CD11b+GR1+ Myeloid Cells Secrete NGF and Promote Trigeminal Ganglion Neurite Growth: Implications for Corneal Nerve Regeneration

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PURPOSE. We characterized fluorescent bone marrow cells (YFP⁺ BMCs) in the *thy1*-YFP mouse and determine if they promote trigeminal ganglion (TG) cell neurite growth.

METHODS. Excimer laser annular keratectomy was performed in *thy1*-YFP mice, and corneas were imaged. BMCs were harvested from femur and tibia, and the expression of surface markers on YFP⁺ BMCs was analyzed by flow cytometry. The immunosuppressive action of BMCs (YFP⁺ and YFP[−]) was evaluated in an allogenic mixed lymphocyte reaction (MLR). Neurotrophic action of BMCs (YFP⁺ and YFP[−]) was determined in compartmental and transwell cultures of dissociated TG cells.

RESULTS. Following annular keratectomy, YFP⁺ BMCs infiltrated the cornea. YFP⁺ BMCs shared surface markers (CD11b+Gr1+Ly6C+Ly6G-F4/80^{low}) with monocytic myeloid-derived suppressor cells (MDSCs), had similar morphology, and suppressed T-cell proliferation in allogenic MLR in a dose-dependent manner. YFP⁺ BMCs, but not YFP[−] BMCs, significantly increased growth of TG neurites in vitro. When cultured in a transwell with TG neurites, YFP⁺ BMCs expressed neurotrophins and secreted nerve growth factor (NGF) in conditioned medium. YFP⁺ BMCs that infiltrated the cornea maintained their phenotype and actions (neuronal and immune).

CONCLUSIONS. YFP⁺ BMCs in *thy1*-YFP mice have immunophenotypic features of MDSCs. They secrete NGF and promote neuroregeneration. Their immunosuppressive and neurotrophic actions are preserved after corneal infiltration. These findings increase our understanding of the beneficial roles played by leukocyte trafficking in the cornea and may lead to therapeutic strategies that use NGF-secreting myeloid cells to repair diseased or injured neurons.

Keywords: cornea, myeloid cell, inflammation, nerve regeneration

The mechanisms responsible for corneal nerve regeneration are not fully determined, but they constitute a high priority in the field of ophthalmology.¹ Because of the unmet clinical need to promote corneal nerve regeneration in neurotrophic corneas, the National Eye Institute (NEI) Cornea Disease Panel has recognized the need to develop agents capable of stimulating appropriate corneal nerve regeneration, as well as the need to increase our understanding of leukocyte trafficking in the cornea to elucidate their beneficial roles.² In this report, we investigated our hypothesis that myeloid cells that traffic in the cornea have neuroregenerative actions.

It is becoming increasingly clear that the nervous and inflammatory (myeloid) systems display considerable overlap in their molecular^{3–5} and cellular⁶ repertoire. Until recently, it was accepted generally that infiltration of immune cells is detrimental for neuroregeneration.⁷ Now, in a paradigm shift, some myeloid cells (e.g., monocyte-derived macrophages) have been

shown to have neuronal actions that are beneficial.^{6,8,9} Recent evidence suggests that, rather than the extent of macrophage presence, their specific phenotype at the site of injury regulates nerve regenerative outcomes.¹⁰ After insult to the retina, CD11b+Ly6C+ myeloid cells have a key role in neuroprotection.⁸ After spinal cord injury in mice, myeloid cells infiltrate the lesion site and contribute to functional recovery.⁹ Currently, therapeutic efforts in various central nervous system pathologies, ranging from acute traumas to chronic neurodegenerative diseases and even inflammatory-mediated disease, are aimed at using multifunctional myeloid cells.⁶

Our findings also have led us in the same direction. We have reported previously that, in the *thy1*-YFP mouse model, YFP⁺ myeloid cells infiltrated the cornea after nerve transecting corneal surgery.^{5,11,12} Experimental manipulations that decreased or increased the number of these myeloid cells in the cornea correspondingly decreased or increased the extent of

nerve regeneration, suggesting that these cells may have beneficial neuronal actions.^{5,11} The discovery of fluorescent nonneuronal cells was unexpected because the *thy1*-YFP mouse was generated using the neuronal *thy1* gene promoter rather than nonneuronal elements.¹³ Feng et al. also observed a small number of YFP-positive mononucleated cells in their transgenic mice lines when they first were generated, but these cells have not been characterized.¹³

The presence of fluorescent myeloid cells in the *thy1*-YFP transgenic mouse, therefore, provides a powerful tool for in situ visualization of interactions between the nervous and inflammatory (myeloid) systems. In our study, we characterized fluorescent bone marrow cells (BMCs) of the *thy1*-YFP mouse and determined their action on trigeminal ganglion (TG) cell neurite growth. Our data suggested that these cells share surface markers with myeloid-derived suppressor cells (MDSCs) and suppress T-cell proliferation in allogeneic mixed lymphocyte reaction (MLR). In addition, we demonstrated for the first time, to our knowledge, that these YFP⁺ BMCs secrete nerve growth factor (NGF) and promote neuroregeneration.

MATERIALS AND METHODS

Animals

All animal experiments were conducted in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. The animal protocol was approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Illinois at Chicago. *Thy1*-YFP neurofluorescent homozygous adult mice (6–8 weeks old) were purchased from Jackson Laboratories (Bar Harbor, ME), and colonies were established by inbreeding. For in vivo experiments, mice were anesthetized with intraperitoneal injections of ketamine (20 mg/kg; Phoenix Scientific, St. Joseph, MO) and xylazine (6 mg/kg; Phoenix Scientific). For terminal experiments, mice were euthanized according to the IACUC protocol.

Excimer Laser Annular Keratectomy

A 193 nm excimer laser (Nidek EX-5000; Nidek, Inc., Fremont, CA) was used to perform an annular keratectomy on mouse corneas. A 1.0 mm diameter metallic occluder was placed on the central cornea, and a 1.2 mm diameter phototherapeutic keratectomy (PTK) was performed to a depth of 65 μ m. The PTK was centered over the occluder. Because the occluder protected the central cornea (1.0 mm diameter), the excimer laser ablated an annular area surrounding the occluder, measuring 0.1 mm in width and 65 μ m in depth. The inner edge of the annular keratectomy had a diameter of 1.0 mm, and the outer edge a diameter of 1.2 mm. Mice were followed up after surgery, and stereomicroscopic pictures were taken at baseline, and days 5, 14, 28, and 42.

In Vivo Stereofluorescent Microscopy

Serial imaging was performed using a fluorescence stereomicroscope (Stereolumar V.12; Carl Zeiss Microscopy, Thornwood, NY) equipped with a digital camera (Zeiss AxioCam MRm; Carl Zeiss Microscopy) and software (Zeiss AxioVision 4.8; Carl Zeiss Microscopy), as described previously.¹⁴ Z-stack images were obtained at 5 μ m intervals and compacted into one maximum intensity projection (MIP) image after alignment using Zeiss AxioVision software (Carl Zeiss Microscopy). The total numbers of YFP⁺ cells were counted in the clear corneal area (2.8 mm diameter) and in the limbal area (0.26 mm width).

Corneal Whole-Mount Immunofluorescence and Confocal Microscopy

Mice were euthanized, and corneal whole mounts were prepared as described previously.¹⁴ Excised corneas were fixed directly in 4% paraformaldehyde (PFA) for 1 hour at room temperature, and overnight at 4°C after which they were washed four times with PBS (15 minutes each). Corneas then were permeabilized and blocked for 1 hour at room temperature in 1% Triton X-100, 1% BSA, and 10% normal donkey serum in PBS. Corneas were washed further and mounted in 4',6-diamidino-2-phenylindole (DAPI)-containing mounting medium on glass slides. Mosaic images of corneal whole-mounts were obtained using an Axio Observer Microscope (Carl Zeiss Meditec, GmbH, Hamburg, Germany). Z-stack images of corneal whole-mounts were obtained using an LSM 710 Confocal microscope (Carl Zeiss Meditec, GmbH).

Harvesting of BMCs and Corneal Cells

Thy1-YFP mice were euthanized, and their femurs and tibia were cleaned carefully from adherent soft tissue. The tips from both ends of each bone were removed, and the marrows were harvested by inserting a syringe needle (25 gauge) into one end of the bone and flushing with RPMI medium containing 10% fetal bovine serum (FBS) (Invitrogen, Carlsbad, CA). The bone marrow cells then were filtered through a 70 μ m nylon mesh filter (BD, Falcon, Franklin Lakes, NJ), and red blood cells (RBCs) were lysed using BD Pharm Lyse Buffer (BD Biosciences, San Jose, CA). After several washes with RPMI medium containing 10% FBS, cells were processed for immunostaining. Corneas were excised and transferred to Dulbecco's modified Eagle's medium (DMEM; Invitrogen) containing 10% FBS, and antibacterial and antimycotic antibiotics (complete medium). The iris and limbus were discarded, and the corneas were washed twice in the above-mentioned RPMI medium. The corneas then were sliced into tiny pieces and transferred to 1.7 mL microcentrifuge tubes containing approximately 9 units of Roche Liberase DL enzyme (Roche Diagnostics Corporation-Roche Applied Science, Indianapolis, IN) prepared in DMEM medium without serum. The corneas were digested at 37°C for 1 hour with intermittent mixing. After digestion, the contents were centrifuged at 250g for 10 minutes and washed three times with complete DMEM medium. The digested contents then were filtered through a 70 μ m filter to separate corneal cells from cell and tissue debris. Filtered isolated corneal cells were transferred to FACS buffer (PBS containing 2% BSA and 2 mM EDTA) and processed by flow cytometry staining or sorting.

Flow Cytometry Analyses of BMCs

Single-cell suspensions of BMCs obtained from *thy1*-YFP mice were stained with appropriate antibodies in flow cytometry buffer (Dulbecco's PBS, Ca²⁺ and Mg²⁺ free, containing 2 mM EDTA and 0.5% BSA; Invitrogen). Blocking antibodies to CD16/CD32 (BD Mouse Fc Block; BD Biosciences) were used to prevent nonspecific binding of antibodies. For staining, BMCs were incubated with fluorescent-conjugated anti-mouse antibodies against the following cell-surface markers of myeloid and lymphoid lineage: PE-CF594-CD45 (Clone 30-F11; BD Biosciences, Inc.) and rat IgG2bk isotype control PE-CF594 (Clone A95-1), APC-eFluor780-CD11b (Clone M1/70; eBioscience, Inc., San Diego, CA) and rat IgG2bk isotype control APC-eFluor780 (Clone eB149/10H5), PerCP-Cy5.5-Gr-1 (Clone RB6-8C5; eBioscience, Inc.) and rat IgG2bk isotype control PerCP-Cy5.5 (clone eB149/10H5), PE-Cy7-Ly6C (Clone AL-21; BD Biosciences) and rat IgMk PE-Cy7-Ly6C (clone R4-22), PE-Ly6G (Clone

1A8; BD Biosciences) and rat IgG2ak isotype control PE (Clone R35-95), eFluor450-F4/80 (Clone BM8; eBioscience) and rat IgG2ak isotype control eFluor450 (Clone eBR2a), Alexa Fluor 700-CD11c (Clone N418; BioLegend, Inc., San Diego, CA) and Armenian hamster IgG isotype control Alexa Fluor 700 (Clone HTK888), and PE-Cy7-CD3e (Clone 145-2C11; eBioscience) and Armenian hamster IgG isotype control PE-Cy7 (Clone 299Arm), Alexa Fluor 647-CD206 (Clone MR5D3; AbD SeroTec, Raleigh, NC) and rat IgG2a isotype control Alexa Fluor 647 (Catalog No. MCA1124A647; AbD SeroTec). BMCs were washed in flow cytometry buffer and simultaneous 8-color staining was run on a Becton Dickinson (Franklin Lakes, NJ) LSRFortessa flow cytometer. Isotype-matched control antibodies served as negative controls. Cells were gated on live cells based on the forward and side scatter properties to exclude debris, doublets, aggregates, and dead cells. Gated cells were assessed for expression levels of various cell surface markers in the total BMC population and the YFP⁺ cell population, and data were analyzed using Summit 4.3 software (Beckman Coulter, Inc., Fullerton, CA). Corresponding single-stained BMCs were used for compensating spectral spillover among fluorophores with overlapping emission spectra.

Flow Cytometry Sorting of BMCs and Corneal Cells

BMCs or corneal cells were sorted to separate YFP⁺ and YFP⁻ fractions using a Beckman Coulter Legacy MoFlo (Beckman Coulter, Inc.) at the UIC RRC Flow Cytometry Core facility. BMCs or corneal cells obtained from C57BL/6 mice were used to control for background fluorescence. BMCs or corneal cells harvested from *thy1*-YFP mice were gated on live cells based on the forward and side scatter properties. A second gate of pulse width versus side scatter was used to gate out aggregates. YFP⁺ cells (FL1 channel) versus autofluorescent cells (FL3 channel) were used to gate out dead cells. YFP⁺ sorted BMCs or corneal cells (>95% purity) were used for in vitro assays.

Cytospin and Nuclear Staining With DAPI

Cytospin preparations were made using the Shandon Cytospin 2 (Thermo Fisher Scientific, Waltham, MA). Sorted YFP⁺ BMCs (10,000) in 250 μ L of medium were transferred to Cytospin tubes and centrifuged for 5 minutes at 100g on high acceleration. Cells were collected on a glass slide through a 2-hole filter card (catalog No. 22030410, Thermo Fisher Scientific) and fixed in 4% PFA for 1 hour. After subsequent washes with PBS, cells were permeabilized for 30 minutes at room temperature in 0.1% Triton X-100 followed by two additional PBS washes. Corneas were mounted onto glass slides with a drop of DAPI-containing mounting medium and covered with a coverslip. Slides were examined using an inverted Axio Observer microscope (Carl Zeiss Meditec, GmbH), and images were captured using a digital camera (Zeiss AxioCam MRm; Carl Zeiss Microscopy) and imaging software (Zeiss AxioVision 4.8; Carl Zeiss Microscopy).

One-Way MLR

Splenocytes were harvested from naive BALB/c and C57BL/6 mice for allogeneic MLR. The BALB/c splenocytes (responder cells) and irradiated C57BL/6 splenocytes (stimulator cells; irradiation dose, 3000 rad) were plated (1×10^5 cells/well each; 1:1 ratio) in sterile 96-well U-bottom plates (Corning, Tewksbury, MA) containing RPMI medium (Invitrogen) with L-glutamine, 2-mercaptoethanol (0.05 μ M) supplemented with 10% FBS (Invitrogen), and an antibiotic-antimycotic solution (100 IU/mL penicillin, 100 mg/mL streptomycin, and 2.5 μ g/mL amphotericin B). Test cells (BMCs or corneal) were added

to responder and stimulator cell-containing wells. Cells tested were: (1) YFP⁺ BMCs (YFP-MDSCs) sorted from bone marrow cells (0.5×10^4 , 1.0×10^4 , or 2.0×10^4 cells/well), (2) YFP⁻ BMCs (2.0×10^4 cells/well) obtained from flow cytometry sorting of total BMCs, (3) unsorted corneal cells (containing a mix of YFP⁺ and YFP⁻ cells) isolated after collagenase digestion of excised *thy1*-YFP mice corneas (8×10^4 cells/well), and (4) YFP⁻ corneal cells obtained from total corneal cells by flow cytometry sorting (2×10^4 cells/well). After 3 days of culture, 1 μ Ci/well of [³H]-thymidine was added to the culture for 16 hours, after which proliferation was stopped by transferring the culture plate to 4°C. Cells were harvested and collected on glass fiber filter membranes using a Packard Cell Harvester System (Perkin-Elmer, Waltham, MA). After drying the filter membranes, they were transferred to a Packard TopCount NXT Microplate Scintillation and Luminescence Counter (Perkin-Elmer), and [³H]-thymidine incorporation was measured in counts per minute (cpm). Stimulation index (SI) was determined by cpm of [³H]-thymidine incorporation of allogeneic responder (BALB/c) and irradiated stimulator cells (C57BL/6) (R+S)/autologous responder cells (BALB/c) (R).

Because the yield of fluorescent BMCs after excimer laser annular keratectomy was low due to cell losses during processing (collagenase digestion and flow cytometry), we elected to use an alternative approach for enhancing corneal infiltration of YFP⁺ cells by applying benzalkonium chloride (0.1%) once a day for 5 days to the *thy1*-YFP mice corneas. We have reported previously that this method causes significant YFP⁺ cell infiltration into the cornea.¹² Others have reported that inflammation increases MDSC levels and suppressive activity as well as resistance to apoptosis^{15,16}; therefore, we expected a greater yield of YFP-MDSCs after BAK-induced corneal inflammation. Because the goal of our experiments was to determine whether YFP⁺ BMCs preserve their phenotype and actions within the corneal microenvironment, using BAK for enhancing corneal infiltration was reasonable. In addition, because the yield of sorted corneal YFP⁺ cells was insufficient to perform all in vitro experiments, we elected to use unsorted corneal cells (mix of YFP⁺ and YFP⁻ cells) to determine YFP⁺ corneal cell action in MLR.

Compartmental Culture of TG Cells With BMCs or Corneal Cells

The TG neurons were isolated from 10-day-old *thy1*-YFP pups, and compartmental cultures were performed as described previously.^{5,11,12} Dissociated TG cells plated in the central compartment (1.5×10^3 /device) were cultured in F12 medium containing 10% FCS, 1% penicillin-streptomycin, NGF, brain-derived neurotrophic factor (BDNF; 1 mg/mL each, diluted to 1:10,000 concentration in the media), and AraC (0.3 μ M). Side compartments were filled with the same medium lacking AraC and BDNF. On day 4, sorted BMCs (YFP⁺ or YFP⁻) or sorted corneal cells (YFP⁺ or YFP⁻) were plated in the side compartments (day 4 of compartmental cell culture will now be referred to as day 0 of co-culture). Corneal cells were isolated, and YFP⁺ and YFP⁻ fractions were sorted by flow cytometry as described for MLR experiments. The yield of YFP⁺ cells after liberase digestion of pooled mouse corneas was low (~2000 cells from 10 corneas) compared to yield of YFP⁺ cells from pooled bone marrows (~100,000 of 6–8 mice). Therefore, 1000 corneal YFP⁺ cells were added in the side compartment compared to 10,000 bone marrow YFP⁺ cells. Control devices (lacking cells in the side compartments) were set similarly. Neurite fiber length (NFL) was calculated on days 0, 4, and 7 of co-culture. For calculating NFL, we included neurites in side compartments that had grown >1.0 mm in length beyond the Teflon barrier on day 0 of co-culture.

Neurites were imaged on days 0, 4, and 7 of co-culture, and the increase in neurite length was determined using Autoneuron and Neurolucida software (MBF Bioscience, Williston, VT). Experiments were terminated on day 7, and RNA was collected for gene expression analysis.

Co-Cultures of TG Cells and BMCs (YFP⁺ or YFP⁻) in Transwells

For Transwell experiments, 0.1×10^4 dissociated TG neuronal cells were plated in the bottom wells of Transwell units (12-well polystyrene plate and 3.0 μ m pore size polytetrafluoroethylene [PTFE] membrane, precoated with collagen types I and IV, Corning-Costar 3494 Transwell; Catalog # 07-200-561; Thermo Fisher Scientific) containing F12 medium with 10% FCS and 1% penicillin-streptomycin. BMCs (1×10^4 cells; YFP⁺ or YFP⁻) were added to the Transwell membrane. Experiments were performed with the following conditions: (1) monocultures, only TG cells in the bottom well or only BMCs (YFP⁺ or YFP⁻) in Transwells, and (2) co-cultures, TG cells in bottom wells and BMCs (YFP⁺ or YFP⁻) in Transwells. The control was F12 medium only. Transwell plates were incubated at 37°C for 72 hours. Bottom wells of the Transwell plates were imaged on day 3 to assess neurite growth. Three random areas were selected from each plate and analyzed to calculate NFL/soma using Autoneuron and Neurolucida software (MBF Bioscience, Williston, VT). Conditioned medium was collected on day 5 and transferred to an anti-NGF-coated 96-well ELISA plate (100 μ L per well, 3 wells per sample). ELISA for NGF was performed according to the manufacturer's protocol (catalog # G7630, NGF Emax Immuno-Assay System; Promega, Madison, WI). NGF levels were calculated from the standard curve prepared for each plate. The standard curves were linear and the quantities of NGF in experimental samples always were within the linear range of the standard curve.

Gene Expression in TG Cells and BMCs

RNA was extracted from TG cells and YFP⁺ BMCs using TRIzol Reagent (Invitrogen) for RNA extraction, which was performed according to the manufacturer's protocol as previously described.¹⁷ Reverse transcription was performed with 1 μ g total RNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. Real-time quantitative PCR (qPCR) was performed with SYBR green (Qiagen-SABiosciences, Valencia, CA) using the StepOnePlus Real-Time PCR system (Applied Biosystems). All primers and reagents were purchased from Qiagen-SABiosciences unless specified otherwise. The primers used were NGF (catalog No. PPM03596B), growth associated protein 43 (Gap43; catalog No. PPM03303B), beta 3 tubulin (Tubb3; catalog No. PPM04754A), small proline-rich protein 1a (Sprr1a, catalog No. PPM40924B), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; catalog No. PPH00150F). Samples were assayed in duplicate in a total volume of 25 μ L using the following cycling conditions: 10 minutes at 95°C, 40 cycles at 95°C for 15 seconds, and 60°C for 60 seconds. A genomic DNA contamination control was used to confirm that amplification reagents were not contaminated with genomic DNA. For data analyses, the cycle threshold (CT) of each gene was normalized to the corresponding value for baseline (day 0 culture) and used to calculate-fold change using the $2^{-\Delta\Delta CT}$ method.

Statistical Analyses

Statistical analyses were performed using Microsoft Excel (Microsoft Corp., Redmond, WA). Student's *t*-test was used to compare

mean values between groups. Results are shown as the mean $\pm$ SEM. Differences were considered significant if $P < 0.05$.

RESULTS

Fluorescent Cells in the Cornea After Annular Keratectomy

In naïve corneas, YFP-fluorescent cells are present primarily in the limbus (Fig. 1). The limbus had significantly greater numbers of fluorescent cells (64.3 ± 13.0 cells, $P < 0.05$) than the cornea (8.2 ± 2.3 cells). After annular keratectomy to transect the corneal nerves, fluorescent cells infiltrated the cornea, initially to the margin of the keratectomy adjacent to the transected nerves (postoperative day 3) and subsequently to the posterior stroma in the central denervated cornea (Figs. 1C-E). The YFP cells were significantly greater in number at day 5 (55.5 ± 18.4 , $P < 0.05$), day 14 (5.8 ± 2.1 , $P < 0.05$), 4 weeks (5.2 ± 0.8 , $P < 0.05$), and 6 weeks (5.4 ± 1.6 , $P < 0.05$) in the annular keratectomy area compared to baseline (1.8 ± 0.7). Regenerating nerves also occupied posterior stroma in the central denervated cornea and are seen as a dense network of interconnected nerves (Figs. 1G, 1I) in approximately the same plane in the cornea as the YFP⁺ cells.

Phenotype of YFP⁺ BMCs

Cytospin preparations of flow cytometry-sorted YFP⁺ BMCs (>95% pure) from naïve *thy1-YFP* mice were stained with DAPI, and their morphology and size were analyzed (Fig. 2). YFP⁺ BMCs were round or oval-shaped and measured 15.63 ± 0.45 μ m in horizontal diameter. The nucleus was large and bean-shaped with a shallow notch (monocytic). BMCs with a ring-shaped nucleus were not fluorescent (Fig. 2E).

We performed FACS analysis of BMCs. YFP⁺ cells were, on average, $0.43 \pm 0.01\%$ of the total BMCs (Fig. 3). All YFP⁺ BMCs were CD45-positive. When gated on YFP⁺ cells, $91.0 \pm 2.2\%$ of YFP⁺ cells were CD11b-positive, $92.8 \pm 1.0\%$ were Gr1-positive, and $92.1 \pm 0.8\%$ were Ly6C-positive. YFP⁺ cells did not show positive staining for Ly6G ($4.4 \pm 0.4\%$). There was low positive expression for F4/80 ($72.7 \pm 5.1\%$). Thus, the YFP⁺ cell signature based on surface markers (CD11b+Gr1+Ly6C+Ly6G-F4/80^{low}) showed similarities with monocytic MDSCs.^{18,19} YFP⁺ BMCs did not show positive staining for MHC-II ($6.9 \pm 0.9\%$), a marker that is expressed on antigen-presenting cells, CD11c ($9.1 \pm 1.6\%$), a marker expressed on dendritic cells, and monocytes, macrophages, and CD3e ($2.3 \pm 2.3\%$), a marker expressed on T-lymphocytes.

Immunosuppressive Action of BMCs (YFP⁺ or YFP⁻)

To determine whether YFP⁺ BMCs possess immunosuppressive capacity that characterizes the MDSC family, we investigated the ability of YFP⁺ BMCs to suppress a murine allogeneic MLR (Fig. 4). Addition of stimulator cells (irradiated C57BL/6 splenocytes) to responder cells (BALB/c splenocytes) increased the stimulation index to 2.4 ± 0.25 , $P < 0.05$. The addition of sorted YFP⁻ BMCs to the MLR of responder and stimulator splenocytes caused a significant increase in the stimulation index (4.3 ± 0.68 , $P < 0.05$). In contrast, addition of YFP⁺ BMCs to the MLR of responder and stimulator splenocytes caused a significant decrease in the stimulation index (0.25 ± 0.08 , $P < 0.05$, Fig. 4A). The immunosuppressive action of YFP⁺ BMCs showed a dose response effect (Fig. 4B). The addition of 5000 YFP⁺ BMCs did not significantly suppress MLR (1.98 ± 0.25 , $P = 0.20$); however, addition of

FIGURE 1. YFP+ cells in naïve corneas and after annular keratectomy. (A) Stereofluorescent microscope image of cornea showing fluorescent nerves and YFP+ cells. *Inset* shows YFP+ cells in the limbal area. (B) Graph showing distribution of YFP+ cells in naïve corneas. (C–E) Stereofluorescent image of a cornea in which excimer laser annular keratectomy was performed. (C) Preoperative image. (D) Six hours postoperative image. The annular keratectomy groove is enclosed between *arrowheads*. (E) Three days postoperative image. A *square area* has been demarcated on these images and shown in the *inset*. *Inset* in (C) shows preoperative appearance of corneal nerves. *Inset* in (D) shows the transection of corneal nerves in the area occupied by the annular keratectomy groove. *Inset* in (E) shows YFP+ cells infiltrating up to the margins of

the annular keratectomy groove. (F) Confocal image showing YFP⁺ cells (arrow) adjacent to transected nerves. (G) Three-dimensional reconstruction of a Z-stack of confocal images shows that the YFP⁺ cells are present in the posterior corneal stroma beyond the transected nerves. The red line indicates the location of the keratectomy groove. (H) Graph showing YFP⁺ cell infiltration into the cornea over a period of 6 weeks. (I) Three-dimensional reconstruction of a Z-stack of confocal images at 6 weeks postoperative showing regenerating corneal nerves in the posterior cornea in approximately the same plane in the cornea as the YFP⁺ cells. E, epithelium; S, stroma. * $P < 0.05$. White scale bars in (A), (C), (D), and (E): 500 μm (inset: 250 μm). White scale bar in (F): 20 μm .

10,000 or 20,000 YFP⁺ BMCs did cause significant suppression (0.87 ± 0.18 and 0.31 ± 0.14 , respectively, $P < 0.05$), which was greatest with the addition of 20,000 cells.

Neuroregenerative Action of BMCs (YFP⁺ or YFP⁻)

Compartmental Cultures. To determine the effect of YFP⁺ BMCs on TG neurite outgrowth, we used compartmental cultures of dissociated TG cells (Fig. 5). Either YFP⁺ BMCs or YFP⁻ BMCs were added to the side compartments and co-cultured with TG neurites. On day 0 of co-culture, the neurite length was similar to control (TG neurite culture without BMCs). On day 4, neurite length was significantly greater with YFP⁺ BMC co-culturing (33.52 ± 2.81 mm, $P < 0.05$) compared to controls (13.76 ± 0.4 mm; Figs. 5B1, 5B2). In contrast, neurite length was similar with YFP⁻ BMC co-cultures (13.13 ± 0.80 mm) compared to controls. On day 7, neurite length was significantly greater with YFP⁺ BMC co-cultures (63.31 ± 13.34 mm, $P < 0.05$) compared to controls (8.12 ± 1.83 mm; Figs. 5C1, 5C2). In contrast, although neurite length was greater with YFP⁻ BMC co-cultures (14.24 ± 2.45 mm) compared to controls, the difference was not statistically significant ($P = 0.06$). At both time points (days 4 and 7), neurite growth with YFP⁺ BMC co-cultures was significantly greater than that with YFP⁻ BMC co-cultures.

On day 7 of co-culturing, expression of regeneration-associated genes (RAGs) was analyzed in TG cells in the central compartment. The expression of *Gap43* (3.49 ± 0.83 -fold), *Tubb3* (1.87 ± 0.26 -fold), and *Spr1a* (2.09 ± 0.64 -fold) in TG cells was significantly increased when YFP⁺ BMCs were co-cultured in the side compartments ($P < 0.05$) compared to TG cell cultures alone (Fig. 5E). YFP⁺ BMCs appeared as round fluorescent spheres at day 0 (Fig. 5F1), but they differentiated to elongated cells (approximately 30 μm) that appeared spindle- or dendritic-shaped on day 7 (Figs. 5F2, 5F3). Several neurites made physical contact with YFP⁺ BMCs (Fig. 6).

Transwell Cultures. To determine if neuronal actions of YFP⁺ BMCs were due to secreted neurotrophic factors, YFP⁺ BMCs and dissociated TG cells were co-cultured in the presence of a semipermeable membrane that separated the two cell populations (with Transwell). On day 3 (72 hours) of co-culture, TG neurite growth was analyzed and compared to neurite growth in TG cell monocultures (Fig. 7). TG neurite outgrowth was significantly greater when co-cultured with YFP⁺ BMCs in the presence of the Transwell (3.26 ± 0.24 mm neurite length/soma, $P < 0.05$) compared to TG cell monocultures (1.52 ± 0.09 mm neurite length/soma, Fig. 7C). In contrast, neurite outgrowth was similar in Transwells with YFP⁻ BMC co-cultures (1.79 ± 0.13 mm neurite length/soma) compared to TG cell monocultures. In Transwell co-cultures, TG neurite outgrowth was significantly greater with YFP⁺ BMCs compared to YFP⁻ BMC co-cultures ($P < 0.05$).

On day 5 of co-cultures, expression of RAGs was analyzed in TG cells. The expression of *Gap43* (7.63 ± 3.88 -fold), *Tubb3* (47.9 ± 13.4 -fold), and *Spr1a* (43.9 ± 5.18 -fold) in TG neurites separated from YFP⁺ BMCs by a semipermeable membrane was significantly increased ($P < 0.05$) compared to TG cell monocultures (Fig. 7D). On day 5 of cultures, expression of NGF was analyzed in YFP⁺ BMCs. The expression of NGF (24.9 ± 4.78 -folds) in YFP⁺ BMCs was significantly increased compared to baseline (day 0, Fig. 7E).

To determine whether TG cells or BMCs (YFP⁺ or YFP⁻) secrete NGF, we performed monocultures or co-cultures (in Transwell) and determined the abundance of NGF in the conditioned medium using ELISA (Figs. 7F, 7G). On day 5, NGF was not detected in F12 medium nor in conditioned medium of TG cell monocultures (Fig. 7F). The abundance of NGF in conditioned medium of YFP⁺ BMC monocultures (399.75 ± 37.77 pg/mL, $P < 0.05$) was significantly greater than YFP⁻ BMC monocultures (16.41 ± 41.37 pg/mL). NGF was detected in only one of six monocultures of YFP⁻ BMCs, whereas abundant NGF was detected in all six monocultures of YFP⁺ BMCs. Consistent with monoculture data, NGF was not detected in co-cultures of YFP⁻ BMCs and TG cells (all six co-cultures, Fig. 7G). In contrast, abundant NGF was detected in conditioned medium when YFP⁺ BMCs were co-cultured with TG cells (321.41 ± 45.42 pg/mL, $P < 0.05$).

Phenotype of YFP⁺ Cells in the Cornea

In naïve bone marrow, 100% of YFP⁺ cells were CD45⁺, and all YFP⁺ cells in naïve cornea were CD45⁺. There was a 4.4-fold increase in the number of YFP⁺ cells in the treated corneas compared to naïve corneas, and nearly all (96%) of the YFP⁺ cells in treated corneas were CD45⁺, suggesting an influx of cells of bone marrow origin within the cornea (Figs. 8A1–A3). The phenotype of YFP⁺ cells in the naïve as well as treated corneas was CD11b+GR1+Ly6C+Ly6G⁻, which was similar to the phenotype of bone marrow YFP⁺ BMCs (Figs. 8A4–A6). Given that YFP⁺ cell numbers increase in treated corneas, nearly all of these cells are CD45⁺ cells, and their phenotype is consistent with YFP⁺ BMCs, the infiltrating corneal cells are of hematopoietic origin, possibly from the bone marrow.

Immunosuppressive Action of YFP⁺ Corneal Cells

To determine whether YFP⁺ BMCs retained their immunosuppressive capacity in the corneal environment, infiltrating YFP⁺ cells were isolated from the corneas and their ability to suppress allogenic MLR was determined (Fig. 8B). Addition of stimulator cells (irradiated C57BL/6 splenocytes) to responder cells (BALB/c splenocytes) increased the stimulation index to 2.96 ± 0.49 , $P < 0.05$. Addition of sorted YFP⁻ corneal cells to the MLR of responder and stimulator splenocytes caused a slight nonsignificant increase in the stimulation index (3.18 ± 0.43 , $P = 0.37$). In contrast, addition of unsorted corneal cells (mix of YFP⁺ and YFP⁻ cells) to the MLR of responder and stimulator splenocytes caused a significant decrease in the stimulation index (1.88 ± 0.88 , $P = 0.05$, Fig. 8B). Addition of sorted YFP⁺ BMCs to the MLR containing YFP⁻ corneal cells caused a much greater and significant decrease in the stimulation index (0.55 ± 0.07 , $P < 0.05$). Therefore, MLR is not suppressed by YFP⁻ corneal cells alone, but is significantly suppressed when YFP⁺ cells are present, either YFP⁺ corneal cells or YFP⁺ BMCs.

Neuroregenerative Action of YFP⁺ Corneal Cells

To determine whether YFP⁺ BMCs retained their neuroregenerative action in the corneal environment, infiltrating YFP⁺ cells were isolated from the corneas and their ability to

FIGURE 2. Fluorescent microscope images of YFP⁺ cells. (A) Mosaic image of corneal whole-mount showing YFP⁺ cells and corneal nerves. *Arrow* points to YFP⁺ cells and *arrowhead* points to nerves in the corneal limbus area. *Inset* shows limbal area at a higher magnification. (B) Confocal image showing YFP⁺ cells (*arrow*) adjacent to corneal nerves (*arrowhead*). (C) Fluorescent YFP⁺ BMCs. *Arrow* points to a cell shown in the *inset* at higher magnification. (D) DAPI staining of YFP⁺ BMC to show nuclear shape. *Arrow* points to a bean-shaped nuclei (monocytic) shown in the *inset* at higher magnification. *Arrowhead* points to YFP⁻ BMC with ring-shaped morphology that is not fluorescent. (E) An overlay with DAPI and YFP to indicate the nucleus and cytoplasm of YFP⁺ BMC. White scale bar: 500 μ m (A) and 200 μ m (*inset* images in [A]); 20 μ m (B–E).

FIGURE 3. (A) Fluorescent microscope image showing YFP⁺ BMCs. (B–J) Flow cytometry analysis of YFP⁺ BMCs. (B1) *Thy1*-YFP mouse BMCs, showing 0.4% to 0.5% of the total BMCs were YFP⁺. (B2) C57BL/6 mouse BMCs used to control for background fluorescence. No fluorescent cells are seen. (B3) CD45 expression in YFP⁺ BMCs. (C1–J1) Top right quadrant of each panel shows YFP⁺ cells in the total BMC population that express the denoted cell surface marker. (C1) CD11b expression. (D1) Gr-1 expression. (E1) Ly6-C expression. (F1) Ly6-G expression. (G1) F4/80 expression. (H1) CD11c expression. (I1) CD3e expression. (J1) CD206 expression. (C2–J2) Corresponding isotype controls. The YFP⁺ BMC signature based on surface markers is CD11b⁺Gr1⁺Ly6C⁺Ly6G⁺F4/80^{low}. White scale bar for (A1): 100 μm.

FIGURE 4. Allogenic MLR to demonstrate immunosuppressive action of bone marrow cells. BALB/c splenocytes (responder cells) and irradiated C57BL/6 splenocytes (stimulator cells) were plated, test cells (YFP⁺ or YFP⁻ BMCs) added, and cell proliferation was determined by [³H]-thymidine incorporation. (A) Graph showing that SI increased after addition of sorted YFP⁻ BMCs to the MLR, but significantly decreased after addition of YFP⁺ BMCs (YFP-MDSCs). (B) Graph showing that YFP⁺ BMCs (5000, 10,000, and 20,000 cells, respectively) when added to the MLR resulted in a dose-dependent decrease in SI. Data shown are means $\pm$ SEM from three independent experiments performed in triplicate. * $P \leq 0.05$.

enhance TG neurite growth was investigated in compartmental culture (Fig. 8C). Flow cytometry-sorted corneal cells (YFP⁺ or YFP⁻) were added to the side compartments and co-cultured with TG neurites. On day 0 of co-cultures, the neurite length was similar to controls (TG neurite culture without corneal cells). On day 4, neurite length was greatest with YFP⁺ corneal

cell co-cultures (15.02 ± 1.90 mm, $P < 0.05$); however, the difference was not significant compared to controls (11.51 ± 0.40 mm) or YFP⁻ corneal cell co-cultures (11.01 ± 1.6 mm). On day 7, neurite length was significantly greater with YFP⁺ corneal cell co-cultures (23.82 ± 1.50 mm, $P < 0.05$) compared to either controls (7.47 ± 1.83 mm) or YFP⁻

FIGURE 5. In vitro compartmental culture of dissociated TG cells showing the effect of BMCs (YFP⁺ or YFP⁻) on neurite outgrowth. Widefield fluorescent images show neurites in the side compartment at day 0 (A1, A2), day 4 (B1, B2), and day 7 (C1, C2) of culture. (A1), (B1), and (C1) are TG cell cultures without BMCs in the side compartments. (A2), (B2), and (C2) are TG cell cultures with YFP⁺ BMCs (YFP-MDSCs) in the side compartments. (D) Graph shows that neurite outgrowth was significantly higher when TG cells were co-cultured with YFP⁺ BMCs compared to YFP⁻ BMCs. (E) Graph showing qPCR analyses of regeneration-associated gene expression in TG cell lysates after 7 days of co-culture. The expression of *Gap43*, *Tubb3*, and *Sprr1a* in TG cells was increased in co-cultures. (F1) Fluorescent microscope image of a YFP⁺ BMC on day 0. Fluorescent microscope image (F2) and Giemsa staining (F3) show YFP⁺ BMC on day 7. Note the change in shape from round (F1) to dendritic cell-like (F2, F3). Scale bar: 250 μ m (A1–C2), scale bar: 10 μ m (F1–F2), scale bar: 20 μ m (F3). * $P \leq 0.05$

FIGURE 6. (A) Wide-field fluorescent image of tracks in the side compartment of TG neurite and YFP⁺ BMC (YFP-MDSC) co-cultures at day 7. (B–G) show zoomed areas from the same three tracks. (B) Neurite with no YFP⁺ BMC contact. (C–G) Physical contact between YFP⁺ BMC and neurites. Scale bars in (A): 200 μ m and in (B–G): 25 μ m.

corneal cell co-cultures (13.60 ± 2.14 mm). In contrast to control or YFP[−] corneal cell co-cultures, significant neurite growth occurred in YFP⁺ corneal cell co-cultures between days 4 and 7.

DISCUSSION

Our study revealed several important findings. We determined that fluorescent cells in the bone marrow of *thy1*-YFP mice (YFP⁺ BMCs) are CD45⁺ hematopoietic cells. Their phenotype is CD11b⁺Gr1⁺Ly6C⁺Ly6G⁺F4/80^{low}, and they inhibit an allogeneic mixed lymphocyte reaction. In mice, the expression

of these surface markers and immunosuppressive ability are characteristics of MDSCs.^{18–21} YFP⁺ BMCs promote neurite growth in TG cells by secreting soluble neurotrophic factors, such as NGF. Finally, YFP⁺ BMCs infiltrate the cornea and retain their phenotype as well as neuroregenerative and immunosuppressive actions within the corneal environment. When depleted of the YFP⁺ fraction, remaining bone marrow and corneal cells were unable to exert neuroregenerative or immunosuppressive actions.

To the best of our knowledge, this is the first report demonstrating that CD11b⁺Gr1⁺ myeloid cells secrete NGF and have neuroregenerative actions. Understanding the interactions between these cells and TG neurites is important for

FIGURE 7. Effect of BMCs (YFP⁺ or YFP⁻) on TG cell neurite growth (in Transwell). Representative mosaic image of a single TG cell after 3 days of monoculture without YFP⁺ BMCs (A) or co-culture with YFP⁺ BMCs (B) in Transwell. (C) Graph shows significantly greater TG neurite length (NFL/soma) when co-cultured with YFP⁺ BMC compared to YFP⁻ BMC. (D) Expression of regeneration-associated genes (*Gap43*, *Tubb3*, and *Sprr1a*) in TG cell lysate obtained after 5 days of culture using qPCR analyses. Expression of RAGs in TG cells was significantly higher in the presence of YFP⁺ BMCs in Transwell than without them (TG cells alone). (E) Expression of NGF in YFP⁺ BMC lysates after 5 days of culture in Transwell. NGF expression was increased significantly in YFP⁺ BMCs but not in TG cells. (F, G) Abundance of NGF in conditioned medium using ELISA after 5 days

of culture. (F) NGF abundance in conditioned medium of monocultures of TG cells, YFP⁺ BMCs, and YFP⁻ BMCs. YFP⁺ BMC monocultures secreted abundant NGF, which was not detectable (ND) in TG cell monocultures. (G) NGF abundance in conditioned medium of co-cultures of TG cells and BMCs (in Transwell). YFP⁺ BMC co-cultures had abundant NGF, which was ND in YFP⁻ BMC co-cultures. White scale bars in (A) and (B): 200 μ m. **P* < 0.05.

developing novel cell-based therapeutic strategies that promote nerve regeneration. For example, one strategy could be to enhance chemotaxis of these cells towards nerve injury sites to facilitate neurite growth. In addition, these strategies also may be relevant in treatment of corneal diseases, such as neurotrophic keratitis. Such cell-based therapeutic strategies have been used in preclinical models of optic nerve injury and spinal trauma where transplantation of activated macrophages promoted axonal regeneration and functional recovery.^{22,23}

In the transgenic *thy1*-YFP mice, YFP is stably expressed in neurons. These mice have been used to study peripheral nerve degeneration and regeneration.^{24–26} The *thy1*-YFP mouse model used in these investigations was generated using the neuronal *thy1* gene promoter, not the nonneuronal elements.¹³ Therefore, in this *thy1*-YFP mouse model, only the nerves are fluorescent. Although wild-type *thy1* is a pan T-cell marker²⁷ and expressed in activated keratocytes in the cornea (fibroblasts and myofibroblasts),²⁸ we have reported previously that, in the transgenic *thy1*-YFP mouse model, corneal epithelial and stromal cells are not fluorescent in their resting or activated phenotypes.¹¹ We also have reported that the fluorescent cells infiltrating the cornea in these mice are CD45⁺ hematopoietic cells.^{11,12} Feng et al. also have observed a small number of YFP-positive mononucleated cells in their transgenic lines when these mice were first generated.¹³

In this report, we have characterized this population of bone marrow-derived YFP⁺ cells and found that they are morphologically, phenotypically, and functionally similar to MDSCs. MDSCs are a group of myeloid cells comprised of precursors of macrophages, granulocytes, dendritic cells, and myeloid cells at earlier stages of differentiation.¹⁸ They are defined in mice on the basis of CD11b and Gr1 marker expression as well as their functional ability to inhibit T lymphocyte activation.²⁹ The morphologic heterogeneity of these cells has been defined based on the expression of Ly6G and Ly6C epitopes of GR1, which are encoded by separate genes.^{18,21} Granulocytic MDSCs have a CD11b+Ly6G+Ly6C- phenotype, whereas MDSCs with monocytic morphology are CD11b+Ly6G-Ly6C^{high}.^{19,21,30} The phenotype and nuclear morphology of YFP⁺ BMCs was similar to monocytic MDSCs.³¹ Therefore, it is possible that YFP⁺ BMCs are a subset of the MDSC family of immunosuppressive CD11b+GR1+ myeloid cells that have neuroregenerative actions. We have not investigated whether neuroregenerative action or NGF secretion is a common feature of all MDSCs. YFP⁺ BMC cell surface markers are different from neutrophils, which are Gr1^{high}F4/80⁻.^{32,33} YFP⁺ BMCs did not express CD3e (expressed on T-lymphocytes), CD11c (expressed on dendritic cells, monocytes, and macrophages), MHC-II (expressed on antigen-presenting cells), and CD206 (marker for M2-type macrophages).

The presence of corneal myeloid cells has been reported previously. The normal murine corneal stroma contains a significant number of CD45⁺ cells. Virtually all of these cells co-express CD11b, but only 50% co-express F4/80, which is consistent with monocyte or macrophage lineage.^{34,35} Monocyte-derived cells infiltrate the cornea and are distributed in the corneal stroma.^{36,37} Although the function of myeloid cells is unclear in the cornea, in the retina, monocyte-derived macrophages have a key role in neuroprotection.

In our report, we demonstrated that YFP⁺ BMCs infiltrate the cornea and that their neuroregenerative action is preserved

within the corneal milieu. Neurite growth due to corneal YFP⁺ cells was lower than bone marrow YFP⁺ cells because fewer corneal YFP⁺ cells were added to the side compartments compared to bone marrow YFP⁺ cells. Our data suggested that one mechanism by which YFP⁺ BMCs promote neuroregeneration is through secretion of NGF, a soluble neurotrophic factor. Myeloid cells other than CD11b+GR1+ cells also secrete NGF and other neurotrophic factors.^{38,39} We found a significant increase in gene expression and protein abundance of NGF in conditioned medium of YFP⁺ BMC monocultures as well as co-cultures (with Transwell). The amount of NGF in the conditioned medium (approximately 400 pg/mL) has been shown to stimulate neurite growth in vitro.⁴⁰ Furthermore, corneal innervation has been reported to be NGF-dependent, and NGF receptor TrkA-knockout mice display reduced corneal nerve density and impaired corneal sensitivity.⁴¹ Topically applied exogenous NGF heals neurotrophic ulcers associated with impairment of sensory innervation of the cornea and improves corneal sensitivity.^{42,43} Given the dependence of corneal innervation on NGF and its therapeutic benefit for corneal neurotrophic disease, infiltration of NGF-secreting YFP⁺ BMCs in the cornea is likely beneficial during nerve disease or injury. Furthermore, NGF secreted by the corneal YFP⁺ BMCs may counteract the neuro-repulsive effects of nerve guidance proteins, such as semaphorin 3A (sema3A), which could be inhibiting neuroregeneration in the cornea. Sema3A guides the growth of neurons during development, and is required for innervation of the corneal stroma and epithelium.^{44–46} It causes neuro-repulsion of trigeminal sensory afferents. Following epithelial wounding and denervation, sema3A inhibits collateral nerve sprouts from innervating the re-epithelialized tissue.⁴⁷

There is another possible mechanism by which YFP⁺ BMCs cells can facilitate neuronal growth. These cells have immunophenotypic characteristics of MDSCs, which are known to secrete soluble factors that produce an immunosuppressive and anti-inflammatory local milieu that is growth permissive. The immunosuppressive and anti-inflammatory action of MDSCs is related to secretion of arginase, nitric oxide (NO), or cytokines (IL-10 and TGF- β) and the production of reactive oxygen species (ROS).¹⁸ MDSCs bear pleiotropic characteristics of M1 and M2 monocytes/macrophages.⁴⁸ M2-type macrophages also express arginase and IL-10 and promote neuroregeneration.^{49,50} Numerous studies in mouse models are consistent with an important role for the monocytic fraction of MDSCs, similar in morphology, phenotype, and function to YFP⁺ BMCs, in restraining the immune response in mice.²¹ In our study, we determined that YFP⁺ BMCs are immunosuppressive. Also, YFP⁺ BMCs express arginase and IL-10 (data not shown). Because the immunosuppressive action of YFP⁺ BMCs is preserved after corneal infiltration, it is possible that they create a growth permissive local milieu in the cornea to facilitate neuroregeneration. Additionally, infiltrating YFP⁺ BMCs may immunosuppress the corneal milieu, thus contributing to immune tolerance and maintaining corneal immune privilege during inflammation or injury. MDSCs have been shown to promote tolerance to alloantigens, and there is direct evidence of a tolerogenic role for MDSCs in heart and islet allografts in mice.^{51,52} Furthermore, MDSCs provide checks-and-balances to counter proinflammatory immune cells in the liver and adipose tissue during obesity to prevent overt immune responses.⁵³ This suggests the intriguing possibility

FIGURE 8. Phenotype and actions of YFP⁺ cells within the corneal environment. (A1–A6) Flow cytometry analyses. (A1–A3) *Top right quadrants* show YFP⁺ BMCs that express CD45 in naïve bone marrow (A1), naïve cornea (A2), and treated corneas (A3). Treated corneas had a 4.4-fold increase in YFP⁺ cells and all of these cells were CD45⁺, confirming hematopoietic origin. (A4–A6) *Top right quadrants* show YFP⁺ BMCs that co-express Cd11b and GR1 in naïve bone marrow (A4), naïve corneas (A5), and treated corneas (A6). All YFP⁺ cells in the treated corneas are CD45⁺ and have the CD11bGR1 phenotype, similar to bone marrow YFP⁺MDSCs. Thus, YFP⁺ corneal cells are infiltrating YFP⁺ BMCs. (B) Graph showing SI

in an allogenic MLR. SI increased upon addition of stimulator cells to responder cells, but did not change with further addition of sorted YFP⁺ corneal cells. However, the addition of unsorted corneal cells (containing a mixture of YFP⁺ and YFP⁻ cells) to the MLR of responder and stimulator splenocytes caused a significant decrease in the SI. Similarly, the addition of YFP⁺ BMCs significantly suppressed MLR. Results represented are means $\pm$ SEM. Three independent experiments were performed. (C) In vitro compartmental culture of dissociated TG cells showing the effect of sorted YFP⁺ corneal cells compared to sorted YFP⁻ corneal cells on neurite outgrowth. Widefield fluorescent images in the side compartment at day 7 co-cultures of TG cell neurites with YFP⁻ corneal cells (C1) and YFP⁺ corneal cells (C2). (D) Graph shows that neurite outgrowth on day 4 and day 7 were significantly greater when TG neurites were co-cultured with YFP⁺ corneal cells compared to YFP⁻ corneal cells. Scale bar: 250 μ m. * $P \leq 0.05$.

that corneal immune privilege and tolerance may have a dynamic (modifiable) component that is related to the extent of infiltration of myeloid cell with immunosuppressive actions, such as YFP⁺ BMCs. This possibility is consistent with other reports that increasingly recognize that immune privilege is maintained actively as a result of the immunoregulatory characteristics of the tissue resident cells and their microenvironment.⁵⁴

The communication between infiltrating myeloid cells and their neuronal counterparts likely is based on molecules with overlapping actions in the hematopoietic and nervous systems,⁵⁵ such as semaphorin 7a (sema7a). Sema7a promotes axon outgrowth in the nervous system,³ and stimulates monocyte and macrophage functions in the immune system.⁵⁶ Therefore, sema7a signaling may be a general mechanism to influence the neuronal and myeloid cells. Activated human T cells, B cells, and monocytes secrete bioactive neurotrophic factors in vitro that support neuronal survival.⁵⁷ Although these examples raise the possibility that, in the nervous system, inflammatory myeloid cells may have a neuroprotective effect,⁵⁷ the contribution of inflammatory cells and their products to axonal injury and repair is not fully understood.⁵⁸ Studies have demonstrated that conditioned medium from cultures of peripheral blood CD14⁺ monocytes inhibit neurite outgrowth.⁵⁸ Activated T cells (CD4⁺ and CD8⁺) align along axons and soma of cultured human fetal brain neurons, leading to substantial neuronal death.⁵⁹ In contrast, T helper type 1 cells enhance neurite outgrowth from embryonic cortical neurons.⁶⁰ These data suggest that the effect of immune cells on neuronal cells (neuroprotection, neuroregeneration, or neurotoxicity) depends in large part on the cell lineage (monocytes, MDSCs, or CD4/CD8⁺ T cells).

We found that YFP⁺ BMCs enhance neurite outgrowth from TG cells. Bone marrow cells depleted of YFP⁺ BMCs (YFP⁻ BMCs) enhanced neurite growth at day 7, albeit to a significantly lesser extent compared to YFP⁺ BMCs. It is possible that the YFP⁻ BMC fraction has some cells that have neuroregenerative actions. These data are consistent with reports that suggest that the overall effect of pooled heterogeneous blood cells on neurite outgrowth depends on distinct functions of immune cell subsets.⁶¹ Pool et al. showed that total peripheral blood mononuclear cells had a significant inhibitory effect on neurite outgrowth, whereas isolated immune cell subsets either enhanced neurite outgrowth (CD4⁺ T cells) or inhibited it (NK cells and CD8⁺ T cells).⁶¹

We have developed surgical methods to transect corneal nerves and denervate the central cornea to investigate the phenomenologic events of reinnervation. We previously reported that nerve regeneration occurs at the flap-stromal interface after lamellar corneal flap surgery.^{14,17} In this report, we have performed excimer laser annular keratectomy to transect corneal nerves; therefore, there is no potential space in the flap interface for nerves to regenerate. Our findings confirmed that regenerating nerves possess the ability to invade denervated stroma and reinnervate it. The regenerating nerves and infiltrating YFP⁺ BMCs are present in

approximately the same plane in the stroma, providing circumstantial evidence of interaction. Our in vitro data clearly demonstrated an interaction between TG neurites and YFP⁺ BMCs, as the neurites make physical contact with the YFP⁺ BMCs. It is possible that the physical contact represents a neuro-immune synapse and there is crosstalk between the two systems. Therefore, our methodology provided a tool for investigating and advancing the emerging neuro-immune concepts.⁶²

In conclusion, to our knowledge this is the first report to provide evidence that CD11b+GR1+ YFP⁺ BMCs secrete NGF, and that their neuroregenerative and immunosuppressive actions are retained within the corneal milieu. Our findings will contribute to emerging neuro-immune concepts, and expand our understanding of the interactions between the myeloid and nervous system that may lead to therapeutic strategies that use the immune system cells to repair diseased or injured neurons.

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