Title: Modulation of microglia activation after human donor retinal ganglion cell transplantation

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Running Head: Modulation of microglia after RGC transplantation

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Abstract

Stem cell-derived retinal ganglion cell (RGC) transplantation therapy offers a promising avenue for restoring vision in patients with significant RGC loss. The major challenge in this therapeutic approach is ensuring the survival of transplanted human donor RGCs within the host retina. Previously, we demonstrated the pivotal role of host retinal microglia and macrophages in the acceptance and survival of human donor cells. This current study aimed to improve the survival and integration of human donor RGCs through modulation of the host retinal microglia. Pretreatment of human donor RGCs with annexin V and soluble FasL (sFasL) before transplantation significantly enhanced their acceptance and survival rate. Detailed analyses of the host mouse retina post-transplantation revealed morphological changes and activation patterns in microglia and macrophages. These findings indicate that sFasL and annexin V activate pro-survival pathways and foster a supportive environment for the acceptance and survival of transplanted human donor RGCs.

Introduction

The degeneration of retinal ganglion cells (RGCs) is a key factor in various eye disorders, including different forms of glaucoma, hereditary optic neuropathies, ischemic optic neuropathies, and demyelinating diseases, all of which result in progressive axonal degeneration and death of RGCs, ultimately leading to irreversible blindness[1,2,3,4,5]. Despite significant advances, the clinical needs for these retinal neurodegenerative conditions remain largely unmet. Consequently, a variety of therapeutic strategies are being actively explored, including stem cell-derived therapy, pharmacologic neuroprotection, and gene therapy. Among these, stem cell-derived RGC transplantation therapy holds considerable promise for restoring vision in patients with RGC loss and axonal degeneration[6,7]. However, one of the primary challenges with this therapeutic approach is ensuring the survival of the transplanted human RGCs within the host retina, due to the active surveillance and subsequent elimination by the host microglia[8].

In the healthy and normally developed retina, microglia reside in the outer plexiform layer (OPL) and inner plexiform layer (IPL), where they perform essential functions in immune defense, homeostasis, and development[9]. When exposed to damaging stimuli, microglia become activated and migrate to the sites of retinal injury[10].

We hypothesize that during cell isolation and transplantation, human donor RGCs undergo significant stress, which leads to the externalization of pro-apoptotic signals on their cell surface. These signals attract tissue microglia and macrophages to the site of injury and initiate clearance of the transplanted cells. By blocking these signals and modulating the host retinal microenvironment, we aim to prevent microglia and

macrophages from recognizing and eliminating the transplanted cells, thereby enhancing the survival of human donor RGCs post-transplantation.

Annexin V emerges as a potential candidate for modulating the retinal microenvironment and preventing microglia and macrophages from recognizing and removing human donor RGCs after transplantation. Annexin V is a calcium-dependent, membrane-bound protein with high affinity for phosphatidylserine (PS), an endogenous phospholipid and a component of the plasma membrane that becomes exposed on the cell surface during cellular stress and early apoptosis[11]. Given the distressing nature of isolation and transplantation, human donor RGCs likely externalize PS under stress. Therefore, we hypothesized that pretreating donor RGCs with annexin V to mask PS would prevent recognition and clearance by microglia and macrophages following transplantation.

Fas ligand (FasL), or CD95 ligand, is another possible candidate for modulating the retinal microenvironment and improving survival of transplanted human donor RGCs. FasL is a 40kDa type II transmembrane protein of the tumor necrosis factor (TNF) family that is capable of inducing apoptosis upon binding to the Fas receptor[12]. Within the eye, FasL can be expressed as a membrane-bound protein (mFasL), which is pro-inflammatory and pro-apoptotic molecule, or cleaved and released as a soluble protein (sFasL), which is non-inflammatory and non-apoptotic[13,14]. Previous studies demonstrated that FasL deficiency or intravitreal administration of sFasL protects RGCs from cell death in a mouse model of glaucoma[15]. Furthermore, sFasL has demonstrated therapeutic benefits in reducing inflammation in experimental models of keratitis[16] and autoimmune disease[17]. Moreover, there is evidence that post injury treatment with

intravitreal AAV2-soluble Fas ligand restores retinal microglia homeostasis and rescues RGC function in a mouse model of glaucoma[18]. We hypothesize that Fas-FasL interaction may protect human donor RGCs from immune attack by host microglia and macrophages, thereby improving graft survival in the host retina.

Taken together, this study demonstrates that pretreating human donor RGCs with annexin V and sFasL before transplantation modulates the host microenvironment and improves graft survival in the host mouse retina. Additionally, our results provide the first quantitative analysis of microglia and macrophages morphology in the mouse retina following human donor RGC transplantation.

Materials and methods

Animals

All procedures conformed to the National Research Council's Guide for the Care and Use of Laboratory Animals, the Association for Research in Vision and Ophthalmology (ARVO) guidelines and were approved by the Institutional Animal Care and Use Committee (IACUC) of the Schepens Eye Research Institute (SERI). All animals were purchased from Jackson Laboratory (Bar Harbor, ME). The breeding of animals was established in the Rodent Barrier Facility at the SERI. At the beginning of the experiment equal numbers of male and female *Brn3b*$^{-/-}$ and *Cx3cr1*$^{-/-}$ mice were group-housed in a temperature-controlled environment with 12-hour light/dark cycle and received a standard diet and water ad libitum.

Organoid dissociation and RGC isolation

RGCs were differentiated from H9:BRN3B:tdTomatoThy1.2-hESCs in retinal organoid 3D culture, as was previously described[19,20]. Mature human retinal organoids were dissociated, and RGCs were isolated using magnetic microbead sorting for Thy1.2+ cells. Following cell isolation, RGCs were formulated in RGC media at 1 – 5 x 10^5 cells/mL. Then, human donor RGCs were pretreated with 5 mg/mL annexin V (BioLegend, San Diego, CA), a calcium-dependent membrane-bound protein with high affinity to PS, and 0.2 mg/mL sFasL (R&D Systems, Minneapolis, MN), a non-apoptotic, non-inflammatory protein, for at least 20 minutes on ice. Following human donor RGC pretreatment, the cells were formulated for transplantation at 2 x 10^4 cell/µL in RGC media.

In vivo human donor RGC transplantation

The *Brn3b*$^{-/-}$ mice were anesthetized intraperitoneally with 100 mg/kg ketamine hydrochloride and 20 mg/kg xylazine hydrochloride solution diluted with saline. Pupils were dilated with 1% tropicamide ophthalmic solution (Bausch + Lomb, Bridgewater, NJ). Following tropicamide application, one drop of 0.5% proparacaine hydrochloride ophthalmic solution (Bausch + Lomb, Bridgewater, NJ) was applied onto the cornea for 5 minutes before transplantation. Up to 2 x 10^4 donor cells per 1 µL were injected into one eye of *Brn3b*$^{-/-}$ mice using a beveled glass microneedle with 100 µm inner diameter at a 1 µL/min flow rate. Each mouse received a single subretinal injection of 1.0 µL of human donor RGCs pretreated with annexin V and sFasL during transplantation. Control group received a single subretinal injection of 1.0 µL of human donor RGCs with no annexin V or sFasL pretreatment. Following human donor RGC transplantation, one

drop of GenTeal® Tears Lubricant eye gel (Alcon Laboratories, Fort Worth, TX) was applied onto the cornea to protect the eye from dryness. Mice were returned to the housing room and maintained on a standard 12-hour light/dark cycle for 3 days. On day 3 mice were euthanized, and eyes were immediately collected and fixed in 4 % paraformaldehyde (PFA) for 12 hours before immunohistochemistry analysis.

Immunohistochemistry of whole retina mounts

After fixation with 4% PFA was complete, retina samples were incubated in blocking buffer [10 % goat serum, 1 % bovine serum albumin (BSA), 0.1 % sodium citrate, 0.1 % Tween 20, and 0.1 % Triton-X in 1X phosphate-buffered saline (PBS)] containing goat anti-mouse antibodies in dilution 1:400 at 4°C for at least 12 hours overnight to limit nonspecific staining. Following blocking, retina flat mounts were incubated with Iba-1 primary antibodies (ab178846, Abcam, Boston, MA) in staining buffer (1 % BSA, 0.25 % Tween 20 and 0.25 % Triton-X in 1X PBS) for 72 hours at 4°C. Then, each retina sample was washed thrice for 15 minutes with washing buffer (0.1 % Tween-20, 0.1 % Triton-X in 1X PBS) at room temperature to remove any unbound primary antibodies before adding secondary antibodies. Secondary antibodies were prepared in dilution 1:500 in staining buffer, and the retina samples were incubated with secondary antibodies for 24 hours at 4°C. Then, each sample was washed with washing buffer thrice for 15 minutes at room temperature and then incubated with 1 µg/mL DAPI (4',6-diamidino-2-phenylindole) in 1X PBS for 20 minutes at room temperature to stain the cell nuclei. Following final rinse with 1X PBS, retina samples were mounted and stored in the dark at room temperature before imaging. Images of retina mounts were acquired

using confocal microscope (Olympus, Tokyo, Japan), and the Iba1-positive microglia and macrophages were imaged within the OPL and IPL.

Quantification and analysis of morphology of retinal microglia and macrophages after human donor RGC transplantation

The image of each retina flat mount was divided into four quadrants with one mid-peripheral region being a representative image of each quadrant; therefore, a total of four images per retina was used for quantitative analysis of retinal microglia and macrophages. The analysis was applied to quantify retinal microglia and macrophages and assess morphology of retinal microglia and macrophages according to metrics such as cell shape, ramification, and complexity. Morphometric analysis of total number of Iba1-positive retinal microglia and macrophages within the OPL and IPL was performed on skeletonized images of Iba1-positive cells from the retinal flat mounts using previously published protocols[21]. Briefly, eight Z-stack images (two images per retinal quadrant) were captured at 20X magnification from one retina per animal and processed using ImageJ software (provided in the public domain). The images were binarized using the “adjust threshold” function and skeletonized using the “skeletonize” function. The plugin “Analyze Skeleton” was then employed to generate the number of branches, number of junctions, and average branch length of the microglia and macrophages in the retina flat mounts. All morphometric parameter values were divided by the total number of cells per unit area of retinal tissue in mm^2.

Results

Donor cell survival is improved by blocking phosphatidylserine residues on human donor RGCs with annexin V

We observed that pretreatment of human donor RGCs with annexin V prior to transplantation improves human donor RGC survival rate and overall transplant success (**Figure 1**). We also observed in the successful grafts, that the axons of human donor RGCs enter the optic nerve head (**Figure 1A**). Specifically, in the pretreated group, eleven out of twelve recipients of human donor RGCs (92%) had approximately 200 donor cells within the retina at day 4, representing more than 1% of all transplanted human donor RGCs in one eye. In contrast, in the control group, only six out of eleven recipients (55%) showed survival of human donor RGCs after transplantation (**Figure 1B**). Furthermore, in the annexin V pretreated group, four out of twelve transplants exhibited neurite outgrowth into the optic nerve head, compared to only one out of eleven transplants in the control group (**Figure 1C**).

Host microglia are activated after transplantation of human donor RGCs in Cx3cr1-GFP mice

To study microglia activation *in vivo*, we imaged the retina repeatedly by scanning laser ophthalmoscopy (SLO)[22] of *Cx3cr1-GFP* heterozygous mice (**Figure 2**). On day 1 post human donor RGC transplantation, host retinal microglia and macrophages become activated and initiate displacement toward injection site to remove the transplanted RGCs (**Figure 2A, 2B**). By days 2 and 3, microglia and macrophages began eliminating human donor RGCs, occupying the area of damage, and by day 7, all human donor RGCs were removed and processed by these activated cells (**Figure 2A, 2B**).

Retinal microglia and macrophages rapidly respond to physiological changes and demonstrate distinct morphology based on their state of reactivity. As shown in **Figure 2C,** activated microglia and macrophages retract their processes and round their cell bodies, whereas in their homeostatic state, they exhibit small cell bodies with ramified processes.

Human donor RGCs are eliminated by primary microglia in vitro

To determine if donor RGCs are eliminated by microglia *in vitro*, we co-cultured primary microglia with human donor RGCs (**Figure 3A**). We observed that primary microglia become activated, move towards human donor RGCs and began eliminating them by day 1 (**Figure 3B**). This finding is consistent with our *in vivo* observation, demonstrating that human donor RGCs are recognized and eliminated by microglia in the host microenvironment. Therefore, modulating this microenvironment might help suppress microglia activity and improve the survival of human donor RGCs.

Host microglia is modulated by exogenous pretreatment of human donor RGCs with annexin V and sFasL before transplantation

To investigate whether the host retinal microenvironment, where microglia and macrophages play an essential role in active surveillance can be modulated, we pretreated human donor RGCs before transplantation. Following dissociation and isolation of human donor RGCs, we pretreated the cells with 5 mg/mL annexin V and 0.2 mg/mL sFasL for at least 20 minutes on ice. Following transplantation, our results

indicate improved survival and axonal elongation in human donor RGCs pretreated with annexin V and sFasL as compared to non-pretreated cells (**Figure 4A, 4B**).

Microglia and macrophages are resident cells in the retina and are localized to the OPL and IPL in the retina. To explore the effect of pretreating human donor RGCs with annexin V and sFasL on microglia and macrophages, we analyzed Iba1-positive cells within OPL and IPL after human donor RGC transplantation. At 3 days post transplantation of human donor RGCs pretreated with Annexin V and sFasL, microglia and macrophages displayed homeostatic morphology with prominent and longer processes as compared to the control group with no pretreatment, where microglia and macrophages displayed a more reactive phenotype with rounded cell bodies and retracted processes (**Figures 4C-F**). Interestingly, we also found that microglia and macrophages become more activated in the OPL as compared to the IPL after subretinal delivery of human donor RGCs (**Figures 4C-F**). Moreover, the total number of microglia and macrophages was reduced in the pretreatment group as compared to the control group with no pretreatment (**Figures 4G, 4J**), again with more significant changes observed in IPL. Additionally, the number of branches per microglia and macrophage was significantly ($P<0.01$) increased in the pretreatment group, indicating less activation when compared to the control group (no pre-treatment) (**Figures 4H, 4K**).

Moreover, microglia and macrophages in the OPL exhibit a significant difference in branch number per cell between pretreated and control groups (**Figure 4H**), while those in the IPL showed lower branch numbers per cell (**Figure 4K**). Similar trend is observed in the branch length per microglia and macrophage cell with longer and more ramified

branches in OPL that in IPL, suggesting that microglia and macrophages are less activated in the OPL than IPL after human donor RGC transplantation (**Figures 4I, 4L**). In summary, pretreating human donor RGCs with annexin V and sFasL leads to morphologic changes in microglia and macrophages within the OPL and IPL of the mouse retina.

Discussion

Optic neuropathies and retinal neurodegenerative diseases, such as glaucoma, diabetic retinopathy, and age-related macular degeneration, result in progressive damage of retinal ganglion cells (RGCs)[23]. If left untreated, these diseases lead to irreversible blindness. For the patients with optic neuropathies and neurodegenerative diseases of the retina, cell replacement therapy[24,25,26] and treatment with neuroprotective agents are promising strategies to halt disease progression and preserve vision[27,28]. One of the key steps for improving cell transplantation success in the retina is to define the factors that positively affect survival of donor RGCs and to modulate the host microenvironment by enhancing the beneficial effect of these factors on donor cells.

The retina is populated by specialized immune resident macrophages called microglia. Normally, retinal microglia reside within the outer plexiform layer (OPL), the inner plexiform layer (IPL), and the retinal ganglion cell (RGC) layer. Microglia represent one of the main barriers the donor cells need to overcome for successful survival and integration within the host microenvironment, as microglia are responsible for elimination of antigens that threaten the retinal integrity[29].

Prior studies demonstrated that phosphatidylserine (PS), a phospholipid that is localized to the inner part of the cell membrane, is crucial for identification of apoptotic cells by macrophages[30,31]. Considering donor RGCs experience a substantial stress during dissociation, isolation, and transplantation events, it is predicted they might generate pro-apoptotic signals and externalize a PS signal on the cell surface, allowing them to be recognized and cleared by retinal microglia and macrophages[32]. Annexin V, a calcium-dependent membrane-binding protein, efficiently and specifically attaches to PS, thus, representing a selective probe for recognition and quantification of apoptotic cells[33,34,35]. By pretreating donor RGCs with annexin V just after isolation and right before transplantation events, a PS signal on the cell surface of donor RGCs can be blocked, and hence preventing donor RGCs from being recognized and attacked by retinal microglia and macrophages.

In addition to blocking the PS signal with annexin V, we previously demonstrated that treatment with sFasL is also neuroprotective and prevents death of RGCs in chronic and inducible mouse models of glaucoma[15,36]. Herein, we determined the combination of annexin V and sFasL significantly improves survival of human donor RGCs in the host mouse retina. FasL is a type II transmembrane protein that exists as both a membrane bound protein (mFasL) that is pro-inflammatory and pro-apoptotic, and a soluble form (sFasL) that is cleaved from the cell surface and released as a soluble fragment that is non-inflammatory and non-apoptotic and can antagonize the activity of mFasL[14,15,37]. In the healthy eye, the anti-apoptotic and anti-inflammatory sFasL is the dominant isoform expressed[36], but with disease there is a shift and the pro-inflammatory

and pro-apoptotic mFasL is the dominant isoform expressed in a variety of ocular pathologies[36,38,39,40].

In inducible and chronic models of glaucoma it has been shown that mFasL mediates destruction of RGCs either by directly killing RGCs and/or by inducing the production of pro-inflammatory chemokines or other mediators in Fas+ glial cells and thereby causing neurotoxic inflammation[15,36,41]. By contrast, intravitreal AAV2-mediated gene delivery of sFasL provided complete and long-term protection of RGCs and their axons[15,36]. Importantly, the sFasL-mediated neuroprotection of RGCs also correlates with reduced microglia activation and reduced production of pro-inflammatory mediators. Therefore, sFasL could be improving survival of donor RGCs by blocking FasL-mediated apoptosis of RGCs and/or by blocking FasL-mediated activation of glia and the induction of neurotoxic inflammation.

Normally, in the healthy eye, retinal microglia display a highly ramified morphology with a small cell body and multiple branching processes. When responding to inflammation or injury, microglia retract their processes and present round cellular morphology. Although microglia are the most abundant immune cells in the retina, the retina also contains a population of perivascular macrophages that are located within the glia limitans of retinal vasculature[42]. The glia limitans acts as a physical barrier against unwanted cells that attempt to enter the retina. During the subretinal delivery of donor RGCs, the microinjury to small retinal blood vessels may occur causing the leakage of perivascular macrophages to the site of injection. Both microglia and macrophages express the ionized calcium-binding adaptor (Iba1) molecule, a 17-kDa actin-binding cross-linking protein that is upregulated during activation of these cells[43].

Here, we investigated the morphology of Iba1+ microglia and macrophages following pretreatment of human donor RGCs with annexin V and sFasL before transplantation. We found that transplantation of human donor RGCs leads to activation of host retinal microglia and macrophages causing them to move towards the injection site to eliminate human donor RGCs. Pretreatment with annexin V and sFasL resulted in reduced activation of microglia and macrophages, with Iba1+ microglia and macrophages displaying a more ramified morphology with longer processes (**Figures 4E, 4F**). In contrast, the Iba1+ microglia and macrophages in the control group exhibited a round ameboid morphology with retracted processes (**Figures 4C, 4D**).

Our results demonstrate that modulating the host retinal microenvironment through the microglia manipulation can improve the survival of human donor RGCs after transplantation.

Statistical analysis

All graphs were generated by GraphPad PRISM 9 (GraphPad software, San Diego, CA). Statistical significance was determined using an unpaired Welch's *t*-test. All data are presented as average ± standard deviation (SD). Where normally distributed, the data were analyzed with a *P* value of <0.05 (*****$p < 0.05$, ******$p < 0.01$, *******$p < 0.001$, ********$p < 0.0001$) as a statistically significant.

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Author contributions

MHP, MGK and PB initiated the project and designed experiments. VVM, MHP, JA performed confocal microscopy, immunohistochemistry experiments. JDR differentiated human donor RGCs. JA analyzed the images of microglia cells. CA performed scanning laser ophthalmoscopy. VVM and PB performed cell transplantation, wrote the manuscript draft. PB supervised the project. All authors reviewed and contributed to editing the manuscript.

Declaration of interests

The authors declare no competing interests.

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Figure legends:

Figure 1. Donor cell survival is improved by blocking phosphatidylcholine (PS) residues on human donor retinal ganglion cells (hRGCs) with annexin V. (**A**) Donor hRGCs survive and send their projections to the optic nerve head (ONH). (**B**) Donor hRGCs survive better after pretreatment with annexin V. (**C**) Donor hRGCs enter ONH more successfully after pretreatment with annexin V.

Figure 2. Studying microglia activation *in vivo* in the retina of *Cx3cr1-GFP* mice after donor human retinal ganglion cells (hRGCs) transplantation. (**A**) Imaging *in vivo* by scanning laser ophthalmoscopy (SLO). Image on day 1 represents injected donor tdTomato-positive hRGCs (red) and host microglia cells (green) after transplantation. Note that by day 7 all injected donor tdTomato hRGCs are eliminated by activated host microglia cells. (**B**) Interaction between host microglia and donor hRGCs on a single cell level. (**C**) Morphological characteristics of a microglia cell.

Figure 3. Elimination of human retinal ganglion cells (hRGCs) by primary microglia *in vitro* on day 0 (**A**) and day 1 (**B**).

Figure 4. Pretreatment of donor hRGCs with annexin V and sFasL inhibits microglia activation and increases survival of hRGCs after transplantation. (**A, B**) Donor hRGCs survive better after pretreatment with annexin V and sFasL. Activation of Iba1-positive microglia and macrophages in the OPL (**C, E**) and IPL (**D, F**) of the retina in the control group and group pretreated with annexin V and sFasL before donor RGC

transplantation. Microglia density (**G, J**), number of branches per microglia and macrophage cell (**H, K**), branch length per microglia and macrophage cell (**I, L**) in the OPL (**G, H, I**) and IPL (**J, K, L**) of the retina in the control group and group pretreated with annexin V and sFasL. hRGCs, human retinal ganglion cells; IPL, inner plexiform layer; OPL, outer plexiform layer; sFasL, soluble Fas ligand.

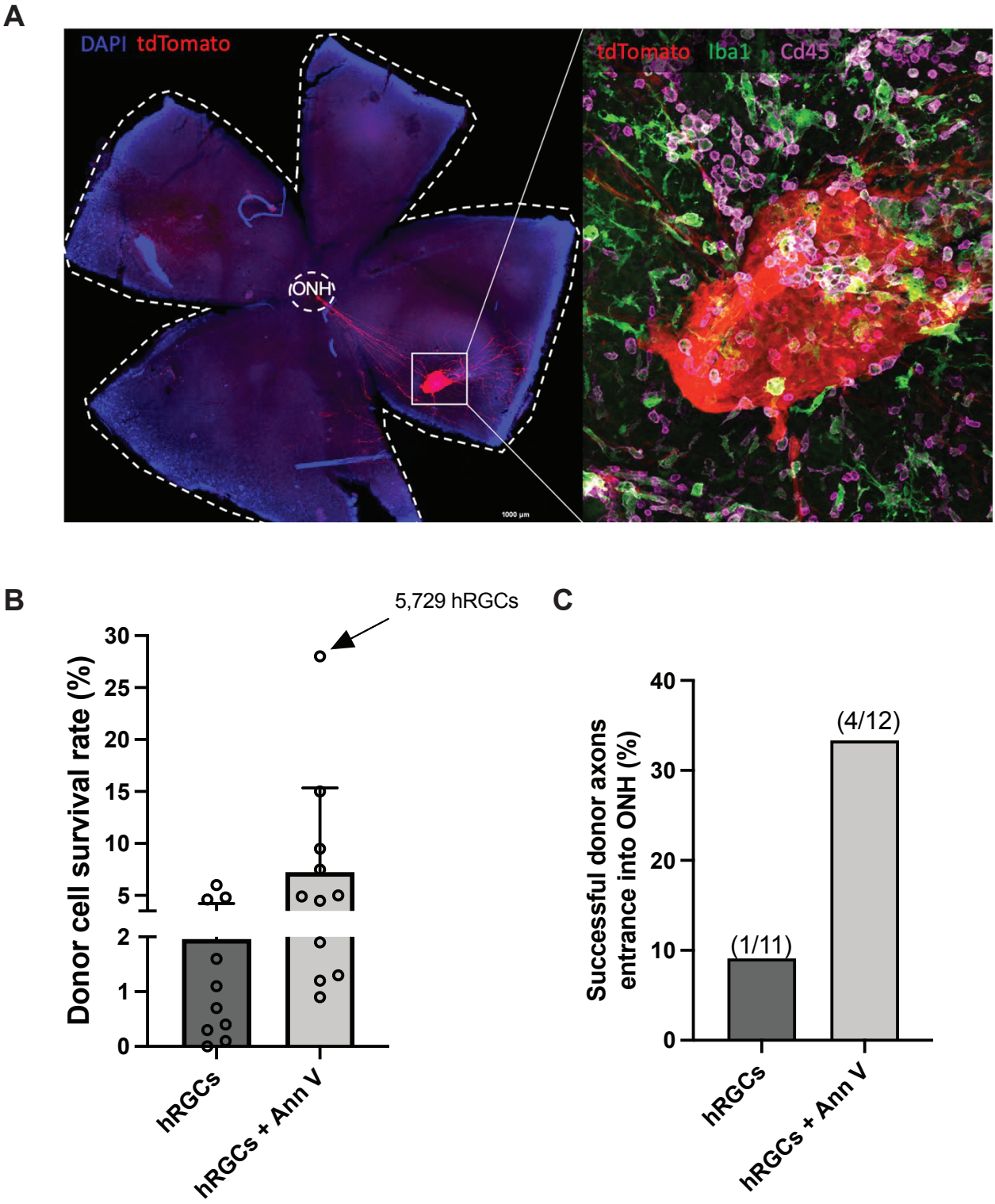


Figure 1. Malechka VV et al. 2024

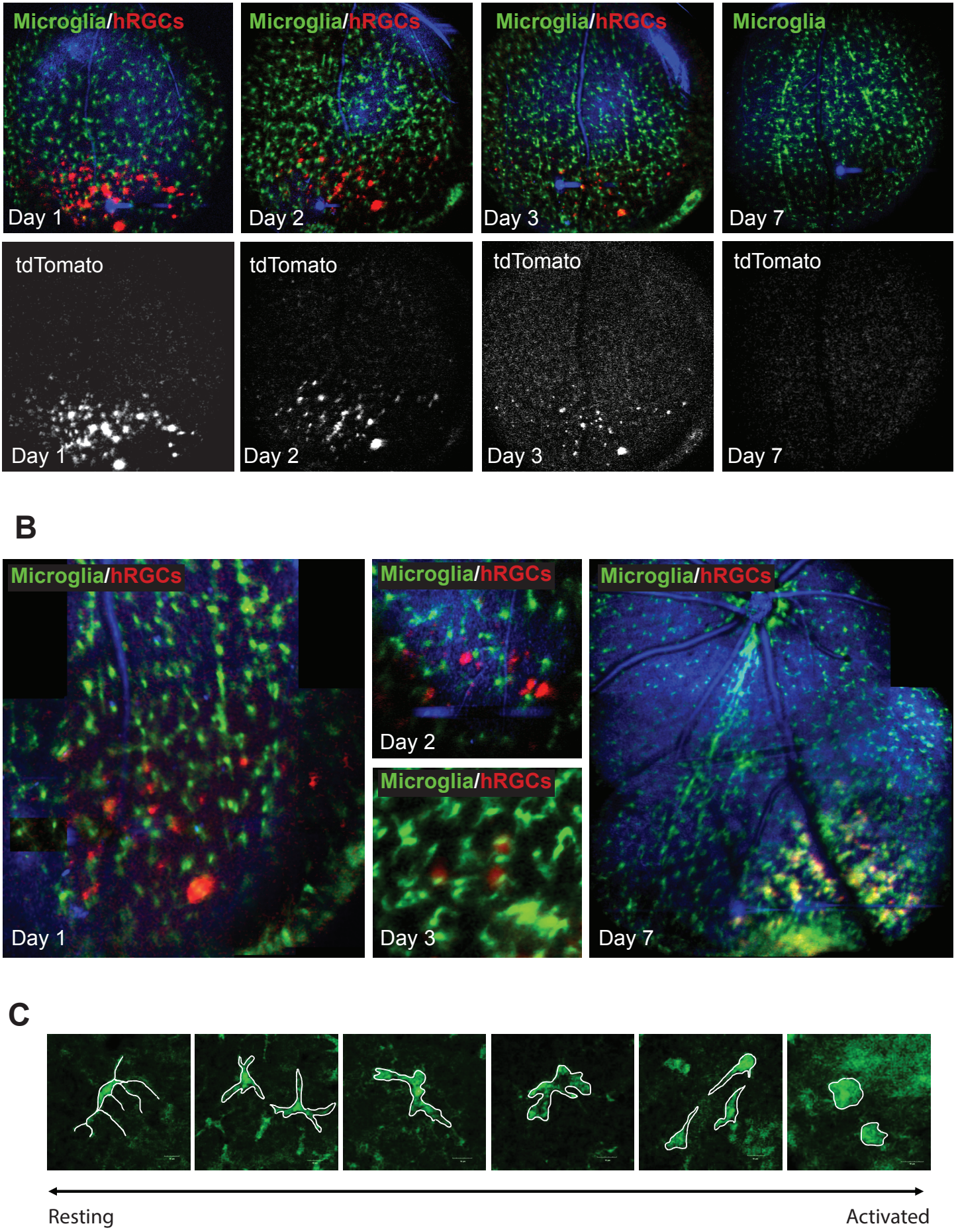
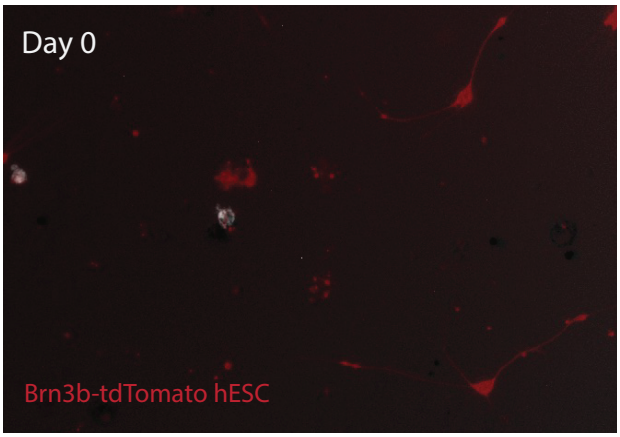


Figure 2. Malechka VV et al. 2024

A



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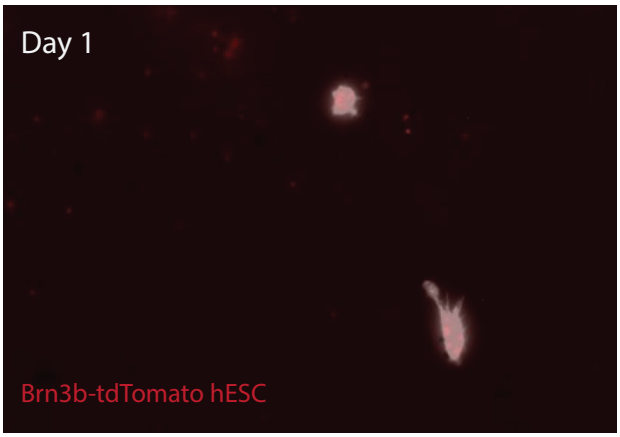

Figure 3. Malechka VV et al. 2024

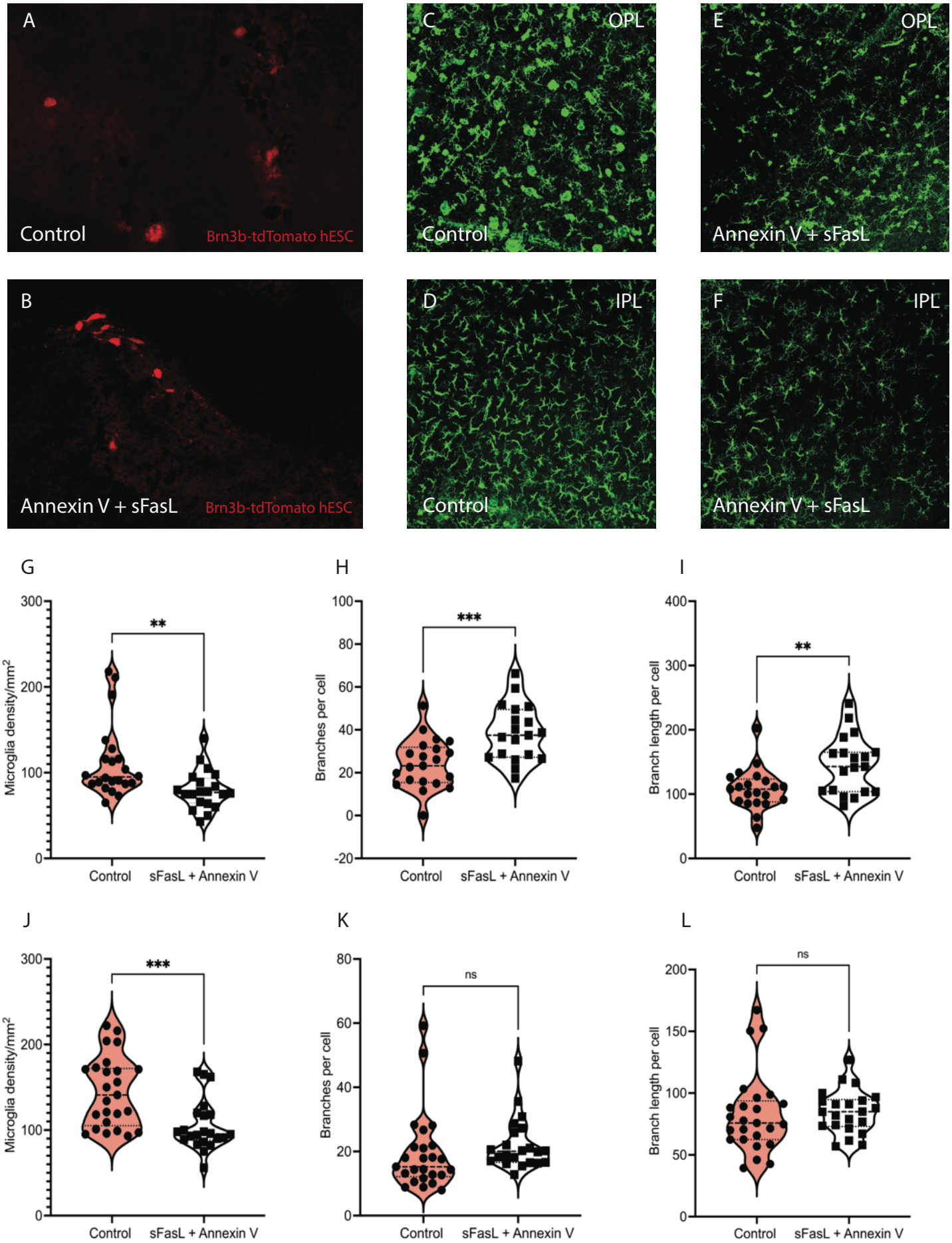

Figure 4. Malechka VV et al. 2024