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Neuron-Interacting Satellite Glial Cells in Human Trigeminal Ganglia Have an APC Phenotype

Monique van Velzen,* Jon D. Laman,†‡ Alex KleinJan,§ Angelique Poot,* Albert D. M. E. Osterhaus,* and Georges M. G. M. Verjans2*

Satellite glial cells (SGC) in sensory ganglia tightly envelop the neuronal cell body to form discrete anatomical units. This type of glial cell is considered neuroectoderm-derived and provides physical support to neuron somata. There are scattered hints in the literature suggesting that SGC have an immune-related function within sensory ganglia. In this study, we addressed the hypothesis that SGC are tissue-resident APC. The immune phenotype and function of a large series (n = 40) of human trigeminal ganglia (TG) were assessed by detailed flow cytometry, in situ analyses, and functional in vitro assays. Human TG-resident SGC (TG-SGC) uniformly expressed the common leukocyte marker CD45, albeit at lower levels compared with infiltrating T cells, and the macrophage markers CD14, CD68, and CD11b. In addition, TG-SGC expressed the myeloid dendritic cell (DC) marker CD11c, the T cell costimulatory molecules CD40, CD54, CD80, and CD86 and MHC class II. However, the mature DC marker CD83 was absent on TG-SGC. Functionally, TG-SGC phagocytosed fluorescent bacteria, but were unable to induce an allogeneic MLR. Finally, TG-infiltrating T cells expressed the T cell inhibitory molecules CD94/NKG2A and PD-1, and the interacting TG-SGC expressed the cognate ligands HLA-E and PD-L1, respectively. In conclusion, the data demonstrate that human TG-SGC have a unique leukocyte phenotype, with features of both macrophages and immature myeloid DC, indicating that they have a role as TG-resident APC with potential T cell modulatory properties. The Journal of Immunology, 2009, 183: 2456–2461.

Sensory ganglia are part of the peripheral nervous system. They contain cell bodies of sensory neurons establishing the connection between the periphery and CNS. Sensory ganglia lack a blood-nerve barrier and enclose a high number of satellite glial cells (SGC) (1–3). SGC are considered to be neuroectoderm-derived and involved in the maintenance of sensory neuron homeostasis by regulating extracellular ion and nutrient levels within sensory ganglia (2). In contrast to CNS-resident glial cells, like astrocytes and microglia, SGC have a distinct interaction with neurons (2, 3). They directly associate with the neuronal soma, so that each neuronal cell body is completely surrounded by a sheet of several SGC providing physical support and a protective barrier (3). The numerous fine invaginations between the neuron and SGC sheath illustrate their intimate association (2, 3). Upon mechanical injury to sensory neurons, SGC undergo morphological changes, proliferate, and up-regulate a variety of growth factors, cytokines, and the glial marker glial fibrillary acidic protein (2, 4, 5).

Human α-herpesviruses, like HSV, are a common threat to human sensory ganglia. HSV establishes a lifelong latent infection in neurons within sensory ganglia, predominantly the trigeminal ganglion (TG), and reactivates intermittently (6). Recent studies in mice and humans emphasized the importance of infiltrating T cells to control latent HSV infections in sensory ganglia (7–9). Virus-specific T cells are directly juxtaposed to latently infected neurons, produce cytokines and cytolytic effector molecules, but do not induce neuronal damage (7, 8, 10–12). Current data suggest that the neurons themselves or hitherto unrecognized resident cells in latently infected sensory ganglia induce and coordinate this nonpathogenic chronic T cell response (8, 10–12).

In this study, we addressed the hypothesis that SGC are tissue-resident APC. The availability of a series of fresh postmortem human TG specimens enabled us to combine ex vivo and in situ analyses for the phenotypic and functional characterization of human TG-resident SGC (TG-SGC).

Materials and Methods

Clinical specimens

Heparinized peripheral blood and TG specimens, i.e., left and right TG, were obtained from 40 subjects (median age 79 years, range 41–94 years) at autopsy with a mean postmortem interval of 6 h (range 2.5–15.5 h). The TG tissue panel consisted of 34 donors with a CNS disease (mainly Alzheimer’s disease and Parkinson’s disease) and six donors without evidence of CNS disease. The cause of death was not related to α-herpesvirus infections. No significant differences in the immunological parameters analyzed were detected between donors with or without a history of CNS disease (data not shown). Specimens were either snap-frozen (n = 23) or transferred to tubes (n = 17) containing culture medium consisting of RPMI 1640 (Lonza) supplemented with heat-inactivated 10% FBS (Greiner) and antibiotics. Written informed consent from the donor or next of kin was obtained. The local ethical committees approved the study, which was conducted according to the tenets of the Declaration of Helsinki.

Generation of TG single cell suspensions

Generation of single cell suspensions from human TG was performed essentially as previously described (12). In brief, the TG were fragmented and subsequently treated with Liberase Blendzyme 3 (0.2 U/ml, Roche) at 37°C for 1 h. Dispersed cells were filtered through a 70-μm pore size cell
strainer (BD Biosciences), and the flow-through was collected in PBS containing 1% FBS. From the same donor, PBMC were isolated from heparinized peripheral blood (~4 ml per donor) by density gradient centrifugation on Ficoll-Hypaque (12). Donor PBMC and TG single cell suspensions were directly used for phenotypic and functional analyses.

Flow cytometry

Donor-matched PBMC and TG cells were subjected to multicolor flow cytometric analysis using the following fluorescent-conjugated mAbs: CD3-allophycocyanin (UCHT1; DakoCytomation), CD14-PE (B-PE; Beckman Coulter), CD11c-PE (S-HCL3; BD Biosciences), CD14-FTC (TUK4; DakoCytomation), CD45-FTC(S53; BD Biosciences), CD45-PerCP (2D1; BD Biosciences), CD54-FTC (6.5B5; DakoCytomation), CD68-PE (Y1/82A; BD Biosciences), CD80-FTC (MAB104; Beckman Coulter), CD83-allophycocyanin (BH15e; BD Biosciences), CD86-PE (FUN-1; BD Biosciences), HLA-DR-PerCP (L243; BD Biosciences), CD94-FTC (DX22; ebioscience), NKGA2-allophycocyanin (131411; ebioscience), programmed death (PD)-1-PE (MIH4; ebioscience), and PD ligand 1 (PD-L1)-PE (MIH1; ebioscience). Cells were labeled according to the manufacturers’ instructions and appropriate isotype- and fluorochrome-matched unrelated mAbs were included as negative controls. Cells and data were analyzed on a BD FACS Calibur flow cytometer and BD CellQuest Pro software (BD Biosciences).

In situ analyses

Snap-frozen TG were embedded in Tissue Tek OCT compound (Sakura) and cut into 6-μm sections on a Leica CM 3050 cryostat. Sections were fixed in acetone for 10 min and incubated with the following unconjugated mAbs according to the manufacturer’s instructions: CD11b (ICRF44; BD Biosciences), CD11c (B-ly6; BD Biosciences), CD14 (TUK4; DakoCytomation), CD16 (3G8; BD Biosciences), CD45 (5D12; Panogenetics), CD45 (2B11 + PD7/26; DakoCytomation), CD54 (LB-2; BD Biosciences), CD64 (32.2; DakoCytomation), CD80 (KPI; DakoCytomation), CD80 (M24; Inogenetics), CD83 (Hb15a; Beckman Coulter), CD86 (1G10; Panogenetics), CD94 (HP-3B1; Immunotech), HLA-E (4D12) by gift from D. E. Geraghty (Fred Hutchinson Cancer Research Center, Seattle, WA), PD-1 (MIH4; ebioscience), and PD-L1 (MIH1; ebioscience). Primary mAb were visualized using the avidin-biotin system (DakoCytomation) and AEC (3-aminon-9-ethylcarbazole; Sigma-Aldrich) as substrate, and sections were counterstained with hematoxylin (Sigma-Aldrich), examined under a Zeiss Axioskop, and photgraphed using a Nikon DC-U1 camera. For each donor and each marker, three sections and three fields per section were analyzed. Human tonsil sections were used as positive control tissue, and appropriate isotype and conjugate-negative control stainings were included.

For double stainings, sections were fixed in acetone, and endogenous peroxidase activity and endogenous biotin were blocked before incubation with the primary antibodies. For CD14 (TUK4) primary stainings, a CD14 FITC (TUK4; DakoCyto) and a CD54 PE (MAB104; Beckman Coulter) gating antibody were used. As first mAb was detected using an avidin-biotin-HRP system (Biogenex). Before substrate incubation, sections were incubated with normal mouse serum (10%) and a CD11c PE mAb (B-ly6), which was visualized using an anti-PE secondary Ab (AbD Serotec) and an alkaline phosphatase-conjugated tertiary Ab (Sigma-Aldrich). Slides were first developed with Fast blue substrate, followed by incubation with AEC substrate solution. Intracellular staining on TG-SGC, which was occasionally associated with T cells, was performed on all TG-SGC. Whereas all TG-SGC of the TG donors expressed both CD80 and CD86 (Fig. 1H and Table I), the mature DC marker CD83 could not be detected (Fig. 1H and Table I), but not at the cell surface of TG-SGC (data not shown). Additionally, TG-SGC selectively expressed Ag uptake receptors like CD11b and CD11c (Fig. 1D and E, Table I), as well as CD16 and CD64 (data not shown). In situ double stainings confirmed the flow cytometry data and demonstrated coexpression of CD14, CD45 and CD11c on TG-SGC (Fig. 2).

Enrichment of peripheral blood- and TG-derived cell populations

Monocytes and TG-SGC were isolated using anti-CD14 microbeads and a MACS magnetic separator (Miltenyi Biotec) according to the manufacturer’s instructions. T cells were isolated from PBMC of healthy blood donors using anti-CD3 microbeads (Miltenyi Biotec). Flow cytometry confirmed that the enriched cell fractions contained >85% CD14+ cells and >95% CD3+ cells, respectively (data not shown).

Phagocytosis assay

TG single cell suspensions were incubated with fluorescein-labeled Escherichia coli K-12 strain bioparticles (Invitrogen) in a cell-to-particle ratio of 1:100 according to the manufacturer’s instructions. After incubation at 37°C for 2 h, cells were washed extensively and subjected to flow cytometry or used for immunocytochemical analyses. For the latter procedure, E. coli–treated TG-SGC were enriched using anti-CD14 beads, spun down onto glass slides, fixed with 4% paraformaldehyde and stained with Alexa Fluor 610-PE-conjugated anti-CD68 mAb (KPI; DakoCytomation). Cytospins were mounted in ProLong Gold anti-fade reagent with DAPI (4',6-diamidino-2-phenylindole; Invitrogen) and analyzed on a confocal laser-scanning microscope (LSM510 Meta; Zeiss). Pictures were made using multitrack recording with a 405 nm diode, 488 nm argon, and 561 nm diode laser to detect DAPI, fluorescein, and Alexa Fluor 610-PE, respectively.

Allogeneic MLR assay

CD14-enriched TG-SGC, peripheral blood-derived monocytes and mature dendritic cells (DC) were used as stimulator cells in allogeneic MLR assays. Due to the low number of monocytes recovered from TG donors’ PBMC, mature DC were generated from peripheral blood samples of healthy blood donors (n = 2). To obtain mature DC, CD14-enriched peripheral blood-derived monocytes were cultured with IL-4 and GM-CSF for 6 days to generate immature monocyte-derived DC, and subsequently matured with a cytokine mixture as previously described (13, 14). The mature DC phenotype, characterized by high CD80, CD83, and CD86 expression (15), was confirmed by flow cytometry (data not shown). The effector cells, i.e., allogeneic peripheral blood T cells, were labeled with CFSE (Invitrogen) at a final concentration of 0.5 μM. The stimulator cells were cocultured with effector cells at a ratio of 1:10 at 37°C. At day 7, cells were harvested for flow cytometric analyses. Cells were stained with CD3-allophycocyanin (UCHT1; DakoCytomation) to discriminate between T cells and stimulator cells.

Results

Human TG-SGC express typical macrophage markers

We have previously shown that TG-SGC uniformly express MHC class II, suggesting that they have a role as APC (12). Tissue-resident APC, including macrophages and DC, express the common leukenocyte marker CD45 enabling their distinction from stromal cells like fibroblasts. Paired TG-derived cells and PBMC samples were assayed for CD45 expression. In contrast to PBMC, the TG-derived CD45+ cell pool included two distinct cell populations: CD45high and CD45low cells (Fig. 1A). Whereas the CD45high cells consisted mainly of T cells (data not shown), all CD45low cells expressed the macrophage/mature macrophage marker CD14 (Fig. 1B and Table I). In situ analyses showed that CD14 was expressed by TG-SGC (Fig. 1B). As hinted upon by a previous report (8), the macrophage-specific marker CD68 was expressed intracellularly (Fig. 1C and Table I), but not at the cell surface of TG-SGC (data not shown). Additionally, TG-SGC selectively expressed Ag uptake receptors like CD11b and CD11c (Fig. 1D, E, and Table I), as well as CD16 and CD64 (data not shown). In situ double stainings confirmed the flow cytometry data and demonstrated coexpression of CD14, CD45 and CD11c on TG-SGC (Fig. 2).

Human TG-SGC have an immature myeloid DC phenotype

The complement receptor CD11c is commonly used as a marker to discriminate between myeloid (DC; CD11c+) and plasmacytoid (CD11c−) DC (16). Maturation of myeloid DC is characterized by up-regulation or induction of surface markers like MHC class II and the costimulatory molecules CD80, CD83, and CD86 essential for T cell interaction and stimulation (15). Surface expression of CD83 is considered characteristic for functionally mature DC (17). The expression of CD11c and MHC class II on TG-SGC prompted us to determine the expression of additional DC markers. Whereas the TG-SGC expressed both CD80 and CD86 (Fig. 1, F and G and Table I), the mature DC marker CD83 could not be detected (Fig. 1H and Table I). Furthermore, TG-SGC coexpressed MHC class II and the T cell adhesion molecule CD54 (Fig. 1I). Except for CD40, all markers determined were expressed uniformly on all TG-SGC. Whereas all TG-SGC of the TG donors (n = 4) analyzed were CD40+ by flow cytometry, in situ analyses revealed interdonor variation of CD40 expression on TG-SGC. Two of six TG donors analyzed showed weak but positive CD40 staining on TG-SGC, which was occasionally associated with T cell clusters (Fig. 1J). The discrepancies observed could be due to the use of two different anti-CD40 mAbs in the separate assays, and, in case of the differential CD40 expression observed in the in
Table I. Marker expression on CD45 low human TG-SGC

<table>
<thead>
<tr>
<th>Marker</th>
<th>Percentage of Positive</th>
<th>No. of Donors</th>
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<tbody>
<tr>
<td></td>
<td>CD45 low human TG-SGC</td>
<td></td>
</tr>
<tr>
<td></td>
<td>± SD</td>
<td></td>
</tr>
<tr>
<td>CD14</td>
<td>95.9 ± 4.7</td>
<td>9</td>
</tr>
<tr>
<td>CD68</td>
<td>97.3 ± 1.1</td>
<td>2</td>
</tr>
<tr>
<td>CD11b</td>
<td>92.3 ± 6.6</td>
<td>2</td>
</tr>
<tr>
<td>CD11c</td>
<td>88.5 ± 8.3</td>
<td>8</td>
</tr>
<tr>
<td>CD80</td>
<td>82.3 ± 18.5</td>
<td>5</td>
</tr>
<tr>
<td>CD86</td>
<td>94.8 ± 5.6</td>
<td>6</td>
</tr>
<tr>
<td>CD83</td>
<td>6.3 ± 4.1</td>
<td>3</td>
</tr>
<tr>
<td>CD40</td>
<td>91.9 ± 7.2</td>
<td>4</td>
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</table>

*Data represent the average of TG-SGC that express the indicated marker determined by flow cytometry.

Human TG-SGC phagocytose bacterial particles

A critical role of macrophages is to phagocytose cellular debris and pathogens. Because the TG-SGC have a macrophage phenotype, we determined their capability to phagocytose bacterial particles. Whole TG cell suspensions were incubated with fluorescein-conjugated E. coli after which the phagocytic cell type was identified by flow cytometry. Bacteria were predominantly associated with the CD45 low TG cells, identified in the experiment as TG-SGC (Fig. 3A). Because this assay does not discriminate between membrane bound and internalized bacteria, the E. coli-treated TG-SGC were isolated using anti-CD14 magnetic beads and subsequently subjected to immunocytochemistry. Confocal laser scanning microscopy revealed that the bacteria colocalized with the late endosome marker CD68 (Fig. 3A), demonstrating that TG-SGC have actively phagocytosed the bacteria.

Human TG-SGC are unable to induce an allogeneic MLR

Although immature myeloid DC primarily function as phagocytes, DC maturation is associated with up-regulation of costimulatory and MHC molecules, secretion of cytokines, down-regulation of phagocytic capacity, and increased ability to induce T cell responses (15). It is well established that mature DC are potent stimulators of an allogeneic MLR, a characteristic that distinguishes them from other APC (18). Because TG-SGC expressed a myeloid DC phenotype, they were used as stimulator cells in allogeneic MLR assays. From the same donor, peripheral blood-derived CD14+ monocytes and CD14+ TG-SGC were cocultured with CFSE-labeled allogeneic T cells. In contrast to mature monocyte-derived DC, both monocytes and TG-SGC were unable to induce T cell proliferation (Fig. 3B), indicating that human TG-SGC resemble immature myeloid DC both phenotypically and functionally.

Human TG-infiltrating T cells express T cell inhibitory molecules and TG-SGC the respective ligands

Although neuron-interacting CD8+ T cells express cytolytic molecules, like perforin and granzyme B, neuronal damage is not observed in type-1 HSV latently infected TG, suggesting that the cytolytic activity of the CD8+ T cells is inhibited (7–12, 19). Recently, Suvas et al. (19) have shown that the NK inhibitory molecule complex CD94/NKG2A prevents CD8+ T cell-mediated TG...
A
euron destruction in mice. Whereas the majority of the TG-infiltrating HSV-specific CD8+ T cells expressed CD94/NKG2A, both neurons and CD11b+ cells expressed the cognate ligand Qa-1b (19). Analogous to the mouse, human TG-infiltrating T cells co-expressed CD94 and NKG2A (Fig. 4A). Moreover, the frequency of CD94/NKG2A+ T cells in TG (mean frequency 13 ± 4%) was higher compared with peripheral blood (mean frequency 3 ± 1%), suggesting selective infiltration or differentiation of T cells to express CD94/NKG2A locally. The cognate receptor HLA-E (20)CD94 expression colocalized with CD3 within neuron-interacting T cell clusters (Fig. 4A).  
In addition to NK inhibitory molecules, several studies have indicated that the molecule PD-1 and its ligand PD-L1 negatively regulate T cell effector functions (21–25). Both CD4+ and CD8+ TG-infiltrating T cells expressed PD-1, but percentages and expression levels did not differ between donor-matched TG-derived T cells (mean 29 ± 7%) and peripheral blood T cells (mean 35 ± 12%) (Fig. 4B). However, in situ analyses revealed that neuron-interacting T cell clusters tended to have a higher PD-1 expression, compared with scattered T cells (Fig. 4B). Notably, PD-L1 expression was confined to TG-SGC and appeared to be higher on TG-SGC in proximity to the T cell clusters (Fig. 4C).

### Discussion
For decades, SGC have been regarded as nursing cells providing physical support to neuron somata in sensory ganglia. The current study demonstrates that human TG-SGC have phenotypic and functional characteristics similar to CNS microglia. The current study also reveals that human TG-SGC have the capacity to induce an allogeneic MLR (24).

#### Table II. Comparison of phenotype and functional characteristics of TG-SGC to other human APCs

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>Macrophage*</th>
<th>Immature DC*</th>
<th>Mature DC*</th>
<th>CNS Microglia*</th>
<th>TG-SGC</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD14 and CD68</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>CD16 and CD64</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>CD11b and CD11c</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>MHC class II</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>+</td>
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<td>CD40 and CD54</td>
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<td>–</td>
<td>++</td>
<td>–</td>
</tr>
<tr>
<td>CD80 and CD86</td>
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<td>–</td>
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<td>+</td>
</tr>
<tr>
<td>CD83</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Function</td>
<td>Phagocytosis</td>
<td>Phagocytosis</td>
<td>Phagocytosis</td>
<td>Allogeneic MLR</td>
<td>Allogeneic MLR</td>
</tr>
</tbody>
</table>

* Results indicate the presence (+), intensity (+ +; high and low), or absence (−) of the markers or functional characteristics indicated.
* Data previously described (14, 24, 28).
* Upon stimulation with LPS, microglia express CD14 and CD83, and are able to induce an allogeneic MLR (24).

**FIGURE 3.** Human TG-resident SGC share functional characteristics with macrophages and immature myeloid DC. A. Human TG-SGC were incubated with fluorescein-conjugated bacteria to determine their phagocytic function by flow cytometry (left) and confocal laser scanning microscopy (right). CD45low and CD45high cells are arbitrarily green and blue in the dot plot, respectively (left). Cytospins of CD14-enriched TG-SGC treated with fluorescein-conjugated bacteria (bacteria in green) were stained for CD68 (late endosomes in red) and DAPI (cellular nuclei in blue) and examined by confocal laser scanning microscopy (right). B. Dot plots of a representative allogeneic MLR using mature monocyte-derived DC generated from peripheral blood-derived monocytes of a healthy blood donor (DC, left), and CD14-enriched peripheral blood monocytes (PB CD14+, middle), and CD14-enriched TG-SGC (TG CD14+, right) recovered from the same TG donor, hereby used as stimulator cells in combination with CFSE-labeled allogeneic T cells. The percentage indicates the frequency of T cells that proliferated upon incubation at 37°C for 7 days. Results are representative of two experiments performed on two TG donors.

**FIGURE 4.** Human TG-infiltrating T cells express inhibitory molecules. A. Dot plot of ex vivo flow cytometry (left) on CD94 and NKG2A expression on gated T cells, and in situ analyses of CD3, CD94, and HLA-E on consecutive sections. B and C. Dot plots of ex vivo flow cytometric (top) and in situ analyses (bottom) of CD3 and PD-1 (B), and CD3 and PD-L1 (C) on consecutive sections. The number for each quadrant in dot plot represents the percentage of cells expressing the indicated marker defined on matched isotype control mAb stainings. Dot plots in A and B are gated on CD3+ cells. Representative data from six TG donors are shown. Sections were developed with AEC (bright red precipitate) and counterstained with hematoxylin (blue nuclei). Original magnification is ×200.
functionally involved in controlling local T cell responses in HSV-1 latently infected mouse sensory ganglia (38).

In addition to CD94/NKG2A, the data on human TG suggest the involvement of the T cell inhibitory molecule PD-1. Human TG-infiltrating T cells and TG-SGC expressed PD-1 and PD-L1, respectively. Notably, the expression of both markers appeared to be higher within neuron-interacting T cell clusters. IFN stimulation up-regulates PD-1 and PD-L1 expression on receptive cells (22, 45). Consequently, the differential PD-1 and PD-L1 expression observed may be attributed to IFN-γ secreted by activated T cells recognizing the latent virus. Functional studies are mandatory to investigate the role of both the HLA-E/CD94-NKG2A and PD-1/PD-L1 pathway to inhibit cytolytic T cell effector function in human HSV-1 latently infected TG. Moreover, elucidation of the T cell inhibitory mechanisms used in the peripheral nervous system may provide tools for the development of future therapeutic intervention strategies to counteract undue cell damage associated with T cell-mediated chronic diseases.

In conclusion, the data presented in this study show that human TG-resident SGC have a unique leukocyte phenotype, sharing properties with macrophages and immature myeloid DC. We hypothesize that TG-SGC are tissue-resident APC involved in sensing the local environment and the control of local T cell responses to protect the irreplaceable neuronal somata in TG.

Acknowledgments

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Disclosures

The authors have no financial conflict of interest.

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