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Immune modulation by MANF promotes tissue repair and regenerative success in the retina

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Abstract

Regenerative therapies are limited by unfavorable environments in aging and diseased tissues. A promising strategy to improve success is to balance inflammatory and anti-inflammatory signals and enhance endogenous tissue repair mechanisms. Here, we identified a conserved immune modulatory mechanism that governs the interaction between damaged retinal cells and immune cells to promote tissue repair. In damaged retina of flies and mice, Platelet-Derived Growth Factor (PDGF)-like signaling induced Mesencephalic Astrocyte-derived Neurotrophic Factor (MANF) in innate immune cells. MANF promoted alternative activation of innate immune cells, enhanced neuroprotection and tissue repair, and improved the success of photoreceptor replacement therapies. Thus, immune modulation is required during tissue repair and regeneration. This approach may improve the efficacy of stem-cell based regenerative therapies.

Graphical Abstract

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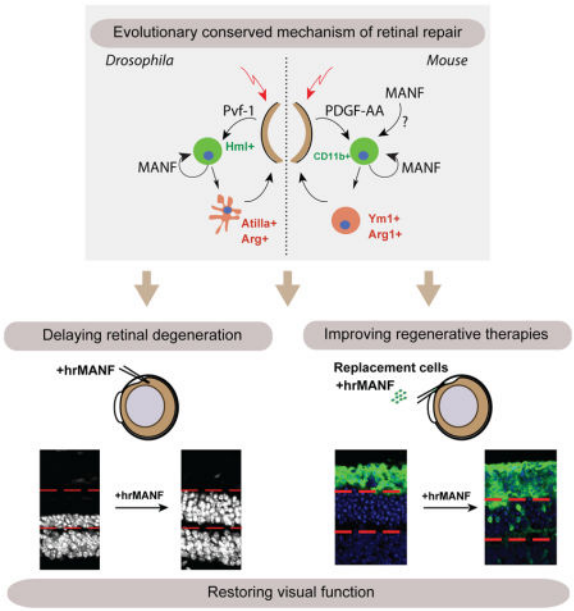

Main Text

Regenerative therapies based on cell replacement hold promise for the treatment of a range of age-related degenerative diseases (1, 2). Moreover, aged and diseased tissues provide a poor microenvironment for integration (3). A case in point are attempts to regenerate the vertebrate retina, a tissue where endogenous repair mechanisms are inefficient and that is subject to a variety of irreversible age-related degenerative pathologies. Human pluripotent stem cells can provide a virtually unlimited source of photoreceptors and retinal pigment epithelial (RPE) cells for replacement and restoration of vision (4), yet the poor integration efficiency of transplanted cells into the host retina has limited clinical applications. Retinal diseases targeted by this therapeutic approach, such as age-related macular degeneration (AMD) or Retinitis Pigmentosa (RP), are characterized by microglial activation and pro-inflammatory microenvironments (5–9) that will negatively affect integration and repair (3, 10).

Microglia, monocyte-derived macrophages, and other innate immune cell types can both promote and resolve inflammation. Managing these inflammatory responses is essential for tissue repair and regeneration (11). In the central nervous system (CNS), resident (microglia) and invading innate immune cells orchestrate a complex response to damage aimed at restoring tissue integrity, but can also promote damaging neuroinflammation (12–15). This antagonism is at least in part a consequence of different states of immune cell activation. Classical or M1 activation is associated with pro-inflammatory conditions that can cause tissue damage, while alternative or M2 activation is associated with resolution of inflammation and tissue repair (16, 17). This M1/M2 paradigm has been used to describe outcomes of in vitro perturbation of macrophages, yet there is evidence that macrophages in vivo can adopt similar phenotypes and functions (18, 19). Because of these opposing effects

of different immune cell phenotypes, immune modulation rather than immune suppression may be an effective way to promote tissue repair and promote regenerative therapies.

Studies in *Drosophila* have significantly advanced our understanding of tissue repair and regeneration in metazoans (20–22). This work has highlighted the critical role of the interaction between hemocytes (*Drosophila* blood cells with macrophage-like activities) and damaged epithelia in the repair process. Hemocytes are activated in response to tissue damage and coordinate localized and systemic repair responses (23–26), but have also been implicated in inflammatory processes in flies (27). A productive model for the genetic dissection of tissue and hemocyte interactions in repair processes is the pupal retina, which responds to UV damage by inducing photoreceptor apoptosis in a dose-dependent manner (28, 29). A paracrine interaction between UV-damaged photoreceptors and hemocytes through the PDGF- and VEGF-related factor 1 (Pvf-1) and PDGF- and VEGF-receptor related (PvR) pathway governs repair of the damaged retina: Damaged photoreceptors secrete Pvf-1 and activate PvR in hemocytes, promoting repair of UV-induced tissue damage (Fig. 1A) (26).

We performed RNA sequencing (RNAseq) on isolated hemocytes to identify PvR-dependent genes encoding secreted proteins that were induced after epithelial damage (Fig. S1 and Table S1). Mesencephalic Astrocyte-derived Neurotrophic Factor (MANF) was found in this screen and, based on its evolutionarily conserved neurotrophic activity (30–32), we decided to explore its potential as a retinal repair factor.

Hemocyte-derived MANF is activated downstream of Pvf-1/PvR paracrine signaling to promote retinal repair in *Drosophila*

We confirmed that MANF is expressed in fly innate immune cells (hemocytes) using immunohistochemistry of hemolymph smears from late 2nd instar larvae (Fig. 1B, left). In these smears, hemocytes were identified by Green Fluorescent Protein (GFP) expression driven by the hemocyte specific driver Hemolectin:Gal4 (HmlΔ:Gal4) (33). MANF was also detected by immuno blot in the plasma fraction of the hemolymph, confirming its secretion (Fig. 1B, right). Consistent with the RNAseq data, Reverse Transcription and Real Time quantitative Polymerase Chain Reaction (RT-qPCR) analysis revealed that MANF mRNA levels were significantly higher in hemocytes from UV treated larvae compared to untreated controls (Fig. 1C, left), and that this induction was PvR dependent (Fig. 1C, right and Fig. S1C and S2A). Over-expression of Pvf-1 in the retina (using GMR:Gal4; Glass Multimer Reporter (34) as a driver) was sufficient to induce MANF mRNA specifically in hemocytes, in the absence of damage (Fig. 1D, left), and was accompanied by a significant increase in MANF protein in the hemolymph (Fig. 1D, right and Fig. S2B).

Flies overexpressing MANF in hemocytes (Fig. S2C, left) showed significant tissue preservation after UV exposure, even after PvR knock-down in hemocytes (26) (Fig. 1E, left and middle, without affecting PvR^{RNAi} knock-down efficiency, Fig. S2A). This protective activity of hemocyte-derived MANF was further confirmed in two genetic models of retinal damage, in which degeneration is induced by retinal (GMR driven) over-expression of the pro-apoptotic gene *grim* or of mutant Rhodopsin ($Rh1^{G69D}$) (35, 36) (Fig. S2D–E).

Null mutations in the *manf* gene (*manf*^{mut96} and *manf*^{mut112}, (31)) are homozygous lethal at early 1st instar larval stages, yet MANF heterozygotes (which express significantly lower levels of MANF in hemocytes compared to wild-types; Fig. S2F) had a significantly increased tissue degeneration response to UV (Fig. 1F, grey dots and Fig. S2G). This increase in tissue loss could be rescued by MANF over-expression in hemocytes (Fig. 1F, black dots) and was recapitulated by hemocyte-specific knock-down of MANF (Fig. 1E, right and Fig. S2C, right).

MANF has immune modulatory properties that are required for retinal repair in *Drosophila*

The protective effect of hemocyte-derived MANF could be caused by direct neuroprotective activity of MANF on retinal cells, or could reflect an indirect effect of MANF on the microenvironment of the damaged retina. To distinguish between these possibilities, we tested if MANF could influence hemocyte phenotypes. Hemocytes can acquire lamellocyte phenotypes, characterized by down-regulation of plasmatocyte markers (*hemolectin*, *hemese*) and expression of Atilla protein (37), during sterile wound healing (38). These phenotypes correlate with hemocyte activation and may influence tissue repair capabilities, and we recapitulated them in our UV damage paradigm (Fig. 2A). Over-expression of MANF in hemocytes in vivo or treatment of hemocytes in culture with human recombinant MANF (hrMANF) significantly increased the proportion of lamellocytes in hemocyte smears, as detected by Atilla expression (Fig. 2A). This correlated with a decrease in the proportion of cells expressing GFP driven by HmlΔ:Gal4 and a decrease in *hml* transcripts (Fig. S3A). Furthermore, MANF was necessary and sufficient to induce the *Drosophila* homolog of the mammalian M2 marker *arginase1* (*arg*) (39) in hemocytes (Fig. 2B and Fig. S3B), suggesting that these cells may be able to acquire phenotypes similar to alternative activation (16, 17). Most MANF expressing hemocytes also expressed Arg, suggesting that there is an association between MANF expression and M2-like activation of hemocytes.

To test whether MANF's immune modulatory function is required for retinal repair, we assessed retinal tissue preservation in conditions in which hemocytes express and secrete high levels of MANF, but are unable to be activated in response to this signal. We generated such a condition by overexpressing MANF in the absence of Kdel Receptors (KdelRs). In human cells, KdelRs modulate MANF secretion and cell surface binding. Intracellular KdelR prevents MANF secretion, while cell surface bound KdelR promotes binding of extracellular MANF (40). Knock-down of the one *Drosophila* KdelR homologue (41) in hemocytes resulted in a significant induction of MANF transcripts and the detection of MANF protein in the hemolymph (Fig. S3C–D), suggesting that KdelR-depleted hemocytes secrete high levels of MANF. In these hemocytes, MANF-induced lamellocyte formation and Arg expression were significantly decreased (Fig. 2C–D). Hemocyte activation by extracellular MANF is thus impaired after KdelR knock-down. This genetic perturbation also resulted in a significant enhancement of UV-induced tissue loss, which could not be rescued by MANF over-expression (Fig. 2E). Thus, immune modulation by MANF is critical for tissue repair.

Damage response-associated PDGF-A/MANF paracrine signaling is conserved in mammals

MANF is an evolutionarily conserved protein (31) and we sought to explore its regulation and its potential to allay retinal degeneration and improve retinal repair in vertebrates. We used focal exposure of the central retina of C57BL/6 mice to 8klux of bright light for 1.5h to induce a retinal innate immune response without generalized photoreceptor apoptosis (C57BL/6 mice carry a protective variant of the Rpe65 gene, preventing excessive retinal damage in response to light (42)). This protocol resulted in a moderate and transient increase in the presence of innate immune cells in the retina (Fig. 3, S4A–B).

PDGF-family and VEGF-family proteins are the mammalian homologs of *Drosophila* Pvf-type ligands (43, 44), and we detected PDGF-A expressing cells in the neural retina six hours after light exposure (Fig. 3B). The induction of PDGF-A was followed by a significant increase in MANF transcripts (Fig. 3C) and the detection of MANF+ innate immune cells, identified by CD11b expression (45, 46), in the vitreous (12 h; Fig. 3D and Fig. S4A). Resting microglia, localized to the plexiform layers (IPL, inner plexiform layer and OPL, outer plexiform layer) in control retinas, did not express MANF (no light exposure; Fig. 3D). Thirty–six hours later, MANF+ innate immune cells were found within the outer nuclear layer (ONL) (36 h; Fig. 3D and Fig. S4A). This innate immune cell activation and/or recruitment was also accompanied by a re-distribution of MANF protein from the cell bodies of Müller glia (where it is detected in control conditions, in the inner nuclear layer, INL) to glial processes (identified by staining against Glial fibrillary acidic protein; GFAP, Fig. S4C). Microglia and/or macrophages recruited and/or activated after light exposure expressed reduced levels of MANF when PDGF-signaling was inhibited using neutralizing antibodies against PDGFRα (47) (Fig. S4D–E). Conversely, intravitreal injection of mouse recombinant PDGF-AA significantly increased CD11b+ innate immune cells in eyes in the absence of light exposure (Fig. 3E). These CD11b+ cells also expressed MANF and were found in the vitreous (Fig. 3F, right, arrowheads) and the choroidal blood vessels (Fig. 3F, left, arrowheads).

Reduction of PDGFRα signaling or MANF levels (in heterozygotes for a null allele (48)) significantly enhanced photoreceptor apoptosis (detected by TUNEL) in response to light exposure (Fig. 3G–H). Homozygotes for this MANF allele are embryonic or perinatal lethal (48). Reduction of MANF expression in bone marrow-derived macrophages (BM-macrophages) of these mice was confirmed by RT-qPCR (Fig. S4F).

Damage signals from retinal cells thus engage a conserved retinal repair response in both flies and mice that involves the Pvf/PDGF-mediated recruitment/activation of MANF-expressing innate immune cells and that is essential to prevent excessive apoptosis in response to light.

MANF has a conserved neuroprotective function in the mammalian retina

To test whether MANF protein supplementation would be sufficient to ameliorate retinal degeneration, we used the light-sensitive BALB/cJ strain, which lacks the protective variant

of the Rpe65 allele, rendering them susceptible to light induced retinal damage (42). This phenotype is accompanied by activation of pro-inflammatory microglia and by chemokine production that modulates photoreceptor degeneration (49). Exposure of these mice to 5klux of bright light for 1h resulted in photoreceptor apoptosis (Fig. S4G). In addition, we used two genetic models of retinal degeneration (*Crx*^{tvrm65}, a slow model of genetically induced retinal degeneration, and *Pde6b*^{Rd1}, a fast model of genetically induced retinal degeneration), whose dynamics of photoreceptor loss have been previously described (50–52).

We injected human recombinant MANF (hrMANF) protein or vehicle (PBS) into the vitreous immediately prior to light exposure or at the onset of retinal degeneration (P14 for *Crx*^{tvrm65} mice and P7 for *Pde6b*^{Rd1} mice), and evaluated photoreceptor apoptosis by TUNEL. MANF injection significantly reduced apoptosis in all three models of retinal degeneration (Fig. 3I and Fig. S5A–B). As photoreceptors degenerate, the number of nuclei in the ONL is reduced, and in *Crx*^{tvrm65} mice there are on average 5–6 rows left at P21. In hrMANF treated eyes, there was a significant preservation of photoreceptors in the ONL (Fig. S5C), suggesting that inhibition of apoptosis effectively slows retinal degeneration in this model. Similar results were observed in the *Pde6b*^{Rd1} mouse model analyzed five days after intravitreal delivery of hrMANF (Fig. S5D–E).

Finally, we asked whether a persistent source of MANF could further delay retinal degeneration in *Crx*^{tvrm65} mice. We infected human fibroblasts with a lentivirus driving the expression of a functional MANF-GFP fusion protein (40). MANF-GFP expression could readily be detected in these fibroblasts and in the media supernatant, confirming that the fusion protein was efficiently secreted (Fig. S5F–G). When MANF-secreting fibroblasts were injected into the vitreous of P14 *Crx*^{tvrm65} mice, their retinas degenerated more slowly than control fibroblast-injected retinas, and a significant amount of photoreceptors were preserved in the ONL (Fig. 3J). Survival of injected fibroblasts was confirmed at the time of analysis (2 weeks after injection) by detecting the presence of GFP expressing cellular aggregates within the vitreous.

MANF can thus prevent photoreceptor apoptosis broadly and delay retinal degeneration, independently of the damaging stimulus.

MANF-dependent modulation of immune cell phenotypes mediates retinal protection

After intravitreal injection of MANF-secreting fibroblasts, MANF+ innate immune cells (CD11b+) could be detected in the vitreous of *Crx*^{tvrm65} mice (Fig. 4A, top panel). These CD11b+ cells with round morphology also expressed markers of alternative activation (16, 17) (Fig. 4A, bottom panel, and Fig. 4B, left – fibroblasts injected into the vitreous were detected by GFP expression at the time of dissection and were completely removed along with the lens). Intravitreal delivery of hrMANF had similar effects on innate immune cell phenotypes in *Crx*^{tvrm65} (Fig. 4B, right) and light-damaged retinas (Fig. 4C and Fig. S6A), supporting an immune modulatory function for MANF. Accordingly, the recruitment of MANF+ innate immune cells in response to PDGF-AA treatment (Fig. 3E–F) was

accompanied by a significant increase in the number of CD11b+ cells co-labelled with Ym1+ (Fig. 4D and Fig. S6B).

The innate immune cell population recruited after MANF delivery was mostly composed of monocytes and monocyte-derived macrophages (60–80%, identified by F4/80 or CD68 expression). Ly6-G+ (Gr-1^{high}) neutrophils represented about 15% of the population. The majority of both macrophages and neutrophils (80%) expressed MANF and Arg1, suggesting that MANF expression is associated with markers of alternative activation (Fig. S6C), similar to what we observed in fly hemocytes (Fig. S3B).

In vitro stimulation with hrMANF for 3 hours was also sufficient to induce markers of alternative activation (Arg1 and Ym1) (16, 17) and Il-13, an anti-inflammatory cytokine (53), in bone marrow-derived macrophages (BM-macrophages) (Fig. 4E) and in a macrophage cell line (RAW264.7, (54), Fig. S7A–B). Silencing of MANF with a targeting siRNA pool in this cell line resulted in the repression of the same set of genes (Fig. 4F). This suggests that MANF has a direct immune modulatory function in macrophages and that at least part of the mechanism is autocrine.

To test whether immune modulation by MANF is required for its neuroprotective activity, we assessed retinal damage after light exposure and following MANF supplementation in mice with impaired immune cell function. We depleted macrophages and microglia using Diphtheria toxin (DT) administration in CD11b:DTR mice (55, 56). DT, but not sham (PBS), injection resulted in a significant reduction in the number of innate immune cells in the retina (Fig. S7C) and induction of photoreceptor apoptosis in response to light exposure (Fig. 4G). Intravitreal supplementation of hrMANF protein did not significantly reduce photoreceptor apoptosis in these mice (Fig. 4G), supporting an essential role for immune cells in mediating the protective effects of MANF.

We further used mice deficient in Cx3Cr1 (57, 58) to test the requirement of immune modulation for the protective effects of MANF. Here we aimed at generating a condition in which immune cells were present but failed to induce alternative activation in response to MANF signaling, similar to KdelR deficiency in flies. Cx3Cr1 is a chemokine receptor expressed in different immune cell populations, including retinal microglia and peripheral monocytes (58). High Cx3Cr1 expression has been associated with a functionally distinct class of monocytes with immune patrolling activity and with a molecular profile of macrophage differentiation resembling alternative activation (18, 19). Loss of Cx3Cr1 results in retinal degeneration in response to several stimuli and is associated with pro-inflammatory activation of immune cells (9, 14, 59). Thus we hypothesized that loss of Cx3Cr1 could be an effective way to impair MANF-induced alternative activation. Indeed, BM-macrophages derived from Cx3Cr1-deficient mice failed to induce genes associated with alternative activation upon MANF stimulation (Fig. 4H), despite expressing normal levels of MANF (Fig. S7D–E). Light-induced photoreceptor apoptosis in Cx3Cr1-deficient mice could not be rescued by intravitreal delivery of hrMANF (Fig. 4I), suggesting that it is not only MANF derived from macrophages that mediates the protective effects, but rather a more complex mechanism that depends on MANF immune modulatory activity. We cannot exclude, however, that Cx3Cr1-deficiency may also result in other alterations that contribute

to the loss of protective effects of MANF observed in these conditions, which may be independent of macrophage functions.

MANF promotes cell integration and restoration of visual function in the mammalian retina

Retinal repair by transplantation of mouse and human photoreceptor precursors can restore vision in mouse models of retinal degeneration (60, 61). Integration efficiency depends on the ontogenetic stage of donor cells (60) and on the status of the degenerative microenvironment (62), and negatively correlates with the presence of classically activated macrophages within the retinal tissue (63). We injected photoreceptors derived from Nrl-GFP mice subretinally into wild-type retinas and found that microglia and/or macrophages located at sites of integration expressed MANF, suggesting a possible role for MANF-mediated immune modulation in promoting integration (Fig. 5A–C). Supporting this hypothesis, integration efficiency was significantly reduced in Cx3Cr1 mice (Fig. 5D).

To further test this hypothesis, we asked whether MANF supplementation would increase integration of subretinal delivered photoreceptors derived from Nrl-GFP mice into a wild-type host. Integration efficiency declines with increased maturity of injected photoreceptors (60, 64). Accordingly, we observed a strong decline in integration efficiency (assessed one week after injection) when using P21 rather than P7 or P14 photoreceptors in a wild-type host (Fig. 5E). hrMANF supplementation rescues this decline while having no effect on P14 cells (Fig. 5E and Fig. S8A), suggesting that MANF may act either directly on refractory photoreceptors to improve their integration capabilities, or indirectly by inducing a more supportive environment for such cells.

To distinguish between these possibilities, we tested the effects of MANF on integration efficiency in degenerating retinas. The inflammatory microenvironment in degenerating retinas is a likely cause for poor integration efficiency (62), and thus a critical limitation in clinical settings. We used Crx^{tvrm65} retinas to model a degenerating environment and found that integration of even young (P7) Nrl-GFP photoreceptors, which efficiently integrated into wild-type retinas (Fig. 5E), was significantly reduced in Crx^{tvrm65} retinas (Fig. 5F). MANF supplementation significantly improved integration in this context (Fig. 5F), supporting the notion that MANF improves the environment for integration even in a disease context.

Importantly, MANF accelerated and improved restoration of visual function, as evaluated by maximal b-wave amplitudes measured in sequential electroretinogram (ERG) testing over the course of four weeks (Fig. 5G–H). Eyes that received MANF-supplemented transplants showed signs of light-responsiveness based on a detectable b-wave as early as 1 week after transplantation, while eyes that received control transplants had the earliest detectable b-wave only at 3 weeks. Comparing ERG b-wave amplitudes of untreated Crx^{tvrm65} mice (no transplant) to treated mice at 1–4 weeks confirmed a functional improvement in vision in the MANF supplemented cohort only, while the PBS supplemented group did not significantly differ from untreated controls. The ERG changes reflected cell integration and not an effect of MANF supplementation alone (Fig. S8B) and represented a recovery of about 60% of

visual function when compared to normal ERG b-wave amplitudes of wild-type mice (Figs. 5H and S8C). This is a significant improvement over non supplemented transplants which yield about 20% of visual function recovery (Figs. 5H and S8C, see also (61)).

Discussion

Our results identify MANF as an evolutionarily conserved immune modulator that plays a critical role in the regulatory network mediating tissue repair in the retina (Fig. 6A). The ability of MANF to increase regenerative success in the mouse retina highlights the promise of modulating the immune environment as a strategy to improve regenerative therapies (Fig. 6B).

The usefulness of immune modulation for regenerative medicine has been anticipated based on studies of tissues where regeneration is sustained endogenously by resident stem cells (3, 11, 65–69). Our study provides strong support for this hypothesis.

MANF has previously been described as a neurotrophic factor (30, 70, 71), and it may also exert a direct neuroprotective effect in the retina, yet our data suggest a more expansive role: because MANF cannot promote tissue repair in flies in which the hemocyte response to MANF is selectively ablated, or in mammalian retinas depleted of innate immune cells or containing macrophages that are unresponsive to MANF, we propose that MANF's role in promoting alternative activation of innate immune cells is central to its function in tissue repair. Further studies will be required to determine the specific contribution of alternative-activated macrophages in mediating these effects. While our data point to an important role of macrophages in mediating the effects it does not exclude the possibility that other cell types are involved in the process, nor that macrophages' functions other than polarization may influence the outcome of MANF's protective effects.

Clinically, MANF may thus have a distinct advantage over previously described neurotrophic factors in both improving survival of transplanted cells directly, as well as in promoting a microenvironment supportive of local repair and integration. Because integration efficiency correlates with the extent of vision restoration (61) it can be anticipated that MANF supplementation will have an important impact in clinical settings.

Further studies involving tissue specific knockdown of MANF in mammals will be required to evaluate the relative contribution of different cellular and tissue sources for MANF in homeostatic and damage conditions. While we found that MANF is strongly expressed in immune cells, we also observed MANF expression in other cell types, in agreement with previous reports (72).

Similarly, the molecular mechanism involved in MANF signaling remains elusive. To date, a signal transducing receptor for MANF has not been identified, although Protein kinase C (PKC) signaling has been described to be activated downstream of MANF (73). MANF can further negatively regulate NF-κB signaling in mammalian cells (74) and loss of MANF in *Drosophila* results in the infiltration of pupal brains with cells resembling hemocytes with high Rel/NFkB activity, potentially representing pro-inflammatory, M1-like phenotypes

(75). The identification of immune cells as a target for MANF in our study may accelerate the discovery of putative MANF receptors and downstream signaling pathways.

Because neurotoxic inflammation has been implicated in Parkinson's disease (76), it is possible that the protective effects of MANF in this context (71) are also mediated by immune modulation, as we show here for retinal disease. Indeed, recent reports suggest that the MANF paralog, cerebral dopamine neurotrophic factor (CDNF), has an anti-inflammatory function in murine models of Parkinson's disease (77) and in nerve regeneration after spinal cord injury (78). A recent study has further shown that loss of MANF leads to beta cell loss in the pancreas (48). Beta cell loss is a commonly associated with chronic inflammation, and it is thus tempting to speculate that MANF is broadly required in various contexts to aid conversion of pro-inflammatory macrophages into pro-repair anti-inflammatory macrophages. Future studies will clarify the role of MANF in resolving inflammation and promoting tissue repair not only in the retina and brain, but also in other tissues. A deeper understanding of MANF-mediated immune modulation and its impact on stem cell function, wound repair and tissue maintenance is thus expected to help in the development of effective regenerative therapies.

Materials and Methods

Mice

All mice used in the described studies were housed and bred at the AAALAC accredited vivarium of The Buck Institute for Research on Aging, in a Specific Pathogen Free (SPF) facility, in individually ventilated cages on a standard 12:12 light cycle. All procedures were approved by the Buck Institute Institutional Animal Care and Use Committee (IACUC). For details on the mouse strains and lines used see Supplementary Materials.

Drosophila stocks and culture

Fly stocks were raised on standard cornmeal and molasses-based food. All experiments were performed at 25°C. Both sexes gave the same result in all experiments, unless otherwise described. For details on the fly lines used see Supplementary Materials.

Intraocular Injections in mice

For intravitreal injection, recombinant proteins or cells in 1μl volume were injected into the right eye using a graduated pulled glass pipet and a wire plunger (Wiretrol II, 5-0000-2005, Durmmond Scientific Company). For details on the test articles injected and procedure for intravitreal injections see Supplementary Materials.

For sub-retinal injection, dissociated GFP-expressing mouse retinal cells from the Nrl-GFP mice were transplanted into the subretinal space of recipient mice using the trans-corneal subretinal injection method. For details on the preparation Nrl-GFP cells and procedure for transplantation see Supplementary Materials.

Light damage in mice

Mice were dark adapted for 18h before the procedure. Test eyes were exposed to 5,000 – 20,000 lux of bright light using a 144-LED microscope ring light (AmScope) for 1–2 hours. After light damage, mice were allowed to recover from anesthesia, returned to their cages and housed in darkness until analysis. Undamaged control mice were housed in regular conditions throughout the experiment. For details see Supplementary Materials.

UV damage in Drosophila Pupae retina and larvae

Pupae retinas were exposed 17.5 mJ of UV light as previously described (26, 28). Second instar larvae were exposed to 50mJ of UV light as previously described (25). For details on the procedures and quantification methods see Supplementary Materials.

Histological analysis, imaging and quantification methods

Retinal sections, macophages and hemocyte smears were analyzed by IHC and other histological methods (see Supplementary Materials for details), imaged using a LSM 700 confocal laser scanning microscope and images were used for quantification purposes. For details on staining methods on the quantification methods see Supplementary Materials.

Electroretinogram (ERG)

All ERGs were carried out under scotopic conditions using a Handheld Multi-species Electroretinograph (HMsERG, Ocuscience) and analyzed using ERGVIEW Version 4.3 (Ocuscience). For details on the procedures see Supplementary Materials.

Cell culture

Raw 264.7 macrophages (ATCC, TIB-71, lot. 61524889) and BM-macrophages were used in hrMANF stimulation experiments and MANF knock down experiments. BM-macrophages were differentiated in culture from bone marrow using 20ng/ml of Macrophage Colony-Stimulating Factor (M-CSF, Sigma, M9170) for 7 days. Raw 264.7 and BM-macrophages were stimulated for 3h with 10μg/ml of hrMANF before analysis. For details on the procedures and on the knock down experiments see Supplementary Materials.

Expression analyses

RNA sequencing and Reverse-Transcription and Real-Time quantitative PCR (RT-qPCR) were used to quantify mRNA levels and Western Blot analysis was used to quantify protein levels. For details on the methods employed in each technique see the Supplementary Materials.

Statistical Analysis

All counts are presented as average and standard error of mean (s.e.m.). Statistical analysis was carried out using Microsoft Excel or GraphPad Prism and student’s t-test or 2-way ANOVA were used to determine statistical significance, assuming normal distribution and equal variance.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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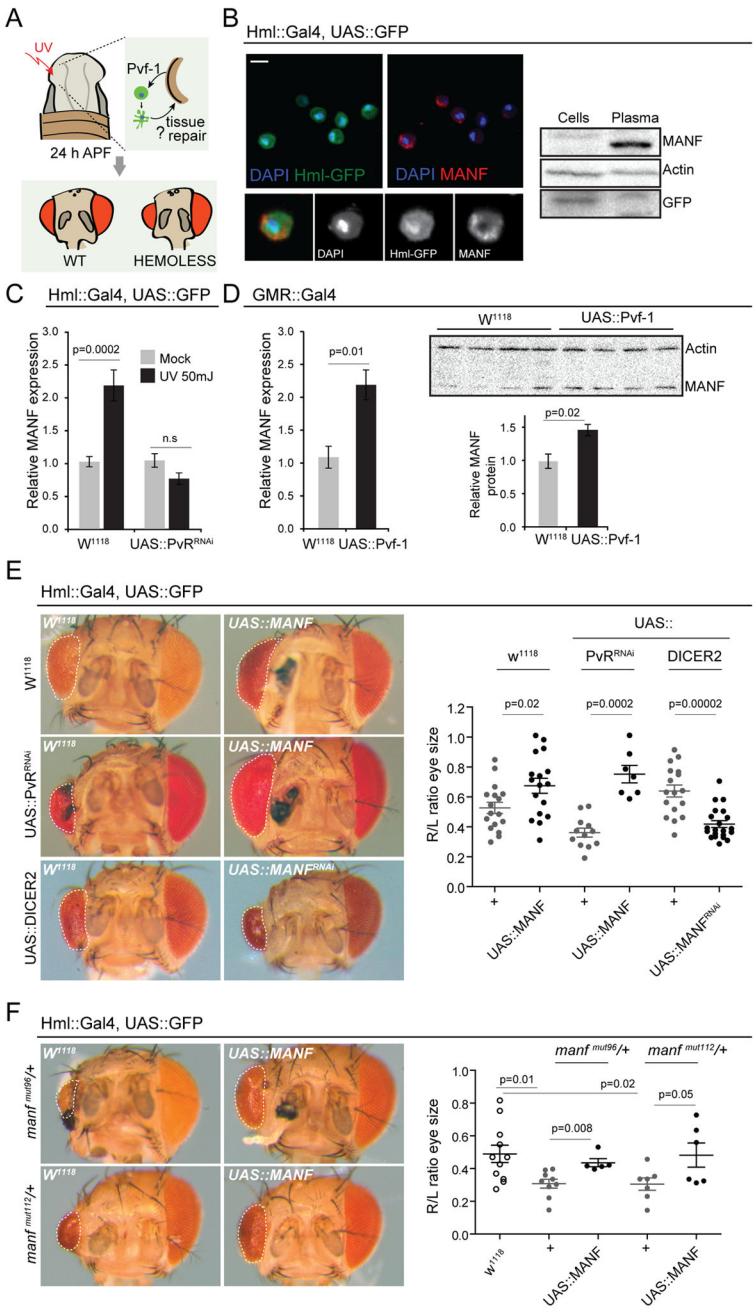

Fig. 1. MANF is a hemocyte-derived damage response factor and promotes retinal repair in *Drosophila*

A, Experimental design and current model for hemocyte mediated retinal repair in *Drosophila*. **B,** Left, representative image of hemocyte smears from 3rd instar larvae (HmlΔ:;Gal4; UAS::GFP) detecting MANF (red) in Hml>GFP+ cells. GFP, green; DAPI, blue. Scale bar 5μm. Right, western blot analysis of MANF and GFP proteins in cellular and plasma fractions from hemolymph of 3rd instar larvae (HmlΔ::Gal4; UAS::GFP). **C,** Relative mRNA levels of MANF detected by RT-qPCR in hemocyte samples collected from 3rd instar larvae of the designated genotypes and treatments (n≥5 for all conditions). For UV

treatments, larvae were exposed to 50mJ of UV at 2nd instar stage and hemocytes collected 24 hours after. **D,** Left, relative mRNA levels of MANF detected by RT-qPCR in hemocyte samples collected from 3rd instar larvae overexpressing Pvf-1 in the retina ($n \geq 5$ for all conditions). Right, western blot analysis of MANF (intracellular in hemocytes and secreted into the hemolymph) and Actin (intracellular in hemocytes) proteins in whole hemolymph collected from 3rd instar larvae overexpressing Pvf-1 in the retina. Bottom graph: average relative levels of MANF in whole hemolymph samples normalized to actin. **E–F,** Left, representative images of adult eye phenotypes from flies with the designated genotypes, after exposure of the right eye of P24 pupae to 17.5mJ of UV light. Right, average relative size of the UV-treated eye when compared to the untreated eye of the same fly ($6<n<17$ for each genotype, each dot represents one fly). For all quantifications error bars represent s.e.m. and p-values are from student's t-test.

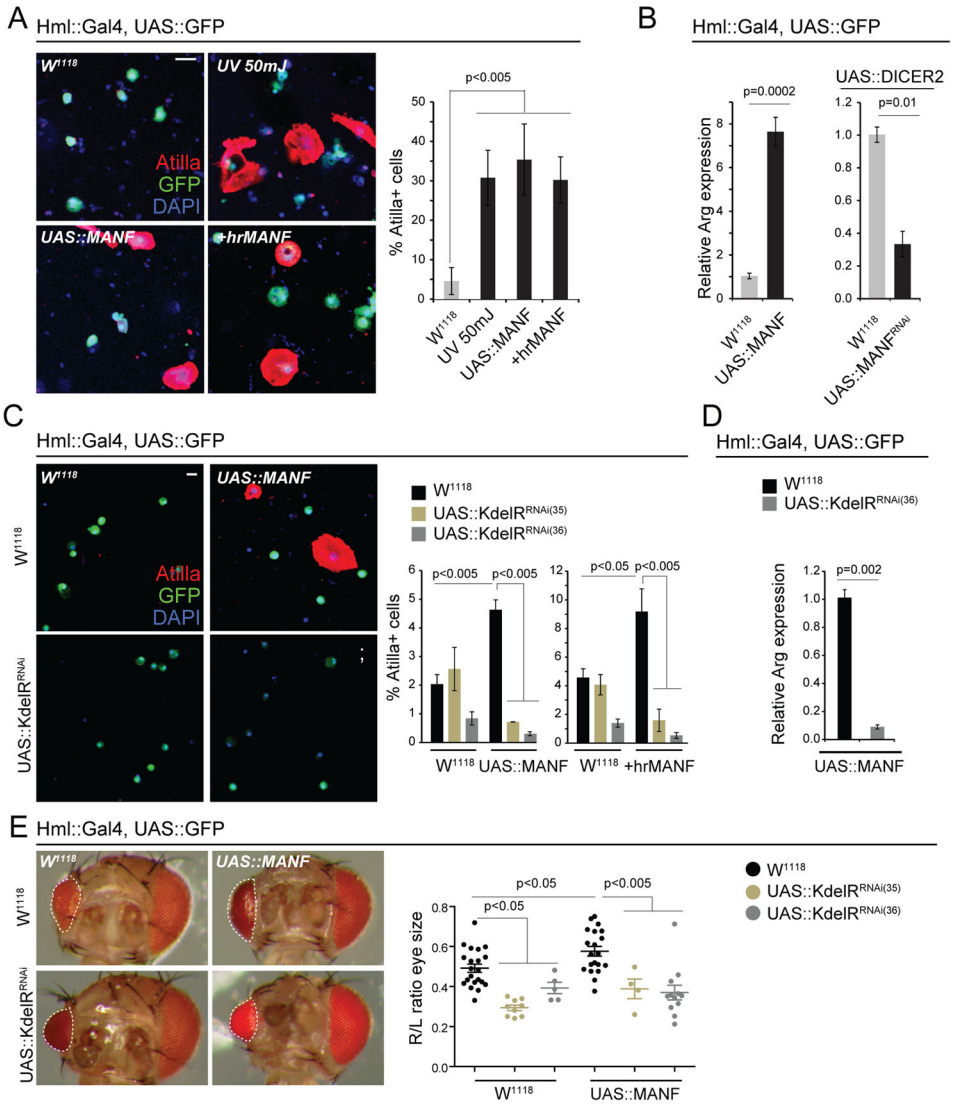

Fig. 2. MANF-dependent hemocyte activation is required for neuroprotection in *Drosophila*

A,C Representative IHC images of hemocyte smears from 3rd instar larvae of the designated genotypes and treatments, detecting Atilla protein in red. Hml+ cells are identified by GFP expression, green; DAPI, blue. Scale bar 5μm. For UV treatments, larvae were exposed to 50mJ of UV at 2nd instar stage and hemocytes collected 24 hours later. In (A) All analysis performed after 24h culture in control media (WT, UV 50mJ and UAS:MANF) or media supplemented with hrMANF protein. In (C), hemocytes were assayed directly after collection and were not cultured (images and left graph) or assayed as in (A). Right graphs: percentage of Atilla+ cells in the hemocyte population collected from 3rd instar larvae of the designated genotypes and treatments is shown (n≥3 for each genotype/treatment). **B,D,** Relative mRNA levels of Arg detected by RT-qPCR in hemocyte samples collected from 3rd instar larvae of the designated genotypes (n≥3 for all conditions). **E,** Representative images of adult eyes from flies with the designated genotypes, after exposure of the right eye of P24 pupae to 17.5mJ of UV light. Right, average relative size of the UV-treated eye when

compared to the untreated eye of the same fly (5<n<20 for each genotype, each dot represents one fly). For all quantifications error bars represent s.e.m. and p-values are from student's t-test. (35) and (36) correspond to two independent dsRNAi expressing lines targeting KdelR transcripts.

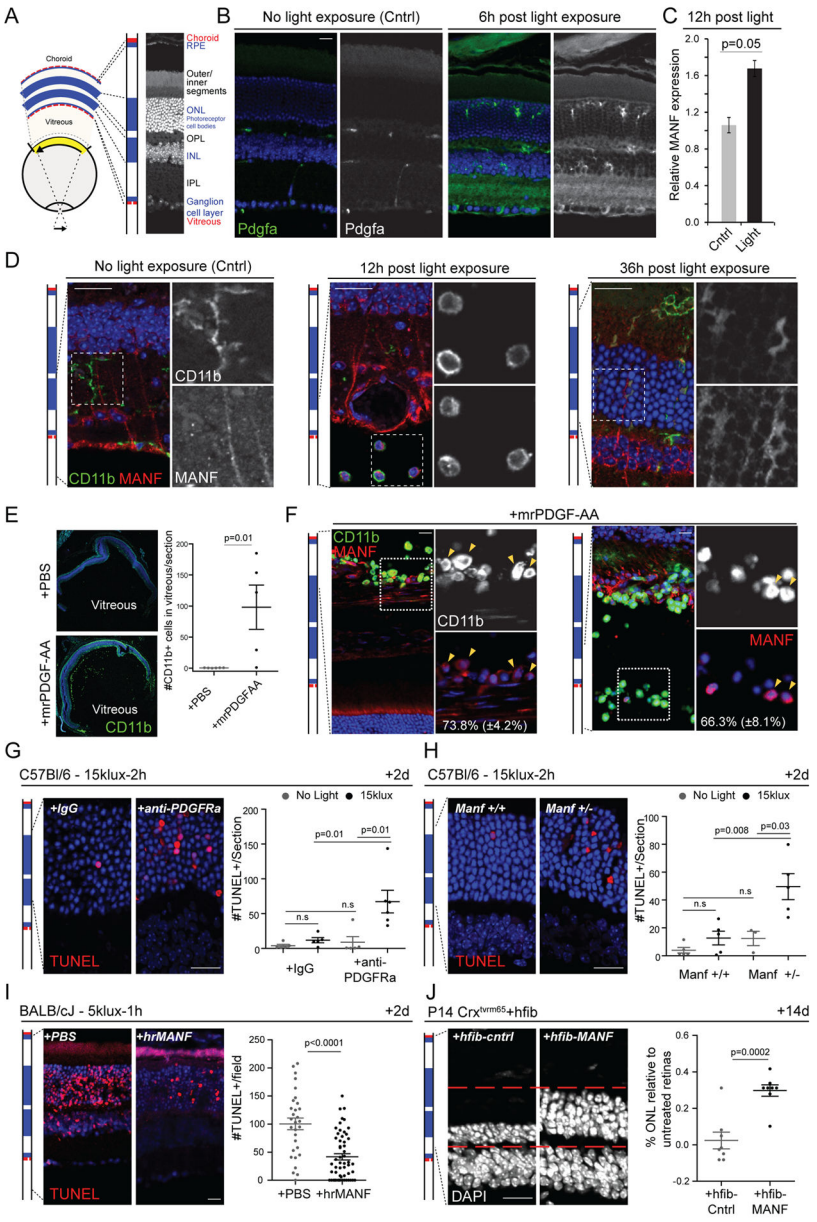

Fig. 3. PDGF-A/MANF damage-associated paracrine signaling is conserved in mammals

A, Cellular layers in the mouse eye. Panels B–H are from C57BL/6 mice. **B,D,** IHC showing expression of PDGF-A, CD11b and MANF after light exposure or in controls. See also Fig. S4A–B. **C,** Retinal mRNA levels of MANF (RT-qPCR) relative to controls (n=3). **E,F,** IHC showing expression of CD11b (E and F) and MANF (F), one day after intravitreal injection of mrPDGF-AA or vehicle (PBS). Details in (F) highlight CD11b+ cells detected in the vitreous (right) and choroid blood vessels (left) and MANF co-expression. (E) Average number of CD11b+ cells in the vitreous (mrPDGF-AA, n=5; PBS, n=6; 3 sections per eye for each animal, each dot represents one animal). **G, H** TUNEL staining, two days after light exposure: **G**, after intravitreal injection of anti-PDGFRα antibody or vehicle (Goat IgG) or **H**, in Manf +/− and Manf +/+ littermates. Average number of TUNEL+ nuclei is quantified

(G: No light: anti-PDGFRα, n=5; IgG, n=5, n=3; Light exposure: anti-PDGFRα, n=6; IgG, n=5; H: No light: Manf +/+, n=5; Manf +/−, n=3; Light exposure: Manf +/+, n=5; Manf +/−, n=5. 12 sections per eye for each animal, each dot represents one animal). **I,** Retina of BALB/cJ mice, stained with TUNEL, two days after intravitreal injection of hrMANF or vehicle (PBS) and exposure to 5klux of bright light for 1h. Average number of TUNEL+ nuclei per retinal field is shown (hrMANF, n=8; PBS, n=8; each dot represents one retinal field). **J,** Retina of P28 Crx^{tvrm65} mice, stained with DAPI, fourteen days after intravitreal injection of hfib-MANF or hfib-Cntrl. Red dashed lines indicate the thickness of the ONL after hfib-MANF delivery for comparison. Quantification of photoreceptor preservation as % of nuclei rows in ONL relative to untreated controls (hfib-MANF, n=8; hfib-Cntrl, n=8; 5 sections per eye, untreated controls for relative quantifications, n=4, 5 sections per eye; each dot represents one animal). For all quantifications error bars represent s.e.m. and p-values are from student's t-test. Scale bars are 20μm.

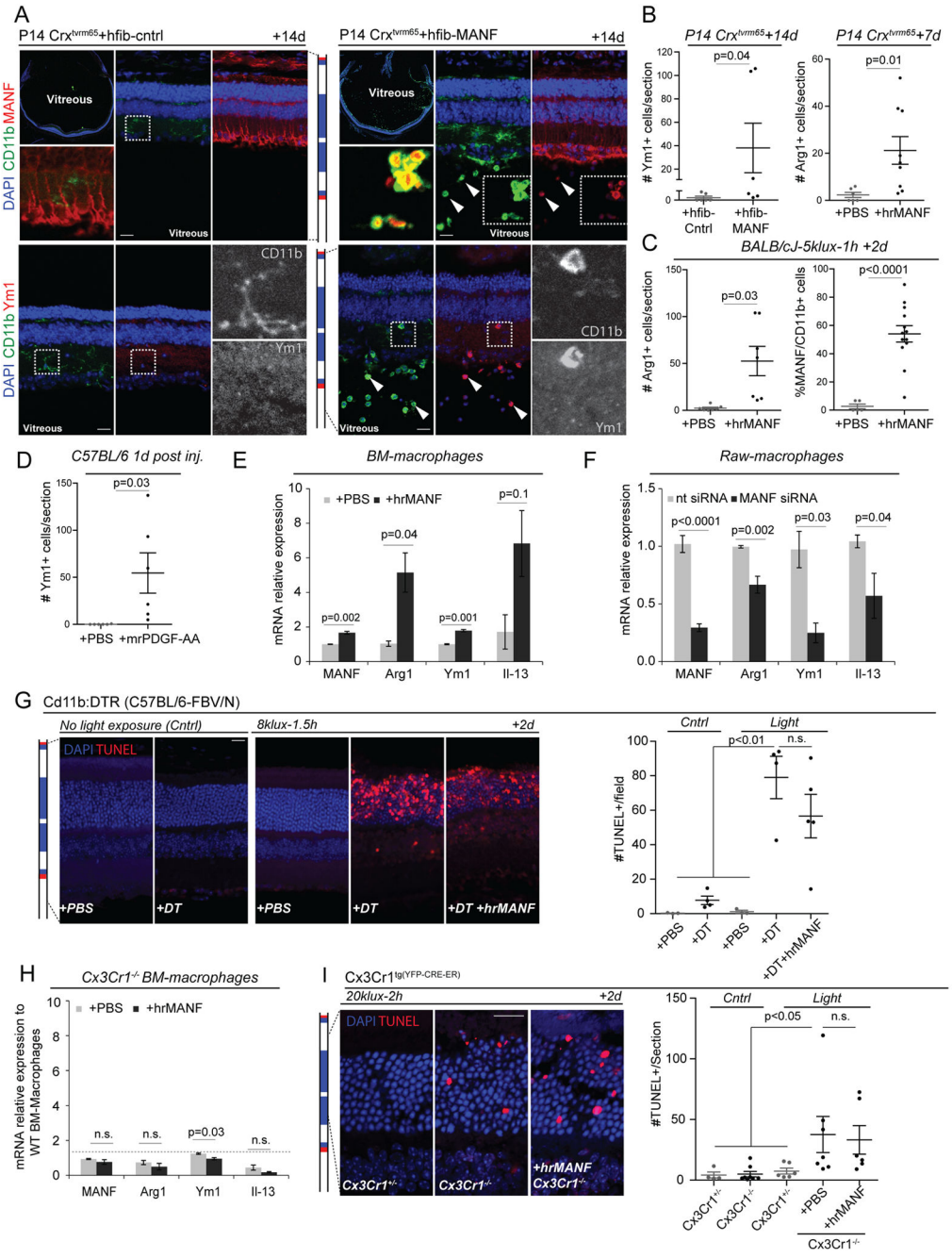

Fig. 4. MANF-dependent immune modulation mediates retinal neuroprotection

A, IHC showing expression of CD11b, MANF and Ym1 in P28 Crx^{tvrm65} mice 14 days after intravitreal injection of hfib-MANF or hfib-Cntrl. Arrowheads indicate co-expression. **B,** Average number of Ym1+ or Arg1+ cells, per eye cryosection, in P28 or P21 Crx^{tvrm65} mice, 14 or 7 days after intravitreal injection of hfib (hfib-MANF, n=6; hfib-Cntrl, n=6; 5 sections per eye; each dot represents one animal) or recombinant protein (hrMANF, n=6; PBS, n=6; each dot represents one section). **C,** Left, average number of Arg1+ cells per eye cryosection, in BALB/cJ mice, 2 days after intravitreal injection of hrMANF or vehicle

(PBS) and light exposure (hrMANF, n=7; PBS, n=7; 3 sections per eye; each dot represents one animal). Right, percentage of CD11b+/MANF+ cells in the retina of BALB/cJ mice after the same treatment (hrMANF, n=13 sections; PBS, n=5 sections, each dot represents one section). See also Fig. S6A. **D,** Average number of Ym1+ cells, per eye cryosection, in C57BL/6 mice, one day after intravitreal injection of mrPDGF-AA or vehicle (mrPDGF-AA, n=5,; PBS, n=6; 3 sections per eye, each dot represents one animal). See also Fig. S6B**. E,F,H** Relative mRNA levels (RT-qPCR) in BM-macrophages from wt (E, n=3) or Cx3Cr1-deficient (H, n=3) mice, stimulated with hrMANF or vehicle (PBS) or Raw macrophages transfected with MANF targeting siRNA pool or a non-targeting siRNA pool (F, n=5). See also Fig. S9. **G, I,** TUNEL staining, 2 days after intravitreal injection of hrMANF or vehicle (PBS) and light exposure of CD11b:DTR (G) or $Cx3Cr1^{tg(YFP\text{-}CRE\text{-}ER)}$ (Cx3Cr1 −/−) mice (I). Average number of TUNEL+ nuclei is shown (G, no light: PBS, n=3; DT, n=4. light: PBS, n=3; DT, n=4, DT+hrMANF, n=5; 4 sections per eye. I, No light: Cx3Cr1+/−, n=4; Cx3Cr1−/−, n=8. Light: Cx3Cr1+/−, n=6; Cx3Cr1-/: PBS, n=7; hrMANF, n=6; 12 sections per eye, each dot represents one animal). For all quantifications error bars represent s.e.m. and p-values are from student’s t-test. Sale bars are 20μm.

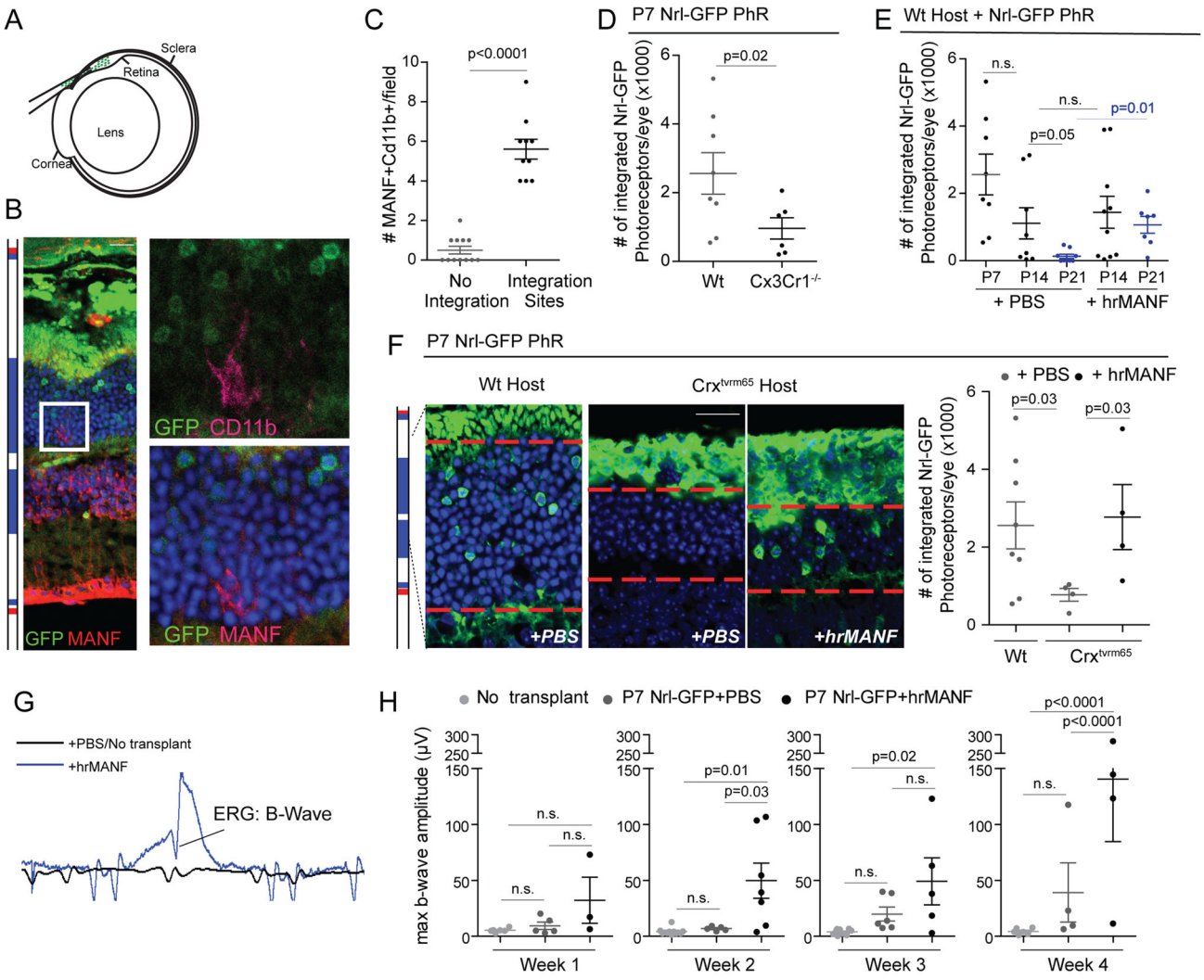

Fig. 5. MANF enhances the efficiency of retinal regenerative therapies

A, Cartoon representing the trans-corneal subretinal injection method. **B,** IHC showing expression of CD11b, MANF and GFP at an integration site of Nrl-GFP donor photoreceptors one week after transplantation. **C,** Average number of MANF+CD11b+ cells/field in integration sites vs. sites of no integration (10 fields per condition, all fields contained cells in the subretinal space, each dot represents one field). **D,** Quantification of integration into wild-type (wt, n=8) or Cx3Cr1−/− (n=6) mice, analyzed by IHC for GFP expression, 7 days after subretinal injection of P7 Nrl-GFP donor photoreceptors (PhR). Each dot represents one animal. **E,** Quantification of integration in C57BL/6 mice, analyzed by IHC for GFP expression, 7 days after subretinal injection of Nrl-GFP donor photoreceptors (PhR) supplemented with hrMANF protein (n=10, P14; n=7, P21) or vehicle (PBS, n=8, P7 and P14; n=9, P21). Each dot represents one animal. See also Fig. S8A for representative images of P21 transplants. **F,** Representative images and quantification of integration in wild-type (wt, n=8, same as in Fig. 5D) or Crx^{tvrm65} mice, analyzed by IHC for GFP expression, 7 days after subretinal injection of P7 Nrl-GFP donor photoreceptors (PhR) supplemented with hrMANF protein (hrMANF, n=4) or vehicle (PBS, n=4). Each dot

represents one animal. **G,** Examples of ERG-waves obtained in MANF supplemented (blue) and PBS supplemented (black) transplants of P7 Nrl-GFP PhRs in Crx^{tvrm65} mice. **H,** maximal b-wave amplitudes measured 1–4 weeks after sub-retinal injections of P7 Nrl-GFP PhRs supplemented with MANF (n=3–7 at each time point), PBS (n=4–6 at each time point) and of eyes that did not receive a transplant (n=6–10), all in Crx^{tvrm65} host. Each dot represents one animal. See also Fig. S8B for b waves after hrMANF or PBS injection without cells and Fig. S8C for b waves of wt eyes. p-values are from a 2-way ANOVA analysis. For all quantifications error bars represent s.e.m. p-values in C–F are from student's t-test. Scale bars are 20μm.

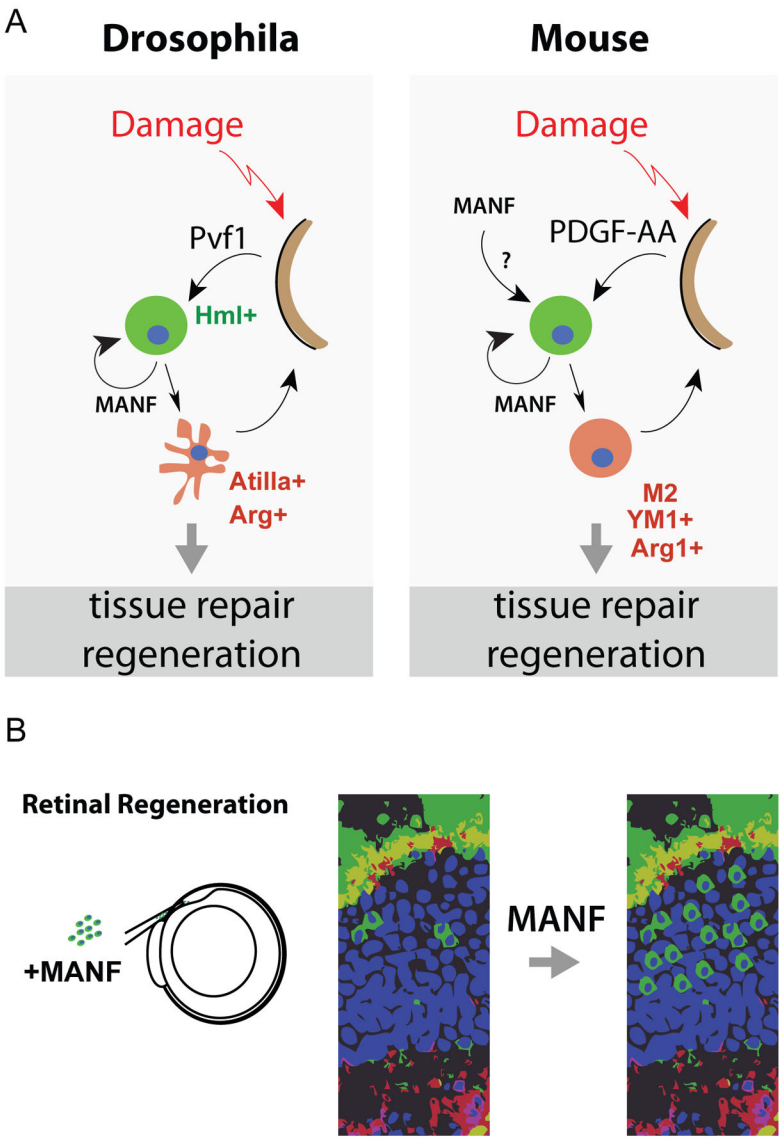

Fig. 6. Model for the evolutionarily conserved immune modulatory function of MANF and its implication in tissue repair and regeneration

A, In *Drosophila* (left) or mouse (right) the damaged retina secretes Pvf-1/PDGF-A which acts on innate immune cells – hemocytes in *Drosophila* or microglia/macrophages in mice. MANF derived from innate immune cells (or other sources) promotes phenotypic changes – atilla and arginase expression in hemocytes or alternative activation of microglia/macrophages – which are part of the mechanism involved in tissue protection. **B,** MANF supplementation is an enhancer of retinal regenerative therapies by increasing the integration efficiency of exogenously supplied photoreceptors for retinal repair.