Treatment of Microglia with Anti-PrP Antibodies Induces Neuronal Allergenicity

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Research article

Keywords: Anti-PrP antibodies, Cellular Prion Protein, PrPC, Microglia, Neurotoxicity, Allergenicity, IgG-mediated Hypersensitivity, Neuroblastoma cell line (N2a), Microglia cell line (N11)

Posted Date: October 27th, 2020

DOI: <https://doi.org/10.21203/rs.3.rs-96576/v1>

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Abstract

Background: Previous reports identified proteins associated with ‘apoptosis’ following cross-linking PrP^C with motif-specific anti-PrP antibodies *in vivo* and *in vitro*. The molecular mechanisms underlying this IgG-mediated neurotoxicity and the role of the activated proteins in the apoptotic pathways leading to neuronal death has not been properly defined. Previous reports implicated a number of proteins, including apolipoprotein E, cytoplasmic phospholipase A2, prostaglandin and calpain with anti-PrP antibody-mediated ‘apoptosis’, however, these proteins are also known to play an important role in allergy. In this study, we investigated whether cross-linking PrP^C with anti-PrP antibodies stimulates a neuronal allergenic response.

Methods: Initially, we predicted the allergenicity of the epitope sequences associated with ‘neurotoxic’ anti-PrP antibodies using allergenicity prediction servers. We then investigated whether anti-PrP antibody treatment of neuronal (N2a) and microglia (N11) cell lines leads to a neuronal allergenic response.

Results: We found that both tail- and globular-epitopes were allergenic. Specifically, binding regions that contain epitopes for ‘neurotoxic’ antibodies such as ICSM18 (146-159), ICSM35 (91-110), POM 1 (138-147), POM 2 (57-88) and POM 3 (95-100) lead to activation of allergenic related proteins. Following direct application of anti-PrP^C antibodies on N2a cells, mass spectrometry analysis identified 4 neuronal allergenic-related proteins when compared with untreated cells. Furthermore, mass spectrometry analysis identified 8 neuronal allergenic-related proteins following cross-linking N11 cells with anti-PrP^C antibodies prior to co-culture with N2a cells, when compared with untreated cells. Of importance, we showed that the allergenic effects triggered by the anti-PrP antibodies were more potent when antibody-treated microglia were co-cultured with the neuroblastoma cell line. Furthermore, in both direct and co-culture with antibody-treated microglia, we demonstrate that the allergenic proteome was part of the PrP^C-interactome.

Conclusions: This study showed for the first time that anti-PrP antibody binding to PrP^C triggers a neuronal allergenic response (we termed ‘*IgG-Mediated Neuronal Allergic Toxicity*’) and highlights the important role of microglia in triggering IgG-mediated neuronal allergenic toxicity. Moreover, this study provides an important impetus for including allergenic assessment of therapeutic antibodies for neurodegenerative to derive safe and targeted biotherapeutics.

Background

Prion diseases or transmissible spongiform encephalopathies (TSE) are invariably fatal diseases characterized by loss of motor control, dementia and paralysis [1, 2]. These disorders are caused by the conversion of a transmembrane cellular prion protein (PrP^C) into a misfolded form (PrP^{Sc}) [3, 4]. PrP^C is a soluble protein rich in alpha helical content while PrP^{Sc} is rich in β -pleated sheets and characterised by its insolubility in detergents and partial resistance to proteases [4–7]. The function of PrP^C has not been completely elucidated but due its conserved nature in a wide range of species, it is believed to play key and vital roles in maintaining cell homeostasis. However, PrP^C was implicated in cell activation, proliferation and differentiation [8–10], copper binding [11], synaptic plasticity and signal transduction [12–15]. Prion diseases immunotherapeutics that directly target PrP^{Sc} elimination and transient inhibition of PrP^C have been efficacious in rodent models [16–20]. However, several reports highlighted potential side-effects caused by anti-PrP^C antibody treatment *in vitro* and *in vivo* [12, 15, 21–25]. Of note, the antibody-mediated ‘neurotoxic’ effects reported previously were made on the basis of microscopic assessments following TUNEL and/or standard histological staining but have not been characterized at a molecular level [23, 24, 26], with the exception of reports by Tayebi *et al.*, Sonati *et al.* and Goniotaki *et al.* that confirmed the association of apolipoprotein E (APOE), cytoplasmic phospholipase A2 (cPLA2), prostaglandin (PG), calpain (CAPN) and group-I metabotropic glutamate receptors (mGluR1 and mGluR5) with anti-PrP mediated ‘neurotoxicity’ [21, 27, 28]. In fact, these proteins are known to play a key role in allergic reactions, and most were identified as human allergy-related proteins by the AllerGAtlas database. For instance, levels of APOE in the bronchoalveolar fluid derived from patients with hypersensitivity pneumonitis were shown to be significantly high and APOE was suggested to play an important role in this allergic disease [29]. Moreover, impaired delayed type hypersensitivity responses were observed in APOE-null mice, demonstrating the important role of APOE in regulating allergy [30]. The role of cPLA2 in allergic response was characterized [31]. Uozumi and colleagues showed that cPLA2-deficient mice displayed marked decrease in the synthesis of eicosanoids (including PG) and platelet activating factor and that the anaphylactic responses were significantly affected. Moreover, cPLA2 was also shown to be essential for fast eicosanoid generation (including PG) by providing arachidonic acid [32]. PGs are synthesized by the cyclooxygenase (COX) enzymes in the arachidonic acid metabolic pathway and have been shown to play an important role in hypersensitivity [33]. The neuron-expressed mGluR7 was shown to regulate histamine and ablation of mGluR7 in mice led to anaphylaxis [34]. Of importance, mGluR7-interactors; mGluR1 and mGluR5 were shown to form complexes with PrP^C and their pharmacological inhibition cancelled the ‘neurotoxic’ effects caused by anti-PrP antibodies [28]. Of interest, the anti-histaminic drug astemizole, a second generation H1-receptor antagonist, was shown to extend the survival of mice infected with prions [35], suggesting a connection between PrP and histamine.

Microglia activation is an important neuropathological feature associated with neurodegenerative disorders, including prion diseases [36–38]. It was previously reported that the cytotoxic effects triggered by a putative toxic PrP peptide were intimately linked to microglia activation [36, 39]. Moreover, a report by Lefebvre-Roque *et al.* demonstrated that a 2-week intraventricular infusion of a full length anti-PrP^C antibody or with its F(ab')₂ and Fab fragment derivative to wild-type and prion-infected mice initiated at the beginning of prion neuroinvasion led to microglial recruitment/activation [25]. Interestingly, the authors show that microglial activation associated with neuronal death was only observed after injection of anti-PrP antibodies and did not seem to be related to prion infection.

In this study, we aimed to verify whether direct treatment of neurons with anti-PrP antibodies or following co-culture with anti-PrP antibody-treated microglia leads to a neuronal allergenic response. In order to investigate the molecular mechanisms underlying antibody-induced toxicity, mass spectrometry analysis was performed to identify allergenic proteins and to characterize possible pathways leading to *IgG-Mediated Neuronal Allergic Toxicity* post-antibody treatment. Here, we used a set of anti-PrP antibodies, including ICSM [40], SAF [41, 42], and POM antibodies [43] with binding specificity for epitopes located on the globular domain (GD) or flexible tail (FT) of PrP^C. We show that direct application of anti-PrP antibodies on N2a causes a neuronal allergic reaction by

activating 4 allergenic-related proteins. Co-culture of N2a with antibody treated N11 led to a more extensive alteration of the proteome and identified 8 allergenic-related proteins. This study demonstrates and for the first time that cross-linking PrP^C with anti-PrP antibodies leads to a neuronal allergic reaction and also highlights the crucial role played by microglia in this *IgG-Mediated Neuronal Allergic Toxicity*.

Methods

The overall methods of the *in silico* study, *in vitro* experimental setup, western blotting, immunofluorescence study and liquid-chromatography mass-spectrophotometry (LC-MS) analysis are illustrated in in Fig. 1.

Prediction and Validation of the Three-dimensional Structure of the Human Major Prion Protein

The I-TASSER server (<https://zhanglab.ccmb.med.umich.edu/I-TASSER/>) [44] was used to predict the three-dimensional (3D) structure of the full-length (253 amino acid) huPrP. I-TASSER is a hierarchical template-based protein three-dimensional structure prediction server which uses the query protein sequence and predicts the structure through multiple threading alignments, iterative structure assembly simulations, and comparative functional modelling approaches using state-of-art algorithms [44]. The best protein model can be selected based on the template modelling score (TM-score), confidence score (C-score), and root mean square deviation (RMSD) score [44]. The predicted structure was visualized using the PyMOL v2.3 (The PyMOL Molecular Graphics System, Version 2.3 Schrödinger, LLC.).

The predicted 3D structure was primarily evaluated based on the Ramachandran plot [45] in PROCHECK [46] that shows mainly most favoured region, additionally allowed regions, and the disallowed regions of the protein structure that was predicted by the PDBsum server (<http://www.ebi.ac.uk/pdbsum>) [46]. The protein 3D model was further assessed by the ProSA (<https://prosa.services.came.sbg.ac.at/prosa.php>), a web server that can recognize the errors of the theoretical protein model and calculates the overall quality of the protein 3D model [47]. The SAVES v5.0 server (<https://servicesn.mbi.ucla.edu/SAVES/>) was used for the Verify3D score of the protein model [48]. Verify3D determines the compatibility of an atomic model (3D) with its own amino acid sequence (1D) by assigning a structural class based on its location and environment (alpha, beta, loop, polar, nonpolar) and comparing the results to good structures [48].

Prediction of Linear and conformational B Cell epitopes in the Human Major Prion Protein

The linear and conformational or discontinuous B Cell epitopes in the huPrP 3D structure were predicted using the Ellipro server (<http://tools.iedb.org/ellipro/>) [49]. Ellipro is a web-server which uses the geometrical properties of the protein structure in combination with MODELLER program and residue clustering algorithm for the prediction of the B Cell epitopes in the protein region protruding from the protein's globular surface [49]. We used the default parameters (minimum score 0.5 and maximum distance 0.6Å) of the Ellipro server for the prediction of both linear and conformational B Cell epitopes.

Prediction of the Toxicity and Allergenicity of the Linear B Cell epitopes

We used ToxinPred server (<http://crdd.osdd.net/raghava/toxinpred/>) [50] for the prediction of toxic/non-toxic nature of the linear B Cell epitopes identified the Ellipro server as described above. We used both the support vector machine (SVM) and quantitative matrix (QM) method in ToxinPred server for non-toxic epitope selection. The ToxinPred server has been developed based on the QM and machine learning technique using different properties of the peptides for the prediction of toxicity or non-toxicity of the peptides with 93.92% and 88.00% accuracy in SVM and QM methods, respectively. This server can also be used to identify the most toxic regions in the protein sequence [50].

For the allergenicity prediction, we used *k* nearest neighbours (kNN) method based server AllerTOP v2.0 (<http://www.ddg-pharmfac.net/AllerTOP/>) [51] and a novel alignment-free descriptor-based fingerprint approach server AllergenFP v1.0 (<http://www.ddg-pharmfac.net/AllergenFP/>) [52]. The AllergenFP v1.0 server predicts the allergenicity and non-allergenicity with an accuracy of 88.00% by analysing the key amino acid features such as β -strand, helix, hydrophobicity forming propensities, size, and relative abundance of the amino acids [52]. Further, AllerTOP v2.0 server shows 88.7% overall accuracy and uses auto and cross-covariance, machine learning approach and a *k*NN method for the classification of allergen and non-allergen proteins and peptides [51].

Treatment of Mouse Neuroblastoma and Microglia Cell Lines with Anti-PrP Antibodies

We used a mouse neuroblastoma (N2a) (American Type Culture Collection, ATCC, USA) [53] and a mouse microglia (N11) [54] cell line to investigate the allergenic effect of anti-PrP antibodies. The N2a cells were used to assess allergenicity following direct application of anti-PrP antibodies (DAT). The N11 cells, initially treated with anti-PrP antibodies, were used to assess their allergenic effects on N2a cells following direct co-culture (DMT) or after separating the antibody-treated N11 and N2a cells by a tissue culture insert (IMT). Both N2a and N11 cells were grown in Dulbecco's Modified Eagle Medium (DMEM) (Thermo Fisher Scientific, Australia), 10% fetal bovine serum (FBS) (Gibco, Fisher Scientific, Australia), and 1% Penicillin-streptomycin (Sigma, USA) at 37°C in 5% CO₂.

The N2a cell line was previously used to investigate anti-PrP toxic activity and prion infectivity [22,55–57]. Furthermore, the N11 cell line is able to produce several cytokines, namely interleukin 1 (IL 1), interleukin 6 (IL 6) and tumour necrosis factor α (TNF- α) [54]. N11 cell line was also previously used in the prion toxicity study [58].

Direct Antibody Treatment (DAT):

N2a cells were plated on 24 tissue culture well plates (Falcon, country) at 200,000 cells/well for 48 hours in tissue culture medium and kept in an incubator at 37°C and 5% CO₂ until optimum growth and adhesion to the surface of the plates were observed. The medium was changed daily. After 48 hours, 3µg of different anti-PrP antibodies, including ICSM18 [40], ICSM35 [40], POM1 [43], POM2 [43], POM3 [43], SAF32 [42], or SAF70 [41] were added daily to the N2a cultures for 3 days. The cells were then removed from the plates and centrifuged at 800 rpm for 5 minutes. The cells were lysed with NP-40 lysis buffer (150mM NaCl, 1.0% Nonidet P-40 and Triton X-100, 50 mM Tris-Cl, adjust PH to 7.4) with addition of AEBSF protease inhibitor (Sigma, USA) and stored at -80°C until further use.

Direct Microglia Treatment (DMT):

The N11 cells were plated and cultured on a Petri dish at 200,000 cells/well for 48 hours in culture medium and incubated at 37°C in 5% CO₂. N11 cells were then treated with 3µg of different anti-PrP antibodies as above daily for 3 days. The antibody-treated N11 cells were centrifuged at 800 rpm for 5 minutes before co-culturing with confluent N2a cells for 3 days. Finally, the N2a/antibody-treated N11 co-culture was centrifuged at 800 rpm for 5 minutes and the pellet was lysed with NP-40 lysis buffer with addition of AEBSF protease inhibitor then stored at -80°C until further use. In another experiment, the antibody-treated N11 cells were centrifuged at 800 rpm for 5 minutes and the pellet was lysed with NP-40 lysis buffer with addition of AEBSF protease inhibitor then stored at -80°C until further use.

Indirect Microglia Treatment (IMT):

In order to verify whether the potential allergenic effect is caused by molecules released from N11 cells following treatment with anti-PrP antibodies, the N11 cells were plated and cultured on tissue culture inserts (Nunc™ Polycarbonate Cell Culture Inserts, 0.4-micron pore size) in 24 well plate at 200,000 cells/well for 48 hours. The N11 cells were treated daily with 3µg of different anti-PrP antibodies as above. The tissue culture inserts containing antibody-treated N11 cells were transferred to 24 well tissue culture plate containing confluent N2A cells and left for 3 days. Finally, the N2a cells were removed from the wells and centrifuged at 800 rpm for 5 minutes and lysed with NP-40 lysis buffer and AEBSF protease inhibitor before storing at -80°C until further use.

Western Blot Analysis

30µl of cell lysate (300 µg/mL) derived from antibody-treated cells was mixed with an equal volume of 1x Laemmli buffer (Bio-Rad, CA, USA). The solution was vortexed then heated for 5 min to 95°C. The solution was left to cool down before loading 30µl of sample into 12% SDS-PAGE gel (Bio-Rad, CA, USA) and run at 200 Volt for 5 min then 1h 30 min at 100V in running buffer (Bio-Rad, CA, USA). Following transfer at 18V for 2h 30 min in transfer buffer (Bio-Rad, CA, USA), the membranes were blocked using 2% bovine serum albumin (BSA) (Sigma-Aldrich, USA) followed by human TrueStain FC_x™ blocker (Biolegend, San Diego, USA) (5µl/blot). The blots were rinsed with TBST and 0.5 µg/ml of primary antibody mouse anti-human CD64 (FcγRI) (Biolegend, San Diego, USA), mouse anti-human CD16 (FcγRIII) (Biolegend, San Diego, USA), monoclonal mouse anti-phosphoserine (Sigma-Aldrich, USA), and monoclonal mouse anti-phosphotyrosine (Sigma-Aldrich, USA) were added for overnight incubation before washing with 0.1% TBST buffer. The secondary antibody goat anti-mouse IgG (Fab specific) (1:80000) (Sigma-Aldrich, USA) was then added for 1hour at room temperature. The blot was washed using 0.1% TBST then visualized using the Clarity Western ECL Substrate (Bio-Rad, CA, USA) in iBright™ CL1000 Imaging System (Thermo Fisher Scientific).

Immunofluorescence Studies

Cover slips were sterilized by immersing in 70% ethanol followed by washing in 100% ethanol, rinsing in autoclaved water, and finally washing with RPMI. The coverslips were then coated with 0.1% gelatin-coating solution in ddH₂O. For direct antibody treatment (DAT), the N2a cells were plated on the 0.1% gelatin-coated coverslip placed in a 24 well plates (Falcon, country) at 200,000 cells/well for 48 hours in tissue culture medium and kept in at 37°C and 5% CO₂ until optimum growth and adhesion to the surface of the coverslip were observed. The medium was changed daily. After 48 hours, 3µg of different anti-PrP antibodies were added daily to the N2a cultures for 3 days. For direct microglia treatment, the N11 cells were plated and cultured on a Petri dish at 200,000 cells/well for 48 hours in culture medium and incubated at 37°C in 5% CO₂. N11 cells were then treated with 3µg of different anti-PrP antibodies as above. The antibody-treated N11 cells were centrifuged at 800 rpm for 5 minutes before co-culturing with confluent N2a cells on a 0.1% gelatin-coated coverslip in a 24 well plates for 3 days. Finally, for indirect microglia treatment, the N11 cells were plated and cultured on tissue culture inserts (Nunc™ Polycarbonate Cell Culture Inserts, 0.4-micron pore size) in 24 well plate at 200,000 cells/well for 48 hours. The N11 cells were treated daily with 3µg of different anti-PrP antibodies as above. The tissue culture inserts containing antibody-treated N11 cells were transferred to 24 well tissue culture plate containing confluent N2a cells on a 0.1% gelatin-coated coverslip and left for 3 days. The coverslips from all above experiments were used for immunofluorescence. Briefly, the coverslips were removed from the wells and transferred to the new 24 well plates containing cold PBS. The coverslips were rinsed two times in cold PBS followed by addition of 300 µL of 4% paraformaldehyde for 20 minutes at room temperature. The coverslips were then washed x3 with cold PBS. 0.01% Triton was added to the coverslips and left to incubate for 1 minute, followed by washing then addition of 2% BSA for 20 minutes. 2 µg/mL of primary mouse anti-human CD64 antibody (FcγRI) (Biolegend, San Diego, USA) and mouse anti-human CD16 (FcγRIII) (Biolegend, San Diego, USA) was added for 1 hour at room temperature followed by secondary anti-mouse IgG (H+L)-Texas Red antibody (Sigma-Aldrich, USA) (1:500 dilution) for 1 hour at room temperature. The coverslips were washed with PBS x3 and finally mounted on glass slides using VECTASHIELD HardSet Antifade Mounting Medium with DAPI (Vector Laboratories, CA, USA) in aqueous mounting media (Agilent Technologies, CA, USA) (1:3 ratio) and sealed with clear nail polish to prevent dehydration.

Sample Preparation for Liquid Chromatography-Mass Spectrometry

The cell lysates prepared above were used for Liquid Chromatography-Mass Spectrometry (LC-MS) sample preparation. For trypsin digestion, 100 µl of protein sample (300 µg/mL) was concentrated using Rotational Vacuum Concentrator (Martin Christ Gefriertrocknungsanlagen GmbH, Germany). 6 µL DTT (Roche Diagnostics Deutschland GmbH, Germany) (200 mM DTT in Tris buffer, pH 7.8) was then added and the mixture was vortexed before addition of 30 µL of 6M Urea into the sample then incubated for 1h at room temperature. 6 µL iodoacetamide (Sigma-Aldrich, Australia) alkylating reagent (200 mM iodoacetamide in Tris buffer, pH 7.8) was then added, the sample mixture vortexed then followed by incubation for 1h at room temperature. The mixture was topped up with 225 µL of distilled water before adding 5 µL of trypsin (Promega Corporation, USA) solution and incubated overnight at 37°C. Finally, the reaction was stopped, and the *pH* of the solution adjusted to <6 with concentrated acetic acid. After trypsin digestion, the solution was purified using Solid Phase Extraction (SPE - Oasis HLB 1 cc Vac Cartridge, 30 mg) vacuum manifold (Waters Milford, Massachusetts, USA) then reconstituted in 15 µL 0.1% formic acid, vortexed and kept for 30 minutes at 25°C. The solution was then vortexed and sonicated for 3 minutes then centrifuged at 14,000 rpm for 10 minutes before transferring into labelled glass vials.

Liquid Chromatography-Mass Spectrometry Analysis

The samples prepared above were carefully placed in a Waters Total Recovery chromatography sample vials for analysis. System specific cleaning protocol was run before loading the sample to avoid contamination in the system. LC-MS was performed using a Waters nanoAcquity UPLC equipped with a Waters nanoEase M/Z Peptide BEH C18 Column, 130 Å, 1.7 µm 75 µm x 100 mm, thermostatted to 40°C (Waters Corporation, USA). Briefly, solvent A consisted of ultrapure water (Milli-Q) plus 0.1% formic acid and solvent B consisted of LC-MS grade acetonitrile (Burdick and Jackson) plus 0.1% formic acid. Samples were injected onto a trapping column (Waters nanoEase M/Z Symmetry C18 Trap Column, 100 Å, 5 µm, 180 µm x 20mm) at 5 µL/min at 99% Solvent A for 3 min before being eluted on the Analytical Column with a flowrate of 0.30 µL/min. An initial solvent composition of 1% B was ramped to 85% B over 50 minutes. Injections of 1 µL were made from sample solutions stored at 4°C.

Mass spectrometry was performed using a Waters SYNAPT G2-Si (HDMS) spectrometer fitted with a nano electrospray ionization source and operating in positive ion mode. Mass accuracy was maintained by infusing at 0.5 µL/min a lock spray solution of 1 pg/µL leucine enkephalin in 50% aqueous acetonitrile, plus 0.1% formic acid, calibrated against a sodium iodide solution. The capillary voltage was maintained at 3 kV, cone voltage at 30 V, source offset at 30 V, ion block temperature 80°C, gas (N₂) flows: purge gas 20 L/hr., cone gas 20 L/hr. MassLynx Mass Spectrometry Software (Waters Corporation, USA) was used to process the data. Each sample was run for three times in the LC-MS system and finally the collected data were run against the mouse proteome using Uniprot database and analysed using Progenesis Q1 software (Waters Corporation, USA).

Functional Analysis and Protein-Protein Interaction Prediction

Functional analysis of the final dataset of DAT, DMT and IMT was performed using DAVID Bioinformatics Resources 6.8 (<https://david.ncifcrf.gov/>) [59,60]. DAVID is an enriched online unified biological knowledge base and analytic tools which thoroughly extract the biological meaning from the expansive gene or protein list [59]. The interaction analysis among all the identified genes or proteins were achieved using STRING v11.0 (<https://string-db.org/>). This online platform is used as functional protein association networks which provides an in-depth assessment and integration of protein-protein interactions including both direct and indirect associations [61].

Identification of the Allergy Related Genes

The final dataset from LC-MS was checked to find out whether there is any allergy related genes or not in our identified gene list. We used AllerGAtlas 1.0 (<http://biokb.ncpsb.org/AllerGAtlas/>), a human allergy-related genes database which has been developed based on the 1195 well-annotated human allergy-related genes, determined by text-mining and manual curation [62]. The objectives of developing this AllerGAtlas database was to look on the pathogenesis and epidemiology of individual cases, novel diagnostic and prognostic biomarker, individual treatment responses and precision medicine [62].

Classification of Gene and Gene Enrichment Analysis

The identified gene dataset was submitted to the online-based PANTHER classification system v14.0 (<http://www.pantherdb.org/>) for the classification of the identified genes based on the biological process, cellular components, molecular function, protein class, and signaling pathways [63]. This is a wide-ranging system that helps assess and analyse extensive genome-wide experimental data [63]. In addition, the gene enrichment analysis was conducted on the identified final dataset of DAT, DMT and IMT using FunRich software v 3.1.3 [64]. FunRich is a stand-alone software tool used mainly for functional enrichment and interaction network analysis of genes and proteins [65].

Statistical analyses

Statistical analyses were assessed by Student's t-test, Chi-Squared test or Anova test. The results were considered significant at $p < 0.05$. However, Bonferroni corrected *p*-value was used in FunRich analysis.

Results

Modelling of the Human Major Prion Protein Three-dimensional Structure

Following assessment with the I-TASSER server [44], five 3D models of the huPrP were predicted, but only the best protein model (Fig. 2A) was selected based on the confidence score (C-score = -4.13), estimated template modelling score (TM-score = 0.28 ± 0.09), and estimated root mean square deviation (RMSD = 16.0 ± 3.1 Å) score. The Ramachandran plot analysis of huPrP by PDBsum server [46] showed 68.4% residues in most favoured region, 24.7% in allowed regions, 3.7% in generously allowed regions, and 3.2% residues in the disallowed regions for the predicted prion protein structure (Fig. 2B). The ProSA server [47] assesses the overall and local model quality of the predicted protein structure. The local model quality is shown by knowledge-based energy score, in which the positive values represent the erroneous or problematic regions of the input structure (Fig. 2C). The sequence position in the negative values of the knowledge-based energy confirms the good quality of our predicted protein structure. Moreover, ProSA also provides the z-score for overall model quality prediction and was - 5.74, indicating that it is located in the acceptable area [47] (Additional file 1: Figure S1A). The Verify 3D determine the compatibility of an atomic model (3D) with its own amino acid sequence (1D) by assigning a structural class based on its location and environment (alpha, beta, loop, polar, non-polar etc.) and compares the results to good structures [48]. For our protein structure, we found 80.63% of the residues have averaged 3D-1D score ≥ 0.2 , well within the acceptable limit to be considered a good quality protein structure (Additional file 1: Figure S1B and Figure S1C).

Identification of Allergenic B Cell Epitopes in the Three-dimensional Structure of the Human Major Prion Protein

We predicted the linear B cell epitopes from the huPrP 3D structure and found 10 B cell linear epitopes ranging from 4 to 26 amino acids long with a protrusion index (PI) of 0.503 to 0.798 (Table 1) [66]. The full-length huPrP is divided into three major parts, including the flexible tail (FT) region (23–123), which comprises the octa-peptide repeats (OR) region (50–90) and the globular domain (GD) regions (124–230). We found four epitopes (epitope L7: 24–49, epitope L9: 56–66 and epitope L10: 76–86, epitope L2: 89–111) in the FT region, two of them located in the OR region. The GD contained three epitopes (epitope L4: 137–153, epitope L3: 167–174 and epitope L6: 189–204). On the contrary, the short epitope 1–4 (epitope L8) is located in the N-terminal region (signal peptide region) and comparatively two longer epitopes L1:222–238 (both in GD and non-structured region) and L5:245–253 (non-structured region) are located in the C-terminal region of the full length huPrP (Table 1). The position of the B cell linear epitopes on the protein structure are illustrated in (Fig. 3A & 3B). We also predicted 9 B cell conformational epitopes, shown in Table 2. The length of the B cell conformational epitopes ranged between 4 and 36 amino acid residues. The protrusion index value for the B cell conformational epitopes ranged between 0.55 and 0.883.

Table 1
Predicted linear B cell epitopes and their toxicity and allergenicity from the three-dimensional structure of the human major prion protein.

Epitope No.	Position	Peptide	Number of Residues	Score	Toxicity		Overall	Allergenicity		Overall
					SVM Method	QM Method		AllergenFP	AllerTop 2.0	
L1	222–238	SQAYYQRGSSMVLFSPP	17	0.798	Non-toxic	Non-toxic		Non-allergenic	Non-allergenic	
L2	89–111	WGQGGGTHSQWNKPSKPKTNMKH	23	0.767	Non-toxic	Toxic	Toxic	Non-allergenic	Allergenic	Allergenic
L3	167–174	DEYSNQNN	8	0.751	Non-toxic	Non-toxic		Allergenic	Non-allergenic	Allergenic
L4	137–153	PIIHFGSDYEDRYREN	17	0.737	Non-toxic	Toxic	Toxic	Allergenic	Non-allergenic	Allergenic
L5	245–253	SFLIFLIVG	9	0.701	Non-toxic	Non-toxic		Non-allergenic	Non-allergenic	
L6	189–204	VTTTTKGENFTETDVK	16	0.685	Non-toxic	Non-toxic		Non-allergenic	Non-allergenic	
L7	24–49	KRPKPGGWNTGGSRYPGQGSPGGNRY	26	0.676	Non-toxic	Toxic	Toxic	Non-allergenic	Non-allergenic	
L8	1–4	MANL	4	0.559	Non-toxic	Non-toxic		Allergenic	Allergenic	Allergenic
L9	56–66	GWGQPHGGGWG	11	0.541	Non-toxic	Toxic	Toxic	Non-allergenic	Allergenic	Allergenic
L10	76–86	PHGGGWGQPHG	11	0.503	Non-toxic	Toxic	Toxic	Non-allergenic	Allergenic	Allergenic

Table 2
Predicted conformational B cell epitopes from the three-dimensional structure of the human major prion protein.

Epitope No.	Residues	Number of residues	Score
C1	Q227, R228, G229, S230, S231, M232, V233, L234, F235	9	0.883
C2	N100, K101, P102, S103, K104, P105, K106, T107, N108, M109, K110, H111, M112	13	0.811
C3	R25, P26, K27, P28, G29, G30, W31, N32, T33, G34, G35, S36, R37, Y38	14	0.747
C4	P137, I138, I139, H140, F141, G142, S143, D144, Y145, E146, D147, R148, Y149, R151, E152, N153, Y157, V189, T191, T192, T193, K194, G195, E196, N197, F198, T199, E200, T201, D202, V203, K204	32	0.717
C5	R164, E168, Y169, S170, N171, Q172, N173, N174, H177	9	0.708
C6	S222, Q223, A224, Y225	4	0.708
C7	S236, S237, P238, L242, S245, F246, L247, I248, F249, L250, I251, V252, G253	13	0.669
C8	P39, G40, Q41, G42, S43, P44, G45, G46, N47, R48, Y49	11	0.579
C9	G55, G56, W57, G58, Q59, P60, H61, G62, G63, G64, W65, G66, Q67, Q75, P76, H77, G78, G79, G80, W81, G82, Q83, P84, H85, G86, W89, G90, Q91, G92, G93, G94, T95, H96, S97, Q98, W99	36	0.55

The B cell linear epitope L2 overlapped with B cell conformational epitopes C2 and C9 (Tables 1 & 2). Further, linear epitope L4 also overlapped with conformational epitope C4 (Tables 1 & 2). On the other hand, rest of the other linear epitopes L1 overlapped with both C1 and C6; L3 overlapped with C5; L5 overlapped with C7; L6 overlapped with C4; L7 overlapped with both C3 and C8; and both L9 and L10 overlapped with C9 (Fig. 3C & 3D).

In this *in silico* analysis, the prediction of the toxicity for each linear epitope was achieved with ToxinPred server (<http://crdd.osdd.net/raghava/toxinpred/>) using Support Vector Machine (SVM) and Quantitative Matrix (QM) method [50]. The toxicity prediction results of the linear epitopes are shown in Table 1. The SVM method did not identify 'toxic' B cell linear epitopes from 3D structure however, the QM method identified epitopes L2 (89–111), L4 (137–153), L7 (24–49), L9 (56–66), and L10 (76–86) as being toxic. Of note, L2 and L4 epitope contain the binding sequences for the 'neurotoxic' anti-PrP antibodies ICSM35/POM3 antibodies [40, 43] and ICSM18/POM1 antibodies [40, 43], respectively. Interestingly, epitopes L9 (56–66) and L10 (76–86) are located in the octa-repeat region of the huPrP protein that contains the binding sequences for SAF 32 (59–89) [42] and POM2 (57–88) antibodies [43], respectively. We also predicted the allergenicity for the B cell linear epitopes using the AllergenFP [52] and AllerTop [51] allergenicity prediction server. L3 (167–174), L4 (137–153), and L8 (1–4) were predicted to be allergenic in AllergenFP, while AllerTop server identified epitopes L2 (89–111), L8 (1–4), L9 (56–66), and L10 (76–86) as allergenic. The allergenicity prediction results are shown in Table 1. Three linear epitopes were shown to be non-toxic and non-allergenic and included L1 (222–238) and L6 (189–204) on the globular domain and L5 (245–253) located on the non-structured region. We therefore investigated whether ICSM18, ICSM35, POM1, POM2, POM3, SAF32, and SAF70 antibodies trigger a neuronal allergenic reaction *in vitro*.

Anti-PrP Antibody Treatment of Mouse Neuroblastoma Cells Leads to Differential Expression of Fcγ receptors

Fcγ receptors (FcγRs) are subdivided into FcγRI (CD64), FcγRII (CD32) and FcγRIII (CD16) according to their structural homology and binding affinity with IgG (reviewed in [67]). Moreover, FcγRs signal through immune tyrosine activating or inhibitory motifs to inhibit (FcγRIIb) or activate (FcγRI, FcγRIIa/c, FcγRIII) immune functions (reviewed in [67]). Here, we wanted to verify whether direct anti-PrP treatment of neurons or via co-culture of pre-treated microglia affects expression of both FcγRI and FcγRIII (FcγRIIa is not expressed in mice). Western blot analysis showed a ~250 kDa CD64 (predicted MW: ~72 kDa) and CD16 (predicted MW: ~16 kDa) band associated with both SAF70 and POM1 when compared to untreated control in DAT (Fig. 4A). Interestingly, no such band was detected with ICSM 18 treatment following DMT, however, two bands ranging between 15–20 kDa were observed with the untreated control but significantly less intense with antibody treatment. Interestingly, a similar band pattern was also observed following probing with anti-phospho-serine and anti-phospho-tyrosine antibodies, signifying an inhibitory effect and/or dephosphorylation by anti-PrP antibodies (Fig. 4A). DMT with anti-PrP antibodies does not appear to affect expression of both CD64 and CD16 (Fig. 4B), however, DMT with ICSM18 showed one band pattern at ~150 kDa as compared with other antibodies that showed a two-band pattern at ~150 and 250 kDa following probing with anti-phospho-tyrosine antibody (Fig. 4B). Finally, IMT with anti-PrP antibodies led to increased CD64 and CD16 expression with ICSM18 and POM1, but lower expression with SAF70 (Fig. 4C). Similar band pattern was also observed when probing with anti-phospho-serine and anti-phospho-tyrosine antibodies (Fig. 4C). Of note, immunofluorescence studies have not been remarkable and did not show differences in the expression levels of FcγRs in N2a cells (data not shown).

Treatment of Mouse Neuroblastoma Cell lines with Anti-PrP Antibodies Leads to Neuronal Allergenicity

Treatment of N2a cells with anti-PrP antibodies ICSM18, ICSM35, POM1 and SAF70 led to the identification of 211 proteins ($p < 0.05$) after LC-MS analysis when compared with untreated N2a cells. Out of the 211 proteins, only the differentially expressed proteins were considered using maximum fold change ≥ 10 , at least 2 identified unique peptides and a confidence score ≥ 40 . The stringent parameters used here led to the identification of 26 proteins (Additional file 2: Table S1). Of note, the stringent parameters used to identify proteins associated with anti-PrP treatment are unusually high and would allow elimination of 'false-negatives' post LC-MS analysis. The 26 proteins were then assessed for allergenicity using AllergAtlas database (<http://biokb.ncpsb.org/AllergAtlas/>) [62] and 4 allergy related proteins, including beta-actin (ACTB), fatty acid-binding protein 5 (FABP5), protocadherin 11 (PCDH11X), and myomegalin

(PDE4DIP). Among the 4 allergenic-related proteins, ACTB, PCDH11X and PDE4DIP were upregulated but FABP5 was found to be down regulated when compared to untreated control (Table 3). The functional annotation of the 4 allergenic-related proteins through DAVID bioinformatics resources [59, 60] showed that ACTB is associated with platelet aggregation and cellular response to electrical stimulus; PCDH11X is involved in the negative regulation of phosphatase activity; FABP5 was found to be associated with phosphatidylcholine biosynthetic process and transport while PDE4DIP was found to be involved in cellular protein complex assembly.

Table 3
Properties of the identified allergenic proteins following direct antibody treatment (DAT) of the neuroblastoma cell line. The properties were identified by Progenesis Software after the LC-MS analysis.

Accession	Gene Id	Similar Protein Family in AllerGAtlas Server	Protein Name	Anova (p)	Max Fold Change	Confidence Score	Peptides	Unique peptides	Highest Mean	Lowest Mean
Q05816	Fabp5	Fabp4	Fatty acid-binding protein 5	0.01718987	16	260	22	3	Control	DAT
B1AZR7	Pcdh11X	Pcdh1	Protocadherin 11	0.00666053	11.7	60.8	9	4	DAT	Control
Q3UBP6	Actb	-	Uncharacterized protein	0.01880576	12.3	802	58	3	DAT	Control
Q3UR03	Pde4dip	Pde4a	Myomegalin (Fragment)	0.02084224	29.9	49.1	7	3	DAT	Control

Protein-protein interaction of the identified 4 allergenic-related proteins with prion protein (PRNP) showed that PrP networks with ACTB via Cofilin-1 (CFL1), while no direct interaction was observed for FABP5, PCDH11X and PDE4DIP (Fig. 5A). Finally, analysis of individual anti-PrP antibody treatments revealed that PDE4DIP was present after DAT with ICSM18 and POM1 treatment, however, DAT with ICSM35 and SAF70 was not found to be associated with allergy related proteins (Table 4).

Table 4
Identification of antibody-specific allergenic proteins following direct antibody treatment (DAT). (✓) Present and (-) Absent

Gene ID	Accession	ICSM Antibodies				SAF Antibody		POM Antibody	
		ICSM18	CTL	ICSM35	CTL	SAF70	CTL	POM1	CTL
Q05816	Fabp5	-	-	-	-	-	-	-	-
B1AZR7	Pcdh11X	-	-	-	-	-	-	-	-
Q3UBP6	Actb	-	-	-	-	-	-	-	-
Q3UR03	Pde4dip	✓	-	-	-	-	-	✓	-

Gene Ontology (GO) Analysis of Allergy Related Proteins Associated with Direct Antibody Treatment

We performed Gene Ontology (GO) analysis using the gene classification server, PANTHER classification system (v.14.0) [63], and the protein gene enrichment software, FunRich (Functional Enrichment analysis tool-Version 3.1.3) [64] (reference list “rodent database”) for the analysis of cellular components, molecular function, biological process, and the signalling pathway. PANTHER identified ACTB and PCDH11X allergenic-related genes involved in cellular components, biological processes and signalling pathways. The cellular component analysis of the 4 allergenic-related proteins showed that both ACTB and PCDH11X are associated with cell and membrane while ACTB is involved in membrane-enclosed lumen, organelle, protein-containing complex, and supramolecular complex (Fig. 5B). The molecular function of ACTB gene was found to be associated with binding and structural molecule activity (data not shown). The biological process of PCDH11X gene was found to be associated with biological adhesion, whereas ACTB was found to be involved in biogenesis, cellular process, developmental process, localization, locomotion, and multicellular organismal process (Fig. 5C). Signalling pathway analysis identified ACTB and PCDH11X association with cadherin signaling and Wnt signaling pathways. In addition, signaling pathway analysis of ACTB was found to be involved in Alzheimer disease-presenilin pathway, cytoskeletal regulation by Rho GTPase, Huntington disease, integrin signalling pathway, nicotinic acetylcholine receptor signaling pathway, and inflammation mediated by chemokine and cytokine signaling pathway (Fig. 5D).

FunRich analysis showed six cellular components such as postsynaptic density (ACTB, FABP5; $p = 0.009$), synapse (ACTB, FABP5; $p = 0.065$), postsynaptic actin cytoskeleton (ACTB; $p = 0.027$), NuA4 histone acetyltransferase complex (ACTB; $p = 0.029$), dense body (ACTB; $p = 0.009$), and tight junction (ACTB; $p = 0.038$) (Fig. 6A). FunRich analysis also identified different biological processes such as regulation of prostaglandin biosynthetic process (FABP5; $p < 0.001$), cellular response to cytochalasin B (ACTB; $p = 0.004$), regulation of norepinephrine uptake (ACTB; $p = 0.01$), regulation of retrograde trans-synaptic signaling by endocannabinoid (FABP5; $p = 0.01$), negative regulation of glucose transmembrane transport (FABP5; $p = 0.025$), protein localization to adherens junction (ACTB; $p = 0.031$), regulation of transepithelial transport (ACTB; $p = 0.031$), and positive regulation of microtubule nucleation (PDE4DIP; $p = 0.038$) (Fig. 6B). On the other hand, reactome pathway analysis through FunRich identified cell-extracellular matrix interactions ($p = 0.014$), interaction between L1 and ankyrins ($p = 0.008$), formation of annular gap junctions ($p = 0.019$), gap junction degradation ($p = 0.021$) for ACTB and triglyceride catabolism ($p = 0.021$) for FABP5 (Fig. 6C).

Co-Culture of Anti-PrP Antibody Treated-Microglia with Mouse Neuroblastoma Cell Line Leads to Neuronal Allergenicity

Co-culture of N2a cells with anti-PrP antibody-treated N11 cells led to the identification of 2346 proteins (only $p < 0.05$) after LC-MS analysis when compared with co-culture of N2a cells with untreated-N11 cells. Out of the 2346 proteins, only the differentially expressed proteins were considered using maximum fold change ≥ 10 , at least 2 identified unique peptides and a confidence score ≥ 40 . The stringent parameters used here led to the identification of 113 proteins (Additional file 2: Table S2). The 113 proteins were assessed for allergenicity using AllergAtlas database and 8 proteins were confirmed to be allergenic (Table 5), including IF rod domain-containing protein (VIM), peroxiredoxin-1 (PRDX1), Legumain (LGMN), cytoskeletal beta-actin (ACTB), V(D)J recombination-activating protein 1 (RAG1), L-lactate dehydrogenase (LDHA), Receptor-type tyrosine-protein phosphatase C (PTPRC), and TIR domain-containing protein (TLR3). Among the identified 8 allergenic-related proteins, 7 (VIM, LGMN, ACTB, RAG1, LDHA, TLR3, PTPRC) showed the highest mean for DMT when compared with N2a cultured with untreated-N11 cells (Table 5). On the other hand, PRDX1 was found to be downregulated (Table 5).

Table 5
Properties of the identified allergenic proteins following direct microglia treatment (DMT) on the neuroblastoma cell line. The properties were identified by Progenesis Software after the LC-MS analysis.

Accession	Gene ID	Protein Name	Anova (p)	Max Fold Change	Confidence Score	Peptides	Unique Peptides	Highest Mean	Lowest Mean
Q3TWW0	Vim	IF rod domain-containing protein	0.000478	221	865	61	2	DMT	Control
B1AXW5	Prdx1	Peroxiredoxin-1 (Fragment)	0.010465	10.7	280	28	3	Control	DMT
A2RTI3	LgmN	Legumain	3.27E-07	443	51.3	8	2	DMT	Control
O89054	Actb	Cytoskeletal beta-actin (Fragment)	2.63E-05	28.2	201	16	2	DMT	Control
Q78NA6	Rag1	V(D)J recombination-activating protein 1	2.91E-13	13.5	95.2	16	5	DMT	Control
Q3UDU4	Ldha	L-lactate dehydrogenase	7.9095E-05	13.5	474	41	6	DMT	Control
Q3TM31	Tlr3	TIR domain-containing protein	0.043612	14.8	43.2	9	2	DMT	Control
P06800	Ptpc	Receptor-type tyrosine-protein phosphatase C	6.11E-07	12.5	41.1	6	2	DMT	Control

Protein-protein interaction of the identified 8 allergenic-related proteins showed that PrP interacts with ACTB via Cofilin-1 (CFL1), while VIM, PTPRC, and LDHA directly interacts with ACTB (node 1, 2, 3, 4 in Fig. 7). It was previously shown that overexpression of PrP^C itself activates the NADPH oxidase (NOS) for reactive oxygen species (ROS) production that initiates the cofilin activation and finally induce cofilin-actin rods in hippocampal neurons [68].

A study by Esue *et al.* demonstrated a direct interaction between actin and vimentin filaments mediated by the tail domain of vimentin [69]. Kristiansen *et al.* showed that mild proteasome impairment in prion-infected cells leads to the formation of aggresomes that contain VIM, HSP70, ubiquitin as well as proteasome subunits [70]. Of interest, PTPRC was found to be upregulated in mice brain following inoculation with prions [71]. A study by Ramljak *et al.* showed that there is a direct interaction between PrP^C and lactate dehydrogenase (LDHA) and revealed that LDHA expression is increased under hypoxic conditions [72]. Protein-protein interaction also showed that both RAG1 (node 6 in Fig. 7) and TLR3 (node 5 in Fig. 7) indirectly interact with ACTB (node 1 in Fig. 7) via PTPRC (node 3 in Fig. 7) while PRDX1 (node 7 in Fig. 7) indirectly interacts with ACTB via LDHA (node 4 in Fig. 7) (Fig. 7). A study by Wagner and co-workers showed that PRDX6 was upregulated in scrapie-infected mice and neuronal cell lines [73]. However, LGMN (node 8 in Fig. 7) did not interact with any of the identified allergenic-related proteins as well as with prion protein (node 9 in Fig. 7).

Among the identified allergenic-related proteins, VIM was found to be involved in the progression of allergic diseases via inflammasome [74, 75] and VIM-P38MAPK complex facilitates mast cell activation via FcεRI/CCR1 activation [76]. LDHA was identified as a potential marker in allergic alveolitis, airway inflammation, allergic encephalomyelitis, asthma disease [77–80]. PRDX1 was found as a negative regulator of inflammation [81], Th2-type airway inflammation, and allergen-related hyperresponsiveness [82]. PTPRC was found to be associated with asthma related phenotypes in a microarray analysis [83]. LGMN was found to be involved in allergic reaction by potentiating antigen processing [84]. A study by Sehra *et al.* showed that RAG1-deficient mice exhibited reduced mast cell infiltration when it was used as a chronic model of allergic inflammation [85].

TLR3 activation in an established experimental allergic asthma mice model increased the release of proinflammatory cytokines and mucus production which was also associated with the increased production of interleukin 17 (IL-17A) by natural killer (NK) cells [86].

The highest versus lowest mean of the allergenic-related protein expression by individual antibody treatments when compared to untreated control is shown in Table 6. Here, we show that ICSM18, ICSM35, and SAF70 share 62.5% allergy related proteins (5 proteins: VIM, LGMN, RAG1, LDHA, and PTPRC). On the other hand, both POM2 and SAF32 showed 75% effect with 6 proteins, but the proteins were found to be different for both POM2 (VIM, ACTB, LGMN, RAG1, LDHA, and TLR3) and SAF32 (VIM, ACTB, LGMN, RAG1, LDHA, and PTPRC). However, the lowest effect of antibody was observed for both POM1 (4 proteins; VIM, ACTB, RAG1, and TLR3) and POM3 (4 proteins; VIM, ACTB, LGMN, and LDHA) with 50% effect.

Table 6
Identification of antibody-specific allergenic proteins following direct microglia treatment (DMT). (✓) Present and (-) Absent

Accession ID	Gene ID	ICSM Antibodies				POM Antibodies				SAF Antibodies					
		ICSM18	CTL	ICSM35	CTL	POM1	CTL	POM2	CTL	POM3	CTL	SAF32	CTL	SAF70	CTL
Q3TWV0	Vim	✓	-	✓	-	✓	-	✓	-	✓	-	✓	-	✓	-
B1AXW5	Prdx1	-	✓	-	✓	-	-	-	✓	-	-	-	✓	-	✓
A2RTI3	Lgmn	✓	-	✓	-	-	-	✓	-	✓	-	✓	-	✓	-
O89054	Actb	-	-	-	-	✓	-	✓	-	✓	-	✓	-	-	-
Q78NA6	Rag1	✓	-	✓	-	✓	-	✓	-	-	-	✓	-	✓	-
Q3UDU4	Ldha	✓	-	✓	-	-	-	✓	-	✓	-	✓	-	✓	-
Q3TM31	Tlr3	-	-	-	-	✓	-	✓	-	-	-	-	-	-	-
P06800	Ptprc	✓	-	✓	-	-	-	-	-	-	-	✓	-	✓	-

In order to verify whether the 8 allergenic-related proteins were specifically stimulated in neurons (and not in both neurons and microglia) following co-culture with antibody-treated microglia, we compared the proteome of the anti-PrP antibody-treated microglia without co-culture with neurons and found that anti-PrP antibody-treated microglia only did not display any common allergy-related proteins with DMT (Additional file 2: Table S3) indicating that our 8 identified allergy-related proteins were specifically activated in neurons.

Gene Ontology analysis of Allergy Related Proteins Associated with Direct Microglia Treatment

PANTHER analysis of the allergy related protein after DMT showed that the allergenic-related proteins are involved in cellular components, molecular functions, biological processes, signaling pathways, and protein classes (Fig. 8). Cellular components were classified into different groups in PANTHER analysis including cell (PRDX1, TLR3, ACTB) and membrane (TLR3, ACTB), membrane-enclosed lumen (ACTB), protein-containing complex (ACTB), and supramolecular complex (ACTB) (Fig. 8A). The molecular function was further classified into 3 different groups where it was observed that ACTB were shown to be involved in both structural molecule activity and binding activity and LGMN, PRDX1 and PTPRC are associated with catalytic activity (Fig. 8B). The biological process analysis showed that PRDX1 and TLR3 are involved in biological regulation, immune system process, and response to stimulus whereas PTPRC, LGMN, PRDX1, TLR3, and ACTB were found to be associated with cellular process (Fig. 8C). On the other hand, ACTB, was shown to be involved in biogenesis, developmental process, localization, locomotion, and multicellular organismal processes (Fig. 8C). The analysis also showed that PTPRC, LGMN, and PRDX1 are associated with metabolic processes while TLR3 is involved in signaling and multi-organism processes (Fig. 8C). The signaling pathway analysis is divided into 11 groups whereas PTPRC is involved in both B cell and T cell activation and JAK/STAT signaling pathway (Fig. 8D). On the other hand, ACTB is found to be associated with Alzheimer disease-presenilin pathway, cadherin signaling pathway, cytoskeletal regulation by Rho GTPase, Huntington disease, inflammation mediated by chemokine and cytokine signaling pathway, integrin signalling pathway, nicotinic acetylcholine receptor signaling pathway, and wnt signaling pathway (Fig. 8D).

Gene enrichment analysis of the 8 allergenic related proteins through FunRich showed 5 cellular components such as focal adhesion (ACTB, PTPRC; $p = 0.02$), ribonucleoprotein complex (VIM, ACTB; $p = 0.018$), bleb (PTPRC; $p = 0.03$), type III intermediate filament (VIM; $p = 0.01$), and membrane microdomain (PTPRC; $p = 0.001$) (Fig. 9A). FunRich analysis of the molecular function identified protein kinase binding (VIM, ACTB, PTPRC; $p = 0.03$), identical protein binding (VIM, PRDX1, ACTB, RAG1, TLR3; $p = 0.001$), and lactate dehydrogenase activity (LDHA; $p = 0.009$) (Fig. 9B).

Furthermore, the biological process analysis was found to be associated with cellular response to extracellular stimulus (LDHA, PTPRC; $p < 0.001$), cellular response to cytochalasin B (ACTB; $p = 0.02$), positive regulation of T cell differentiation (RAG1, PTPRC; $p = 0.03$), positive regulation of hematopoietic stem cell migration (PTPRC; $p = 0.03$), immunoglobulin biosynthetic process (PTPRC; $p = 0.01$), glucose catabolic process to lactate via pyruvate (LDHA; $p = 0.004$), negative regulation of ERBB signaling pathway (LGMN; $p = 0.004$), vacuolar protein processing (LGMN; $p = 0.02$), plasma membrane raft distribution (ACTB; $p = 0.004$), positive regulation of antigen receptor-mediated signaling pathway (ACTB; $p = 0.004$), positive regulation of protein tyrosine phosphatase activity (ACTB; $p = 0.01$), positive regulation of T cell mediated immunity (ACTB; $p = 0.01$), regulation of humoral immune response mediated by circulating immunoglobulin (ACTB; $p = 0.004$), regulation of interleukin-8 production (ACTB; $p = 0.004$), and regulation of Schwann cell migration (VIM; $p = 0.004$) (Fig. 9C).

Contactless Co-Culture of Anti-PrP Antibody Treated-Microglia and Mouse Neuroblastoma Cell Lines Leads to Neuronal Allergy.

Contactless co-culture of anti-PrP antibody treated-microglia N11 and N2a cells was designed to verify whether the allergenic effects caused by DMT were due to a direct cognate interaction of N2a and N11 or via indirect release of microglial factors which in turn might have led to allergenicity. N11 cells were initially treated with anti-PrP antibodies, including ICSM18, ICSM35, POM1, POM2, POM3, SAF32 or SAF70 on tissue culture inserts before placing the inserts

containing antibody-treated microglia on tissue culture plate containing untreated N2a cells (IMT). IMT resulted in an initial dataset of 292 proteins ($p < 0.05$) after LC-MS analysis. Differentially expressed proteins ($p < 0.05$) were considered with a maximum fold change ≥ 10 and at least 2 identified unique peptides and a confidence score ≥ 10 and identified a total of 11 proteins (Additional file 2: Table S4). Out of the 11 proteins, AllergAtlas database identified Integrin beta-4 (ITGB4) (upregulated, $p = 0.034$, maximum fold change 45, confidence score 33.6, peptide 6, unique peptide 3) as being allergenic.

The protein-protein interaction analysis showed that ITGB4 indirectly interacts with PRNP via ITGB6 and NCAM 1 (Additional file 1: Figure S2A). Santucci and co-workers showed that activation of p59fyn was achieved via PrPC recruitment NCAM to lipid rafts [87]. Ghodrati *et al.* also showed that NCAM directly interacts with PrP and identified the transforming growth factor β and integrin signaling as prion interactors via gene ontology analysis [88].

Liu and co-workers revealed that ITGB4 is involved in airway hyper-responsiveness and lung inflammation in allergic asthma. ITGB4-deficient mice displayed increased infiltration of lymphocyte, neutrophil, and eosinophil as well as expression of IL-4, IL-13, and IL-13A in lung tissue [89]. Tang *et al.* showed that ITGB4-deficiency causes spontaneous exaggerated lung inflammation in early life [90]. A study by Yuan and co-workers showed that p53 pathway activation in ITGB4-deficiency prompts the senescence of airway epithelial cells [91]. Yuan and co-workers also demonstrated that the lack of ITGB4 is responsible for increased Th2 responses in allergic asthma by down-regulation of CCL17 and EGFR pathway in airway epithelial cells [92].

Gene Ontology analysis of Allergy Related Proteins Associated with indirect Microglia Treatment

The Gene classification through PANTHER showed that ITGB4 is associated with cellular components (cell junction, membrane, and protein-containing complex) (Additional file 1: Figure S2B), molecular function (binding) (Additional file 1: Figure S2C), biological process (biological adhesion, biological regulation, cellular process, localization, locomotion, response to stimulus, and signaling) (Additional file 1: Figure S2D), and signaling pathway (Integrin signalling pathway) (Additional file 1: Figure S2E). The Gene enrichment analysis ITGB4 through FunRich showed that the cellular components involved, included hemidesmosome ($p < 0.001$), integrin complex ($p < 0.001$), basal plasma membrane ($p = 0.022$), and cell leading edge ($p = 0.032$) (Additional file 1: Figure S3A). The biological process was found to be associated with trophoblast cell migration ($p < 0.001$), peripheral nervous system myelin formation ($p < 0.001$), hemidesmosome assembly ($p = 0.002$), cell adhesion mediated by integrin ($p = 0.023$), and cell motility ($p = 0.036$) (Additional file 1: Figure S3B). On the other hand, the reactome pathway analysis of ITGB4 identified type I hemidesmosome assembly ($p = 0.002$), syndecan interaction ($p = 0.006$), laminin interaction ($p = 0.009$), and assembly of collagen fibrils and other multimeric structures ($p = 0.038$) (Additional file 1: Figure S3C).

Discussion

Hypersensitivity reactions are triggered by the immune response. Little is known about the so-called IgG-mediated neuronal hypersensitivity, however, a body of new emerging studies suggest that hypersensitivity is an important feature in response to IgG immunotherapy or disease-associated auto-antibodies [93–95]. Fc γ receptors (Fc γ R) are known to mediate protective immune functions via binding of IgG molecules to the Fc domain in addition to modulating the adaptive immune response. Fc γ R have been implicated in hypersensitivity reactions; for instance, Fc γ -chain-deficient mice were protected against a number of autoimmune disorders (reviewed in [67]), suggesting an important role for Fc γ RI in hypersensitivity reactions. Furthermore, functional polymorphism in Fc γ R genes was shown to play an important role in the pathogenesis of allergy [96]. A number of *in vitro* and *in vivo* studies have demonstrated the presence of Fc γ R in neurons. Previous reports implicated IgG in inducing a neuronal hypersensitivity reaction [94]. Of importance, Fuller *et al.* highlighted the importance of increased expression and ligation of Fc γ R in the CNS as a result of administration of therapeutic antibodies or by endogenous IgG which resulted in vascular damage and exacerbation of neurodegeneration [97]. Furthermore, experimental treatment with anti-PrP antibodies directed against PrP^C led to neuronal apoptosis based on non-molecular microscopic assessments [23, 24, 26]. Upon further analysis, some of these studies also revealed that anti-PrP antibodies induced activation of allergenic-related proteins identified by the AllergAtlas database [21, 27, 28]. We therefore sought molecular confirmation of a neuronal hypersensitivity/allergenic process associated with anti-PrP antibody treatment of neuroblastoma and microglia. Initially, we performed *in silico* analysis to predict the most antigenic epitopes from the huPrP 3D structure and to verify whether some of the predicted motifs overlap with those recognized by the reported 'neurotoxic' anti-PrP antibodies such as ICSM and POM antibodies [40, 43]. The *in silico* analysis revealed a set of antigenic B cell linear epitopes located on the flexible tail (FT) region: L2 (89–111), L7 (24–49), L9 (56–66), and L10 (76–86) of which L9 and L10 are on the octa-peptide repeats (OR) in the FT region. Interestingly, epitopes L2 and L9/L10 were mapped to the 'neurotoxic' antibodies ICSM35 [26, 40], POM3 and POM2 [23, 24, 27, 43]. We also confirmed that L2, L9 and L10 were toxic following assessment with the ToxinPred server [50] by quantitative matrix based method (QM method). The *in silico* analysis also revealed a set of antigenic B cell linear epitopes located on the globular (GD) region: L4 (137–153), L3 (167–174), L6 (189–204), L1 (222–238) and L5 (245–253), of which L4 was mapped to the 'neurotoxic' antibodies ICSM18 [24, 26, 40] and POM1 [24, 27, 43]. The ToxinPred server also identified L4 as being toxic. While performing *in silico* analysis to assess antigenicity and toxicity of epitopes located on the huPrP 3D structure, we also noticed that some antigenic epitopes were predicted to be allergenic by AllergenFP [52] and AllerTop [51] allergenicity prediction servers. A total of 7 B cell linear epitopes were identified as allergenic, including L3 (167–174), L4 (137–153), and L8 (1–4) with AllergenFP and L2 (89–111), L8 (1–4), L9 (56–66) and L10 (76–86) with AllerTop server. Of interest, L4 was mapped to the neurotoxic antibodies POM1 and ICSM18 and L2 was mapped to ICSM35 and POM3 while L9 and L10 were both mapped to POM2. POM1, a similar antibody to ICSM18 and mapped to the L4 epitope was previously shown to induce neurotoxicity via calpain [27]. Our LC-MS data also shows that calpain 1 is activated by ICSM18 treatment, but on the contrary led to inhibition of calpain 3 (data not shown), probably via negative feedback following cross-linking PrP^C. In addition to its newly characterized role in antibody-induced neuronal apoptosis, calpains have a well-established role in allergy [98–100]. For instance, a study by Wu *et al.* showed that calpain 1 contributes to mast cell degranulation [98]. Furthermore, inhibition of mGluRs, known to regulate histamine [34], abolished the anti-PrP antibody toxic effects [28]. Taken together and in addition to the allergenic-related proteins associated with ICSM35 treatment reported by Tayebi and colleagues [21], this provides sufficient evidence to investigate the allergenic pathways potentially induced by treatment with anti-PrP antibodies which we refer to as "*IgG-Mediated Neuronal Allergenic Toxicity*".

To that end, we treated mouse neuroblastoma (N2a) and microglia (N11) cell lines with anti-PrP antibodies then assessed the neuronal allergenic proteome following mass spectrometry analysis as well as expression of neuronal FcγRI and FcγRIII. Initially, we applied the anti-PrP antibodies directly (DAT) on N2a cells and showed that SAF70 and POM1 but not ICSM18 led to a high molecular band associated with both FcγRI and FcγRIII but not in untreated control, indicating that antibody-treated N2a cells are positively primed to regulate an immune response [101]. DMT with anti-PrP antibodies does not appear to affect expression of both CD64 and CD16, however, IMT with anti-PrP antibodies led to increased CD64 and CD16 expression with ICSM18 and POM1. These results indicate that despite a possible role for FcγRs in inducing allergenicity via antibody treatment of microglia following binding of the Fc portion, it is more probable that this was caused by cross-linking of PrP^C with anti-PrP antibodies via binding with the Fab region. A report by Lunnon *et al.* demonstrated increased activation of microglial FcγRs in a mouse model of prion diseases, suggesting that this increase is directly linked to PrP alteration [102]. The FcγRs activation profile as shown by western blotting contrasted with the outcome observed following LC-MS analysis. Failure of DMT to alter FcγRs activation was not reflected in the profile of the allergenic proteome as this identified 8 upregulated allergenic proteins, further proving that this allergenic reaction is caused following cross-linking of PrP^C and not via binding to the Fc portion of the antibody. This dichotomy is also reflected by the DAT where FcγRI and III was activated but LC-MS identified only 4 proteins associated with allergy. Amongst the 4 proteins, ACTB was shown to interact with PrP^C via cofilin 1 by STRING. Walsh and co-workers demonstrated that overexpression of PrP^C activates the NADPH oxidase (NOS) for reactive oxygen species (ROS) production which initiates cofilin activation and finally induce cofilin-actin rods in hippocampal neurons [68]. Moreover, DAVID analysis showed that ACTB was involved in platelet aggregation. Blood platelets play an active and essential role in allergic inflammation and pathogenesis of the allergic diseases [103, 104]. The cytoplasmic FABP5 is found in adipocytes and was previously shown to be with allergic asthma where FABP5 is upregulated in sputum of allergic asthmatics and had positive correlation with the vascular endothelial growth factor (VEGF) [105]. Lee *et al.* also observed an association between FABP5 and atopic march or allergic march where knockdown of FABP5 dramatically reduced IL-17A in T cells from atopic march patients [106]. Another study by Shum *et al.* revealed that FABP controls allergic airway inflammation [107]. Ge *et al.* showed that FABP4 is involved in the regulation of eosinophil recruitment and plays a proinflammatory role during allergic asthma development [108]. Of note, FABP5 is an intracellular lipid carrier protein involved in the regulation of inflammation and its inhibition decreases levels of prostaglandin E₂ and proinflammatory cytokines [109]. PCDH11X and PCDH1 are cell adhesion molecules and belong to the cadherin protein family that are highly expressed within the CNS [110–112]. PCDH1 was shown to be associated with childhood asthma, bronchial hyperresponsiveness as well as eczema and other atopic phenotypes [113–115]. Variation in the PCDH11X gene is associated with late-onset Alzheimer's disease [116, 117]. Finally, PDE4 plays an essential regulatory role in immune and inflammatory cells [118]. The involvement of PDE4 in allergy and asthma has been widely studied in human blood leukocytes and a positive regulatory role of the IL-4 has also been established [118–121]. In addition, inhibition of PDE4 leads to reduction of hyperresponsiveness, airway inflammation as well as phosphodiesterase activity [122–124].

The PANTHER analysis showed that DAT-associated allergenic-related proteins are associated with Wnt and integrin, inflammation mediated by chemokine and cytokine and nicotinic acetylcholine receptor signaling pathway. The Wnt/β-catenin signaling pathway is involved in airway remodeling in chronic asthma and enhances the development of allergic airway disease [125–127]. The recruitment of eosinophils is a prominent feature of asthma and integrin is involved in the regulation of extravasation of eosinophils [128, 129]. The Nicotinic acetylcholine receptors modulate the synaptic and cellular functions in the brain and are important for the regulation of cytokine release [130]. Impairment of the α7- nicotinic acetylcholine receptor leads to high production of cytokine that enhances the possibility of tissue damage [131, 132]. FunRich analysis of DAT-associated allergenic-related proteins highlighted the association between regulation of prostaglandin biosynthetic process and allergic lung inflammation [133, 134]. Similarly, cytochalasin B was shown to be associated with asthma through enhancing release of platelet-activating factor (PAF)-induced histamine [135]. During the course of prion disease, mouse microglia are highly activated and express TGF-β and PGE₂, both known mediators of allergy [136, 137]. Moreover, anti-PrP therapeutic antibodies also led to strong microglial activation associated with neuronal loss [25]. Due to the important role played by microglia in the exacerbation of neuropathology in the prion and other related disorders, we sought to verify whether co-culture of N2a with anti-PrP antibody-treated N11 (DMT) leads to a neuronal allergenic reaction. We have identified 8 allergenic-related proteins following DMT, 7 of which were upregulated with the exception of PRDX1 that was found to be downregulated. The protein-protein interaction analysis showed that VIM, interacts with ACTB. VIM is an intermediate filament protein which plays an important role in stabilizing intracellular architecture and a regulatory role in the NLRP3 inflammasome where IL-1β and caspase-1 were decreased in VIM deficient macrophage cells [74]. Of note, IL-1β and NLRP3 inflammasome are involved in the progression of allergic diseases [75]. A study by Toda *et al.* revealed that VIM-P38MAPK complex facilitates mast cell activation via FcεRI/CCR1 activation [76]. Amongst the DMT associated allergenic-related proteins, LDHA was found to interact with PRDX1 by STRING. A study by Ramljak and co-workers revealed that PrP^C and LDHA are direct interactors and that LDHA expression increased under hypoxic conditions [72]. Plasma LDHA was found to be a potential marker for cryptogenic fibrosing alveolitis and extrinsic allergic alveolitis [77]. In three separate study, serum LDHA was also found to be a potential marker for airway inflammation [78], atopic dermatitis [138] and experimental allergic encephalomyelitis [79]. Al Obaidi and co-workers showed that sputum LDHA is a potential and accurate marker in asthma disease [80]. Taken together, the above studies clearly highlight the important role played by LDHA in allergy. Interestingly, PRDX6, a protein that directly networks with our downregulated PRDX1, was shown to be upregulated in scrapie-infected mice and neuronal cell lines and controls expression of PrP^C and PrP^{Sc} in neuronal cells [73]. PRDX1 is a ubiquitous antioxidant enzyme known to act as a negative regulator for protection against inflammation [81]. Inoue *et al.* showed that PRDX1 protects against allergen-related hyperresponsiveness and Th2-type airway inflammation and involved in the inhibition of allergen-specific T-cell proliferation through immunological synapse [82]. In protein-protein interaction analysis PTPRC directly interacted with ACTB, TRL3, RAG1 and RAG2. PTPRC was upregulated in brain of mice following infection of prion [71]. PTPRC is associated with asthma related phenotypes [83] and its ligation enhances the frequency of constitutive apoptosis in human eosinophils [139]. LGMN helps to destroy the ASNase activity (degraded asparaginase produced by *Escherichia coli*) leading to an allergic reaction by potentiating antigen processing [84]. RAG1 plays an important regulatory role in the reorganization and recombination of T cell receptor (TCR) and immunoglobulin (Ig) genes [140]. A study by Sehra *et al.* showed that Rag1-deficient mice exhibited reduced mast cell infiltration when it was used as a chronic model of allergic inflammation [85]. Moreover, Rag1-deficient zebrafish showed that the immunity expression and apoptosis associated genes are increased and showed greater prevalence of cell cycle arrest and oxidative stress [141]. Of interest, rag1-mice were shown to be highly resistant to oral challenge with prions [142]. Another allergenic-protein identified following DMT is TLR3 that directly interact with PTPRC. TLR3 is a membrane protein which act as a pathogen recognition receptor and is expressed in the CNS and other cell types [143]. TLR3 activation by poly (inosinic-cytidylic) acid in an

established experimental allergic asthma mice model increased the release of proinflammatory cytokines and mucus production which was also associated with increased production of IL-17A by NK cells [86]. Starkhammar and colleagues showed that combined stimulation of TLR3 and TLR4 causes airway hyperresponsiveness which is increased during an ongoing allergic inflammation [144]. ACTB, which was found in both DAT and DMT, is associated with platelets aggregation [145] and might have a role in allergic diseases [103, 104].

Analysis of individual anti-PrP antibody treatment identified that the highest allergenic effect, as assessed by the number of allergenic-related proteins, was associated with the GD and FT targeting antibodies ICSM18/SAF70 and ICSM35 which shared 5 common proteins. ICSM18 and 35 were produced in PrP-null against truncated hrPrP^{91–231} while SAF70 was raised in hamsters using SAF preparation. However, both ICSM18 and SAF70 bind to a similar epitope on the GD but ICSM35's epitope is located on the FT domain. ICSM35 and SAF70 are of the same Ig isotype (IgG2b). It remains a challenge to pinpoint which of these antibody characteristics led to activation of the common proteins, however, there is strong indication that inherent antibody properties (e.g. epitope; isotype etc.) trigger similar allergenic pathways. POM2 and SAF32, two antibodies that bind to an epitope on the octa-repeat activated 6 proteins separately where 5 proteins were common and the protein TLR3 and PTPRC was found to be activated by POM2 and SAF32, respectively. SAF32, similar to SAF70 was raised in hamsters using SAF preparation and is an IgG2b, while POM2 was raised in PrP-null mice against full-length mrPrP^{23–231} and is an IgG1. In this case, the common octa-repeat epitope appears to be playing a key role in triggering similar allergenic pathways by these 2 antibodies, however, all molecular aspects should also be considered, including antibody affinity for instance. It is noteworthy that among the 5 common proteins shared by POM2 and SAF32, 4 proteins were also common with ICSM18, ICSM35 and SAF70 treatments, possibly reflecting the involvement of several antibody properties. Finally, POM1 and POM3, raised in PrP-null mice against full-length mrPrP^{23–231} and of IgG1 isotype with binding motifs located on GD and FT domains respectively activated only 4 proteins with very little commonality with the other antibody treatments.

The PANTHER analysis of the 8 allergy-related proteins identified pathways that were common between DAT and DMT. However, DMT also includes JAK/STAT signalling pathway, B cell and T cell activation, and toll receptor signaling pathway. The JAK/STAT signaling pathway was found to be associated with the chronic inflammatory skin disease atopic dermatitis [146]. Toll-like receptors are also involved in allergy pathogenesis [147], asthma [148], and allergic rhinitis [149]. Functional gene enrichment analysis identified positive regulation of protein tyrosine phosphatase activity besides the other immune response related function such as positive regulation of T cell mediated immunity, regulation of humoral immune response, regulation of interleukin-8 production, and positive regulation of antigen receptor-mediated signaling pathway. Protein tyrosine phosphatases is involved in the regulation of allergic asthma where the protein tyrosine phosphatase inhibition in allergen-challenge phase or allergen-sensitization phase helps decrease the development of asthma which correlated with increased T helper 1 (Th1) response [150]. Finally, IMT activated one allergy-related protein. ITGB4 expression reduces antigen presentation and regulates airway inflammation reaction in allergic asthma [151]. Yuan and co-workers demonstrated that lack of ITGB4 is responsible for increasing of Th2 responses in allergic asthma by down-regulation of CCL17 and EGFR pathway in airway epithelial cells [92]. ITGB4 deficiency was also found to be associated with airway inflammation where ITGB4-deficient mice showed increase of microglia and pro-inflammatory cytokines, TNF- α , IL-6, and IL-1 β in the hippocampus and prefrontal cortex [152]. The effect by IMT, albeit limited as it led to activation to a single allergenic protein, might have been triggered by release of cytokines such as IL-1, IL-6, and TNF- α antibody-treated N11 [54] and possibly induced a neuronal allergenic reaction.

Conclusion

Antibody-mediated therapy for prions attracted intense debate and controversy as some of the reported were contradictory partly because these relied on a somewhat superficial assessment using microscopy and also due to the failure of investigating the fine molecular events caused by cross-linking PrP^C with anti-PrP antibodies. Luckily, this controversy related to prion antibody treatment did not lead to fatalities in humans affected with CJD. However, Alzheimer's disease trials have led the death of individuals administered with therapeutic antibodies. This study led to unique discovery showing that anti-PrP antibodies led to neuronal allergenicity via different pathways but also highlights the key role played by microglia in causing the allergenic reaction. This study also emphasizes the need to include a screening 'allergenicity' step during development of therapeutic antibodies to avoid potential side-effects.

Abbreviations

TSE: Transmissible Spongiform Encephalopathies; APOE: Apolipoprotein E; cPLA2: Cytoplasmic Phospholipase A2; PG: Prostaglandin; CAPN: Calpain; mGluR: Group-I Metabotropic Glutamate Receptors; COX: Cyclooxygenase; GD: Globular Domain; FT: Flexible Tail; OR: Octa-peptide Repeats; LC-MS: Liquid-Chromatography Mass-Spectrophotometry; DAT: Direct Antibody Treatment; DMT: Direct Microglia Treatment; IMT: Indirect Microglia Treatment; SVM: Support Vector Machine; QM: Quantitative Matrix; RMSD: Root Mean Square Deviation; TM-score: Template Modelling Score

Declarations

Availability of data and materials

Detailed results of our analyses are included with this article and its additional files.

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Competing interests

The authors declare no competing interest.

Funding

This work was supported by an Ainsworth Medical Research Innovation Fund Grant awarded to MT. TK receives funding from two project grants from the National Health and Medical Research Council [#1102012 and #1141789] and the NHMRC dementia research team initiative [#1095215] as well as the Ainsworth Medical Research Innovation Fund and the Australian Research Council [#DP18010473].

Authors' contributions

U.K.A performed experiments and wrote draft manuscript; ES. Performed experiments; U.H performed experiments; S.K performed experiments; M.M performed experiments; G.G, L.O, M.D, and T.K reviewed manuscript; M.T designed, managed, wrote and revised manuscript. All authors read and approved the final manuscript

Acknowledgements

UK was awarded an Australian Government Research Training Program Stipend Scholarship (RTP) for PhD support. The authors would like to acknowledge the Mass Spectrometry Facility (MSF) of Western Sydney University for access to its instrumentation.

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Figures

Figure 1

Overall workflow of the in silico and in vitro study for the identification of allergenic proteins following antibody treatment and LC-MS analysis.

Figure 2

Evaluation of the predicted protein three-dimensional structure of the human major prion protein. A) three-dimensional (3D) structure of the human major prion protein which is enriched with alpha-helix and random coils B) Ramachandran plot for the predicted 3D structure of the human major prion protein (68.4% residues in most favoured region, 24.7% in allowed regions, 3.7% in generously allowed regions, and 3.2% residues in the disallowed regions) C) ProSA energy plot for the predicted protein model. Herein 0.0 is the baseline, the sequence position under the baseline (0.0) indicates the good quality of the protein model.

Figure 3

Predicted linear and conformational B cell epitopes and their position in the human major prion protein structure. Here, the protein structures have shown as cartoon structure and the linear and conformational B cell epitopes have shown as spheres on the protein structures. A) Linear B cell epitopes where L1 (222-238), L2 (89-111), L3 (167-174), L4 (137-153), L5 (245-253), L6 (189-204), L7 (24-49), L8 (1-4), L9 (56-66), and L10 (76-86) representing different epitope position. B) all the linear B cell epitopes have been shown as spheres by different colours to see the overlaps of the identified linear B cell epitopes. C) Conformational B cell epitopes where C1(227-235), C2 (100-112), C3 (25-38), C4 (137-149, 151-153, 157, 189, 191-204), C5 (164, 168-174, 177), C6 (222-225), C7 (236-238, 242, 245-253), C8 (39-49), and C9 (55-67, 75-86, 89-99) representing different epitope position. D) all the conformational B cell epitopes have been shown as spheres by different colours to see the overlaps of the identified conformational B cell epitopes. Here, liner B cell epitope L1 was found in the conformational B cell epitope position C1 and C6; C2 and C9 representing the linear epitope L2; C5 representing the linear epitope L3; C4 (two position where one is in the left side and the other one is in the middle position of the structure) representing the linear epitope L4 and L6; C7 representing the linear epitope C5; C3 and C8 representing the linear epitope L7; and the conformational epitope C9 (two position in the bottom of structure) representing both the linear epitope L9 and L10.

Figure 4

Expression of different FC γ receptors for the identification of allergenicity following different anti-PrP antibody treatment to the mouse neuroblastoma cell line. Western blotting of A) direct antibody treatment (ICSM18, SAF70, and POM1), B) direct microglia treatment (anti-PrP antibody treated microglia directly applied to the mouse neuroblastoma cells), and C) indirect microglia treatment (anti-PrP antibody treated microglia indirectly applied (in inserts) to the mouse neuroblastoma cells) samples using human TrueStain FC blocker, different antibodies FC γ I (CD64), FC γ III (CD16), anti-phosphoserine (anti-phos-S), and anti-phosphotyrosine (anti-phos-T) as primary antibodies, and anti-mouse IgG (Fab-specific) antibody was used as secondary antibody. Herein "NoT" indicating the control which is not antibody treated. 30 μ L (300 μ g/mL) of the sample was loaded in each well of the 12% Mini-PROTEAN $^{\circ}$ TGX $^{\text{TM}}$ Precast Protein Gels and Precision Plus Protein $^{\text{TM}}$ Kaleidoscope $^{\text{TM}}$ Standards was used as a marker.

Figure 5

Protein-protein interaction and classification of the identified allergenic genes following direct antibody treatment (DAT). Protein-protein interaction analysis was performed using STRING v11.0 server. The Mus Musculus database was used as the host organism for the identification of protein interactions. A) Interaction among the allergenic proteins (numbered 1, 2, 3, & 4) and with PrPC (numbered 5) following direct antibody treatment. Classification of the B) cellular components; C) biological processes; and D) signalling pathways of the identified allergenic genes using PANTHER server analysis.

Figure 6

Gene enrichment analysis of the 4 identified allergenic genes following direct anti-PrP antibody treatment (DAT). Classification of the A) cellular components; B) biological processes, and C) signalling pathways of the identified genes using functional gene enrichment analysis tool, FunRich (v3.1.3). The p-value in the plot is the Bonferroni corrected p-value and the reference p-value is $p=0.05$.

Figure 7

Protein-protein interaction analysis among the 8 identified allergenic genes following direct microglial treatment (DMT) with anti-PrP antibodies. The analysis was performed using STRING v11.0 server. The Mus Musculus database was used as the host organism for characterising the protein interactions. Herein, the numbered purple circles (numbered 1, 2, 3, 4, 5, 6) and numbered red circle refer to allergenic-related proteins identified following DMT and shown here to be part of the same inetractome. The red circle (numbered 7) was shown to be downregulated and the orange circle (numbered 8) is not part of the inetractome with other allergenic-related proteins. Finally, the green circle (numbered 9) represents the prion protein, PrPC.

Figure 8

Classification of the identified allergenic genes following direct microglial treatment (DMT). Classification of the A) cellular components; B) molecular functions; C) biological processes, and D) signalling pathways of the 8 identified allergenic genes using PANTHER server analysis.

Figure 9

Gene enrichment analysis of the 8 identified allergenic genes following direct microglial treatment (DMT). Classification of the A) cellular components; B) molecular functions, and C) biological processes of the 8 identified genes using functional gene enrichment analysis tool, FunRich (v3.1.3). The p-value in the plot is the Bonferroni corrected p-value and the reference p-value is $p=0.05$.

Supplementary Files

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- [SupplementaryFile.pdf](#)