)** Fluorescein leakage in CNV lesions was graded on late images. The grading criteria of FFA images were as follows: Grade I indicated the absence of hyperfluorescence, Grade II indicated the presence of hyperfluorescence without leakage, Grade III indicated hyperfluorescence in the early or mid-transit images and late leakage, and Grade IV indicated bright hyperfluorescence in the transit images and late leakage beyond the treated areas. The data are mean ± SD, one-way ANOVA, $n = 6$. *$P < 0.05$, **$P < 0.01$, and ****$P < 0.0001$.

tonin might play diverse regulatory roles on IL-10 and STAT3 in different diseases. Our results found that melatonin significantly inhibited the IL-10/STAT3 pathway activation in macrophage/microglia of old mice, and the STAT3 activator colivelin reversed the suppressive effect of melatonin on M2 polarization of senescent macrophage/microglia and laser-induced CNV in old mice, suggesting that the IL-10/STAT3 signaling pathway was involved in the beneficial effect of melatonin on senescence-associated CNV.

Furthermore, it is important to realize that IL-10 can be produced via the NF-κB signaling pathway. Indeed, Cao et al.[65] found that NF-κB1 (p50) homodimers bind to the proximal IL-10 promoter and form a complex with coactivators to initiate the process of IL-10 transcription. However, they also observed that NF-κB1-KO mice can still generate a certain amount of IL-10, implying the existence of p50-independent mechanisms for facilitating IL-10 transcription. Constitutively expressed transcription factors, such as Sp-1 and Sp-3, could be candidates for these mechanisms because both have been shown to participate in IL-10 regulation.[66,67] These studies indicated that the intricate and precise regulation of IL-10 production is attributable to the involvement of numerous transcription factors and the existence of multiple levels of gene regulation. Additionally, it has been reported that the NF-κB signaling in macrophages does not play a role in CNV, and macrophage-specific knockout of IKKβ showed no effect on CNV, confirming that in macrophages, the NF-κB signaling is not critical for the regulation of CNV.[19]

The baseline expression of suppressor of cytokine signaling 3 (SOCS3) is also important for predicting macrophage polarization in an IL-10 rich environment.[19] In fact, SOCS3 is widely recognized as a prominent target of STAT3-mediated signaling after IL-10 stimulation, functioning as a feedback regulator for the production of IL-10.[68] Analysis of SOCS3 expression in macrophages of different age groups by Nakamura et al.[19] discovered that old macrophages showed a significant decrease in SOCS3 expression and an increase in M2 markers expression, suggesting an inverse relationship between SOCS3 and M2 marker expression in old macrophages. Moreover, they also demonstrated that impaired SOCS3 feedback led to permissive IL10/STAT3 pathway that promoted M2 polarization and pathological CNV. Therefore future studies are planned to further investigate the role of SOCS3 in the pathogenesis of senescence-associated CNV more comprehensively.

In summary, our findings indicated that melatonin effectively prevented the polarization of senescent macrophage/microglia toward a proangiogenic M2 type by inhibiting the IL-10/STAT3 signaling pathway and eventually attenuated CNV and vascular leakage. These results will be valuable for a better understanding of the pathogenesis of age-related diseases and provide evidence for the potential therapeutic benefit of melatonin in the treatment of senescence-associated CNV.

Acknowledgments

Supported by the National Natural Science Foundation of China (82271099, 81970807), the Research Grant from Guangzhou Municipal Science and Technology Bureau in China (202201020507, 202102010324), and the Guangdong Basic and Applied Basic Research Foundation (2022A1515010355, 2022A1515110213).

Disclosure: **K. Cui**, None; **X. Tang**, None; **A. Hu**, None; **M. Fan**, None; **P. Wu**, None; **X. Lu**, None; **J. Lin**, None; **F. Yang**, None; **X. Zhao**, None; **J. Huang**, None; **S. Yu**, None; **Y. Xu**, None; **X. Liang**, None

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