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Human microglia-derived proinflammatory cytokines facilitate human retinal ganglion cell development and regeneration

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SUMMARY

Microglia (μG), the resident immune cells in the central nervous system, surveil the parenchyma to maintain the structural and functional homeostasis of neurons. Besides, they influence neurogenesis and synaptogenesis through complement-mediated phagocytosis. Emerging evidence suggests that μG may also influence development through proinflammatory cytokines. Here, we examined the premise that tumor necrosis factor alpha (TNF-α) and interleukin-1β (IL-1β), the two most prominent components of the μG secretome, influence retinal development, specifically the morphological and functional differentiation of human retinal ganglion cells (hRGCs). Using controlled generation of hRGCs and human μG (hμG) from pluripotent stem cells, we demonstrate that TNF-α and IL-1β secreted by unchallenged hμG did not influence hRGC generation. However, their presence significantly facilitated neuritogenesis along with the basal function of hRGCs, which involved the recruitment of the AKT/mTOR pathway. We present *ex vivo* evidence that proinflammatory cytokines may play an important role in the morphological and physiological maturation of hRGCs, which may be recapitulated for regeneration.

INTRODUCTION

Microglia (μG) are the resident immune cells in the retina, surveilling the environment to maintain structural and functional homeostasis (Ahmad and Subramani, 2022). They are immigrant cells from the embryonic yolk sac, and the timing of the entry of their precursors in the developing retina coincides with the early stages of neurogenesis, when the fate of retinal ganglion cells (RGCs), the projection neurons, is determined. RGCs are generated in close proximity to μG. Emerging evidence suggests that like elsewhere in the central nervous system (CNS) (Prinz et al., 2019), μG influence neurogenesis. For example, the elimination of μG from the developing retina is associated with an increased number of RGCs, suggesting that μG remove nascent RGCs by complement-mediated phagocytosis, thus regulating the neurogenic output (Anderson and Vetter, 2019). Besides, recent observations suggest that the influence of μG on neurogenesis may also involve cytokine-mediated regulation of cell proliferation and survival (Cserep et al., 2022; Diaz-Aparicio et al., 2020), neurite outgrowth (Lilienberg et al., 2022), and functional differentiation (Cserep et al., 2021).

Here, we examined the premise that proinflammatory cytokines, tumor necrosis factor alpha (TNF-α) and interleukin-1β (IL-1β), the two most prominent components of the μG secretome, influence retinal development, specifically the morphological and functional differentiation of RGCs. Given the wide-ranging influence of cytokines, we examined the effects of TNF-α and IL-1β on the development, function, and regeneration of pluripotent stem cell-derived human RGCs (hRGCs) in controlled conditions. The rationale for using TNF-α and IL-1β together was based on the likelihood that the developing RGCs will be exposed to both, due to their simultaneous secretion by μG. We observed that the proinflammatory cytokines had no effect on the efficiency of the generation of hRGCs. However, their presence significantly improved neuritogenesis along with the basal function of hRGCs. The facilitatory effects of cytokines on neuritogenesis and function were recapitulated when hRGCs were generated in the presence of human induced pluripotent stem cell (iPSC)-derived μG and involved the recruitment of the AKT/mTOR pathway. Furthermore, we observed that the cytokine-mediated recruitment of the AKT/mTOR pathway promotes hRGC axon regeneration. We have presented *ex vivo* evidence that proinflammatory cytokines, components of unchallenged human μG (hμG) secretome, may play an important role in the morphological and physiological maturation of hRGCs toward efficient targeting and networking. This developmental mechanism may be recapitulated to promote regeneration.

RESULTS

Recombinant TNF-α and IL-1β do not influence hRGC generation

Evidence suggests that μG play a role in RGC generation through the phagocytotic removal of nascent postmitotic cells (Anderson and Vetter, 2019). However, whether μG could influence RGC differentiation through TNF-α and

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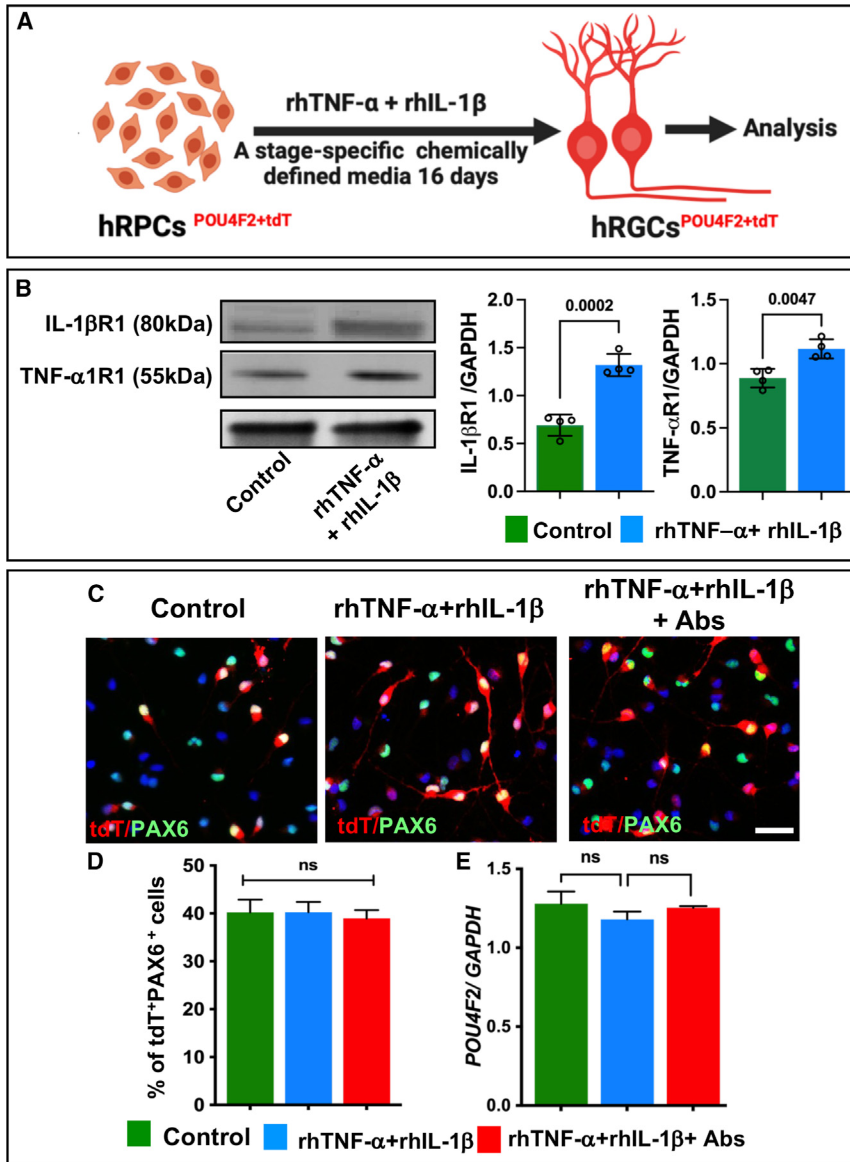

Figure 1. rhTNF-α and rhIL-1β do not influence the generation of hRGCs

Schematic representation of hRGCs generation from human retinal progenitor cells (hRPCs$^{POU4F2+tdT}$) using the stage-specific differentiation protocol involving chemically defined medium (CDM) in the presence of rhTNF-α and rhIL-1β (A). Western blot analysis revealed expression of immunoreactive bands corresponding to TNFα-1R1 and IL-1βR1 in hRGCs. The levels of their expression increased in the presence of rhTNF-α and rhIL-1β (B). There was no significant difference in the number of tdT$^+$ and PAX6$^+$ hRGCs (C and D) and expression levels of hRGCs regulatory gene *POU4F2* (E) in different culture conditions, demonstrating that the presence of rhTNF-α and rhIL-1β does not affect hRGC generation. Experiments were carried out in triplicates per group from 3 to 4 independent biological replicates. Values expressed as mean ± SEM. Scale bar: 50 μm.

IL-1β, important components of the μG secretome, remains poorly understood. We began by examining the effects of recombinant human TNF-α (rhTNF-α) and IL-1β (rhIL-1β) on the generation of hRGCs from the RGC reporter human ES line, hESC$^{POU4F2-tdT}$ (Sluch et al., 2017; Subramani et al., 2023a) (Figure 1A). We first determined if hRGCs expressed receptors for TNF-α and IL-1β. Western blot analysis revealed that hRGCs expressed both TNF-αR1 and IL-1βR1 and their levels increased when cells were cultured in the presence of rhTNF-α and rhIL-1β (Figure 1B). Since proinflammatory cytokines have concentration-dependent effects (Zipp et al., 2023), we selected a low concentration (50 ng/mL) for both cytokines, based on dose-dependent effects on hRGC survival (Figure S1). Next, we generated hRGCs in the presence of rhTNF-α and rhIL-1β and examined if they influenced hRGC generation. The specificity of cytokine action was determined by their neutralization using anti-TNF-α and anti-IL-1β antibodies. hRGCs were characterized by the co-expression of POU4F2-tdT and PAX6 immunoreactivities (Subramani et al., 2023b; Xiao et al., 2020), corroborated by the co-expression of POU4F2-tdT and RBPMS (Figures S1C and S1D). Quantification of tdT$^+$ and PAX6$^+$ cells revealed no significant difference in the number of hRGCs generated in the presence of cytokines or when they were neutralized versus controls (Figures 1C and 1D). qPCR analysis of RGC markers revealed no significant influence of rhTNF-α and rhIL-1β on their expression compared to controls,

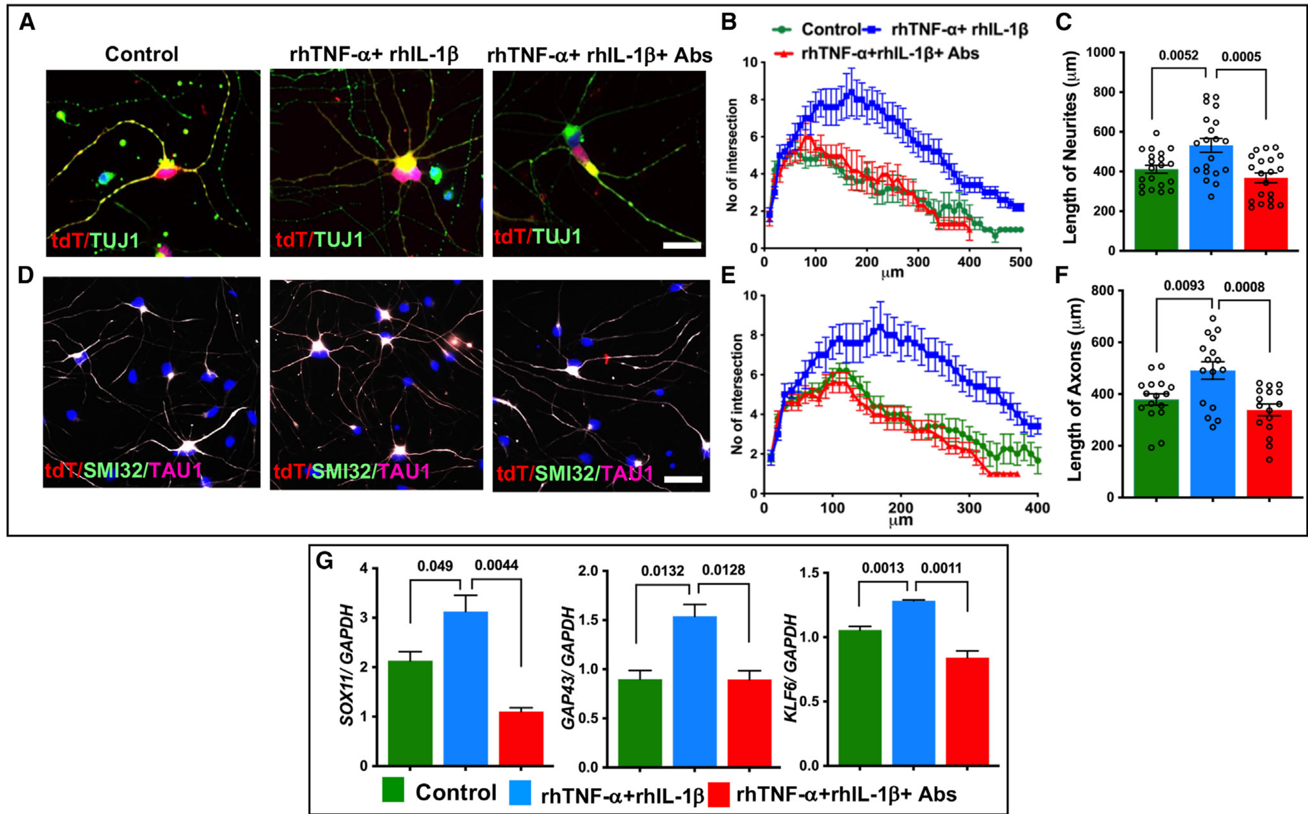

Figure 2. rhTNF-α and rhIL-1β influence hRGC neuritogenesis

Analysis of TUJ1$^+$ neurites of hRGCs generated in three different conditions revealed a significant increase in the complexities (A and B) and length (A and C) of the processes in the presence of rhTNF-α and rhIL-1β, versus control and their abrogation in antibody-neutralized conditions. Similarly, analysis of SMI32$^+$ and TAU1$^+$ hRGCs axons revealed a significant increase in their complexities (D and E) and length (D and F) in the presence of rhTNF-α and rhIL-1β, versus control and their abrogation in antibody-neutralized conditions. Examination of the expression of transcripts corresponding to *GAP43*, *SOX11*, and *KLF6* revealed their levels increased in the presence of rhTNF-α and rhIL-1β, versus control and its abrogation in antibody-neutralized conditions (G). Experiments were carried out in triplicates per group from 3 independent biological replicates. Values expressed as mean ± SEM. Scale bar: 20 μm.

corroborating immunocytochemical analysis that the presence of cytokines do not affect hRGC generation in the defined condition (Figure 1E).

Recombinant TNF-α and IL-1β influence hRGC neuritogenesis

Next, we examined if the presence of rhTNF-α and rhIL-1β during hRGC generation influenced neuritogenesis. Proinflammatory cytokines have been observed to influence neurite outgrowth in the peripheral nervous system (PNS) and CNS (Lilienberg et al., 2022). First, we determined the overall complexity of neurites in general by staining hRGCs for TUJ1 immunoreactivities. A significant increase, in both the complexity and length of the neurites, was observed in hRGCs, generated in the presence of rhTNF-α and rhIL-1β versus control (Figures 2A and 2B). Their influence on neurites was abrogated when cytokines were neutralized by anti-TNF-α and anti-IL-1β antibodies (Figures 2A–2C). Second, we specifically examined the influence of the cytokines on the axons (SMI32$^+$ TAU1$^+$ processes), as it would have the implication in target innervation. A significant increase in the complexity and length of the axons was observed when hRGCs were generated in the presence of cytokines versus controls, their effects abrogated in the presence of the neutralizing antibodies (Figures 2D–2F). In subsequent experiments, the length and complexity of SMI32$^+$ and TAU1$^+$ axons were examined as a measure of neurite outgrowth. Next, we wanted to know if the normal mechanisms of axon growth were recruited by the recombinant cytokines. qPCR analysis revealed that the expression of genes having demonstrable effect on axon generation and growth, the growth-associated protein 43 (*GAP43*) (Meyer et al., 1994; Schaden et al., 1994), Kruppel-like factor 6 (*KLF6*) (Moore et al., 2009), and *SOX11*, a Sry-related high-mobility box superfamily of transcription factor (Jiang et al., 2013; Kuwajima

et al., 2017; Norsworthy et al., 2017), increased significantly when hRGCs were generated in the presence of the cytokines, compared to controls (Figure 2G). The positive effects of the cytokines on transcript levels were significantly decreased in the presence of neutralizing antibodies (Figure 2G). We observed similar facilitatory effects of cytokines on neuritogenesis in hRGCs generated from two different iPSC clones demonstrating that the effects of cytokines on neuritogenesis are not hES cell specific but include those on iPSC-derived hRGCs across different clones (Figure S2). Together, these observations suggested that proinflammatory cytokines facilitate neuritogenesis during hRGC differentiation, likely through the recruitment of intrinsic mechanism of neurite growth.

hμG influence RGC neuritogenesis and function through TNF-α and IL-1β

Next, we examined if hμG facilitated neuritogenesis in RGCs and the effects were mediated through the cytokines as observed for rhTNF-α and rhIL-1β. This premise was examined where hRGC generation was carried out in the presence of condition medium (CM) derived from hμG. hμG were generated from human iPSCs (Teotia et al., 2017) through a stage-specific protocol based on the recapitulation of the developmental mechanism (Abud et al., 2017; McQuade et al., 2018) (Figure 3A). Phenotype characterization revealed that more than 80% of cells expressed μG markers, including (1) fractalkine receptor CX3CR1, (2) the purinergic receptor, P2RY12, and (3) triggering receptor expressed on myeloid cells 2 along with ionized calcium-binding adapter molecule 1 (Figures 3B and 3C), and secrete TNF-α and IL-1β when challenged with lipopolysaccharide (Figure 3D). That the iPSC-derived hμG were functional was demonstrated by P2RY12-mediated migration in response to ADP (Figure 3E) and phagocytosis of fluorescent $A\beta_{1-42}$ (Figure 3F). In order to minimize the variability in the concentration of cytokines elaborated by unchallenged iPSC-derived hμG, we derived CM from a defined number of microglia (10^5 cells/cm^2) in culture for each experiment (Figure S3). Next, we generated hRGCs in the presence or absence of hμG CM (Figure 4A). In a parallel culture, hRGCs were generated in the presence of hμG CM, incubated with anti-TNF-α and -IL-1β antibodies to neutralize TNF-α and IL-1β in the hμG CM. hRGCs were examined on day *in vitro* (DIV) 16 of generation. Quantification tdT$^+$ and PAX6$^+$ cells revealed no significant difference in the number of hRGCs generated in the presence of hμG CM or when in the presence of neutralizing antibodies compared to controls (Figures 4B–4E), suggesting the lack of influence of cytokines on hRGC generation. In contrast, analyses of the hRGC processes revealed that the length and complexity of SMI32$^+$ and TAU1$^+$ axons were significantly increased in the presence of hμG CM compared to controls (Figures 4F–4J). The positive influence of hμG CM on hRGC neurites was abrogated in the presence of neutralizing antibodies, suggesting that the facilitation of neuritogenesis was due to hμG-derived TNF-α and IL-1β. Next, we were interested in knowing if the cytokines influenced hRGC function, which along with neuritogenesis, may help facilitate neuronal activity and target innervation. This premise was supported by observations that TNF-α and IL-1β directly impact the basal activities of neurons and neuronal transmission (Zipp et al., 2023). The functional impact of hμG-derived cytokines on hRGC neuronal activity was examined using the multi-electrode array (MEA) system, a non-invasive *in vitro* approach to analyze neural network activities dependent on synaptic inputs (Obien et al., 2014). For this experiment, DIV 16 hRGCs were plated onto an MEA plate with hμG CM, and recordings were carried out for 6 days post-plating. In a parallel culture of hRGCs, activities were recorded in the presence of hμG CM with the neutralizing antibodies against TNF-α and IL-1β. In MEA, raw voltage of neurons is detected by adjacent electrodes and converted into extracellular action potential. We observed that the number of active electrodes and the mean firing rate of neurons were significantly higher in the presence of hμG CM at day 6 after plating, compared with control (Figures 4K and 4L). These physiological indices were significantly decreased in the presence of CM with neutralizing antibodies (Figures 4K and 4L). Raster plots and spike waveforms showed the comparative neuronal activities, enhanced in the presence of hμG CM and decreased in the antibody-neutralized groups, compared to controls (Figures 4M–4O). Similar effects on hRGC function were observed in the presence of rhTNF-α and rhIL-1β (Figure S4). Next, we examined if the physiological response involved sodium channel subunit, SCN2A (NaV 1.2), involved in the generation of action potential (Beacham et al., 2007). Western blot analysis revealed that the expression of SCN2A increased in the presence of hμG CM and decreased in the antibody-neutralized groups versus controls (Figures 4P and 4Q). Together, these results suggested that hμG can influence RGC neuritogenesis and basal function through TNF-α and IL-1β, which may involve facilitating the expression of voltage-gated sodium channels.

Proinflammatory cytokine-mediated influence of hμG on neuritogenesis and function involves the mTOR pathway

We have previously demonstrated that the differentiation of hRGCs including neuritogenesis and functional maturity are facilitated by the mTOR pathway (Subramani et al., 2023b; Teotia et al., 2019). Recent evidence suggests that both TNF-α (Diem et al., 2001; Liu et al., 2012) and IL-1β (Diem et al., 2003; Wyszynski et al., 2016) could recruit the AKT/mTOR pathway toward diverse functions. Therefore, we next examined if hμG-derived TNF-α and

Figure 3. hμG are generated from human iPSCs by stage-specific recapitulation of developmental mechanism
Schematic representation of stage-specific hμG differentiation protocol with bright-field representation of cells in different stages of differentiation (A). Mature cells expressed immunoreactivities corresponding to μG-specific markers CX3CR1, P2RY12, and TREM2 along with IBA1 (B), and the number of cells expressing these markers was in the excess of 80% of total cells (C). Mature cells elaborated TNF-α and IL-1β when challenged with LPS (D) and migrated in response to μG chemoattractant ADP, which is abrogated in the presence of P2RY12 receptor antagonist, PSB0739 (E). Mature cells phagocytosed fluorescently tagged $A\beta_{1-42}$ peptide (F). Experiments were carried out in triplicates per group from 3 independent biological replicates. Values expressed as mean ± SEM. Scale bar: 25 (AIV, B, F) 50 (AIII, E), and 100 μm (AI, AII).

IL-1β recruited the AKT/mTOR pathway for neuritogenesis and the functional maturation of hRGCs. hRGC generation was carried out in the presence and absence of hμG CM as described earlier, in parallel cultures of hμG CM with anti-TNF-α + anti-IL-1β antibodies or rapamycin, an acute inhibitor of mTOR complex 1 (mTORC1) (Lamming, 2016). Our primary readout for activated mTOR signaling was phosphorylated mTOR (pmTOR) and S6 (pS6), a ribosomal protein that facilitates translation of mRNAs into proteins (Saxton and Sabatini, 2017). Western blot analysis

Figure 4. hμG-derived TNF-α and IL-1β influence neuritogenesis in and function of developing hRGCs
Schematic representation of the hμG-hRGCs co-culture to examine the effect of the former on hRGC generation, neurite growth, and function (A). Immunocytochemical analysis revealed no difference in the number of tdT$^+$ and PAX6$^+$ hRGCs, generated in three different culture conditions (control, hμG CM, and hμG CM with anti-TNF-α and anti-IL-1β antibodies) demonstrating that proinflammatory cytokines secreted by hμG have no effects on hRGC generation (B–E). Analysis of SMI32$^+$ TAU1$^+$ axons of hRGCs revealed a significant increase in the complexities and length (F–J) of the axons in the presence of hμG CM versus controls and their abrogation in antibody-neutralized hμG CM. Analysis of MEA recording at day 7 of hRGCs revealed a significant increase in the number of active electrodes (K) and mean firing rate (L); when cells are exposed to hμG CM versus controls, the effects significantly abrogated in antibody-neutralized hμG CM (K, L). Raster plots of representative hRGC recordings demonstrated the effects of the three different culture conditions on the spontaneous activities (M–O). Representative extracellular spike waveforms revealed the facilitatory effects of hμG CM on the neural function versus control, which is abrogated in antibody-neutralized hμG CM (M–O). Western blot analysis carried out on the cell lysates of hRGCs showed hμG CM influences the sodium channel (SCN2A) expression (P). SCN2A levels increased significantly in the presence of hμG CM versus controls, which is abrogated in antibody-neutralized hμG CM (Q). Experiments were carried out in triplicates per group from 3 to 4 independent biological replicates. Values expressed as mean ± SEM. Scale bar: 50 μm. MEA, microelectrode array.

carried out on cell lysates revealed that levels of pAKT (Figures 5A and 5B), pmTOR (Figures 5A and 5C), and pS6 (Figures 5A and 5D) increased when hRGCs were co-cultured with hμG. The increase in their levels was significantly decreased in the presence of rapamycin demonstrating the recruitment of the mTORC1 (Figures 5A–5D). That the recruitment of the mTOR pathway was due to hμG-derived cytokines was demonstrated by a decrease in pAKT, pmTOR, and pS6 levels in the presence of hμG CM with anti-TNF-α and -IL-1β antibodies (Figures 5A–5D). The presence of rapamycin in the CM abrogated the increase in axon length and complexity demonstrating the

Figure 5. Proinflammatory cytokine-mediated influence of hμG on hRGC neuritogenesis involves the mTOR pathway
Western blot analysis carried out on the cell lysates of hRGCs in four different culture conditions to examine the specificity of TNF-α and IL-1β influence and recruitment of the AKT/mTOR pathway (A). Levels of immunoreactivities corresponding to pAKT, pmTOR, and pS6 increased significantly in the presence of hμG CM versus controls, which is abrogated in antibody-neutralized hμG CM (B–D), demonstrating the engagement of the AKT/mTOR pathway by TNF-α and IL-1β in hμG CM. Inclusion of rapamycin in hμG:hRGC co-culture decreased the levels of pAKT, pmTOR, and pS6 more than twice the levels observed in the presence of hμG CM/hμG CM with neutralizing antibody, corroborating the engagement of mTORC1 (A–D). Both the length and the complexities of SMI32^{+} TAU1^{+} hRGC axons decreased significantly in the presence of rapamycin compared to those cultured in the presence of hμG CM and antibody-neutralized hμG CM demonstrating that proinflammatory cytokine-mediated influence of hμG on neuritogenesis involves the AKT/mTOR pathway (E-J). That the AKT/mTOR-mediated effects on neuritogenesis may engage intrinsic mechanism for neurite growth was demonstrated by a decrease in the levels of *GAP43*, *SOX11*, and *KLF6* transcripts in the presence of rapamycin versus all other groups (K-M). Experiments were carried out in triplicates per group from 3 independent biological replicates. Values expressed as mean ± SEM. Scale bar: 50 μm.

involvement of mTOR pathway in TNF-α and IL-1β-mediated neuritogenesis (Figures 5E–5J). That it may involve mTOR-mediated recruitment of intrinsic mechanisms of neurite growth was demonstrated by complete abrogation of the cytokine-dependent increase in *GAP43*, *SOX11*, and *KLF6* transcript levels in the presence of rapamycin (Figures 5K–5M). Similar results corroborating the cytokine-mediated recruitment of the mTOR pathway for neuritogenesis were obtained when hRGCs were generated in the presence of rhTNF-α and rhIL-1β (Figures S5).

Next, we examined if the cytokine-mediated functional maturity involved the mTOR pathway. We observed that all indices of neural activities in MEA recordings—mean firing rates (Figures 6A and 6C), active electrodes (Figures 6B–6D), and extracellular spike waveform amplitude (Figures 6E–6H)—which were increased in the presence of hμG-derived TNF-α and IL-1β were significantly decreased in the presence of rapamycin, including the expression of sodium channel, SCN2A (Figures 6I and 6J). Together, these observations suggested that the TNF-α and IL-1β-mediated

Figure 6. Proinflammatory cytokine-mediated influence of hμG on hRGC function involves the mTOR pathway

A temporal representation of number of active electrodes (A) and mean firing rate (B) revealed by MEA recordings of hRGCs cultured in four different conditions over 22 days. Analysis of MEA recordings at DIV17 after plating revealed that the increase observed in the number of active electrodes (C) and mean firing rate (D) in the presence of hμG CM decreased significantly when rapamycin was included in the culture demonstrating the involvement of AKT/mTOR pathway in proinflammatory cytokine-mediated influence of hμG on hRGC function, which is displayed in the Raster plots of representative hRGC recordings showing the effects of four different culture conditions on the spontaneous activities (E–H). Representative extracellular spike waveforms revealed the facilitatory effects of hμG CM on the neural function versus control (E and F), which is abrogated in antibody-neutralized hμG CM (F and G) or CM with rapamycin (F and H). Western blot analysis carried out on the cell lysates of hRGCs in different cultured conditions revealed that the expression of sodium channel subunit, SCN2A, which increased significantly in the presence of hμG CM versus controls, was abrogated in the presence of neutralizing antibodies and rapamycin (I and J). Experiments were carried out from 6 technical replicates per group. Values expressed as mean ± SEM.

influence of hμG on the developing hRGCs may involve the AKT/mTOR pathway.

hμG influence axon regeneration through proinflammatory cytokine-mediated recruitment of the mTOR pathway

Evidence has emerged that the mTOR pathway plays an important role in optic nerve regeneration (Park et al., 2008; Teotia et al., 2019). Therefore, we were interested in knowing if unchallenged hμG could promote axon regeneration and if that involved cytokine-mediated recruitment of the mTOR pathway. We tested this premise in the microfluidic model of axon regeneration developed in our lab (Subramani et al., 2023b; Teotia et al., 2019). hRGCs were seeded in the soma chamber of the microfluidic device, and axons were allowed to

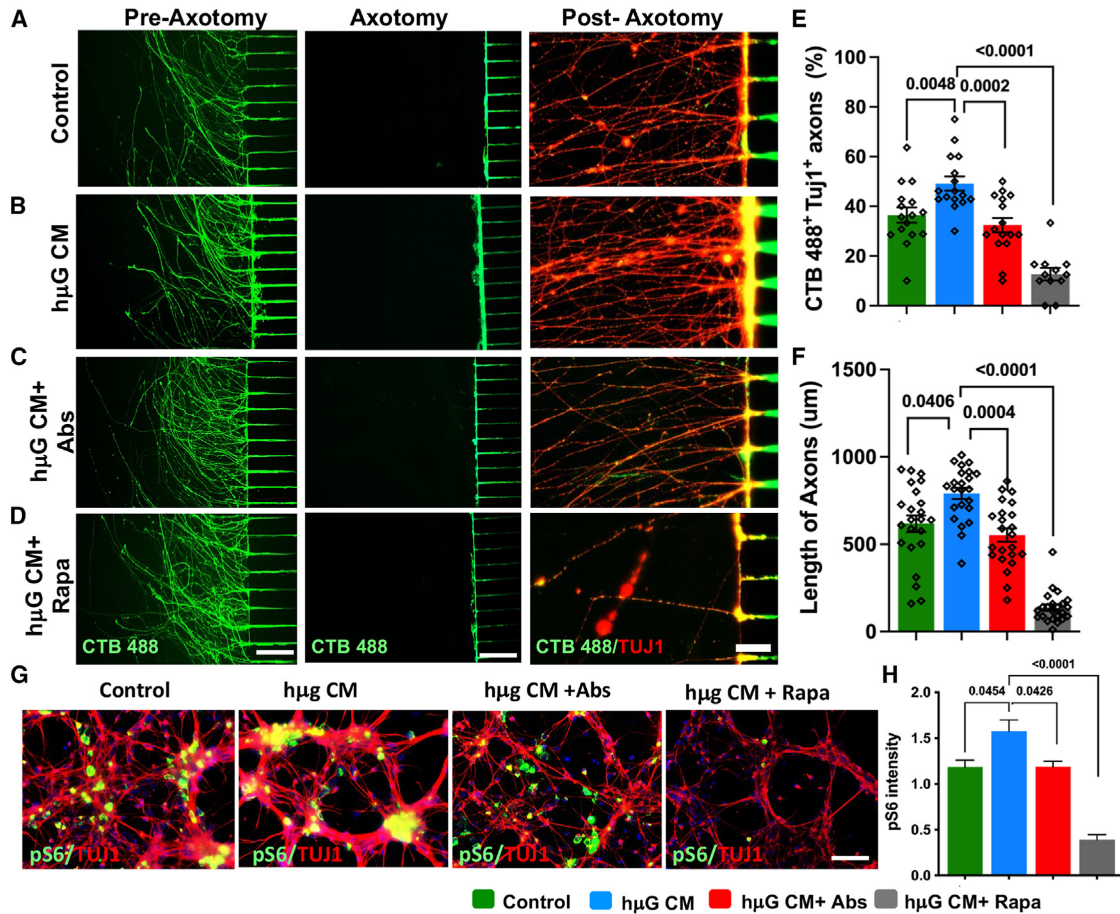

Figure 7. hμG influence axon regeneration through proinflammatory cytokine-mediated recruitment of the mTOR pathway

hRGCs were cultured in the soma chambers of microfluidic devices till they elaborated axons into the axon chambers and divided in 4 groups. Axons were retrogradely labeled with CTB 488 (pre-axotomy lane) followed by axotomy (axotomy lane) and regeneration was followed for five days post axotomy (post-axotomy lane) (A–D). Only CTB 488$^+$ tdT$^+$ processes in the axon chambers were regarded regenerative and counted. The proportion of regenerated axons (E) and their length (F) were significantly increased in hμG CM group versus antibody-neutralized hμG CM group demonstrating the influence of cytokines on axon regeneration. That the facilitatory influence of hμG-derived TNF-α and IL-1β involved AKT/mTOR pathway was demonstrated by remarkable abrogation of increase in axon regeneration in the presence of rapamycin (E, F). pS6 staining showed higher levels when exposed to hμG CM, an effect abrogated by neutralization and abolished by rapamycin (G and H). Experiments were carried out in triplicates per group from 3 independent biological replicates. Values expressed as mean ± SEM. Scale bars: 20 (post-axotomy group) and 50 μm (pre-axotomy, Axotomy, pS6).

grow through the microgrooves into the axon chamber. The retrogradely labeled axons were chemically axotomized, and their regeneration was examined in the presence of hμG CM alone, hμG CM with anti-TNF-α + anti-IL-1β antibodies, or hμG CM with rapamycin (Figure 7). Axon regeneration was observed in all groups. However, the number and length of axons regenerated in the presence of hμG CM were significantly higher versus controls (Figures 7A, 7B, 7E, and 7F). The number and length of regenerated axons decreased significantly in antibody-neutralized hμG CM group versus hμG CM group (Figures 7C, 7E, and 7F), demonstrating the facilitatory role of hμG-derived cytokines on axon regeneration. There was a significant decrease in the number of regenerating axons in the rapamycin-containing group compared to the hμG CM group, suggesting the involvement of the mTOR pathway in the hμG cytokine-mediated axon regeneration (Figures 7D, 7E, and 7F) accompanied by a decrease in the levels of pS6, compared to hRGCs exposed to hμG CM (Figures 7G and 7H). Similar results corroborating the proinflammatory cytokine-mediated recruitment of the mTOR pathway were obtained when hRGC axon regeneration was cultured in the presence of rhTNF-α and rhIL-1β (Figure S6). Together, these results suggested that TNF-α and IL-1β secreted by unchallenged hμG

recruit the mTOR pathway to facilitate hRGC axon regeneration.

DISCUSSION

In the vertebrate retina, cells are generated in an evolutionarily conserved temporal sequence, where RGCs are the first cell types born during early neurogenesis (Rapaport et al., 2004; Young, 1985). The differentiation of RPCs into RGCs takes place in the close proximity of immigrant μG. Recent evidence suggests that, like elsewhere in the CNS, μG also regulate the neurogenic output in the retina. For example, when μG were experimentally eliminated after they had colonized the retina, the number of nascent RGCs increased suggesting that μG regulate the number of RGCs by complement receptor 3-mediated phagocytosis of postmitotic precursors (Anderson and Vetter, 2019; Anderson et al., 2019). The phagocytotic regulation of neurogenesis by μG is also observed in the developing cortex (Cunningham et al., 2013), and in the sub-granular zone (SGZ) in adult hippocampus (Klein et al., 2016). However, the role of proinflammatory cytokines, particularly TNF-α and IL-1β, the key components of the μG secretome, during neuronal differentiation in general and RGC in particular is poorly understood.

We demonstrate, in controlled conditions of generating hRGCs from pluripotent stem cells, that low levels of TNF-α and IL-1β, and either recombinant or secreted from unchallenged hμG, did not affect the differentiation of hRPCs into hRGCs. The lack of hμG effect on hRGC differentiation in contrast to that observed *in vivo* (Anderson and Vetter, 2019) may be attributed to the two-dimensional culture system where the diverse cellular interactions of the developing retina are not properly recapitulated. The proinflammatory cytokines on the other hand had a significant positive effect on the length and complexity of hRGC neurites similar to that observed in the culture of entorhinal cortex and cerebellar granule neurons in the presence of IL-1β (Boato et al., 2011; Temporin et al., 2008). These results are in contrast with those observed in the embryonic hippocampal neurons *in vitro* where exposure to TNF-α inhibited neurite outgrowth and branching (Neumann et al., 2002). These may represent a contextual response of pleiotropic cytokines depending upon receptor types and recruitment of cell-type-specific intracellular signaling pathway (see further). The positive influence of TNF-α and IL-1β on hRGC neuritogenesis observed here may be due to the expression of genes that are known regulators of neurite and axon growth in the developing CNS in general and RGC in particular (Bradke, 2022). These include *GAP43* (Meyer et al., 1994; Schaden et al., 1994), *KLF6* (Moore et al., 2009), and *SOX11* (Jiang et al., 2013; Kuwajima et al., 2017; Norsworthy et al., 2017). Therefore, it is likely that the constitutive levels of proinflammatory cytokines recruit the developmental mechanism during *ex vivo* generation of hRGCs to facilitate neuritogenesis.

μG influence neural transmission and network through complement-mediated synaptic pruning (Lui et al., 2016; Schafer et al., 2012). In addition, proinflammatory cytokine-mediated facilitation of synaptic efficacy and synaptic scaling (Beattie et al., 2002; Cserep et al., 2021; Stellwagen and Malenka, 2006), and long-term potentiation (LTP) (Avital et al., 2003; Hewett et al., 2012; Ross et al., 2003; Schneider et al., 1998) may help regulate basal neural transmission, which is corroborated by our observations. For example, hμG-derived TNF-α and IL-1β had a significant effect on hRGC function, demonstrated by an increase in their mean firing rate, and the functionality of the synapses as depicted by an increase in the synchronous generation of action potentials. The improved neural transmission in the presence of hμG-derived TNF-α and IL-1β is likely due to the altered activities of ion channels as proinflammatory cytokines have been observed to affect activities of a variety of ion channels in the CNS and PNS. For example, TNF-α decreases outward K^+ currents in rodent RGCs (Diem et al., 2001), increases L-type Ca^{2+} currents in hippocampal neurons (Furukawa and Mattson, 1998), and increases voltage-dependent Na^+ currents in dorsal root ganglion neurons (Czeschik et al., 2008; Jin and Gereau, 2006). Similarly, IL-1β potentiates voltage-dependent Na^+ currents in trigeminal nociceptor neurons (Liu et al., 2006). It is therefore likely that cytokine-mediated influence on channel activities may underlie the improved hRGC neural transmission given the TNF-α and IL-1β-mediated increase in voltage-dependent Na^+ currents and Na^+ channel subunit SCN2A ($Na_V1.2$).

Proinflammatory cytokines facilitate axonal regeneration in lower vertebrates. For example, both TNF-α and IL-1β have been observed to promote axonal regeneration in the injured zebrafish spinal cord (Cavone et al., 2021; Tsarouchas et al., 2018). The regenerative effects could be direct (Tsarouchas et al., 2018) or indirect through TNF-α-mediated recruitment of ependymo-radial glial progenitor cells (Cavone et al., 2021). In mammals, where adult neurogenesis is confined to the subventricular zone (SVZ), and SGZ in the hippocampus (Ming and Song, 2011), the involvement of TNF-α and IL-1β has not been reported although TNF-α-induced neurogenesis in SVZ neurospheres has been observed *in vitro* (Bernardino et al., 2008). Recently, it has been demonstrated that another proinflammatory cytokine, IL-6, covalently linked to soluble IL-6 receptor can facilitate axonal regeneration in mouse RGCs (Fischer, 2017), pluripotent stem cell-derived hRGCs (Teotia et al., 2019), and corticospinal tract in adult mice (Leibinger et al., 2021). Our observation demonstrates

the pro-regenerative effect of hμG-derived TNF-α and IL-1β on axotomized hRGC axons. However, factors other than TNF-α and IL-1β in the hμG secretome that may also facilitate regeneration cannot be excluded. For example, insulin-like growth factor 1 (IGF-1), a pleiotropic protein with neurotrophic and neuro-modulatory functions, is known to be secreted by μG (Labandeira-Garcia et al., 2017). IGF-1 enhances mTOR signaling via IGF-1R-mediated activation of the PI3K-AKT pathway (Feng and Levine, 2010; Wrigley et al., 2017) and has been observed to promote the regeneration of axotomized hRGC axons (Subramani et al., 2023b). The near collapse of the regenerative potential of hRGCs in the presence of rapamycin as compared to modest but significant decrease in the presence of anti-proinflammatory cytokine antibodies suggests that factors in addition to TNF-α and IL-1β may support the regeneration and that could be hμG-derived IGF-1. A limitation to our observation regarding the proinflammatory cytokine-mediated regeneration is the fact that the assay was carried out on relatively immature hRGCs, where the developmental mechanism may still be operational and recruited to rescue degenerative changes.

The pleiotropy of the proinflammatory cytokines is due to differential engagement of their receptors and/or recruitment of different intracellular signaling pathways. For example, TNF-α biological action is mediated through two separate receptors, TNF-α receptor 1 (TNFR1) and TNF-α receptor 2 (TNFR2), the former mediating the proinflammatory and the latter the growth and survival effects of TNF-α (Carpentier and Palmer, 2009). In the context of the CNS, the differential actions of TNF-α on progenitor proliferation and neuron survival are mediated through TNFR1 and TNFR2, respectively (Iosif et al., 2006). In contrast to TNF-α, IL-1β signals through a single receptor, IL-1βR1 suggesting that the pleiotropic effect of this cytokine primarily involves cell-type-specific recruitment of different intracellular signaling pathways. For example, while the nuclear factor κB pathway (Liu et al., 2017; Srinivasan et al., 2004) is involved in proinflammatory actions, MAPK (Coogan et al., 1999; Srinivasan et al., 2004), JNK (Wang et al., 2007), and AKT (Han et al., 2016) pathways are recruited for diverse non-inflammatory effects such as LTP in hippocampal neurons, regulation of neural progenitor proliferation, and synaptic plasticity, respectively. Evidence has emerged for proinflammatory cytokines recruiting the AKT/mTOR pathway for influencing functions in the CNS. For example, IL-1β-mediated increase in dendritic length and soma size of neonatal hippocampal neurons *in vitro* involves the PI3K/AKT-mTOR pathway (Xiao et al., 2015). TNF-α recruits the AKT pathway to promote RGC survival following optic nerve crush injury; however, whether the downstream target of AKT included mTOR remains unknown (Diem et al., 2001). In retinal pigmented epithelial cells, a non-neuronal derivative of neural progenitors, TNF-α-mediated signaling involves the AKT/mTOR pathway to promote cell migration (Liu et al., 2012). The AKT/mTOR pathway, which is observed to regulate hRGC neurite growth, functional maturity, and facilitate axon regeneration (Teotia et al., 2019), is recruited by hμG-derived TNF-α and IL-1β for neuritogenesis and functional maturity during the generation of hRGCs and their regeneration following axotomy.

In summary, our observations through *ex vivo* model of hRGC development suggest that hμG play an evolutionarily conserved facilitatory role in neuritogenesis and the functional maturity of RGCs through proinflammatory cytokines, which can be recapitulated to promote axonal regeneration. Our results posit the AKT/mTOR pathway as one of the prominent intracellular pathways, which mediates the developmental and regenerative influence of the constitutively expressed proinflammatory cytokines. Though these results should be viewed under the caveat of controlled conditions that lacked complex and diverse *in vivo* cellular interactions and involved relatively immature hRGCs, they do point out at the possibility of the pathological recruitment of the cytokine-mediated mechanism under proinflammatory condition, exacerbating degenerative changes.

EXPERIMENTAL PROCEDURES

Resource availability

Lead contact

Information and requests should be directed to the lead contact, Iqbal Ahmad (iahmad@unmc.edu).

Materials availability

This study does not generate new unique reagents.

Data and code availability

This study does not generate new unique datasets or code.

Differentiation of hRPCs into hRGCs

CF-iPSC and CJ-iPSC (Teotia et al., 2017) and $hESC^{POU4F2-tdT}$ generation into hRPCs, their banking, and differentiation into hRGCs were carried out as previously described (Subramani et al., 2023a). Briefly, banked hRPCs were thawed and suspended in 2 mL of retinal induction media (RIM), and 1 mL of cell suspension was plated per well of a Matrigel-coated 6-well plate and incubated for 2 h at 37°C to allow the cells to adhere to the plate. Each well received an additional 1 mL of RIM and was incubated overnight at 37°C. hRPCs were sequentially treated with stage-specific chemically defined medium (Subramani et al., 2023a; Teotia et al., 2019) to differentiate them into hRGCs in the presence and absence of rhIL1-β (50 ng/mL) plus rhTNF-α (50 ng/mL). In a parallel group, the proinflammatory cytokines were neutralized with anti-TNF-α antibodies (100 ng/mL) and anti-IL1-β antibodies (100 ng/mL). All reagents were purchased from R&D Systems, Sigma-Millipore, and Gibco-Thermo Fisher Scientific).

Differentiation of human iPSCs into hμG

CF-iPSC cell line (Teotia et al., 2017) was differentiated into hμG using kits per manufacturer's instructions (Abud et al., 2017). Briefly, 40–100 colonies per well were seeded into a Matrigel-coated (Corning) 12-well plate in mTeSR Plus (STEMCELL Technologies) the day prior to starting differentiation. On day 0, mTeSR was replaced with hematopoietic medium A from the Hematopoietic Differentiation Kit, and on day 2, a half-media change was performed. On day 3, media was replaced with hematopoietic medium B with half-media changes on days 5, 7, and 10. On day 12, 1–2 x 10^6 floating cells were seeded into one well of Matrigel-coated 6-well plate containing 2 mL microglia differentiation medium. 1 mL of microglia differentiation media was added every other day until day 36. On day 24, cells were replated in 1 mL of fresh and 1 mL of old differentiation media. On day 36, 1 × 10^6 cells were seeded into a new Matrigel-coated well containing 2 mL of microglia maturation medium. 1 mL of media was supplemented every other day until day 40–46. All kits were purchased from STEMCELL Technologies.

Microglia characterization

A detailed description is provided in the supplemental experimental procedures.

hRGCs and hμG co-culture

6 DIV hRGCs (1 × 10^6) were co-cultured with huG (2 × 10^5) for 10 days. A detailed description is provided in the supplemental experimental procedures.

MEA recording of hRGCs

16 DIV hRGCs were seeded into a 48-well MEA plate (Cytoview MEA48, Axion Biosystems) and recorded over 22 days. A detailed description is provided in the supplemental experimental procedures.

Immunofluorescence staining

A detailed description is provided in the supplemental experimental procedures.

Western blot

A detailed description is provided in the supplemental experimental procedures.

qPCR

A detailed description is provided in the supplemental experimental procedures.

TUNEL

A detailed description is provided in the supplemental experimental procedures.

Statistical analysis

The data were analyzed and plotted using GraphPad Prism (GraphPad, La Jolla, CA, http://www.graphpad.com) and Windows Excel (Microsoft, Redmond, USA). For several groups, statistical significance was determined using a paired Student's t test (two-tailed) or a one-way analysis of variance (ANOVA). The data are presented as mean ± SEM; statistical differences with p values <0.05 were considered significant. Statistical analysis was performed once each group had at least three replicate samples from three different experiments.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.stemcr.2024.06.009.

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AUTHOR CONTRIBUTIONS

I.A. designed the study, interpreted the data, wrote and edited the manuscript, and provided financial and administrative assistance. M.S. performed the experiments, analyzed the data, interpreted the results, and wrote the manuscript. B.L. performed the experiments and wrote the manuscript. All authors have read and approved the final version of the paper.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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