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14. ABSTRACT

Microglia are the innate immune cells of the brain. They share cell lineage with macrophages, which have been divided into two major subgroups: (i) classical or "M1" macrophages, which promote inflammation and express IL-12, and (ii) alternatively activated or "M2" macrophages, which phagocytose apoptotic cells, promote wound repair, and (in mice) express arginase-1. We proposed that microglia might be also reflect these functional subsets and that activation of microglia by TBI would be detrimental to the extent that it involves M1-like (pro-inflammatory) microglia, but beneficial to extent that it involves the activation of M2-like (reparative) microglia. To test this, we are studying TBI in "reporter" mice that express the fluor YFP under control of the promoter for either IL-12 or arginase-1. One day after TBI, we see little activation of microglia or macrophages. By day 4, ~20% of the combined macrophage/microglia population is activated, as assessed by marked enlargement. It is not yet clear whether these activated cells derive from macrophages or microglia. In this dramatic response, the enlarged cells have intrinsic fluorescence, requiring us to turn to additional means to fully characterize them, but our preliminary results indicate that they more readily express N-arginase than IL-12.

15. SUBJECT TERMS
traumatic brain injury, microglia, macrophages

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INTRODUCTION

Prior studies have shown that traumatic brain injury results in extensive activation of microglia, which phagocytose apoptotic neurons and initiate the process of brain repair. Activated microglia, however, can also initiate an inflammatory response, extending brain damage (*1*). Our studies seek to define if these opposing functions of microglia are mediated by different microglial subtypes that could be selectively activated following TBI. This hypothesis is suggested by studies of peripheral macrophages, which have demonstrated at least two major macrophage subtypes, called M1 and M2 (*2*, *3*).

M1 macrophages are pro-inflammatory, while M2 macrophages (also called "alternative" macrophages) are generally anti-inflammatory and have an increased capacity for phagocytosing apoptotic cells. In mice, M2 cells are also marked by the expression of arginase-1. Of particular importance to our work, the activation of M2 macrophages is promoted by peroxisome proliferator-activated receptor γ (PPARγ), a nuclear receptor, and activation of PPARγ has anti-inflammatory effects both in the periphery and the brain (*4*, *5*). Recent *in vitro* studies of microglia suggest that like macrophages they may be either proinflammatory or phagocytic, and that expression of the surface receptor TREM-2 promotes phagocytosis while suppressing the production of the inflammatory cytokine TNF-α (*6*).

Based on these findings, we seek to test the central hypothesis that subtypes of microglia activated by trauma regulate the extent of TBI. To test this, we are defining the subtypes of microglia that are activated by TBI in mice, as assessed first by the expression of cytokines and other cell markers and second by function. We will then define methods for promoting the expansion of M2 cells *in vivo* following TBI. Finally, we will test the effects of altering microglial subsets on two important consequences of TBI, the recruitment of T cells into the brain and the induction of neuronal cell death by apoptosis.

Our Specific Aims are:

1. Determine the development of microglia into M1 and M2 subtypes in response to TBI at multiple timepoints *in vivo* and establish the function of these microglial subtypes *ex vivo*.
2. Skew the microglial response to TBI towards an M2 phenotype using PPARγ and TREM-2 agonists as treatments given post-TBI.
3. Define the effects of PPARγ activation with and without and stimulation of TREM-2 on T cell infiltration and neuronal death in response to TBI.

BODY

Our primary approach to our analysis of microglial subsets is the use of reporter cell mice provided by our collaborator, Richard Locksley (UCSF). There are two strains: (i) yet40 mice, which express the fluor YFP under the control of the IL-12p40 promoter, a marker for M1 macrophages, and (ii) YARG mice, which express YFP under the control of the promoter for arginase-1, a marker for M2 macrophages.

The approved Statement of Work includes the following discussion of timelines:

> Specific Aim 1. Determine the development of microglia into M1 and M2 subtypes in response to TBI at multiple timepoints *in vivo* and establish the function of these microglial subtypes *ex vivo.* As detailed in the application, these studies involve the isolation of microglia from mice at intervals after they are subjected to TBI and assessing their subtypes as determined in particular by flow cytometry to assess the activation of IL-12 or arginase-1, using the yet40 and YARG reporter mice for IL-12 and arginase, respectively. Additionally, the overall production of cytokines by microglia will be assessed by the production of cytokines, as determined by ELISA. Microglia will be assessed for the expression of TREM-2 and will be subjected to functional assays, including phagocytosis of neurons. We will conduct more experiments in YARG mice than in yet40 mice, because these will be the primary focus of subsequent therapeutic studies. These studies will be conducted in years 1 and 2. As detailed in the IACUC protocol, the estimated use of mice for experiments in year 1 is 110 yet40 mice and 280 YARG mice. In year 2, about half of the designated mice will be used for this specific aim, i.e., 55 yet40 and 140 YARG mice. At present, for all studies with mice we must breed twice the number of mice needed for experimentation, because there are no available studies to define whether we can mix the use of male and female mice in TBI. We will test this, but our current plan is to use only male mice, as these have been the usual focus of study of TBI. Specific Aim 1 will be completed in Year 2.
>
> Specific Aim 2. Skew the microglial response to TBI towards an M2 phenotype using PPARγ and TREM2 agonists as treatments given post-TBI. These studies test the use of a PPARγ agonist to drive microglia to an M2 phenotype, as assessed by use of the yet40 and YARG reporter cell mice. Two dosing schedules will be tested, with examination following a schedule determined by Specific Aim 1. Next, mice will be treated both with the PPARγ agonist, to activate M2 microglia, and with antibody to TREM2, to test whether this will augment and/or sustain the M2 phenotype of microglia. Additionally, effects on the brain will be assessed by immunohistochemistry and staining for microglial activation and neuronal death. Work on this Aim will begin in year 2 and continue into year 3. In year 2, about half of the designated mice will be used for this specific aim, i.e., ~60 yet40 and 120 YARG mice. In year 3, yet40 mice will still be bred in case they are needed, but all experiments will be on YARG mice, including 60 for this Specific Aim, which will be completed in year 3.
>
> Specific Aim 3. Define the effects of M2-like microglial activation by PPARγ and TREM2 on T cell infiltration and neuronal death in response to TBI. TBI invokes an invasion of T lymphocytes. This Specific Aim will test whether the activation of PPARγ and/or anti-TREM2 will alter the influx of T cells. We will also correlate this with neuronal cell death, as assessed by TUNEL assay. These experiments will be entirely on YARG mice. They will begin in year 3, using 60 mice, and continue into year 4, using 120 mice. The Specific Aim will be completed in year 4.

In accord with the above, our work in year 1 has been devoted to Specific Aim 1. We have spent considerable effort in establishing the procedures for TBI within our own lab. We began our studies of TBI by using the equipment of our collaborator, Jialing Liu, who has considerable

expertise in this area and who has helped us considerably in our work. It became clear, however, that our need for equipment would outpace the availability of Dr. Liu's equipment, which is also shared by others. Therefore, with support from other sources, including our division, we have assembled a high-quality device for TBI within our own lab. This includes a pneumatic (cortical) impact device from AmScien Instruments (Richmond, VA), a stereotactic platform, modified for use with mice and for the use of gas anesthesia, from Stoelting (Wood Dale, IL), as well as all accessories necessary for anesthesia and surgery (Figure 1). Delays in obtaining this and in correcting malfunctions caused delays, but we do not anticipate any significant overall delays in the work, and no reduction in scope.

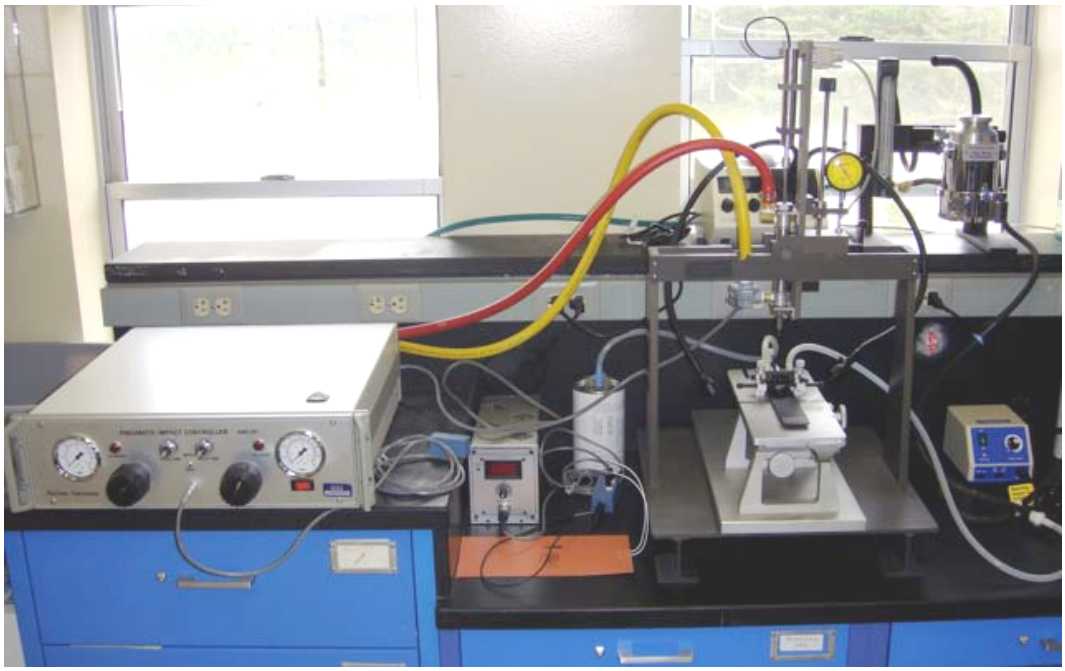

Figure 1. Station established in the Seaman lab for performing TBI in mice. This includes a custom-made stereotactic platform (lower right), adapted for gas anesthesia (upper right). The tanks for compressed nitrogen driving the impactor are out of sight to the left.

We have obtained, bred, and are studying TBI in the two mouse reporter cell lines, yet40 (IL-12 reporter) and YARG (arginase-1). In studies of brain cells from these mice, microglia are identified by flow cytometry as expressing CD11b together with intermediate levels of CD45, while CNS macrophages are identified as expressing CD11b together with high levels of CD45. We first established that freshly obtained, unstimulated microglia from neither strain express detectable levels of YFP, as assessed by flow cytometry, indicating that resting microglia are not activated toward either the M1 or the M2 phenotype. CNS macrophages from either strain similarly do not express YFP in the resting state. When yet40 mice are given lipopolysaccharide (LPS) intraperitoneally, however, a portion of microglia and CNS macrophages are activated to express YFP, confirming that microglia, like macrophages, can be activated to express IL-12. We have not yet found a control stimulus that will activate the expression of YFP in brain cells (microglia or macrophages) from YARG mice, despite testing combinations of cytokines (IL-4

and IL-13) both intraperitoneally and intrathecally, as well as systemic infection with *Strongyloides*. These stimuli did not activate YFP in microglia from YARG mice even though systemic cytokines and *Strongyloides* activated YARG in peritoneal macrophages. As discussed below, however, we do see expression of YFP in YARG mice following TBI, confirming that CNS myeloid cells can be activated to express arginase-1.

Turning to studies of TBI, we have tested wild-type, yet40, and YARG mice for their response to TBI. A summary of mice studies to date is in Table 1.

	Day 1	Day 4	Day 7	Total mice/group
WT sham	1	10	2	13
WT TBI	1	12	2	15
YARG sham	0	3	2	5
YARG TBI	0	4	2	6
Yet40 sham	1	4	2	7
Yet40 TBI	1	4	2	7
Total mice/day	4	37	12	53

Table 1. Number of mice analyzed by flow cytometry as of July 2009

One day following TBI, there is no significant activation of YFP or other evidence for activation of either microglia or CNS macrophages, as assessed by flow cytometry. Four days after TBI, however, flow cytometry reveals a substantial population of large cells that express CD11b and high levels of CD45, and this is sustained at day 7 (Figure 2, next page).

There is not a similar enlargement of cells expressing intermediate levels of CD45, the usual marker for microglia. We are nonetheless uncertain at this point whether these enlarged (activated) cells are macrophages or, instead, activated microglia that have increased their expression of CD45. Based on size alone, it is possible to identify this group of activated cells in wild-type mice, and in all mice their number is equivalent to about 20% of the combined number of microglia and CNS macrophages—a remarkable level of activation. Prior histologic studies of TBI have shown both widespread activation of microglia as well as a localized response of cells that are either macrophages or microglia (*7-9*). The large number of activated cells seen in our studies suggests that at least a portion of them derive from resident CNS cells, but we will seek to define both their nature and their source.

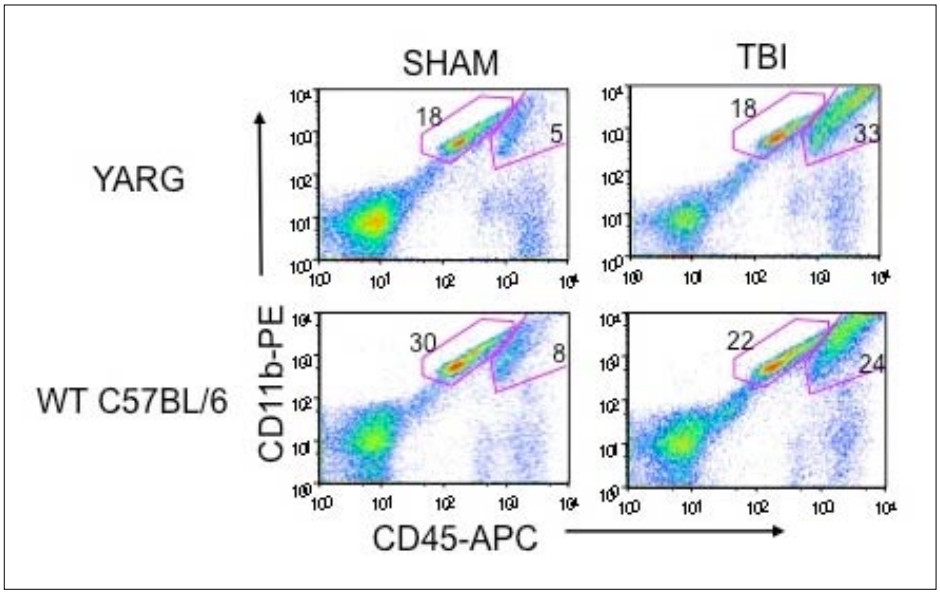

Figure 2. Gating of CD45high and CD45intermediate cells from the brain. In untreated mice, the former has been shown to represent macrophages, while the latter has been shown to represent microglia. Four days following TBI ("TBI"), there is a substantial increase in cells that express high levels of CD45. It is yet unclear whether these are macrophages or microglia that have been activated to express high levels of CD45.

These cells are not only large, as assessed by forward scatter, but also complex, as assessed by side scatter. Activation by LPS causes a similar increase in size, but not in complexity. The size and complexity of these cells, however, present a new challenge because the cells are consequently autofluorescent; even in the wild-type mice, they fluoresce in the YFP channel, as they do to some degree in all channels. In the yet40 mice, we have not yet been able to detect a level of YFP fluorescence following TBI that is greater than that in wild-type mice, but in the YARG mice it is usually possible to detect a significant population of cells that expresses YFP at higher levels than are seen wild-type mice (Figure 3). Thus, our results to date indicate that TBI activates a large number of myeloid cells, and at least a portion of these express arginase-1, while expression of IL-12 is not yet established. If these results are confirmed, they suggest that the activation of these cells may favor brain repair more than inflammation, although concomitant exposure to LPS (as with infection by LPS+ bacteria) might skew this response.

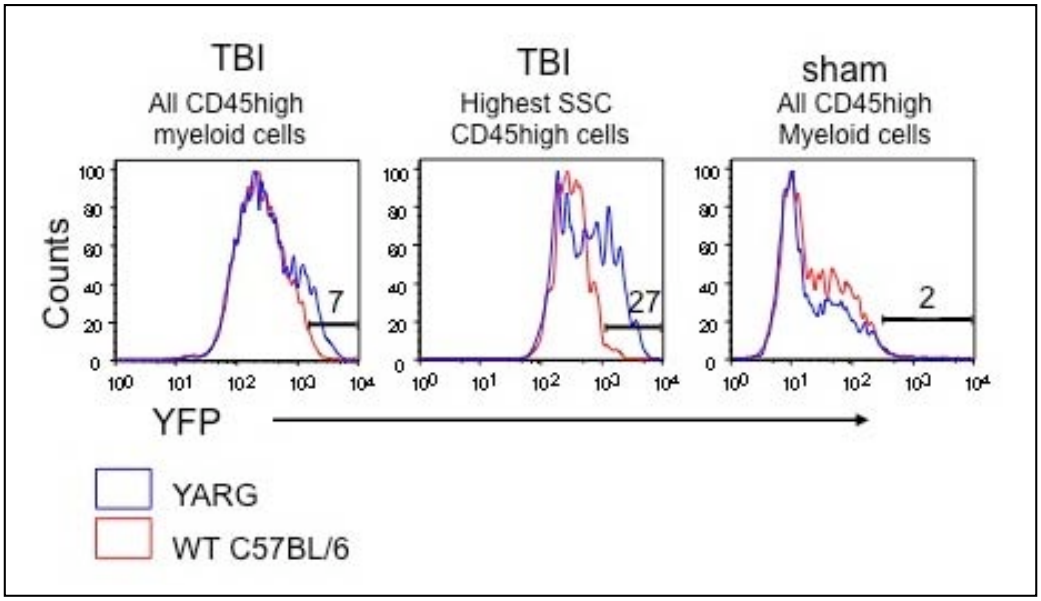

Figure 3. Expression of YFP in YARG vs WT mice 4 days following TBI. The first panel shows levels of fluorescence in all activated CD45high cells. A small subpopulation of cells is identified in YARG mice in which YFP is expressed at levels above the autofluorescence seen in WT mice, indicating that arginase-1 is activated in these cells. When the gate is set to analyze only cells with very high side scatter (SSC), second panel, a larger fraction of cells is identified in YARG mice that express YFP. The third panel shows fluorescence by myeloid cells from sham-treated mice, in which the activated cells are not seen and there is consequently relatively little autofluorescence.

We are planning several approaches to define these cells and their source. Briefly, they include the following:

1. Isolate the cells by cell sorting and examine the phenotype of the cells by microscopy and by expression of transcripts for cytokines as assessed by RT-PCR.
2. Perform fluorescence microscopy on sections from TBI mice to examine the location of activated cells. For these studies, expression of YFP can be examined by antibodies to YFP, allowing use of any stain or fluor, so we can overcome issues of autofluorescence.
3. Perform both flow cytometry and microscopy on brains from mice subjected to bone marrow reconstitution with cells from CD45.1 mice (C57BL/6 mice are CD45.2). Turnover of microglia following bone marrow reconstitution requires many months, so this will allow us to distinguish microglia from macrophages.

In addition to these studies, we have studied the expression of TREM2 in adult mice as planned. Our studies are based on our prior studies of TREM2 expression and function in microglia, which were sponsored by the DoD through the VA (W81XWH-05-2-0094). This work was submitted for publication shortly after the prior grant expired, and the reviewers called for studies of TREM2 expression that were within the scope of the current grant. They were

therefore pursued and the paper was published this year in the Journal of Neurochemistry. Support from the current grant was acknowledged in the paper, but through an error we listed the application number (PT075779) rather than the award number (W81XWH-08-2-0055).

Presented below are the deliverables included in our approved Statement of Work, with notation on their status:

End of year 1

1. Quantification of microglial subsets in normal mice by use of reporter cell mouse lines yet40 and YARG. *Completed. Resting microglia do not express detectable transcripts for YFP in either reporter strain.*
2. Quantification of the expression of TREM2 on microglial subsets in normal mice as identified by use of reporter cell mouse lines yet40 and YARG. *Completed. Resting microglia do not express detectable transcripts for YFP in either reporter strain, and only rare cells are detected that express detectable levels of surface TREM2. By immunofluorescence microscopy, however, we detect TREM2 in most microglia, leading to the conclusion that most TREM2 is inside the microglia rather than on the surface. These studies are presented in the attached publication (Hsieh, et al., J Neurochem 109:1144-56, 2009).*
3. Quantification of the production of the cytokine TNFα, as determined by ELISA, by microglial subset from normal mice, as identified by use of reporter cell mouse lines yet40 and YARG. *Not yet completed, but we are working on this now and expect to achieve this within the next 60 days.*

End of year 2

4. Quantification of microglial subsets following TBI by use of reporter cell mouse lines yet40 and YARG. *Studies initiated, as discussed above.*
5. Quantification of TREM2 expression on microglial subsets following TBI, as identified by use of reporter cell mouse lines yet40 and YARG. *These studies will be achieved within the next 60 days.*
6. Quantification of the expression of the cytokine TNFα as determined by ELISA, by microglial subset following TBI, as identified by use of reporter cell mouse lines yet40 and YARG. *Not yet completed, but we are working on this now and expect to achieve this within the next 60 days.*
7. Quantification of phagocytosis by microglial subset following TBI, as identified by use of reporter cell mouse lines yet40 and YARG. *Not yet initiated.*

End of year 3

8. In YARG mice, define the effects of the PPARγ agonist 15-deoxy$\Delta^{12,14}$-prostaglandin J_2 (15d-PGJ_2)on microglial activation and neuronal death *in vivo*, as assessed by flow cytometry and immunohistochemistry. *Not yet initiated.*

End of year 4

9. In YARG mice, define the effects of 15d-PGJ_2 with or without antibody to TREM2 (delivered intracranially) on the response to TBI, as assessed by T cell infiltration of the brain and neuronal death. *Not yet initiated.*

KEY RESEARCH ACCOMPLISHMENTS

- TREM2 is expressed intracellularly but only to a limited extent on the surface of freshly obtained microglia from untreated adult mice, though they appear on the surface following activation in culture.
- Equipment and procedures for TBI have been established in our laboratory
- TBI in mice causes appearance of a large and complex population of activated cells of myeloid lineage
- TBI in mice causes activation of arginase-1 in a subpopulation of activated myeloid cells

REPORTABLE OUTCOMES

Hsieh CL, Koike K, Spusta S, Niemi E, Yenari M, Nakamura MC, Seaman WE. A role for TREM2 ligands in the phagocytosis of apoptotic neuronal cells by microglia. J Neurochem. 109:1144-56, 2009.

CONCLUSIONS

Perhaps our most important achievement to date is that we have brought the tools of flow cytometry to the study of TBI. We can clearly identify a large number of activated cells that are either microglia or macrophages, and we will be able to use flow cytometry to isolate these cells for further study. Although we have found that arginase-1 is activated in at least some of these cells, we are still at an early stage in identifying their nature and their source, but we have the tools to determine these.

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APPENDICES

Publications. Paper by Hsieh, et al., attached

List of Personnel. This is unchanged. Personnel include:

William E. Seaman, M.D. Principal Investigator
Mary C. Nakamura, M.D. Investigator
Christine L. Hsieh, Ph.D., Postdoctoral Fellow
Erene Niemi, Research Technician IV

SUPPORTING DATA

All figures and/or tables are imbedded in the report.

Journal of Neurochemistry

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A role for TREM2 ligands in the phagocytosis of apoptotic neuronal cells by microglia

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Abstract

Following neuronal injury, microglia initiate repair by phagocytosing dead neurons without eliciting inflammation. Prior evidence indicates triggering receptor expressed by myeloid cells-2 (TREM2) promotes phagocytosis and retards inflammation. However, evidence that microglia and neurons directly interact through TREM2 to orchestrate microglial function is lacking. We here demonstrate that TREM2 interacts with endogenous ligands on neurons. Staining with TREM2-Fc identified TREM2 ligands (TREM2-L) on Neuro2A cells and on cultured cortical and dopamine neurons. Apoptosis greatly increased the expression of TREM2-L. Furthermore, apoptotic neurons stimulated TREM2 signaling, and an anti-TREM2 mAb blocked stimulation. To examine the interaction between TREM2 and TREM2-L in phagocytosis, we studied BV2 microglial cells and their engulfment of apoptotic Neuro2A. One of our anti-TREM2 mAb, but not others, reduced engulfment, suggesting the presence of a functional site on TREM2 interacting with neurons. Further, Chinese hamster ovary cells transfected with TREM2 conferred phagocytic activity of neuronal cells demonstrating that TREM2 is both required and sufficient for competent uptake of apoptotic neuronal cells. Finally, while TREM2-L are expressed on neurons, TREM2 is not; in the brain, it is found on microglia. TREM2 and TREM2-L form a receptor–ligand pair connecting microglia with apoptotic neurons, directing removal of damaged cells to allow repair.

Keywords: apoptotic neurons, microglia, phagocytosis.
J. Neurochem. (2009) **109**, 1144–1156.

Microglia are resident myeloid-derived cells in the CNS that provide constant surveillance of the brain and spinal cord. In a resting state, microglial dendrites display a divergent and branched phenotype, with their protruding processes dynamically sampling and monitoring their environment (Nimmerjahn *et al.* 2005). As part of the innate immune system, microglia can defend against microbial pathogens, clear injured neurons and cellular debris, and provide sustenance to other cells in the CNS (Aloisi 2001; Napoli and Neumann 2009). Microglia, however, can also promote inflammation, which may exacerbate neurodegenerative diseases, such as Alzheimer's disease and Parkinson's disease, as well as ischemic brain injury (Kempermann and Neumann 2003; Minghetti 2005; Yenari *et al.* 2006; Block *et al.* 2007). Proinflammatory microglia and macrophages also play a detrimental role during multiple sclerosis, where the importance of specifically inhibiting inflammatory signals from CNS myeloid cells has been clearly elucidated (Prinz *et al.* 2008). Thus, the functional differentiation of microglia has important consequences for disease.

Triggering receptor expressed by myeloid cells-2 (TREM2) is an immunoglobulin-like orphan receptor of the TREM family that is expressed on activated macrophages, immature dendritic cells, osteoclasts, and at least some

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Abbreviations used: APC, allophycocyanin; CHO, Chinese hamster ovary; CN, cortical neurons; DAP12, DNAX-adaptor protein; DMEM, Dulbecco's modified Eagle's medium; EAE, experimental autoimmune encephalitis; FCS, fetal calf serum; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GFP, green fluorescent protein; NeuN, neuronal nuclei; NFAT, nuclear factor of activated T cells; PBS, phosphate-buffered saline; PMA, phorbol 12-myristate 13-acetate; PE, phycoerythrin; TH, tyrosine hydroxylase; TREM2, triggering receptor expressed by myeloid cells-2; TREM2-L, TREM2 ligand; VMN, ventral midbrain neurons.

microglia (Colonna 2003). TREM2 associates with DNAX adaptor protein-12 (DAP12), a signaling molecule that contains an immunoreceptor tyrosine-based activation motif. Loss-of-function mutations in either TREM2 or DAP12 cause Nasu-Hakola disease, a rare and fatal neurodegenerative disease also known as polycystic lipomembranous osteodysplasia with sclerosing leukoencephalopathy (Paloneva *et al.* 2000, 2002). Symptoms and consequences of Nasu-Hakola disease include late-onset dementia, demyelination, and cerebral atrophy, with widespread activation of microglia, demonstrating that both TREM2 and DAP12 are critical in maintaining homeostasis of the CNS. The mechanisms of neurodegeneration in this disorder are unknown, but one hypothesis is that lack of either TREM2 or DAP12 impairs the clearance of apoptotic neurons by microglia, leading to the accumulation of necrotic debris (Thrash *et al.* 2008). Phagocytosis of apoptotic cells is important to prevent leakage of noxious contents, to avert immune responses against self-antigens, and to suppress unwanted immune responses (Ravichandran and Lorenz 2007).

DAP12, the signaling partner for TREM2, was originally described as transducing conventional activation signals, but the TREM2–DAP12 complex inhibits some macrophage functions. Depletion of TREM2 either by RNAi or by targeted gene deletion amplifies inflammatory cytokine responses by macrophages following stimulation of toll-like receptors (Hamerman *et al.* 2005, 2006; Piccio *et al.* 2007). Furthermore, TREM2 expression in microglia impairs tumor necrosis factor-α and nitric oxide synthase-2 transcript expression even as it increases phagocytosis in response to apoptotic neurons (Takahashi *et al.* 2005). In mice with experimental autoimmune encephalitis (EAE), blockade of TREM2 with a mAb exacerbates disease, while treatment with TREM2-expressing myeloid cells reduces inflammation and improves disease (Piccio *et al.* 2007; Takahashi *et al.* 2007).

In sum, these findings support a model in which TREM2 suppresses inflammation and promotes tissue repair through removal of apoptotic cells. Although loss of either TREM2 or DAP12 does not usually have detectable clinical consequences until adulthood, studies in mice also implicate DAP12 in CNS development, as neonatal mice lacking DAP12 have reduced capacity for mediating neuronal cell death during hippocampal development (Wakselman *et al.* 2008).

Although these clinical and experimental studies demonstrate the importance of TREM2 in the brain, ligands for TREM2 have not been identified. In addition, the functional recognition of apoptotic cells by TREM2 has not been described. We have previously shown that TREM2 recognizes anionic patterns of ligands on bacteria and some eukaryotic cells (Daws *et al.* 2003). We demonstrate here the finding of an endogenous cellular ligand for TREM2 on neurons, and thus have identified a novel pathway of direct communication between microglia and neurons. We show that TREM2 can bind directly to neuronal cells, with increased binding to apoptotic neuronal cells. TREM2 ligands (TREM2-L) on apoptotic neurons mediate signal transduction by TREM2 on microglia and promote phagocytosis. Further, blockade of this interaction between microglia and apoptotic neurons using a TREM2 mAb impairs phagocytosis of apoptotic neurons by microglia. As direct evidence of a role for TREM2 in the phagocytosis of apoptotic neuronal cells we also show that TREM2 transfected Chinese hamster ovary (CHO) cells have increased phagocytosis of dying Neuro2A cells. Our findings support the hypothesis that phagocytosis of apoptotic neurons by microglia is promoted by a novel interaction of TREM2 on microglia with TREM2-L on apoptotic neurons.

Materials and methods

Animals and surgical procedures

Wildtype C57BL/6 mice were obtained from Charles Rivers Laboratories (Wilmington, MA, USA). Male mice (8–12 weeks) were used for histological sections and for isolation of adult microglia. Neonatal mice (days 1–4) were also used to obtain microglia. For experiments examining green fluorescent protein (GFP^+) neonatal microglia, cells were derived from heterozygous $CX3CR1^{GFP/+}$ mice. $CX3CR1^{GFP/GFP}$ mice, which are homozygous knockouts for CX3CR1 with GFP knocked-in were previously described (Jung *et al.* 2000; Cardona *et al.* 2006) and obtained from Drs Li Gan and Sharon Haynes (UCSF) with the kind permission of Dr Dan Littman (Skirball Institute). $CX3CR1^{GFP/GFP}$ mice were crossed with wildtype C57BL/6 mice to generate $CX3CR1^{GFP/+}$ heterozygous animals, which coexpress CX3CR1 and GFP in their microglia. Animals were housed at the San Francisco VA Animal Facility, an AAALAC-approved facility, and were used under approved protocols.

Antibodies

To detect microglia, allophycocyanin (APC) or phycoerythrin-conjugated anti-CD11b (Clone M1/70) and anti-CD45 (Clone Ly5) FITC antibodies were used (eBioscience, San Diego, CA, USA). Our production of rat anti-mouse TREM2 mAbs has been described (Humphrey *et al.* 2006). Isotype controls for the TREM2 antibodies were rat IgG2a (for Clones 67.8, 69.2, 150.1, and 181.1) and functional grade rat IgG1 (for Clone 78.18) (BD Biosciences, San Jose, CA, USA). For detection of TREM2 on primary cells, primary antibody staining was amplified with a biotin-conjugated goat anti-rat Fab antibody (1 : 400; Jackson Immuno Research, West Grove, PA, USA) followed by either streptavidin–Cy3 (1 : 150) (Sigma, St Louis, MO, USA) for microscopy, or by streptavidin–APC (Caltag, Carlsbad, CA, USA) for flow cytometry. To detect TREM2 on cell lines by flow cytometry, a secondary donkey anti-rat phycoerythrin or APC-conjugated $F(ab')_2$ antibody (Jackson Immunolabs) was used. TREM1 on CHO cells was detected by using an anti-TREM1 biotin-conjugated antibody (R&D Systems, Minneapolis, MN, USA) followed by streptavidin–APC. Dopaminergic neurons were identified by antiserum to tyrosine hydroxylase (TH) (Chemicon, Billerica, MA, USA), and all neurons were detected by staining for alexa-fluor488-conjugated neuronal nuclei (NeuN) (1 μg/mL; Chemicon) or for neuronal class III β-tubulin (Covance, Princeton, NJ, USA). To stain for TREM ligands by flow cytometry, TREM2-

Fc or TREM1-Fc proteins (R&D Systems) were used and detected by a donkey anti-human $F(ab')_2$ APC-conjugated antibody (Jackson Immunolabs). Both TREM fusion proteins were validated by western blot to contain both their respective TREM receptor and the Fc domain of human IgG1.

Cell culture and isolation

BV2, Neuro2A, BWZ, WEHI-231, P388D1, and Chinese Hamster Ovary (CHO) cells were cultured in Roswell Park Memorial Institute 1640 (Cellgro, Manassus, VA, USA) with 10% fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA, USA), 1% penicillin/streptomycin (Gibco, Carlsbad, CA, USA), and 50 μM 2 mercaptoethanol (Sigma). The derivation of CHO cells expressing a chimeric TREM2/DAP12 receptor or a chimeric TREM1/DAP12 receptor has been previously described (N'Diaye *et al.* 2009). Cell death was induced in Neuro2A cells by treatment with either the kinase inhibitor staurosporine (0.5 μM) or the neurotoxin MPP^+ (7.5–15 mM) for 16 h (Sigma). Apoptosis was induced in BWZ, WEHI-231, and P388D1 cells with 0.25–0.4 μM staurosporine for 16 h. Following treatment with staurosporine or MPP^+, cells were 40–60% Annexin V^+ and propidium iodide$^+$ (BD Biosciences) as assessed by flow cytometry. TREM2-L expression was analyzed on Annexin V^- PI^-, Annexin V^+ PI^-, and Annexin V^+ PI^+ cell populations.

For isolation of primary cultured neonatal microglia, whole brains were harvested from mouse pups and were stripped of meninges. Tissue was mechanically dissociated through a 100 μm cell strainer and the resulting cell suspension was washed and plated in culture medium [Dulbecco's modified Eagle's medium (DMEM) high glucose; Cellgro], 10% fetal bovine serum, 1% penicillin/streptomycin, 1% GlutaMAX (Invitrogen, Carlsbad, CA, USA), 8 μM HEPES, and 10 μg/mL insulin (Invitrogen). Supernatants were transferred after 3–4 days to a new flask, and fresh medium was added to all cells. Mixed glial cells were cultured for 10–21 days, with 1–2 media changes/week. Where indicated, neonatal microglia were sorted to 99% purity using an anti-CD11b antibody and a FACSAria (BD Biosciences).

Primary adult microglia were isolated according to previously published methods (Sedgwick *et al.* 1991). Briefly, following perfusion, brains and spinal cords were obtained and gently dissociated through a 100 μm cell strainer. Washed cells were treated at 37°C for 30 min with 400 U/mL Dnase (Sigma) and 0.5 mg/mL collagenase type I (Worthington, Lakewood, NJ, USA). Leukocytes were isolated by separation on a Percoll gradient (Amersham Biosciences, Piscataway, NJ, USA).

Primary cortical neurons (CN) were isolated from mice at embryonic day 16. Cortices were incubated with 0.12% trypsin for 10 min at 37°C and washed three times with DMEM containing 10% fetal calf serum (FCS). Tissue was triturated and cells were plated at 0.3×10^6 cells/mL onto poly-D-lysine-coated plastic in Neurobasal medium supplemented with B27 (Invitrogen), 1% penicillin/streptomycin, and 1% GlutaMAX. In some cases, 3 μM araC was used at day 1 for 24 h to inhibit the growth of glia. CN were cultured for 7–10 days and were greater than 90% $NeuN^+$.

Ventral midbrain neurons (VMN) were isolated from embryos at day 13.5. Ventral midbrain tissue was trypsinized (1%; Worthington) for 15 min at 37°C, quenched with medium containing 20% horse serum, washed, and triturated. Cells were plated at 30 000 or 100 000 cells/well in 96- or 24-well plates, respectively, onto poly-D-lysine and laminin (Sigma) coated surfaces. VMN were cultured in DMEM/F12 containing 2.2% Albumax (Invitrogen), and 1% N1 additive (Sigma). VMN were used for experiments at day 1. The cultures contained 3–4% dopaminergic neurons and the entire culture was nearly 100% neuronal.

The generation of CHO cells transfected with chimeric TREM2/DAP12 or TREM1/DAP12 has been described (N'Diaye *et al.* 2009).

Cytochemistry, immunofluorescence confocal microscopy, and histology

For examination of TREM2-L expression on isolated CN and VMN, cells were fixed with 3.7% *p*-formaldehyde (Ted Pella, Inc., Redding, CA, USA) in phosphate-buffered saline (PBS) for 15 min, blocked with 5% goat and mouse sera, and stained with either TREM2b-Fc or TREM1-Fc chimeras (1 μg/mL). To detect the human IgG1 Fc domain of the TREM-Fc fusion proteins, a biotin-conjugated goat anti-human $F(ab')_2$ antibody (Jackson Immunolabs) followed by an immunoperoxidase reaction (ABC elite kit; Vectorlabs, Burlingame, CA, USA) was used. Images were taken using an inverted microscope.

To analyze TREM2 expression by immunofluorescence microscopy of cultured cells, sorted neonatal microglia were plated onto poly-D-lysine-coated glass coverslips (BD Biosciences) and fixed with 3.7% *p*-formaldehyde. Cells were blocked with 5% goat serum and stained for TREM2 using a cocktail of five anti-TREM2 antibodies (1 μg/mL of each antibody) overnight at 4°C. After washing in PBS and a second round of blocking, a secondary antibody was applied for 1 h at 20–25°C. After washing, cells were stained with streptavidin–Cy3 for 1 h. Cells were stained with 4′,6-diamidino-2-phenylindole for 5 min and mounted onto glass slides with Permount (Fisher, Pittsburgh, PA, USA). Images were visualized with an LSM510 laser scanning confocal microscope (Zeiss, Thornwood, NY, USA). Single optical sections with a thickness of < 1.0 μm were imaged with a 60× magnification lens with oil. White scale bars represent a 10 μm distance.

For histology, mice were killed and subjected to cardiac perfusion. Harvested brains and spinal cord tissues were incubated in increasing concentration (15–30%) of sucrose until saturated and then frozen in tissue-freezing medium. Sections (10 μm) were obtained and mounted onto Superfrost glass slides (Fisher). Tissue was fixed and permeabilized with 75% ethanol/25% methanol for 10 min and stained and imaged as above for TREM2. Tissue was incubated in a blocking solution containing 5% rat and mouse serums, and stained for CD11b and NeuN.

Triggering receptor expressed by myeloid cells-2 reporter assay

BWZ thymoma reporter cells, which express lacZ under control of the promoter for nuclear factor of activated T cells (NFAT), were a generous gift from Dr Nilabh Shastri (UC Berkeley). BWZ cells were transfected to coexpress TREM2 and DAP12. One positive clone was designated BWZ.TREM2/DAP12. BWZ.TREM2/DAP12 cells or, as a control, parental BWZ cells were plated at 1×10^6 cells/mL on top of neuronal target cells that had been left untreated or treated with the apoptotic stimulus MPP^+ in triplicate in assay medium [Roswell Park Memorial Institute, 1% FCS, and 20 ng/mL phorbol 12-myristate 13-acetate (PMA)]. Neuro2A cells were treated with 7.5–15 mM MPP^+ and primary neurons were treated with 300 μM MPP^+ overnight and washed. As a positive control,

ionomycin (3 μM) was added to reporter cells alone. Reporter cells were stimulated by fresh or apoptotic cells for 16 h at 37°C, washed, and lysed in a buffer containing 100 mM 2-mercaptoethanol, 9 mM $MgCl_2$, 0.125% NP-40 (Sigma), and 30 mM chlorophenol red galactosidase. Plates were developed for 24 h at 37°C, and lacZ activity was measured as previously described (Sanderson and Shastri 1994). Statistical significance was determined using unpaired two-tailed Student's *t*-test and PRISM software (GraphPad, San Diego, CA, USA).

Lentiviral-mediated shRNA

Lentivirus encoding for a TREM2 shRNA sequence (5′-GA-AGCGGAATGGGAGCACA-3′) (TREM2 shRNA-GFP 3.7) or a control empty virus (GFP 3.7) was produced by Fugene (Roche, Indianapolis, IN, USA)-mediated co-transfection of 293T cells with pREV, pVSVg, pMDL, and the pLenti-GFP 3.7 plasmid containing the shRNA sequence. Effector BV2 cells were plated in 24-well plates at 100 000 cells/well overnight. Medium was replaced and cells were treated with 8 μg/mL polybrene. Filtered and concentrated virus was applied to cells that were then spun at 1000 *g* for 90 min at 20–25°C and then cultured for 24 h at 37°C. Knockdown of TREM2 was determined at 72 h, and cells were used for phagocytosis assays.

Phagocytosis assay

BV2 or CHO effector cells (100 000 cells/well) were plated in 24-well plates overnight. Cytochalasin D (2 μM; Sigma), rat IgG1 (100 μg/mL), or anti-TREM2 blocking antibody (100 μg/mL, Clone 78.18) was added for 20 min at 20–25°C in fresh medium. Target Neuro2A cells were labeled with CM-DiI (Invitrogen) and cultured overnight in low-cluster wells with or without 0.5 μM staurosporine to induce apoptosis. Untreated or apoptotic Neuro2A cells were washed several times, and then plated with effector cells at 1 : 10 E : T. Red fluorescent polystyrene microspheres (1.0 μm) (Invitrogen) were washed and plated on BV2 cells at a 1 : 50 E : T. Plates were spun for 3 min at 400 *g* and incubated for ≥ 1 h at 37°C. Cells were washed thrice with ice-cold PBS and harvested with 0.25% trypsin. Cells were immediately transferred into ice-cold flow cytometry buffer (PBS, 0.02% azide, and 1% FCS) and kept on ice. In all samples, BV2 cells were distinguished from neuronal cells and beads by anti-CD11b APC antibody (eBioscience), and histogram gates for $CD11b^+$ cells were drawn based on effector cells cultured without target cells (not shown). Phagocytosis was quantified as follows: (percent CM-DiI^+ or red effector cells − percent of CM-DiI^+ or red effector cells treated with cytochalasin D)/percent CM-DiI^+ or red effector cells) ± SEM. Statistical significance was determined using unpaired two-tailed Student's *t*-test and PRISM software.

Semi-quantitative real-time PCR

Adult murine microglia were sorted by flow cytometry for $CD45^{lo}CD11b^+$ parameters. Total RNA was isolated by using TRIzol (Invitrogen), and cDNA was synthesized using Superscript III reverse transcriptase with oligo dT primers (Invitrogen). Amplification of TREM2 cDNA used the following primers: 5′-GCACCTCCAGGAATCAAGAG-3′, 5′-GGGTCCAGTGAGGAT-CTGAA-3′. For glyceraldehyde 3-phosphate dehydrogenase (GAPDH) primers were: 5′-ATTCAACGGCACAGTCAAGG-3′, 5′-TGGTTCACACCCATCACAAA-3′. PCR was performed using an ABI 7500 (Applied Biosystems, Foster City, CA, USA) real-time PCR machine. SYBR green (New England Biolabs, Ipswich, MA, USA) was used to quantify the amplifications, and levels of TREM2 transcripts were normalized to GAPDH controls.

Results

Neurons express ligands for TREM2 that are increased by apoptosis

Receptors and ligands involved in phagocyte recognition of apoptotic cells are still being unveiled. Because TREM2 on microglia has been shown to be important for the phagocytosis of apoptotic neurons (Takahashi *et al.* 2005), we tested the hypothesis that TREM2 directly recognizes a ligand on neurons that facilitates engulfment. To address this, we studied the neuronal cell line, Neuro2A and primary cultured embryonic mouse cortical and VMN. We first examined these cells for the expression of TREM2-L by staining them with a TREM2-Fc fusion protein or, as a control, a TREM1-Fc control fusion protein. The chimeric proteins consist of the extracellular domains of the TREM receptor fused to the Fc domain of human IgG1, mutated to reduce binding to Fc receptors. By cytochemistry, staining with these soluble receptors demonstrated that both Neuro2A cells and fresh neuronal cells bind to TREM2-Fc but not TREM1-Fc (Fig. 1). Microgliosis has been implicated in the pathogenesis of Parkinson's disease, a neurological disorder characterized by degeneration of TH^+ dopaminergic neurons in the substantia nigra pars compacta (Minghetti 2005; Block *et al.*

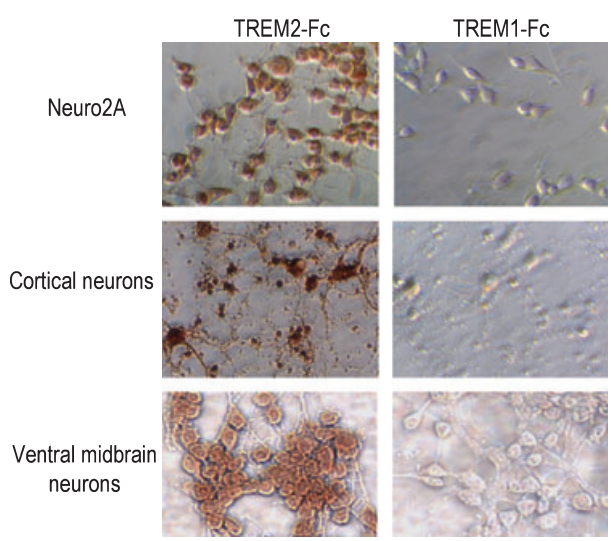


Fig. 1 Neuronal cells express ligands for TREM2. Neuro2A cells (top), primary cortical neurons (middle), and primary ventral midbrain neurons (bottom) were cultured and stained for TREM2-L expression by cytochemistry, using a soluble TREM2-Fc fusion protein. All neuronal cells examined bound soluble TREM2 (brown), but not TREM1 (right column). Data are representative of at least three experiments for each cell type.

2007). To specifically pursue the possibility that dopaminergic neurons might communicate with microglia through TREM2, we assessed whether dopaminergic neurons in ventral midbrain cultures expressed TREM2-L. By fluorescence microscopy, TREM2-L was detected on all VMN, including the TH^+ neurons (data not shown). These data suggest that multiple cultured neuronal cells express a potential ligand for TREM2.

To assess the effects of apoptosis on the expression of TREM2-L by neuronal cells, we used flow cytometry to stain Neuro2A cells with soluble TREM2-Fc before and after induction of apoptosis by MPP^+ or staurosporine. Consistent

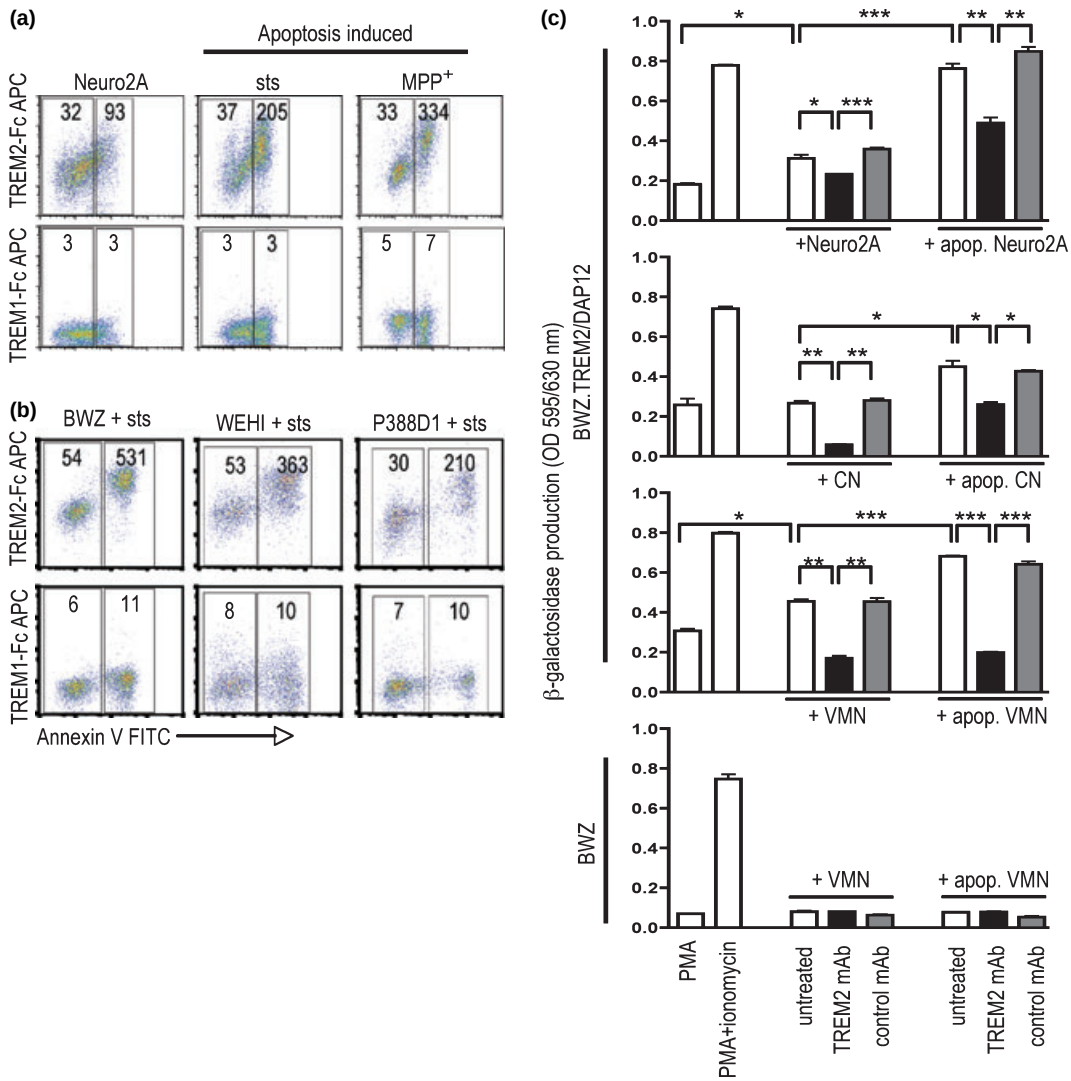


Fig. 2 Apoptosis increases the expression of functional TREM2-L on neuronal and non-neuronal cells. (a) TREM2-L expression was quantified by flow cytometry before (left) and after (right) induction of apoptosis in Neuro2A cells by staurosporine (sts) or MPP^+. The median fluorescence intensity of TREM2-Fc or TREM1-Fc binding of each gate is shown. Apoptotic Neuro2A cells (Annexin V^{hi}) express 3- to 10-fold greater levels of TREM2-L compared with Annexin V^{lo} Neuro2A cells, but do not express TREM1-L ($n = 7$). (b) TREM2-L expression is increased on non-neuronal cells during apoptosis. BWZ, WEHI-231, and P388D1 cells bind to TREM2-Fc, but not TREM1-Fc. Treatment with staurosporine boosts TREM2-Fc binding to Annexin V^{hi} cells by 7- to 10-fold ($n = 3$). (c) Neuronal cells activate TREM2/DAP12 signal transduction as assessed by BWZ.TREM2/DAP12 reporter cells. Healthy Neuro2A cells induce a modest level of cellular activation in the BWZ.TREM2/DAP12 cell line, and this is increased to near-maximal levels when the Neuro2A are treated with MPP^+ to induce apoptosis (top panel). Pre-treating the BV2 cells with a blocking TREM2 mAb (black bars), but not with a control rat IgG1 mAb (gray bars), significantly reduces cellular activation in response to both untreated and apoptotic Neuro2A cells. Ventral midbrain neurons (VMN) elicit similar responses (third panel), while cortical neurons (CN) demonstrate little stimulation unless they are apoptotic (second panel). BWZ.TREM2/DAP12 reporter cell activation in response to primary neurons was effectively blocked with the TREM2 mAb. None of the neuronal cells activate the parental BWZ reporter cell line (representative results for VMN are shown in the bottom panel); $^*p < 0.05$, $^{**}p < 0.005$, and $^{***}p < 0.0005$.

with our cytochemistry data, Neuro2A cells bound TREM2-Fc but not TREM1-Fc (Fig. 2a). Notably, induction of apoptosis in Neuro2A cells with MPP^+ or staurosporine resulted in a 5- to 10-fold increase in the median fluorescence intensity of TREM2-L expression on the Annexin V^{hi} cells (Fig. 2a). These data indicate that apoptosis increases the expression of TREM2-L on neurons, presenting a potential mechanism for enhancing their clearance by $TREM2^+$ microglia.

TREM2-L is also increased on non-neuronal apoptotic cells

Ligands for TREM2 are not exclusively expressed on neurons. We have previously shown that mouse TREM2 binds to human astrocytoma cell lines, and our colleagues have previously reported potential TREM2-L expressed on macrophages (Daws *et al.* 2003; Hamerman and Lanier 2006). To test whether cells other than neurons also have enhanced TREM2-L expression during apoptosis, we induced cell death in multiple murine cell lines using staurosporine. Untreated BWZ thymoma cells, WEHI-231 B cell lymphoma cells, and P388D1 macrophage-derived cells all expressed low levels of TREM2-L on the Annexin V-negative population (Fig. 2b). Similar to the Neuro2A cells, administration of staurosporine to BWZ, WEHI-231, or P388D1 cells heightened their binding of TREM2-L by 7- to 10-fold, while TREM1-Fc binding remained unaffected (Fig. 2b). Other murine cell lines such as B16s melanoma and RAW 264.7 macrophages were also tested and showed similar results (not shown). These data suggest that up-regulation of TREM2-L during apoptosis is a common cellular phenomenon.

TREM2-L on neuronal cells activate the TREM2/DAP12 receptor complex

To determine if TREM2-L on neuronal cells can functionally engage TREM2 and initiate intracellular signaling, we utilized a TREM2 reporter cell line. This was constructed from BWZ cells, a thymoma expressing the gene for β-galactosidase under the control of multiple copies of the NFAT promoter element (Sanderson and Shastri 1994). We expressed both TREM2 and DAP12 in this line (BWZ.TREM2/DAP12 cells), anticipating that functional perturbation of TREM2 by ligands would lead to the phosphorylation of DAP12, and the consequent activation of the NFAT reporter, and production of β-galactosidase.

We first assessed stimulation of the BWZ.TREM2/DAP12 reporter line by healthy or apoptotic Neuro2A cells. Untreated Neuro2A cells stimulated the BWZ.TREM2/DAP12 reporter cell above the PMA alone control (Fig. 2c). Strikingly, however, apoptotic Neuro2A cells stimulated the reporter cell line much more, to a level comparable to maximal excitation by PMA and ionomycin, whether cell death was induced by the MPP^+ neurotoxin or by serum-starvation (apoptotic vs. untreated Neuro2A, $p = 0.0001$). This response was specifically mediated by TREM2 as assessed by two means. First, BWZ cells lacking TREM2 and DAP12 did not respond to healthy or apoptotic neuronal cells. Second, stimulation of the TREM2/DAP12 reporter cells by Neuro2A or apoptotic Neuro2A cells was partially blocked by one of our anti-TREM2 mAb (Clone 78.18) (black bars), but stimulation was unaffected by an isotype control mAb (rat IgG1) (gray bars) (p-values of blockade < 0.05). Stimulation of the reporter cells with the anti-TREM2 antibody alone did not induce activation (not shown). We also attempted to block TREM2 by using other anti-TREM2 mAbs in our panel (data not shown), but only Clone 78.18 inhibited TREM2 activation. These results suggest that the 78.18 mAb may specifically block a binding site engaged by TREM2-L. Alternatively, this mAb may inactivate TREM2 in a manner not mimicked by our other mAbs.

We next tested whether primary neurons, particularly apoptotic primary neurons, could also activate TREM2. VMN activated TREM2 and this activity was fully impaired by the anti-TREM2 mAb (Fig. 2c). Healthy cortical neurons had less effect on TREM2 stimulation, although activation was again completely inhibited with the anti-TREM2 mAb ($p < 0.005$) (Fig. 2c). Like apoptotic Neuro2A cells, apoptotic primary neurons, either cortical or from the ventral midbrain, more effectively activated the TREM2/DAP12 reporter cells, and this activation was fully impaired by the anti-TREM2 mAb ($p < 0.05$) (Fig. 2c). None of the neuronal cells activated the parental BWZ cell line (Fig. 2c, results for VMN are shown). In addition, reporter cell activation required cell–cell contact, because supernatants from apoptotic neuronal cells did not activate the BWZ.TREM2/DAP12 reporter (not shown). Thus, apoptotic neuronal cells bind to TREM2, and they activate signal transduction through the TREM2–DAP12 complex.

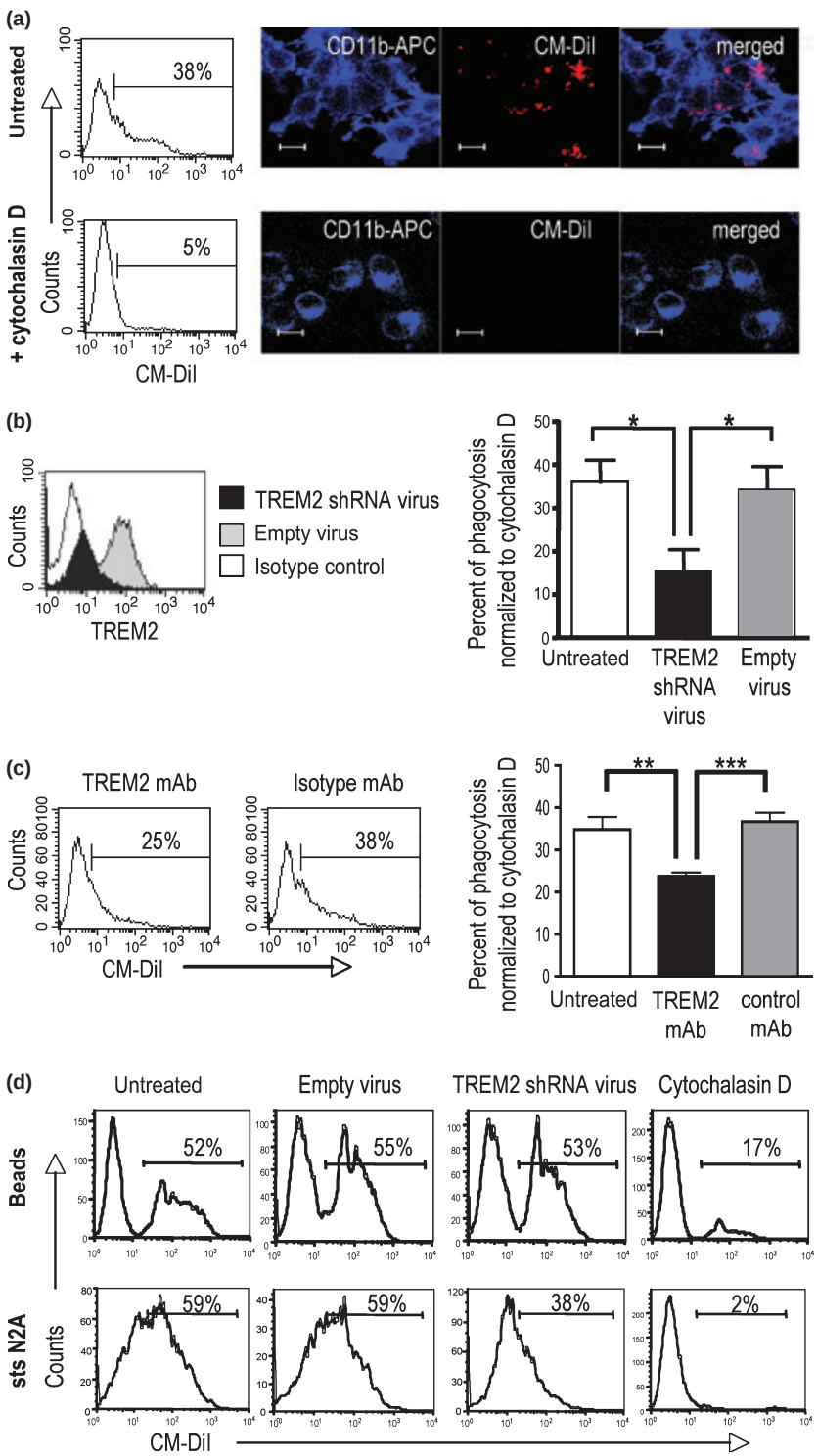
Phagocytosis of apoptotic neuronal cells is inhibited by antibody to TREM2

As a model for microglial phagocytosis of neurons, we studied the uptake of Neuro2A neuroblastoma cells by BV2 murine microglial cells. For these studies, Neuro2A cells were labeled with the red fluorescent dye CM-DiI, and apoptosis was initiated by treatment with staurosporine. After 1 h, about 30% of the BV2 microglial cells engulfed fluorescent material from the Neuro2A cells as detected by flow cytometry (Fig. 3a). This did not reflect non-specific binding, because pre-treatment of the BV2 cells with 2 μM cytochalasin D, a cytoskeletal inhibitor, abrogated phagocytosis. To confirm by microscopy the uptake of fluorescent cell particles by BV2 microglia (labeled with CD11b-APC, blue), confocal images of single optical sections were taken of the effector cells following coincubation with CM-DiI-labeled Neuro2A cells at a 1 : 10 E : T ratio. Untreated BV2 cells showed uptake of $CM\text{-}DiI^+$ particles, but BV2 cells treated with cytochalasin D did not (Fig. 3a). Using sections of < 1 μm transversing the nuclei, the neuronal particles were intracellular and they were not

removed by washing, evidence that they did not represent particles on the cell surface.

It has previously been shown that TREM2 expression on microglia promotes phagocytosis (Takahashi *et al.* 2005). To confirm this in our system, BV2 microglial cells were transduced with shRNA targeted for TREM2, which reduced surface expression of TREM2 by 50–84% as detected by flow cytometry (Fig. 3b, left), and it reduced phagocytosis compared with empty virus infected BV2 cells (Fig. 3b, right) ($n = 6$).

To determine if the requirement for TREM2 in phagocytosis reflects engagement of the extracellular TREM2 domain



with putative TREM2-L on neuronal cells, we first performed phagocytosis assays using BV2 microglial cells in the presence of the blocking TREM2 mAb or with an isotype control mAb. BV2 cell uptake of CM-DiI-labeled apoptotic Neuro2A cells was diminished in the presence of the TREM2 mAb, but not with the isotype control (Fig. 3c). Representative flow cytometric histograms are shown (Fig. 3c, left), and a summary of multiple experiments ($n = 6$) examining the effect of blocking TREM2 and TREM2-L interactions on phagocytosis is also shown (Fig. 3c, right). Untreated BV2 cells or isotype control BV2 cells showed comparable levels of phagocytosis, 39 ± 3% and 37 ± 2%, respectively. Pretreatment of the BV2 cells with the anti-TREM2 blocking mAb decreased the number of cells in which phagocytosis could be detected to 24 ± 1%, which is 38% less than untreated cells and 35% less than cells treated with control mAb ($p = 0.005$ and 0.0002, respectively). Blockade of phagocytosis by an anti-TREM2 mAb supports the hypothesis that direct recognition by TREM2 of its ligands on neuronal cells is important for efficient phagocytosis by microglial cells. Blockade of phagocytosis by the anti-TREM2 mAb is incomplete, however. This may be partially explained by the inability of the antibody to completely block the activation of TREM2 by its ligands on Neuro2A cells as shown in the reporter cell assays (Fig. 2c). Alternatively, other interactions may contribute to microglial phagocytosis of apoptotic neuronal cells in a manner that is independent of TREM2.

To test whether TREM2 expression directs only clearance of apoptotic cells or whether it broadly enhances phagocytosis, we performed phagocytosis assays examining the uptake of red fluorescent microspheres by BV2 cells with and without TREM2. TREM2 expression in BV2 cells was inhibited by using the TREM2 shRNA lentivirus. In contrast to the reduction in phagocytosis of apoptotic neurons (bottom row), the phagocytosis of microspheres at an E : T of 1 : 50 (top row) remained unchanged in the same experiments following reduction of TREM2 expression (Fig. 3d). Phagocytosis of beads was also examined at 1 : 80, 1 : 100, and 1 : 250 ratios with similar results (data not shown). Thus, TREM2 is not essential for all phagocytosis but is important for the efficient clearance of apoptotic neurons.

TREM2 is sufficient to induce phagocytosis of apoptotic neuronal cells by Chinese hamster ovary cells

CHO cells do not express known phagocytic receptors, and gene transfection into these cells has been used to demonstrate the engulfment activity of phagocyte receptors, such as CR3 and FcR gamma (Nagarajan *et al.* 1995; Le Cabec *et al.* 2002). To determine whether the presence of TREM2 is sufficient for phagocytosis of neuronal cells, we analyzed the phagocytic activity of CHO cells that had been transfected to express either TREM2 or TREM1. In this system, TREMs are directly coupled to the cytoplasmic domain of DAP12, and these TREM/DAP12 chimeric receptors were previously shown to permit TREM-mediated signaling through DAP12 (Hamerman *et al.* 2006). The TREM2-transfected CHO cells have recently been used to demonstrate that TREM2 is sufficient to bestow CHO cells with the ability to internalize bacteria (N'Diaye *et al.* 2009). We therefore tested whether the over-expression of TREM2 or TREM1 in CHO cells would similarly confer phagocytic activity of apoptotic neuronal cells. The plasmids also express GFP, and GFP expression was a faithful marker of TREM receptor expression, as both the CHO.TREM2/DAP12 and CHO.TREM1/DAP12 cell lines expressed their respective receptor on cells gated for GFP (Fig. 4, left). Assessment of phagocytosis by CHO.TREM2/DAP12, CHO.TREM1/DAP12, and the parental CHO cell line for engulfment of apoptotic Neuro2A cells demonstrated that

Fig. 3 TREM2/TREM2-L interactions are important for phagocytosis, and TREM2 is required for efficient phagocytosis of apoptotic neuronal cells but not of beads. (a) Phagocytosis of apoptotic Neuro2A cells (> 40% Annexin V^{hi}) by BV2 microglial cells cocultured at an E : T of 1 : 10 as assessed by flow cytometry (left column) or fluorescence confocal microscopy (right panels). Histograms indicate the percent of BV2 cells that have internalized CM-DiI-labeled staurosporine-treated Neuro2A cells during 1 h assays. Images of single optical sections (< 1 μm) were obtained by confocal microscopy with a 60× magnification lens of BV2 cells labeled with an APC-conjugated anti-CD11b mAb (blue) and cocultured with CM-DiI^{+} apoptotic Neuro2A cells (red). Images indicate uptake of neuronal debris by BV2 cells. Phagocytosis is inhibited by cytochalasin D in both assays. Scale bars, 10 μm. (b) Phagocytosis of apoptotic Neuro2A cells is reduced in BV2 cells following lentiviral-mediated RNAi against TREM2. RNAi reduced the surface expression of TREM2 up to 84% as detected by flow cytometry (left). Quantification of 6 experiments shows that phagocytosis by BV2 cells deficient in TREM2 is reduced to 15 ± 5% (mean ± SEM) from 36 ± 5% for untreated BV2 cells and from 34 ± 5% for BV2 cells transduced with empty virus (*$p < 0.05$) (right). (c) A mAb to TREM2 partially but significantly inhibits phagocytosis of apoptotic Neuro2A cells by BV2 microglia. Representative flow cytometric histograms assessing phagocytosis in the presence of a blocking TREM2 mAb (Clone 78.18) or an isotype control mAb (rat IgG1) is shown (left). Summary of 6 experiments, showing a reduction to 23.7 ± 0.9% of effector cells engulfing targets compared with untreated (34.8 ± 2.9%) and control mAb (36.7 ± 2.1%) treated BV2 cells (right). The TREM2 mAb partially decreases phagocytosis by 32–35%. (**$p \leq 0.005$ and ***$p \leq 0.0005$). (d) Reduction of microglial TREM2 by RNAi does not reduce BV2 phagocytosis of microspheres (here at 1 : 50 E : T ratio, top row), although the same cells again show a loss of phagocytosis of apoptotic Neuro2A cells at 1 : 10 E : T (sts N2A, bottom row) during 1 h assays. BV2 cells were left untreated or subjected to cytochalasin D or infected with TREM2 shRNA or empty virus. Representative flow cytometric histograms are shown ($n = 2$).

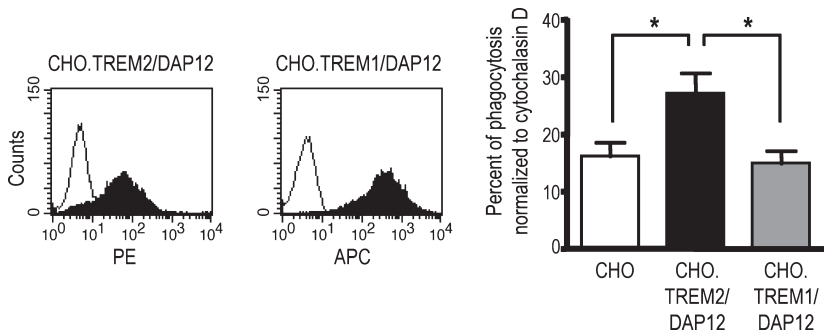


Fig. 4 TREM2 is sufficient to confer phagocytosis of apoptotic Neuro2A. CHO cells stably transfected to express TREM2/DAP12 or TREM1/DAP12 chimeras were assessed for their phagocytosis of apoptotic Neuro2A cells. Flow cytometry confirmed that the transfected CHO cells express either TREM2 (filled histogram, left) or TREM1 (filled histogram, middle) on cells gated for GFP, while isotype controls (open histograms) did not bind to the CHO cells. The expression of TREM2/DAP12 (black bar) was sufficient to increase phagocytosis of apoptotic Neuro2A cells by 1.7-fold over untransfected cells CHO cells (white bar), while expression of TREM1/DAP12 did not (gray bar) ($n = 8$, $^*p < 0.05$).

expression of TREM2/DAP12 increased phagocytosis, but expression of TREM1/DAP12 did not (Fig. 4, right). These findings strongly support the hypothesis that a direct interaction between TREM2 and ligands on neuronal cells mediates phagocytosis.

TREM2 is found only on microglia in the normal murine CNS

The cellular profile of TREM2 expression is important in understanding both the role of TREM2 in the CNS, and the defects leading to Nasu-Hakola disease. All studies of microglia confirm the expression of TREM2 on at least a portion of microglia *in vivo* (Schmid *et al.* 2002) and *in vitro* (Takahashi *et al.* 2005; Piccio *et al.* 2007). Some studies, however, have also found expression of TREM2 on cortical neurons and oligodendrocytes (Sessa *et al.* 2004; Kiialainen *et al.* 2005). Having shown that neurons express a ligand for TREM2, we therefore examined both cell lines and tissue sections to determine whether neurons or other cells in the CNS indeed also expressed the TREM2 receptor.

Semi-quantitative real-time PCR analysis for TREM2 transcripts was performed on Neuro2A cells, BV2 cells, and fresh adult murine microglia sorted to 99% purity for $CD45^{lo}CD11b^{+}$ parameters. Neuro2A cells did not express TREM2, while BV2 cells and primary microglia expressed abundant amounts of TREM2 transcript (Fig. 5a).

TREM2 protein expression in the CNS *in vivo* was determined by histological analysis of fresh-frozen healthy wildtype C57BL/6 adult mouse brain sections using a panel of specific rat anti-mouse TREM2 mAbs. Of 123 $CD11b^{+}$ cells examined, 111 (90%) expressed detectable TREM2 and these were the only $TREM2^{+}$ cells found. TREM2 (Fig. 5b, red) was found to colocalize with $CD11b^{+}$ (green) cells in the cortex, hippocampus (CA1 and dentate gyrus regions), putamen, and spinal cord, but TREM2 did not colocalize with neuronal soma that stained with an antibody against the neuronal-specific nuclei (NeuN) marker (blue). Thus, of 349 $NeuN^{+}$ neurons analyzed none of them expressed detectable TREM2.

To examine brain cells for surface expression of TREM2, we used flow cytometry to stain isolated neonatal brain cells with anti-TREM2 mAbs. Neonatal brain cells were examined because microglia can be more easily cultured from neonatal brains, and because of evidence that DAP12 and possibly TREM2 may play an important role in the developing CNS (Wakselman *et al.* 2008). To facilitate the identification of microglia, and to isolate microglia without the use of antibodies that could affect the cells, we used $CX3CR1^{GFP/+}$ mice in which one of the genes for CX3CR1 (also known as the fractalkine receptor) is replaced by the gene for green fluorescence protein (GFP). It has previously been shown in these mice that all microglia coexpress functional CX3CR1 and GFP (Jung *et al.* 2000). Cultured neonatal microglia (GFP^{+} $CD11b^{+}$) expressed low, but detectable levels of TREM2 on the surface (Fig. 5c, right). Notably, the expression of TREM2 showed a single peak, i.e. TREM2 did not distinguish two or more distinct populations of microglia, although the lower end of the TREM2 expression profile was not distinguishable from background. TREM2 expression was clearly not found on the non-microglial cells, which include astrocytes. Staining of TREM2 on cultured microglia was also confirmed by using a different anti-TREM2 mAb (Clone 150.1) and the expression profiles were nearly identical (not shown). To confirm the expression profile of TREM2 on cultured microglia, GFP^{+} cells from $CX3CR1^{GFP/+}$ mice were sorted by flow cytometry, as gated in Fig. 5c, allowed to adhere to coverslips overnight, and examined by immunofluorescence confocal microscopy for TREM2 expression. In accord with the results obtained by flow cytometry, most neonatal microglia had detectable TREM2 on the surface, and on some this was relatively abundant (Fig. 5d). Although TREM2 protein was expressed

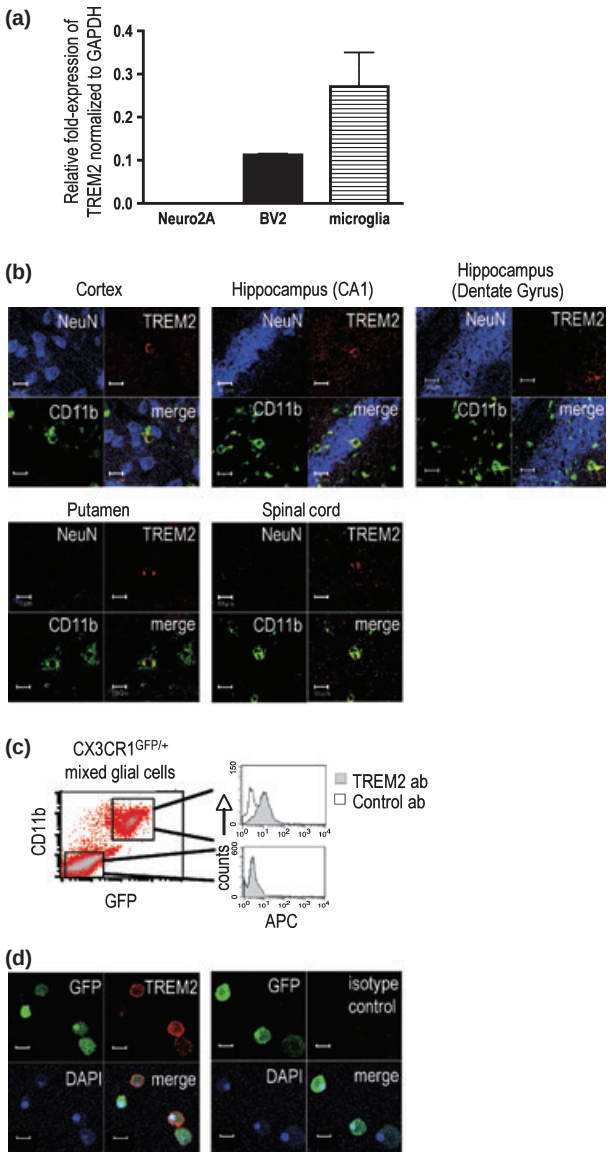


Fig. 5 TREM2 is not expressed by normal adult neurons but is expressed by adult and neonatal microglia. (a) Semi-quantitative RT-PCR analysis for TREM2 using cDNA from Neuro2A neuroblastoma cells, BV2 microglial cells, and sorted adult microglia ($CD45^{lo}$ $CD11b^{+}$). TREM2 transcript levels are normalized to GAPDH RNA levels. Transcripts for TREM2 are not found in Neuro2A cells, but are strongly expressed in BV2 cells and microglia. (b) Immunofluorescence confocal microscopy of histologic sections from brains of normal adult C57BL/6 mice to determine TREM2 expression in the brain. Single optical sections of < 1 μm were imaged at 60× magnification from the cortex, hippocampus (CA1 and dentate gyrus regions), putamen, and spinal cord tissues. A TREM2 mAb cocktail (red) colocalized with CD11b (green) on microglia, but not with neuronal soma as detected by an antibody to NeuN (blue) in neurons. Scale bars, 10 μm. (c) Flow cytometric analysis of TREM2 on cultured neonatal microglia. Mixed glial cells were cultured from $CX3CR1^{GFP/+}$ mice. TREM2 is expressed on microglia ($GFP^{+}CD11b^{+}$), but not on non-microglial (GFP^{-} $CD11b^{-}$) cells, which include astrocytes. (d) Immunofluorescence confocal microscopy images of neonatal microglia isolated from $CX3CR1^{GFP/+}$ mice and sorted for GFP. TREM2 is detected on nearly all microglia, although the expression level varies from one small patch of TREM2 on the cell membrane to expression around the entire cell membrane. Single optical sections (< 1 μm) at 60× magnification were imaged. Scale bars, 10 μm.

along the entire cell membrane on a majority of the microglia, on a portion of the cells TREM2 was detectable only in patches. Almost all cultured neonatal microglia express some level of TREM2 on the cell surface.

Discussion

In microglia, it has been previously demonstrated that TREM2 promotes phagocytosis of apoptotic neurons without up-regulation of antigen presentation molecules or tumor necrosis factor-α transcripts, but a role for target recognition by TREM2 had not been elucidated (Takahashi *et al.* 2005). Thus, TREM2 might have bound to a stimulatory molecule on the microglia themselves, or may simply have facilitated the recruitment of DAP12 to a signaling complex during phagocytosis. In contrast, our current studies provide compelling evidence that microglial phagocytosis of apoptotic neurons involves direct recognition by TREM2 on microglia with ligands that are up-regulated on apoptotic neurons. When neuronal cells undergo apoptosis, they increase the expression of TREM2-L with a corresponding increase in their phagocytosis by BV2 cells, which is blocked at least in part by our antibody to TREM2. The up-regulation of TREM2-L on apoptotic neurons appears to reflect a phenomenon that is generalizable to multiple cell types; conditions that induce apoptosis, as assessed by staining with Annexin V, increase binding by soluble TREM2 5- to 10-fold. TREM2 may thus be generally important in clearing apoptotic cells.

The engulfment of apoptotic cells is essential in the CNS to clear cell debris without eliciting an inflammatory response (Ravichandran and Lorenz 2007; Napoli and Neumann 2009). The up-regulation of TREM2-L on apoptotic neuronal cells provides a means by which microglia can be directed to the phagocytic removal of these cells, and when this interaction is blocked with an anti-TREM2 mAb, phagocytosis is diminished. TREM2-L were also found at lower levels on non-apoptotic cultured neuronal cells, but we could not detect TREM2-L *in vivo* on neurons in tissue sections from healthy adult mice (not shown) suggesting that the mere stress of being in culture may be enough to up-regulate low levels of TREM2-L on neurons. Regardless, apoptotic cells expressed much more TREM2-L and they more effectively activated signaling through TREM2/DAP12.

Although TREM2 is important for phagocytosis of apoptotic neurons, TREM2 is not likely to be the only

engulfment receptor on microglia that can recognize injured neurons. Other phagocyte receptors that may be involved include CD36, receptors for phosphatidylserine, and/or the vitronectin receptor ($\alpha_v\beta_3$ integrin) (Savill *et al.* 2002). Interestingly, a recent report indicates that another member of the TREM receptor family, TREM-like 4, also recognizes apoptotic cells (Hemmi *et al.* 2009).

In our studies, transfection of TREM2 into CHO cells conferred phagocytic capacity for apoptotic cells while TREM1 did not, strongly supporting the evidence that recognition of apoptotic cells by TREM2 directly activates phagocytosis. Moreover, in BV2 microglia, while loss of TREM2 led to reduced phagocytosis of apoptotic cells, it did not lead to reduced phagocytosis of microbeads, indicating that TREM2 does not non-specifically promote phagocytosis.

The ligands on apoptotic cells that are recognized by TREM2 are unknown. We have previously shown that TREM2 also binds broadly to bacteria and that this binding is inhibited by anionic glycans (Daws *et al.* 2003). We hypothesize that TREM2 binds to glycans on both bacteria and apoptotic cells. In this regard, it would be similar to other pattern recognition receptors, such as the mannose receptor, the Siglec receptors, and toll-like receptor 4, all of which recognize ligands on pathogens as well as endogenous ligands (Akira and Hemmi 2003; Allavena *et al.* 2004; Crocker *et al.* 2007).

Our studies also further clarify the expression of TREM2 on mouse microglia. TREM2 is selectively expressed by microglia *in vivo* in the normal adult mouse brain in multiple regions of the CNS, including the cortex, hippocampus, spinal cord, and putamen, but TREM2 is not expressed by neurons in the adult mouse. Our data are thus consistent with findings from others that TREM2 is expressed on microglia, but we did not find evidence for its presence in neurons. It has previously been suggested that TREM2 marks a subset of microglia with variation in brain regions as determined at the RNA level by *in situ* hybridization of TREM2, which costained with tomato lectin, a protein that binds both microglia and blood vessels (Schmid *et al.* 2002). The highest previously reported percent of murine $TREM2^+$ microglia *in vivo* was $57 \pm 5.8\%$ in the cortex (Schmid *et al.* 2002). Our studies using a cocktail of specific TREM2 mAbs, which do not bind myeloid cells derived from TREM2 knockout mice, suggest that TREM2 is expressed in the majority ($\sim$90%) of $CD11b^+$ microglia *in vivo* at the protein level, though the expression levels may vary. *In vitro*, nearly all cultured microglia from neonatal mice expressed low levels of TREM2 on the surface.

The expression of TREM2 on microglia is in contrast to circulating monocytes on which TREM2 is not detected. It is consistent, however, with evidence that TREM2 is expressed by tissue macrophages as well as by other cells of the monocyte/macrophage lineage, including immature dendritic cells and osteoclasts (Bouchon *et al.* 2001; Colonna 2003; Turnbull *et al.* 2006).

Macrophages have been classified into at least two main subtypes. Classical (M1) macrophages develop in response to cytokines that promote Th1 immune responses while the development of alternatively activated (M2) macrophages is promoted by the Th2 cytokines interleukin-4 and -13 (Gordon 2003). M1 macrophages are proinflammatory while M2 macrophages inhibit inflammation and instead promote tissue repair in part through the phagocytosis of apoptotic cells. It is tempting to suggest that $TREM2^+$ microglia in their resting state are similar to alternatively activated macrophages and are already poised to respond to TREM2-L. The failure of this recognition may impair the clearance of neuronal debris by microglia, resulting in the degenerative brain disease, Nasu-Hakola disease.

The recognition of TREM2-L on neuronal cells may also regulate microglial functions in addition to phagocytosis. In this regard, blockade of TREM2 has been shown to exacerbate EAE, while infusion of $TREM2^+$ cells improves it (Piccio *et al.* 2007; Takahashi *et al.* 2007). Inflammation contributes not only to multiple sclerosis, but also to Alzheimer's disease and Parkinson's disease. With regard to Alzheimer's disease, TREM2 was found to be specifically up-regulated in amyloid plaque-associated microglia in amyloid precursor protein 23 transgenic mice (Frank *et al.* 2008). Further studies are warranted to examine the function of microglia, TREM2, and TREM2-L in Alzheimer's disease.

TREM2 and TREM2-L join other receptor/ligand pairs that mediate crosstalk between microglia and neurons. Neurons express CD200, which tonically inhibits microglia through interaction with its receptor, CD200R. Mice deficient in CD200 have augmented microglial responses following transection of the facial nerve, and they have accelerated onset of EAE (Hoek *et al.* 2000). Similarly, microglia are inhibited by the fractalkine receptor, CX3CR1, through the expression and release of fractalkine by neurons, and mice lacking CX3CR1 have increased neuronal loss in Parkinson's disease and amyotrophic lateral sclerosis (Cardona *et al.* 2006). While neurons are capable of inhibiting microglia through CD200R and CX3CR1, apoptotic neurons may engage TREM2 to influence microglial differentiation towards an 'alternative' phenotype that facilitates phagocytosis of neurons. Interestingly, DAP12, the adapter protein associated with TREM2, has been implicated in microglia-mediated neuronal cell death in the developing hippocampus exclusively in postnatal days 1–2 mice (Wakselman *et al.* 2008). TREM2 and DAP12 may thus provide important functions at different developmental stages as well as during disease states.

In sum, our data suggest that TREM2 is a phagocyte receptor that is stimulated by an unknown 'eat-me' signal on

apoptotic neurons. This unknown signal may be commonly expressed on all apoptotic cells. The nature of the ligands for TREM2, however, has not yet been identified. Characterization of TREM2-L will greatly facilitate studies about TREM2 and its role in the CNS. In the meantime, understanding the mechanisms by which TREM2-L regulate microglial activity may prove important to ameliorate neurodegenerative diseases and brain injury.

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