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Preparation, Crystallization, and Preliminary Crystallographic Analysis of Wild-type and Mutant Human TREM-2 Ectodomains Linked to Neurodegenerative and Inflammatory Diseases

Daniel L. Kober^{1,2}, Kelsey M. Wanhainen², Britney M. Johnson³, David T. Randolph², Michael J. Holtzman^{2,5,6}, and Tom J. Brett^{2,4,5,6}

¹Molecular Microbiology and Microbial Pathogenesis Program, Washington University School of Medicine, St. Louis, Missouri 63110

²Department of Internal Medicine, Washington University School of Medicine, St. Louis, Missouri 63110

³Biochemistry Program, Washington University School of Medicine, St. Louis, Missouri 63110

⁴Biochemistry and Molecular Biophysics, Washington University School of Medicine, St. Louis, Missouri 63110

⁵Cell Biology and Physiology, Washington University School of Medicine, St. Louis, Missouri 63110

⁶Drug Discovery Program in Pulmonary and Critical Care Medicine, Washington University School of Medicine, St. Louis, Missouri 63110

Abstract

TREM-2 (triggering receptor expressed on myeloid cells- 2) is an innate immune receptor expressed on dendritic cells, macrophages, osteoclasts, and microglia. Recent genetic studies have reported the occurrence of point mutations in TREM-2 that correlate with a dramatically increased risk for the development of neurodegenerative diseases, including Alzheimer's disease, frontotemporal dementia, and Parkinson's disease. Structural and biophysical studies of wild-type and mutant TREM-2 ectodomains are required to understand the functional consequences of these mutations. In order to facilitate these studies, we undertook the production and crystallization of these proteins. Here we demonstrate that, unlike many single Ig domain proteins, TREM-2 could not be readily refolded from bacterially-expressed inclusion bodies. Instead, we developed a mammalian-cell based expression system for the successful production of wild-type and mutant TREM-2 proteins in milligram quantities and a single-chromatography-step purification scheme that produced diffraction-quality crystals. These crystals diffract to a resolution of 3.3 Å and produce data sufficient for structure determination. We describe herein the procedures to produce wild-type and mutant human TREM-2 Ig domains in sufficient quantities for structural and biophysical studies. Such studies are crucial to understand the functional consequences of TREM-2 point mutations linked to the development of neurodegenerative diseases and, ultimately, to develop patient-specific molecular therapies to treat them.

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Address correspondence to: Tom J. Brett, PhD., Campus Box 8052, 660 S. Euclid, St. Louis, MO 63110. Phone: (314) 747-0018; Fax: (314) 362-8987; (tbrett@wustl.edu).

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Introduction

The molecular mechanisms contributing to age-related neurodegenerative disease pathogenesis are not well understood. Genetic studies released within the last year have identified specific mutations in *TREM2* that are linked to a significantly increased risk for the development of late-onset Alzheimer's Disease (AD) [1, 2]. The greatest risk is associated with the point mutant R47H, and is roughly equivalent to that associated with mutations in *APOE4*, currently the strongest known risk factor for late-onset AD [3]. This mutation has now been found in cohorts from Iceland [2], USA, Germany, Netherlands, Norway [1], Spain [4], and France [5]. Further, this mutation has recently been identified as a determinant in Parkinson's disease (PD) patients [6, 7]. Additional variants including D87N and T96K have been linked to AD development [1] and two other point mutations in *TREM2* (Y38C and T66M) have been linked to the development of frontotemporal dementia (FTD) [8–11]. These findings highlight a crucial homeostatic role for *TREM2* in maintaining neuronal health and represent the first molecular link between innate inflammatory signaling and neurodegenerative diseases [12]. However, it is currently unknown how these mutations affect *TREM2* function and thereby contribute to neurodegenerative disease pathogenesis.

The *TREM2* gene encodes the protein triggering receptor expressed on myeloid cells 2 (TREM-2). TREM-2 is an innate immune receptor expressed on dendritic cells, macrophages, osteoclasts, and microglia [13]. The protein has a minimal intracellular sequence and therefore traffics and signals through association with the adaptor protein DAP12 [14]. The ectodomain consists of a single Ig fold anchored to a transmembrane domain via a short stalk. The major role of the Ig domain is presumed to be ligand engagement, though the endogenous signaling ligand(s) of TREM-2 are uncertain. Several different types of ligands have been suggested, including various anionic bacterial carbohydrates [15], apoptotic cell membrane components [16], the immune receptor Plexin-A1 [17], and the chaperone Hsp60 [18]. A proper understanding of the function of TREM-2 has been constrained by a lack of structural information and the elusive nature of TREM-2 ligands.

A crucial role for TREM-2 in neuronal health was first suggested through association with neurodegenerative Nasu-Hakola disease (NHD) [19]. The major mutations associated with NHD produce premature termination of TREM-2 protein and are also phenocopied by deletion of DAP12. In contrast, all of the point mutations associated with AD, PD, and FTD are located within the Ig domain, suggesting these mutations alter function of full-length TREM-2. Proper understanding of how the newly identified point mutations of TREM-2 could modulate function and contribute to disease requires not only functional studies but also structural and biophysical comparisons of wild-type (WT) and mutant TREM-2 ectodomains. In order to facilitate this approach, we developed a mammalian cell-based expression and purification system to address the difficulties with generating TREM-2 protein suitable for structural and biophysical studies. Indeed, we were able to identify experimental conditions that produce crystals of human TREM-2 that diffract to 3.3 Å resolution and can be phased by molecular replacement. The studies outlined here provide a methodological framework for understanding the molecular basis of TREM-2 mutations and their contribution to the development of neurodegenerative diseases.

Materials and Methods

Expression constructs

For expression in *E. Coli*, the Ig domains of human TREM-2 (NCBI ref NP_061838; amino acids 19–134; MW_{calc}[with tag] = 14387 Da; pI_{calc} = 8.5) and mouse TREM-2 (NCBI ref NP_112544; amino acids 19–136; MW_{calc}[with tag] = 14440 Da; pI_{calc} = 8.4) were amplified using PCR and cloned into pET23b as tagless constructs using standard molecular biology techniques. Full-length human *TREM-2* in pMX-3p plasmid (a gift from Marco Colonna) and mouse *Trem-2* cDNA were used as PCR templates, respectively. Mammalian cell expression constructs of human TREM-2 wild-type, R47H mutant, and mouse TREM-2 Ig domains were produced similarly by cloning into the pHLSEC vector [20], which contains an optimized signal sequence and C-terminal 6-histidine tag for purification. All constructs were confirmed by sequencing.

Expression and purification of TREM-2 inclusion bodies in *E. Coli*

Human and mouse TREM-2 Ig domains in pET23b were transformed into Rosetta2 (DE3) cells (Novagen) for expression. Cultures were grown to O.D.₆₀₀ = 1.0, induced with 0.5 mM IPTG, and expressed with shaking at 37° C for 4 hours. Cells were then harvested by centrifugation and lysed in buffer containing 50 mM Tris pH 8.0, 12.5% sucrose, 50 mM NaCl, 0.5 mM EDTA, 0.5 % Triton X-100, and 10 mM DTT supplemented with lysozyme (0.5 mg/mL) and DNase. The inclusion bodies were harvested by centrifugation and washed 3 times in buffer containing 50 mM Tris pH 8.0, 100 mM NaCl, 1mM EDTA, 1mM DTT and 0.5% Triton X-100. A final wash without Triton X-100 was performed and the purified inclusion bodies were solubilized in 6M guanidine hydrochloride containing 20 mM β-ME and 50 mM Tris pH 8.0.

Refolding trials for human and mouse TREM-2 Ig domains

Initial refoldings were attempted by rapid dilution of the solubilized inclusion bodies into a buffer containing 100 mM Tris pH 8.5, 400 mM arginine, 2 mM EDTA, 5 mM reduced glutathione, and 0.5 mM oxidized glutathione, as similar conditions had previously been used to refold human and mouse TREM-1 [21–23], human TREM-like transcript 1 (TLT-1) [24], human immune receptor expressed on myeloid cells (IREM-1) [25], and human NK cell receptor NKp44 [26]. Solutions were initially clear upon addition of protein, but after about 1 hour the protein would precipitate. Subsequent workup revealed no properly folded soluble protein. Therefore we performed screening of refolding conditions. A refolding screen was developed based on the QuickFold Protein Refolding Kit (Athena Enzyme Systems) (Table 1). For this screen, 50 μL of each solubilized TREM-2 inclusion body (at 2 mg/mL) was added to 950 μL of each refolding condition, rocked overnight at 4° C, and then centrifuged at high speed to pellet insoluble protein. The presence of soluble protein in the supernatant was assessed by SDS-PAGE.

Expression and purification of TREM-2 Ig domains from mammalian cells

Wild-type human and mouse TREM-2 Ig domain and human R47H mutant were expressed in mammalian cells by large-scale transient transfection. Briefly, plasmid DNA (1 μg/mL final concentration) was diluted in Opti-MEM (Gibco) and mixed 1:2 with 25 kDa branched polyethylenimine chemically modified with trimethylene carbonate (termed PEI-TMC25) [27] (2 μg/mL final concentration) and allowed to form micellar complexes for 30 minutes. Transfection was achieved by adding these mixtures to FreeStyle 293F cells (Invitrogen) at a ratio of 1 μg DNA : 1×10⁶ cells. Cells were allowed to grow in serum-free 293 FreeStyle media (Invitrogen) with shaking in vented flasks at 37° C and 8% CO₂. The TREM-2 Ig domain has two conserved N-glycosylation sites (Fig. 1a). To facilitate removal of N-linked

glycans, kifunensine (Sigma; 1 μ g/mL), an inhibitor of α -mannosidase I, was added to the culture to inhibit maturation of N-linked glycans and leave them as high-mannose glycans that can be removed with EndoH [28]. Culture supernatants were collected at 72 and 144 hours post-transfection, modified by addition of 50 mM phosphate, 10 mM Tris pH 8.5, 300 mM NaCl, and 10 mM imidazole, and then purified using NiNTA-affinity chromatography. Proteins were washed on column using the same buffer containing 20 mM imidazole and eluted in 250 mM imidazole. After elution, EndoHf, (NEB), a fusion protein of EndoH and maltose binding protein, was used to deglycosylate TREM2 to homogeneity. This reaction cleaves the high-mannose glycan leaving a single glucose attached to the asparagine and was carried out by addition of 50 mM sodium citrate pH 5.5 and approximately 2000 units of EndoHf per mg of TREM-2 Ig domain. Reactions were incubated overnight at 25 °C. EndoHf was then removed by incubation with amylose resin for 1 hour at 4 °C, followed by pelleting of the resin by centrifugation and harvesting of the supernatant. The deglycosylated TREM-2 Ig domains were then exchanged into buffer containing 20 mM Hepes pH 7.5 and 150 mM NaCl with a concentrator (Vivaspin, Sartorius) for use in experiments. The resulting proteins were >90% pure as assessed by SDS-PAGE.

Crystallization, data collection, and initial crystallographic analysis

Human and mouse TREM-2 Ig domains at 10 mg/mL were screened for crystallization by hanging drop vapor diffusion. Initial hits were only obtained for human TREM-2 Ig domain. Optimal crystals were obtained when protein solution was mixed 1:1 with well solution containing 100 mM Tris pH 7.1, 2.1 M NaCl, 200 mM $MgCl_2$, 200 mM NDSB-201. Crystals appeared over the course of 2–5 days and had approximate dimensions of 300 $\times$ 300 $\times$ 50 μ m. For data collection, crystals were cryopreserved in reservoir solution containing 25% ethylene glycol and flash-frozen in a nitrogen stream at 100 °K. A native data set was collected at a wavelength of 1.0007 Å with 5 second exposure times and 1° oscillations per image at beamline ID19 (Advanced Photon Source, Argonne). Data were processed with HKL-2000 [29]. Scaling and analysis of systematic absences revealed the space group to be either P6₂22 of P6₄22. Molecular replacement was carried out in PHASER [30] using various similar single Ig domain probes including human TREM-1 (PDB ID: 1SMO) [22], mouse TREM-1 (1U9K) [21], human NKp44 (1HKF) [26], human TLT-1 (2FRG) [24], human IREM-1 (2NMS) [25], mouse CNRF35-like molecule 1 (CLM-1) (1ZOX), and ensembles of these structures. The best rotational and translational Z scores were obtained with mouse TREM-1 (1U9K). The translational search revealed the correct space group to be P6₄22. Two copies of the probe were placed in the asymmetric unit (ASU). Data collection and molecular replacement statistics are provided in Table 2. Patterson and self-rotation function were calculated with GLRF [31] using a 25 Å radius of integration.

Western blot analysis

Recombinant human TREM-2 Ig domain proteins were analyzed on Western blot using an antibody directed against the C-terminal 6-histidine tag (HRP conjugated rabbit α -6-His antibody, Bethyl Labs, A190-114A). The antibody was used at a 1:5000 dilution in binding buffer (PBS containing 0.1 % TWEEN-20 and 0.5 % powered milk). Blocking, binding, and washing steps were carried out in a SNAP i.d. system (Millipore) according to product recommendations.

Results and Discussion

TREM-2 Ig domain refolding screen

Initial experiments were directed at obtaining the large amounts of purified protein that are required for structural and biophysical studies. Structure predictions estimate that the

TREM-2 ectodomain consists of a single immunoglobulin (Ig) domain followed by an approximately 40 amino acid unstructured stalk (Fig 1a). Most of the mutations linked to increased risk of neurodegenerative diseases lie in the Ig domain [12], which is most likely to be involved in ligand engagement for signaling. A number of Ig domains from TREM family receptors have previously been crystallized, including human TREM-1 [22, 23], mouse TREM-1 [21], human TLT-1 [24], human IREM-1 [25], mouse CLM-1, and human NKp44 [26]. Each of the proteins for these studies was produced by bacterial expression of insoluble inclusion bodies and refolding. Thus, we initiated our structural studies of the TREM-2 Ig domain by attempting to refold both the human and mouse TREM-2 Ig domains from bacterially expressed inclusion bodies. Although the inclusion body preparations were very homogeneous (Fig 1b), we were unable to refold these proteins by rapid dilution into a buffer similar to that used for other family members (100 mM Tris pH 8.5, 400 mM arginine, 2 mM EDTA, 5 mM reduced glutathione, and 0.5 mM oxidized glutathione). The protein would initially be soluble, but after about one hour, heavy precipitation was observed in the refolding buffer. We then attempted to screen numerous potential refolding conditions (Table 1). This screening produced a number of potential conditions (Fig 1c). A number of potential hits, including conditions #4 and #14, were expanded to large-scale refoldings in 500 mL of refolding buffer. However, these conditions produced similar results (heavy precipitation after one hour) when scaled-up. In addition, given that NDSB-201 increased solubility and crystal size for the mammalian cell-produced protein, we attempted large-scale refolding in buffers incorporating that reagent as the solubilizer (50 mM Hepes pH 7.5, 0.4 – 1.0 M NDSB-201, 150 mM NaCl, 5 mM EDTA, 5 mM reduced glutathione, and 0.5 mM oxidized glutathione). This attempt gave similar unsuccessful results. Some possible interpretations of these observations could be a requirement for post-translational modification that is unable to occur in *E. coli*, the need of chaperones for proper folding, or instability due to absence of a ligand.

Production and Purification of TREM-2 from mammalian cells

To circumvent the protein production problems found in the bacterial system, we next pursued expression and purification of natively folded and secreted protein from mammalian cells. We previously incorporated a large-scale transient transfection system using mammalian cells in suspension to produce secreted proteins [32]. The advantages of this transient transfection system are: expression constructs can be quickly optimized as there is no requirement to produce expression viruses or stably expressing cell lines; the 293 Freestyle cells are readily transfected and can be grown to high densities (up to around 4×10^6 /ml); and the cells can be cultured in serum-free media, simplifying purification from media supernatants (Fig 2a). Using this system, we transfected 293 Freestyle cells at a density of 1×10^6 /ml with expression plasmids encoding mouse TREM-2 Ig domain, human WT TREM-2 Ig domain, and human TREM-2 Ig domain R47H variant. To inhibit glycan maturation, kifunensine was added to the culture medium at the time of transfection. This treatment allows for removal of the resulting high-mannose glycans by post-purification addition of Endo Hf, which is advantageous for crystallization. Media supernatants were harvested 72-hours post-transfection and adjusted to pH 8.5 with phosphate/Tris, 300 mM NaCl, and 10 mM imidazole for efficient retrieval of the 6-histidine-tagged proteins using Ni-NTA superflow resin. Bound proteins were eluted from the column in a buffer consisting of 50 mM phosphate pH 8.0, 300 mM NaCl, and 250 mM imidazole. The eluted proteins ran at approximately 28 kDa on SDS-PAGE and displayed laddering due to inhomogeneous glycosylation (calculated monomeric weight is approximately 14 kDa) (Fig. 2b). The eluted proteins were then adjusted to acidic pH (50 mM sodium citrate pH 5.5) and treated with Endo Hf overnight at 25 °C. The deglycosylated proteins collapsed to a single band of about 15 kDa on SDS PAGE (Fig 2b) and this was confirmed by Western blot for the C-terminal

6-histidine tag (Fig 2c). Typical yield was 5–10 mgs of pure TREM-2 Ig domains per liter of culture medium.

Crystallization of the human TREM-2 Ig domain

Both human and mouse WT TREM-2 Ig domains were exchanged into a buffer consisting of 20 mM Hepes pH 7.5 and 150 mM NaCl and concentrated to 10 mg/mL prior to crystallization screening. Crystallization screening was carried using a Mosquito crystallization robot (TTP Labtech, UK). Screening was carried out by hanging drop vapor diffusion with crystallization drops containing 150 nL protein solution plus 150 nL precipitant solution. The most promising hits were obtained with the human TREM-2 Ig domain. The best initial condition produced multiple small hexagonal crystals per drop and consisted of 100 mM Bis-Tris pH 7.0, 2.0 M NaCl, 200 mM MgCl₂. Crystallization conditions were optimized by fine varying of buffer pH and NaCl concentration, and the addition of additives (96 condition Additive Screen, Hampton Research, CA, USA). The best additives produced only a few large crystals per drop and consisted of sulfobetaines (NDSB-201 and NDSB-211) and divalent cations (CsCl₂ and BaCl₂). The final optimized conditions for the best crystals consisted of 100 mM Tris pH 7.0–7.2, 2.0–2.2 M NaCl, 200 mM MgCl₂, and 200 mM NDSB-201 with crystallizations drops setup in a 1:1 protein:precipitant ratio. Large hexagonal crystals appeared after 2–3 days, reaching maximum dimensions of around 300×300×50 μm after 3–5 days (Fig. 2d).

Preliminary Crystallographic Data

Initial diffraction data was collected at the Advanced Photon Source, Argonne National Lab, beamline ID-19. Crystals of most of the previously analyzed TREM family members produced diffraction beyond 2.0 Å resolution. In contrast, the best crystals of human TREM-2 purified from mammalian cells diffracted to a resolution of 3.3 Å (Fig 3a). This decreased resolution is partially because the protein crystallizes in a large unit cell (Table 2). Even given the high symmetry space group (P6₄22), six to seven TREM-2 Ig domains would be required per ASU for the crystals to have a solvent content near the commonly observed average of about 50%. However, molecular replacement using numerous suitable probes was only able to locate two molecules in the ASU, for a solvent content of 83%. This result suggested that a co-purified protein enriched in the crystallization process might have co-crystallized with the human TREM-2 Ig domain. In order to determine if this was the case, we extracted single crystals from crystallization drops, washed the crystals, dissolved them in SDS loading buffer, and finally analyzed the crystal contents by silver-stained SDS PAGE. This analysis revealed that the only protein in the crystals was human TREM-2 Ig domain and was confirmed by Western blot for the C-terminal 6-histidine tag (Fig 3b). Furthermore, analysis of the Patterson self-rotation function revealed only peaks consistent with the crystallographic symmetry on the $\kappa = 60^\circ$, 120° , and 180° sections [33], and a unique peak on the $\kappa = 90^\circ$ section (Fig 3c), indicative of two-fold noncrystallographic symmetry, and thus consistent with the observation of two TREM-2 Ig domains in the ASU. Thus, the limited resolution observed from this crystal form of human TREM-2 Ig domain is mainly due to the large unit cell (limiting the number of unit cells per diffracting volume) and high solvent content. However, the data are sufficient to solve the structure to 3.3 Å resolution using molecular replacement.

Conclusions

TREM-2 is an innate immune receptor that is implicated in regulating inflammatory responses as well as acute and chronic inflammatory diseases [34]. In this capacity it has been suggested to contribute to and/or represent a biomarker for a number of diseases including multiple sclerosis [35, 36], stroke [37], inflammatory bowel disease [38], and

chronic inflammatory lung disease [39]. Recent genetics studies have uncovered point mutations in the TREM-2 Ig domain that correlate with a significantly increased risk for the development of neurodegenerative diseases such as AD, FTD, and PD. To begin to understand the influence of TREM-2 polymorphisms on TREM-2 function will require structural and biophysical studies of TREM-2 protein. These types of studies require generous amounts of purified protein and production of protein crystals that diffract to a resolution reasonable for structure determination. Single Ig domains often readily refold from bacterial inclusion bodies, however, we were unable to identify conditions under which the TREM-2 Ig domain would refold. There may be a functional basis for this observation, such as the lack of a stabilizing binding partner, post-translational modification, or folding chaperone. Nonetheless, we abandoned this strategy in favor of developing a mammalian cell-based expression system. Using this system, we were able to produce both WT and mutant TREM-2 Ig domains in sufficient quantities for structural and biophysical studies. This protein readily crystallizes and these crystals produce diffraction data sufficient for structure determination at 3.3 Å. It is worth noting that we were able to perform a one-step purification to obtain these crystals, which are homogeneous by gel analysis and by preliminary LC/MS analysis of solvent extractions from the purified protein (data not shown). This is significant as further purification steps can often lead to losses in protein yield that then prohibit or limit the number of crystallization trials that can be attempted in order to identify crystallization conditions. Thus, this study is hallmark example of minimal purification that produces crystals sufficient for structure determination. In our future efforts, we will attempt to manipulate these crystals to diffract to higher resolution. High solvent content crystals such as the ones reported here usually respond well to dehydration [40, 41], desiccation [42], or annealing [43–45]. The current results provide a basis for structural and biophysical studies of the TREM-2 variants associated with neurodegenerative diseases, which will be required to understand the functional consequences of these point mutations. Ultimately, these studies of TREM-2 structure and function have the potential to lead to the development of patient-specific treatments for neurodegenerative and related inflammatory diseases.

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References

1. Guerreiro R, Wojtas A, Bras J, Carrasquillo M, Rogaeva E, Majounie E, Cruchaga C, Sassi C, Kauwe JS, Younkin S, Hazrati L, Collinge J, Pocock J, Lashley T, Williams J, Lambert JC, Amouyel P, Goate A, Rademakers R, Morgan K, Powell J, St George-Hyslop P, Singleton A, Hardy J. TREM2 variants in Alzheimer's disease. *N Engl J Med*. 2013; 368:117–127. [PubMed: 23150934]
2. Jonsson T, Stefansson H, Steinberg S, Jonsdottir I, Jonsson PV, Snaedal J, Bjornsson S, Huttenlocher J, Levey AI, Lah JJ, Rujescu D, Hampel H, Giegling I, Andreassen OA, Engedal K, Ulstein I, Djurovic S, Ibrahim-Verbaas C, Hofman A, Ikram MA, van Duijn CM, Thorsteinsdottir U, Kong A, Stefansson K. Variant of TREM2 associated with the risk of Alzheimer's disease. *N Engl J Med*. 2013; 368:107–116. [PubMed: 23150908]
3. Saunders AM, Strittmatter WJ, Schmechel D, George-Hyslop PH, Pericak-Vance MA, Joo SH, Rosi BL, Gusella JF, Crapper-MacLachlan DR, Alberts MJ, et al. Association of apolipoprotein E allele epsilon 4 with late-onset familial and sporadic Alzheimer's disease. *Neurology*. 1993; 43:1467–1472. [PubMed: 8350998]

4. Benítez BA, Cooper B, Pastor P, Jin SC, Lorenzo E, Cervantes S, Cruchaga C. TREM2 is associated with the risk of Alzheimer's disease in Spanish population. *Neurobiol Aging*. 2013; 34:1711, e1715–e1717. [PubMed: 23391427]
5. Pottier C, Wallon D, Rousseau S, Rovelet-Lecrux A, Richard AC, Rollin-Sillaire A, Frebourg T, Campion D, Hannequin D. TREM2 R47H variant as a risk factor for early-onset Alzheimer's disease. *J Alzheimers Dis*. 2013; 35:45–49. [PubMed: 23380991]
6. Benítez BA, Cruchaga C. TREM2 and neurodegenerative disease. *N Engl J Med*. 2013; 369:1567–1568. [PubMed: 24131187]
7. Rayaprolu S, Mullen B, Baker M, Lynch T, Finger E, Seeley WW, Hatanpaa KJ, Lomen-Hoerth C, Kertesz A, Bigio EH, Lippa C, Josephs KA, Knopman DS, White CL 3rd, Caselli R, Mackenzie IR, Miller BL, Boczarzka-Jedynak M, Opala G, Krygowska-Wajs A, Barcikowska M, Younkin SG, Petersen RC, Ertekin-Taner N, Uitti RJ, Meschia JF, Boylan KB, Boeve BF, Graff-Radford NR, Wszolek ZK, Dickson DW, Rademakers R, Ross OA. TREM2 in neurodegeneration: evidence for association of the p.R47H variant with frontotemporal dementia and Parkinson's disease. *Mol Neurodegener*. 2013; 8:19. [PubMed: 23800361]
8. Guerreiro RJ, Lohmann E, Bras JM, Gibbs JR, Rohrer JD, Gurunlian N, Dursun B, Bilgic B, Hanagasi H, Gurvit H, Emre M, Singleton A, Hardy J. Using exome sequencing to reveal mutations in TREM2 presenting as a frontotemporal dementia-like syndrome without bone involvement. *JAMA Neurol*. 2013; 70:78–84. [PubMed: 23318515]
9. Guerreiro R, Bilgic B, Guven G, Bras J, Rohrer J, Lohmann E, Hanagasi H, Gurvit H, Emre M. A novel compound heterozygous mutation in TREM2 found in a Turkish frontotemporal dementia-like family. *Neurobiol Aging*. 2013; 34:2890, e2891–e2895. [PubMed: 23870839]
10. Borroni B, Ferrari F, Galimberti D, Nacmias B, Barone C, Bagnoli S, Fenoglio C, Piaceri I, Archetti S, Bonvicini C, Gennarelli M, Turla M, Scarpini E, Sorbi S, Padovani A. Heterozygous TREM2 mutations in frontotemporal dementia. *Neurobiol Aging*. 2013
11. Cuyvers E, Bettens K, Philtjens S, Van Langenhove T, Gijssels I, van der Zee J, Engelborghs S, Vandenbulcke M, Van Dongen J, Geerts N, Maes G, Mattheijssens M, Peeters K, Cras P, Vandenbergh R, De Deyn PP, Van Broeckhoven C, Cruts M, Sleegers K. Investigating the role of rare heterozygous TREM2 variants in Alzheimer's disease and frontotemporal dementia. *Neurobiol Aging*. 2014; 35:726, e711–e729. [PubMed: 24119542]
12. Golde TE, Streit WJ, Chakrabarty P. Alzheimer's disease risk alleles in TREM2 illuminate innate immunity in Alzheimer's disease. *Alzheimers Res Ther*. 2013; 5:24. [PubMed: 23692967]
13. Ford JW, McVicar DW. TREM and TREM-like receptors in inflammation and disease. *Curr Opin Immunol*. 2009; 21:38–46. [PubMed: 19230638]
14. Bouchon A, Hernandez-Munain C, Cella M, Colonna M. A DAP12-mediated pathway regulates expression of CC chemokine receptor 7 and maturation of human dendritic cells. *J Exp Med*. 2001; 194:1111–1122. [PubMed: 11602640]
15. N'Diaye EN, Branda CS, Branda SS, Nevarez L, Colonna M, Lowell C, Hamerman JA, Seaman WE. TREM-2 (triggering receptor expressed on myeloid cells 2) is a phagocytic receptor for bacteria. *J Cell Biol*. 2009; 184:215–223. [PubMed: 19171755]
16. Hsieh CL, Koike M, Spusta SC, Niemi EC, Yenari M, Nakamura MC, Seaman WE. A role for TREM2 ligands in the phagocytosis of apoptotic neuronal cells by microglia. *J Neurochem*. 2009; 109:1144–1156. [PubMed: 19302484]
17. Takegahara N, Takamatsu H, Toyofuku T, Tsujimura T, Okuno T, Yukawa K, Mizui M, Yamamoto M, Prasad DV, Suzuki K, Ishii M, Terai K, Moriya M, Nakatsuji Y, Sakoda S, Sato S, Akira S, Takeda K, Inui M, Takai T, Ikawa M, Okabe M, Kumanogoh A, Kikutani H. Plexin-A1 and its interaction with DAP12 in immune responses and bone homeostasis. *Nat Cell Biol*. 2006; 8:615–622. [PubMed: 16715077]
18. Stefano L, Racchetti G, Bianco F, Passini N, Gupta RS, Panina Bordignon P, Meldolesi J. The surface-exposed chaperone, Hsp60, is an agonist of the microglial TREM2 receptor. *J Neurochem*. 2009; 110:284–294. [PubMed: 19457124]
19. Paloneva J, Manninen T, Christman G, Hovanes K, Mandelin J, Adolfsson R, Bianchin M, Bird T, Miranda R, Salmaggi A, Tranebjærg L, Kontinen Y, Peltonen L. Mutations in two genes encoding different subunits of a receptor signaling complex result in an identical disease phenotype. *Am J Hum Genet*. 2002; 71:656–662. [PubMed: 12080485]

20. Aricescu AR, Lu W, Jones EY. A time- and cost-efficient system for high-level protein production in mammalian cells. *Acta Crystallogr D Biol Crystallogr*. 2006; 62:1243–1250. [PubMed: 17001101]
21. Kelker MS, Debler EW, Wilson IA. Crystal structure of mouse triggering receptor expressed on myeloid cells 1 (TREM-1) at 1.76 Å. *J Mol Biol*. 2004; 344:1175–1181. [PubMed: 15561137]
22. Kelker MS, Foss TR, Peti W, Teyton L, Kelly JW, Wuthrich K, Wilson IA. Crystal structure of human triggering receptor expressed on myeloid cells 1 (TREM-1) at 1.47 Å. *J Mol Biol*. 2004; 342:1237–1248. [PubMed: 15351648]
23. Radaev S, Kattah M, Rostro B, Colonna M, Sun PD. Crystal structure of the human myeloid cell activating receptor TREM-1. *Structure*. 2003; 11:1527–1535. [PubMed: 14656437]
24. Gattis JL, Washington AV, Chisholm MM, Quigley L, Szyk A, McVicar DW, Lubkowski J. The structure of the extracellular domain of triggering receptor expressed on myeloid cells like transcript-1 and evidence for a naturally occurring soluble fragment. *J Biol Chem*. 2006; 281:13396–13403. [PubMed: 16505478]
25. Marquez JA, Galfre E, Dupeux F, Flot D, Moran O, Dimasi N. The crystal structure of the extracellular domain of the inhibitor receptor expressed on myeloid cells IREM-1. *J Mol Biol*. 2007; 367:310–318. [PubMed: 17275839]
26. Cantoni C, Ponassi M, Biassoni R, Conte R, Spallarossa A, Moretta A, Moretta L, Bolognesi M, Bordo D. The three-dimensional structure of the human NK cell receptor Nkp44, a triggering partner in natural cytotoxicity. *Structure*. 2003; 11:725–734. [PubMed: 12791260]
27. Yang C, Cheng W, Teo PY, Engler AC, Coady DJ, Hedrick JL, Yang YY. Mitigated cytotoxicity and tremendously enhanced gene transfection efficiency of PEI through facile one-step carbamate modification. *Adv Healthc Mater*. 2013; 2:1304–1308. [PubMed: 23505024]
28. Chang VT, Crispin M, Aricescu AR, Harvey DJ, Nettleship JE, Fennelly JA, Yu C, Boles KS, Evans EJ, Stuart DI, Dwek RA, Jones EY, Owens RJ, Davis SJ. Glycoprotein structural genomics: solving the glycosylation problem. *Structure*. 2007; 15:267–273. [PubMed: 17355862]
29. Otwinowski Z, Minor W. Processing of X-ray diffraction data collected in oscillation mode. *Methods Enzymol*. 1997; 276:307–326.
30. McCoy AJ, Grosse-Kunstleve RW, Adams PD, Winn MD, Storoni LC, Read RJ. Phaser crystallographic software. *J Appl Crystallogr*. 2007; 40:658–674. [PubMed: 19461840]
31. Tong L, Rossmann MG. Rotation function calculations with GLRF program. *Methods Enzymol*. 1997; 276:594–611. [PubMed: 9048382]
32. Yurtsever Z, Sala-Rabanal M, Randolph DT, Scheaffer SM, Roswit WT, Alevy YG, Patel AC, Heier RF, Romero AG, Nichols CG, Holtzman MJ, Brett TJ. Self-cleavage of human CLCA1 protein by a novel internal metalloprotease domain controls calcium-activated chloride channel activation. *J Biol Chem*. 2012; 287:42138–42149. [PubMed: 23112050]
33. Sawaya MR. Characterizing a crystal from an initial native dataset. *Methods Mol Biol*. 2007; 364:95–120. [PubMed: 17172762]
34. Paradowska-Gorycka A, Jurkowska M. Structure, expression pattern and biological activity of molecular complex TREM-2/DAP12. *Hum Immunol*. 2013; 74:730–737. [PubMed: 23459077]
35. Piccio L, Buonsanti C, Cella M, Tassi I, Schmidt RE, Fenoglio C, Rinker J 2nd, Naismith RT, Panina-Bordignon P, Passini N, Galimberti D, Scarpini E, Colonna M, Cross AH. Identification of soluble TREM-2 in the cerebrospinal fluid and its association with multiple sclerosis and CNS inflammation. *Brain*. 2008; 131:3081–3091. [PubMed: 18790823]
36. Takahashi K, Prinz M, Stagi M, Chechneva O, Neumann H. TREM2-transduced myeloid precursors mediate nervous tissue debris clearance and facilitate recovery in an animal model of multiple sclerosis. *PLoS Med*. 2007; 4:e124. [PubMed: 17425404]
37. Sieber MW, Jaenisch N, Brehm M, Guenther M, Linnartz-Gerlach B, Neumann H, Witte OW, Frahm C. Attenuated inflammatory response in triggering receptor expressed on myeloid cells 2 (TREM2) knock-out mice following stroke. *PLoS One*. 2013; 8:e52982. [PubMed: 23301011]
38. Correale C, Genua M, Vetrano S, Mazzini E, Martinoli C, Spinelli A, Arena V, Peyrin-Biroulet L, Caprioli F, Passini N, Panina-Bordignon P, Repici A, Malesci A, Rutella S, Rescigno M, Danese S. Bacterial sensor triggering receptor expressed on myeloid cells-2 regulates the mucosal inflammatory response. *Gastroenterology*. 2013; 144:346–356. e343. [PubMed: 23108068]

39. Poliska S, Csanky E, Szanto A, Szatmari I, Mesko B, Szeles L, Dezso B, Scholtz B, Podani J, Kilty I, Takacs L, Nagy L. Chronic obstructive pulmonary disease-specific gene expression signatures of alveolar macrophages as well as peripheral blood monocytes overlap and correlate with lung function. *Respiration*. 2011; 81:499–510. [PubMed: 21430361]
40. Heras B, Edeling MA, Byriel KA, Jones A, Raina S, Martin JL. Dehydration converts DsbG crystal diffraction from low to high resolution. *Structure*. 2003; 11:139–145. [PubMed: 12575933]
41. Heras B, Martin JL. Post-crystallization treatments for improving diffraction quality of protein crystals. *Acta Crystallogr D Biol Crystallogr*. 2005; 61:1173–1180. [PubMed: 16131749]
42. Abergel C. Spectacular improvement of X-ray diffraction through fast desiccation of protein crystals. *Acta Crystallogr D Biol Crystallogr*. 2004; 60:1413–1416. [PubMed: 15272164]
43. Harp JM, Hanson BL, Timm DE, Bunick GJ. Macromolecular crystal annealing: evaluation of techniques and variables. *Acta Crystallogr D Biol Crystallogr*. 1999; 55:1329–1334. [PubMed: 10393299]
44. Harp JM, Timm DE, Bunick GJ. Macromolecular crystal annealing: overcoming increased mosaicity associated with cryocrystallography. *Acta Crystallogr D Biol Crystallogr*. 1998; 54:622–628. [PubMed: 9761858]
45. Yeh JI, Hol WG. A flash-annealing technique to improve diffraction limits and lower mosaicity in crystals of glycerol kinase. *Acta Crystallogr D Biol Crystallogr*. 1998; 54:479–480. [PubMed: 9761934]

- TREM-2 Ig domains were expressed and purified from a mammalian expression system
- This protein could be readily deglycosylated and crystallized
- Crystals can be obtained from protein purified in a single chromatography step
- Crystals diffract to 3.3 Å and a molecular replacement solution has been determined
- Results allow structural and biophysical study of disease-linked TREM-2 mutations

Figure 1. Production of human and mouse TREM-2 ectodomain inclusion bodies in *E. coli* and refolding screening

(a) Schematic representation of human TREM-2 protein. Conserved N-linked glycosylation sites are shown as branched trees. Structural features are labeled as follows: SS = signal sequence; Ig = immunoglobulin domain; ST = stalk; TM = transmembrane; IC = intracellular region. (b) SDS-PAGE analysis of human (lane 1) and mouse (lane 2) TREM-2 solubilized inclusion bodies (M = molecular weight marker). Proteins in 6M guanidine were diluted at a ratio of 1:50 into SDS-PAGE loading buffer (to prevent precipitation of guanidine by SDS). Final protein concentration in the loading buffer was 1 mg/ml and approximately 15 μ g of protein were loaded on each lane of the gel. (c) SDS-PAGE analysis of refolding screen outcome for human TREM-2 and (d) mouse TREM-2 Ig domains. The lane numbers correlate to the refolding screen conditions in Table 1.

Figure 2. Expression, purification, and crystallization of mammalian expressed TREM-2 Ig domains

(a) Schematic of protein production and purification from mammalian cells. (b) SDS-PAGE analysis of purified TREM-2 Ig domains before (–) and after (+) endoHf treatment. Proteins were diluted in SDS-PAGE loading buffer to a concentration of 1 mg/ml, and 15 µg of protein were loaded on each lane of the gel. (c) α-6-His Western blot of WT TREM-2 Ig domain before (–) and after (+) endoHf treatment. Bands corresponding to TREM-2 Ig domain modified with 2, 1, of 0 glycans are highlighted. (d) Hexagonal crystals of human TREM-2 WT Ig domain grown in 100 mM Tris pH 7.0–7.2, 2.0 M NaCl, 200 mM MgCl₂, 200 mM NDSB-201.

Figure 3. Preliminary diffraction data from human TREM-2 WT Ig domain crystals
 (a) Representative oscillation diffraction image from human TREM-2 WT Ig domain crystals (b) Left: Silver-stained SDS-PAGE of human TREM-2 Ig WT crystals; Right: α -6-His Western blot of the TREM-2 Ig WT crystals (c) Patterson self-rotation function kappa sections from the 3.3 Å resolution data set.

Table 1

Refolding screen conditions

	Buffer	Salt	Cation/ Chelator	Guanidine	Stabilizing Agent(s)	Additives	Detergent	1 mM DTT	1mM GSH/ 0.1mM GSSH
1	MES pH 6.0	9.6 mM NaCl, 0.4 mM KCl	2 mM MgCl ₂ , 2 mM CaCl ₂	0.75 M			0.5% Triton X-100	•	
2	MES pH 6.0	9.6 mM NaCl, 0.4 mM KCl	2 mM MgCl ₂ , 2 mM CaCl ₂		0.5 M L-Arginine	0.05% PEG 3,350			•
3	MES pH 6.0	9.6 mM NaCl, 0.4 mM KCl	1 mM EDTA	0.75 M	0.4 M Sucrose	0.05% PEG 3,350	0.5% Triton X-100	•	
4	MES pH 6.0	240 mM NaCl, 10 mM KCl	2 mM MgCl ₂ , 2 mM CaCl ₂		0.5 M L-Arginine		0.5% Triton X-100		•
5	MES pH 6.0	240 mM NaCl, 10 mM KCl	1 mM EDTA	0.75 M	0.4 M Sucrose			•	
6	MES pH 6.0	240 mM NaCl, 10 mM KCl	1 mM EDTA		0.4 M Sucrose 0.5 M L-Arginine	0.05% PEG 3,350	0.5% Triton X-100		•
7	MES pH 6.0	240 mM NaCl, 10 mM KCl	2 mM MgCl ₂ , 2 mM CaCl ₂	0.75 M		0.05% PEG 3,350		•	
8	Tris pH 8.5	9.6 mM NaCl, 0.4 mM KCl	2 mM MgCl ₂ , 2 mM CaCl ₂		0.4 M Sucrose	0.05% PEG 3,350	0.5% Triton X-100		•
9	Tris pH 8.5	9.6 mM NaCl, 0.4 mM KCl	1 mM EDTA	0.75 M	0.5 M L-Arginine	0.05% PEG 3,350		•	
10	Tris pH 8.5	9.6 mM NaCl, 0.4 mM KCl	2 mM MgCl ₂ , 2 mM CaCl ₂	0.75 M	0.4 M Sucrose 0.5 M L-Arginine				•
11	Tris pH 8.5	9.6 mM NaCl, 0.4 mM KCl	1 mM EDTA				0.5% Triton X-100	•	
12	Tris pH 8.5	240 mM NaCl, 10 mM KCl	1 mM EDTA			0.05% PEG 3,350			•
13	Tris pH 8.5	240 mM NaCl, 10 mM KCl	1 mM EDTA	0.75 M	0.5 M L-Arginine		0.5% Triton X-100	•	
14	Tris pH 8.5	240 mM NaCl, 10 mM KCl	2 mM MgCl ₂ , 2 mM CaCl ₂	0.75 M	0.4 M Sucrose 0.5 M L-Arginine	0.05% PEG 3,350	0.5% Triton X-100		•
15	Tris pH 8.5	240 mM NaCl, 10 mM KCl	2 mM MgCl ₂ , 2 mM CaCl ₂		0.4 M Sucrose			•	

Table 2

Crystallographic data and initial molecular replacement statistics.

Space Group	P6 ₄ 22
Unit Cell (Å)	a=125.76 b=125.76 c=183.66
X-ray source	APS, ID-19
Wavelength(Å)	0.979180
Resolution(Å)	50.00-3.30 (3.42-3.30) *
R _{sym} (%)	0.133(0.920)
Completeness(%)	99.9 (100)
Redundancy	13.6(14.1)
I/ σ(I)	21.8(3.0)
Molecular Replacement Statistics	
Probe PDB ID	1U9K
No. Molecules/ASU	2
Program	PHASER
TFZ	13.8
Rigid Body R _{work}	0.3909
Rigid Body R _{free}	0.4602

* Values in parenthesis are for the highest-resolution shell.