Journal of Visualized Experiments

A cell-based assay to study antibody-mediated tau clearance by microglia

--Manuscript Draft--

Article Type:	Invited Methods Article - JoVE Produced Video
Manuscript Number:	JoVE58576R1
Full Title:	A cell-based assay to study antibody-mediated tau clearance by microglia
Keywords:	Alzheimer's, tauopathy, tau, aggregation, microglia, BV-2, uptake, clearance; Alzheimer's, tauopathy, tau, aggregation, microglia, BV-2, uptake, clearance
Corresponding Author:	Wouter Koudstaal Janssen Prevention Center Leiden, NETHERLANDS
Corresponding Author's Institution:	Janssen Prevention Center
Corresponding Author E-Mail:	WKoudsta@its.jnj.com
Order of Authors:	Wouter Koudstaal
	Donata De Marco
	Renske Taggenbrock
	Rosa Crespo
	Elizabeth Ramsburg
	Adrian Apetri
Additional Information:	
Question	**Response**
Please indicate whether this article will be Standard Access or Open Access.	Open Access (US$4,200)
Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations.	Archimedesweg 6, Leiden, The Netherlands, 2333CN

TITLE:
Cell-Based Assay to Study Antibody-Mediated Tau Clearance by Microglia

AUTHORS & AFFILIATIONS:
Donata De Marco[1], Renske Taggenbrock[2]*, Rosa Crespo[2], Wouter Koudstaal[2], Elizabeth Ramsburg[3] and Adrian Apetri[2]

[1]Neurosceince Discovery, Janssen Pharmaceutical Company of Johnson & Johnson, Leiden, The Netherlands
[2]Janssen Prevention Center, Janssen Pharmaceutical Company of Johnson & Johnson, Leiden, The Netherlands
[3]Neurosceince Discovery, Janssen Pharmaceutical Company of Johnson & Johnson, Spring House, Pennsylvania, USA
*Current affiliation: Department of Hematopoiesis, Sanquin research and Landsteiner laboratory, AMC, Amsterdam, The Netherlands

Corresponding Authors:
Donata De Marco
Email Address: ddemarc2@its.jnj.com
Tel: +31 (0) 646562068

Adrian Apetri
Email Address: aapetri@its.jnj.com
Tel: + 31 (0) 655123522

KEYWORDS:
Alzheimer's, tauopathy, tau, aggregation, microglia, BV-2, uptake, clearance.

SHORT ABSTRACT:
Here we describe a cell-based assay to quantitatively assess tau uptake by microglia with the aim of creating an investigational tool to better characterize the mechanisms of action of anti-tau antibodies.

LONG ABSTRACT:
Alzheimer's disease (AD) is a progressive neurodegenerative condition in which aggregated tau and amyloid proteins accumulate in the brain causing neuronal dysfunction which eventually leads to cognitive decline. Hyperphosphorylated tau aggregates in the neuron are believed to cause most of the pathology associated with AD. These aggregates are assumed to be released into the extracellular compartment and taken up by adjacent healthy neurons where they induce further tau aggregation. This "prion-like" spreading can be interrupted by antibodies capable of binding and "neutralizing" extracellular tau aggregates as shown in preclinical mouse models of AD. One of the proposed mechanisms by which therapeutic antibodies reduce pathology is antibody-mediated uptake and clearance of pathological aggregated forms of tau by microglia. Here, we describe a quantitative cell-based assay to assess tau uptake by microglia. This assay

uses the mouse microglial cell line BV-2, allows for high specificity, low variability and medium throughput. Data generated with this assay can contribute to a better characterization of anti-tau antibody effector functions.

INTRODUCTION:

Alzheimer's disease (AD) is a progressive neurodegenerative condition characterized by the conformational change and self-assembly of amyloid β peptide and tau protein into pathological aggregates. The normal soluble amyloid β peptide is converted into oligomeric and fibrillar amyloid β, while abnormally phosphorylated tau accumulates as oligomers and neurofibrillary tangles[1, 2]. These protein aggregates cause neuronal death leading to memory loss and subsequent progressive cognitive decline. Other factors, including non-productive neuroinflammation and a reduced ability to clear misfolded proteins, may exacerbate and accelerate disease. Currently, intervention strategies against AD provide largely symptomatic relief, but there is no disease-modifying cure or prevention.

Increasing evidence suggests a key role of hyperphosphorylated tau aggregates in the pathology of AD. In its non-pathological state, tau is a natively unfolded protein that binds to microtubules and promotes their assembly into the neuronal cytoskeleton. When tau becomes hyperphosphorylated, it detaches from the cytoskeleton and clusters into tau aggregates in the neuron, which are believed to cause most of the pathology associated with AD[3]. Aggregated tau starts accumulating first intracellularly, but as disease progresses, it is assumed to be released from affected neurons into the extracellular space, from which it can be taken up by adjacent or synaptically connected healthy neurons in a "prion-like manner". Once internalized, the tau aggregate induces further tau aggregation *via* templated conformational change[4].

According to this hypothesis, therapies capable of interrupting tau seeding might slow down or reverse the course of tau-mediated neurodegenerative disease. In support of this, mice made susceptible to tauopathy by genetic mutation and passively injected with anti-tau antibodies show reduced tau pathology and improved cognitive function[5–9]. However, the mechanisms by which therapeutic antibodies reduce pathology still remain elusive.

One of the proposed mechanisms is antibody-mediated uptake and clearance of pathological aggregated forms of tau by microglia, the brain's resident immune cells. Recent publications suggest that microglia can efficiently internalize and degrade pathological tau species and this ability is enhanced by anti-tau antibodies *via* an Fc-dependent mechanism involving Fc receptors expressed on the surface of microglia and receptor mediated phagocytosis[10, 11]. These data identify microglia as potentially important effectors of therapeutic antibodies.

We describe herein a cell-based assay to quantitatively assess tau uptake by microglia. Data generated with this assay can help elucidating the mechanisms of action of anti-tau antibodies thus representing a useful tool to advance anti-tau antibodies to further the steps of their development as potential AD treatment.

PROTOCOL:

1. BV-2 Cells Culture

Note: Handle BV-2 cells under Biosafety Level 2 containment. The BV-2 cell line produces an enveloped recombinant ecotropic retrovirus (capable of infecting murine cells only)[12]; such viruses are known for their *in vitro* transforming ability and *in vivo* tumorigenic potential.

1.1. Culture BV-2 cells in high glucose Dulbecco's modified eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, 100 µg/mL streptomycin and 2 mM L-Glutamine (referred to as culturing medium from now on) by seeding cells at 4 x 10^4 cells/mL.

1.2. Maintain cultures in a humidified atmosphere of 5% CO_2 at 37 °C.

Note: the cells grow loosely attached and in suspension.

2. Label Recombinant Tau Aggregates with pH-Sensitive Fluorescent Dye

Note: Tau aggregates were prepared as described in Apetri *et al.*[13] with the difference that no Thioflavin T (ThT) was added to the reaction buffer. Aggregated samples were collected in 1.5 mL centrifuge tubes. Final fluorescence signal was checked by mixing 118 µL of the pool sample with 12 µL of a 50 µM ThT solution. Aggregates were separated by centrifuging the aggregation reaction mixture at 20,000 x *g* for 1 h at 4 °C. The supernatant was analyzed by S MALS to confirm that all the monomeric tau was converted into aggregates. Pellets (tau aggregates) were snap frozen and stored in a freezer at -80 °C.

2.1. Resuspend tau aggregates in 0.1 M sodium bicarbonate buffer ($NaHCO_3$) at pH 8.5 to a concentration of 1 mg/mL (~ 20 µM).

Note: Concentration of tau aggregates is based on the initial monomers concentration as assessed by the absorption of tau monomers at 280 nm using an extinction coefficient of 0.31 $mLmg^{-1}cm^{-1}$.

2.2. Sonicate the resuspended aggregates using a probe sonicator while keeping on ice to avoid over-heating.

2.2.1. Use an amplitude of 65% (with sonicator of power 250 W).

2.2.2. Perform 8 pulses of 3 s with pauses of 15 s between pulses to avoid overheating.

2.3. Prepare an 8.9 mM stock solution of pHrodo dye (henceforth reference to as pH dye) in dimethyl sulfoxide (DMSO) following manufacturer's instruction.

Note: Always prepare a fresh solution and use it only on the day it is prepared.

2.4. Add 10 moles of dye per mole of protein to a final dye concentration of 0.2 mM.

2.5. Mix by gently pipetting up and down.

2.6. Incubate the reaction mixture for 45-60 min at room temperature, protected from light.

2.7. In the meantime, assemble a cross-linked dextran gel desalting column following manufacturer's instructions.

2.8. Equilibrate the column with 25 mL elution buffer (100 mM $NaHCO_3$ pH 5, 3% DMSO). Discard the flow through.

2.9. Add the product of the tau aggregate labeling reaction to the column in a total volume of 2.5 mL. If the sample is less than 2.5 mL, add buffer until a total volume of 2.5 mL is achieved.

2.10. Let the sample enter the packed gel completely, discard the flow-through.

2.11. Elute with 3.5 mL elution buffer and collect the eluate in 4 equivalent fractions in 2 mL tubes.

2.12. Determine protein concentrations of the 4 fractions by bicinchoninic acid (BCA) assay.

2.13. Store the labeled protein in a -20 °C freezer.

3. Uptake Assay with Fluorescence-Activated Cell Sorting (FACS) Read-Out

3.1. Day 1 – Seed the Cells

3.1.1. Wash BV-2 cells in the flask with phosphate-buffered saline (PBS) 1x.

Note: Washing volume will vary based on the size of the cell flask used. For example, for a T175 flask, wash with 10 mL of PBS 1x.

3.1.2. Detach cells by incubating with trypsin-ethylenediaminetetraacetic acid (EDTA) 0.05% at 37 °C and 5% CO_2 until the cells detach from the flask (approximately 5 min).

Note: Volume of trypsin-EDTA 0.05% depends on the size of the cell flask used. For example, for a T175 flask, use 2 mL of trypsin-EDTA 0.05%.

3.1.3. Resuspend cells in culturing medium containing 200 µg/mL heparin to a final concentration of 1 x 10^5 cells/mL.

3.1.4. Plate 250 µL of cell suspension (2.5 x 10^4 cells) per well in a 96-well tissue culture flat bottom plate.

3.1.5. Incubate plate overnight at 37 °C with 5% CO_2.

3.2. Day 1 – Prepare Immunocomplexes

3.2.1. Thaw pH dye-tau on ice.

3.2.2. Prepare a 250 nM solution of pH dye-tau aggregates in serum-free medium (SFM) (high glucose DMEM supplemented with 100 U/mL penicillin, 100 µg/mL streptomycin and 200 µg/mL of heparin).

3.2.3. Dilute antibodies in 250 nM pH dye-tau solution to desired concentrations in a 96-well plate. Seal the dilution plate and incubate over night at 37 °C.

3.3. Day 2 – Immunocomplexes Uptake

3.3.1. Remove culturing medium from BV-2 cells. Wash cells once with 100 µL room temperature PBS 1x.

3.3.2. Transfer 125 µL of immunocomplexes to the cells using a multichannel pipette. Incubate the cells with the immunocomplexes for 2 hours at 37 °C with 5% CO_2.

3.3.3. Remove the incubation medium from the cells and discard it. Wash cells once with 100 µL room temperature 1x PBS.

3.3.4. Treat cells with 50 µL trypsin-EDTA 0.25% for 20 min at 37 °C with 5% CO_2.

3.3.5. Add 200 µL of culturing medium and resuspend well to detach the cells. Transfer cells to a 96-well U-bottom plate. Centrifuge plate at 400 x *g* for 5 min at 4 °C.

3.3.6. Put the plate on ice, remove culturing medium and resuspend the cell pellets in 150 µL ice cold 1x PBS.

3.3.7. Centrifuge plate at 400 x *g* for 5 min at 4 °C.

3.3.8. Put the plate on ice and wash cells by adding 150 µL cold PBS. Centrifuge plate at 400 x *g* for 5 min at 4 °C.

3.3.9. Put cells on ice and wash them by adding 150 µL cold FACS buffer (1x PBS, 0.5% bovine serum albumin (BSA), 2 mM EDTA). Centrifuge plate at 400 x *g* for 5 min at 4 °C.

3.3.10. Put cells on ice and resuspend them in 200 µL cold FACS buffer.

3.3.11. Analyze samples immediately by FACS acquiring 2 x 10^4 events in the live cells gate (see step 4.1).

4. FACS analysis

Note: Refer to **Figure 1** for the gating strategy.

4.1. Using the forward scatter area (FSC-A) versus side scatter area (SSC-A) density plot, gate on live cells by excluding events with lower forward scatter levels (*i.e.,* debris and dead cells).

4.2. Within the live cell population, use FSC-A versus forward scatter height (FSC-H) to exclude cell doublets and aggregates. This is the singlet gate.

4.3. Using the events in the singlet gate, generate a pH dye single parameter histogram.

4.4. Determine mean fluorescence intensity. Determine percentage of pH dye-tau positive cells by excluding negative cells as determined using BV-2 only control.

5. Day 2 – Immunocomplexes Uptake with Microscopy Read-Out

5.1. Day 1 – Seed the Cells

5.1.1. Wash BV-2 cells in the flask with PBS 1x. Detach cells using trypsin-ethylenediaminetetraacetic acid (EDTA) 0.05% and incubating at 37 °C with 5% CO_2 until the cells detach from the flask (approximately 5 min).

Note: Volume of trypsin-EDTA 0.05% depends on the size of the cell flask used (*e.g.*, for a T175 flask, use 2 mL of trypsin-EDTA 0.05%).

5.1.2. Resuspend cells in culturing medium to a final concentration of 2.6 x 10^4 cells/mL.

5.1.3. Plate 150 µL of cell suspension (3.9 x 10^3) per well in a poly-D-Lysine coated 96-well black plate with clear flat bottom.

5.1.4. Incubate plate overnight at 37 °C with 5% CO_2.

5.2. Day 3 – Prepare Immunocomplexes

Note: Mild sonication of labelled tau aggregates prior to incubation with antibody, was performed to improve microscopy results.

5.2.1. Thaw pH dye-tau on ice and sonicate using a probe sonicator while keeping on ice. Use an amplitude of 15% (sonicator power of 250 W). Perform 30 pulses of 2 s and wait 20 s between pulses.

5.2.2. Prepare a 250 nM solution of pH dye-tau aggregates in SFM.

5.2.3. Dilute antibodies in 250 nM pH dye-tau solution to the desired concentrations in a 96-well plate. Seal the dilution plate and incubate over night at 37 °C.

5.2.4. Remove medium from cell plate and replace with 150 µL of culturing medium supplemented with 200 µg/mL heparin.

5.2.5. Incubate plate overnight at 37 °C with 5% CO_2.

5.3. Immunocomplexes Uptake

5.3.1. Remove culturing medium from the BV-2 cells. Transfer 125 µL of immunocomplexes to the cells using a multichannel pipette.

5.3.2. Incubate the cells with the immunocomplexes for 1 h and 45 min at 37 °C with 5% CO_2. Stain cell nuclei and acidic organelles with Hoechst and Lysotracker Red respectively. Incubate the cells 15 min at 37 °C with 5% CO_2.

Note: Dilute the dyes in SFM.

5.3.3. Perform live-cell imaging using a high content screening confocal system. Set temperature set to 37 °C and 5% CO_2. For high quality images, use a 63X water immersion objective and acquire 0.5 µm planes (20 per Z-stack) per imaged field.

REPRESENTATIVE RESULTS:

Aggregated recombinant tau was covalently labelled with pHrodo green dye. This dye dramatically increases its fluorescence upon its internalization in acidic organelles, thereby allowing for intracellular quantification. Labeled tau aggregates were incubated with anti-tau monoclonal antibodies. In particular, we used a chimeric version (mouse IgG1 Fc region) of CBTAU-28.1. This human antibody binds to the N-terminal insert region of tau and is able to bind *in vitro* generated tau fibrils[13]. In this assay, we also tested an affinity-improved version of CBTAU-28.1 – dmCBTAU-28.1. Fab fragments of CBTAU-28.1 in the parental and high-affinity mutant format and a mouse IgG1 isotype control were used as controls.

BV-2 cells were incubated with the pre-formed immunocomplexes or aggregated tau alone for 2 h in the presence of heparin to block antibody-independent uptake. After incubation, cells were trypsinized to remove the tau bound to the extracellular membrane, and were analyzed for tau uptake by flow cytometry. As we recently described[13], we observed that CBTAU-28.1 variants promoted uptake of tau in BV-2 cells in a dose-dependent manner. The uptake was Fc mediated since CBTAU-28.1 Fab fragments did not increase basal tau uptake (**Figure 2**). Moreover, the high affinity dmCBTAU-28.1 antibody mediated tau uptake into BV-2 cells to a higher extent than the wild-type antibody (**Figure 2**).

Antibody-mediated tau uptake and localization of tau aggregates in the endolysosomal compartment was confirmed by confocal microscopy (**Figure 3**) where the acidic cellular compartment was stained using the LysoTracker dye. Intracellular puncta of green pH dye labeled tau aggregates were observed inside the cells that were incubated with CBTAU-28.1. Moreover, intracellular tau aggregates often colocalized with LysoTracker red thus suggesting presence of tau aggregates in the acidic organelles. CBTAU-28.1 Fab fragments did not increase tau uptake again indicating an Fc-receptor mediated internalization mechanism (**Figure 3**).

FIGURE LEGENDS:

Figure 1. Gating strategy used in flow cytometry analysis to detect tau internalization by BV-2 cells. Sample data from BV-2 only control (**A-C**), isotype control (**D-F**) and dmCBTAU-28.1 (**G-I**) are shown. BV-2 cell population was gated on a FSC-A vs SSC-A density plot excluding debris and dead cells (A, D). BV-2 cells were then further gated on a FSC-A vs FSC-H density plot to exclude cell doublets and aggregates (B, E). Single cell gate was used to generate a pH dye (FITC in these representative results) single parameter histogram and determine geometric mean fluorescence intensity. Alternatively, percentage of pH dye-tau positive cells was calculated excluding negative cells as determined by using BV-2 only control.

Figure 2. CBTAU-28.1 mediates uptake of tau aggregates into microglial BV-2 cells. Aggregated recombinant tau was covalently labelled with green fluorescence pH-sensitive dye and incubated with a mouse chimeric version of the human anti-tau antibody CBTAU-28.1, its affinity improved format, dmCBTAU-28.1, the corresponding Fab fragments, a mouse IgG1 isotype control antibody or no antibody (tau aggregates alone). Immunocomplexes were subsequently incubated with BV-2 cells for 2 h in the presence of heparin to block antibody-independent uptake. Uptake of immunocomplexes was assessed by flow cytometry and expressed as the geometric mean (GM) of fluorescence intensity (**A**) or percentage of tau positive (tau+) cells (**B**). Error bars in (A) indicate the standard deviation of two independent experiments, while (B) shows a single experiment.

Figure 3. Tau aggregates are internalized by BV-2 cells and localize in cellular acidic organelles. Preformed tau/antibody immunocomplexes were incubated with BV-2 cells for 2 h in the presence of heparin to block antibody-independent uptake. After incubation, nuclei were stained with Hoechst (blue) and the acidic cellular compartment with LysoTracker Red dye. Live-cell imaging revealed intracellular puncta of labeled tau aggregates (green) inside the cells that were incubated with CBTAU-28.1 and dmCBTAU-28.1, but not with the isotype control. Moreover, intracellular tau aggregates often colocalized with the red dye (yellow) thus suggesting presence of tau aggregates in the acidic cellular compartment. CBTAU-28.1 Fab fragments did not increase tau uptake indicating an Fc-receptor mediated internalization mechanism. Images represent maximum intensity projections of a 20 planes Z-stack (0.5 µm planes) acquired with a 63X water immersion objective.

DISCUSSION:

Microglia, the resident brain's immune cells, have been recently identified as important players in antibody-mediated therapeutic approaches for tauopathies[10, 11]. Antibody-mediated tau

clearance by microglia, together with blocking of neuronal uptake[9], inhibition or destabilization of fibril formation[13, 14] and clearance of intraneuronal fibrils *via* the lysosomal pathway[15], might all contribute to the anti-tau antibody efficacy observed in mouse model of tauopathy[5–9].

We described here a cell-based assay to quantitatively assess tau uptake by microglia with the aim of creating an investigational tool to better characterize the mechanisms of action of anti-tau antibodies.

This assay, adapted from Funk *et al.*[11], uses BV-2 cells, which are immortalized murine microglial cells. While they cannot fully be compared to primary microglial cells, they feature many of the characteristics of primary microglia, including the ability of robustly phagocytose both Aβ and tau fibrils[11, 16–19]. Moreover, they showed a reproducible behavior *in vitro* which made them highly suitable for assay development and quantitative studies, which require minimal experimental variability. Beside this, immortalized cell lines allow higher assay throughput and eliminate the need for animal sacrifice compared to the use of primary microglia.

The tau aggregates we used in this assay were obtained using the highly reproducible *in vitro* aggregation procedure that we recently described[13], and show similar morphology to paired helical filaments (PHFs) isolated from brains of AD patients. While we did not observe any unexpected results that might have been caused by tau aggregates adherence to plastic or glass surfaces, the use of stable and well characterized tau aggregates played a crucial role in the reproducibility of this assay.

Another aspect that significantly contributed to assay reproducibility was cell density. The numbers of cells per well described in the protocol represent the optimal cell density in the described conditions.

Differently than what Funk *et al.*[11] described, we labeled tau aggregates with a pH sensitive dye, pHrodo, which significantly increases its fluorescence upon internalization in acidic organelles, thus allowing for intracellular quantification. This, together with trypsin digestion of surface bound immunocomplexes and/or tau, guarantees that fluorescence signal measured by flow cytometry is the result of tau uptake rather than binding to the cellular surface. Moreover, the use of a pH sensitive dye eases detection of internalized tau aggregates in microscopy experiments without the need of digesting surface bound immunocomplexes/tau aggregates which would then requires cell re-plating and recovery.

We also further optimized the microscopy read-out of our assay, compared to what has previously been described[11], by using a highly selective dye for acidic organelles (LysoTracker) in our microscopy experiments which allowed us not only to confirm antibody-mediated tau uptake, but also localization of tau aggregates in the endolysosomal compartment.

The assay we developed, has optimal specificity which results in a good experimental window allowing a strong separation between positive and negative samples. Interestingly, the assay

indirectly detects differences in antibody affinity thus representing a powerful tool to study anti-tau antibody effector functions.

ACKNOWLEDGMENTS:

We would like to thank Alberto Carpinteiro Soares for his valuable technical assistance.

DISCLOSURES:

The authors have nothing to disclose.

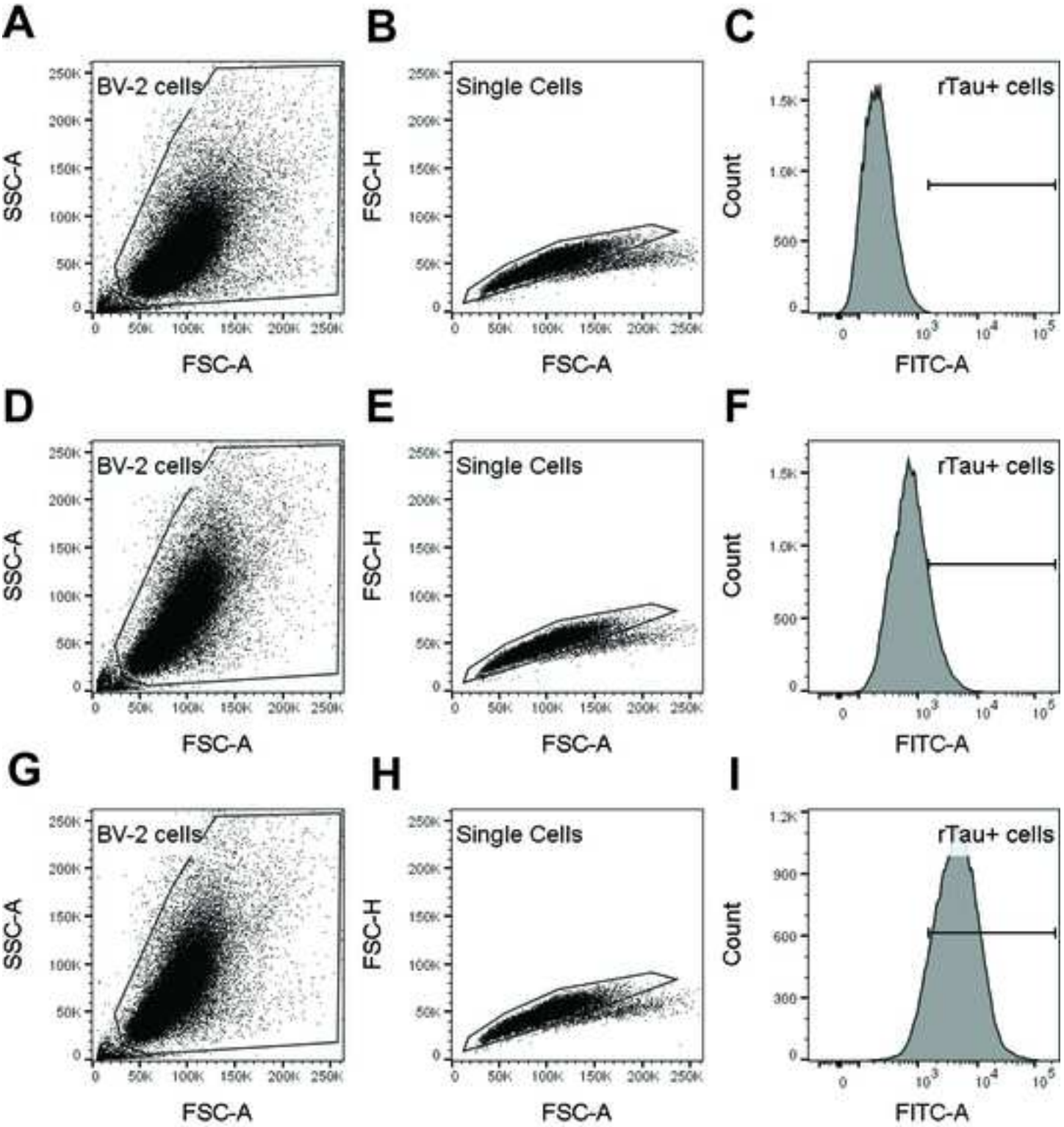
REFERENCES:

1. Hefti, F., Goure, W.F., Jerecic, J., Iverson, K.S., Walicke, P.A., Krafft, G.A. The case for soluble Aβ oligomers as a drug target in Alzheimer's disease. *Trends in Pharmacological Sciences*. **34** (5), 261–266, doi: 10.1016/j.tips.2013.03.002 (2013).
2. Castillo-Carranza, D.L., Lasagna-Reeves, C.A., Kayed, R. Tau aggregates as immunotherapeutic targets. *Frontiers in bioscience (Scholar edition)*. **5** (8), 426–38, doi: S381 [pii] (2013).
3. Martin, L., Latypova, X., Terro, F. Post-translational modifications of tau protein: Implications for Alzheimer's disease. *Neurochemistry International*. **58** (4), 458–471, doi: 10.1016/j.neuint.2010.12.023 (2011).
4. Holmes, B.B., Diamond, M.I. Prion-like properties of Tau protein: The importance of extracellular Tau as a therapeutic target. *Journal of Biological Chemistry*. **289** (29), 19855–19861, doi: 10.1074/jbc.R114.549295 (2014).
5. Giacobini, E., Gold, G. Alzheimer disease therapy--moving from amyloid-beta to tau. *Nature Reviews Neurology*. **9** (12), 677–686, doi: 10.1038/nrneurol.2013.223 (2013).
6. Asuni, A.A., Boutajangout, A., Quartermain, D., Sigurdsson, E.M. Immunotherapy targeting pathological tau conformers in a tangle mouse model reduces brain pathology with associated functional improvements. *Journal of Neuroscience*. **27** (34), 9115–9129 (2007).
7. Boutajangout, A., Ingadottir, J., Davies, P., Sigurdsson, E.M. Passive immunization targeting pathological phospho-tau protein in a mouse model reduces functional decline and clears tau aggregates from the brain. *Journal of Neurochemistry*. **118** (4), 658–667, doi: 10.1111/j.1471-4159.2011.07337.x (2011).
8. Chai, X. *et al.* Passive immunization with anti-Tau antibodies in two transgenic models: reduction of Tau pathology and delay of disease progression. *Journal of Biological Chemistry*. **286** (39), 34457–34467, doi: 10.1074/jbc.M111.229633 (2011).
9. Yanamandra, K. *et al.* Anti-tau antibodies that block tau aggregate seeding *in vitro* markedly decrease pathology and improve cognition *in vivo*. *Neuron*. **80** (2), 402–414, doi: 10.1016/j.neuron.2013.07.046 (2013).
10. Luo, W., Liu, W., Hu, X., Hanna, M., Caravaca, A., Paul, S.M. Microglial internalization and degradation of pathological tau is enhanced by an anti-tau monoclonal antibody. *Scientific Reports*. **5**, 11161, doi: 10.1038/srep11161 (2015).
11. Funk, K.E., Mirbaha, H., Jiang, H., Holtzman, D.M., Diamond, M.I. Distinct Therapeutic Mechanisms of Tau Antibodies: Promoting Microglial Clearance *Versus* Blocking Neuronal Uptake. *Journal of Biological Chemistry*. **290** (35), 21652–21662, doi: 10.1074/jbc.M115.657924 (2015).

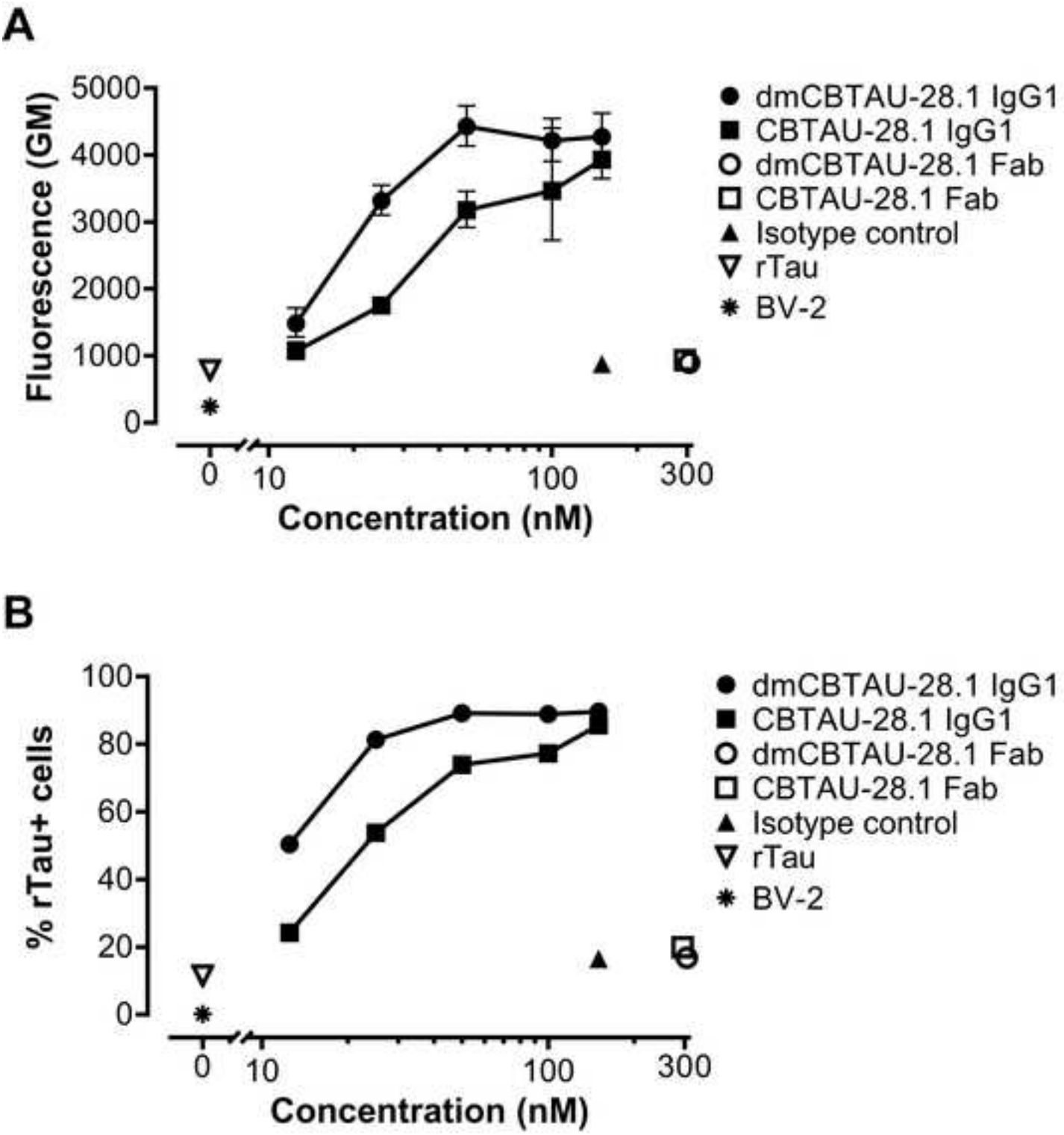
12. Blasi, E., Barluzzi, R., Bocchini, V., Mazzolla, R., Bistoni, F. Immortalization of murine microglial cells by a v-raf/v-myc carrying retrovirus. *Journal of neuroimmunology*. **27** (2–3), 229–37, at <http://www.ncbi.nlm.nih.gov/pubmed/2110186> (1990).
13. Apetri, A. *et al.* A common antigenic motif recognized by naturally occurring human VH5-51/VL4-1 anti-tau antibodies with distinct functionalities. *Acta neuropathologica communications*. **6** (1), 43, doi: 10.1186/s40478-018-0543-z (2018).
14. Schneider, A., Mandelkow, E. Tau-based treatment strategies in neurodegenerative diseases. *Neurotherapeutics*. **5** (3), 443–457, doi: 10.1016/j.nurt.2008.05.006 (2008).
15. Congdon, E.E., Gu, J., Sait, H.B., Sigurdsson, E.M. Antibody uptake into neurons occurs primarily *via* clathrin-dependent Fcgamma receptor endocytosis and is a prerequisite for acute tau protein clearance. *Journal of Biological Chemistry*. **288** (49), 35452–35465, doi: 10.1074/jbc.M113.491001 (2013).
16. Bocchini, V., Mazzolla, R., Barluzzi, R., Blasi, E., Sick, P., Kettenmann, H. An immortalized cell line expresses properties of activated microglial cells. *Journal of Neuroscience Research*. **31** (4), 616–621, doi: 10.1002/jnr.490310405 (1992).
17. Koenigsknecht, J. Microglial Phagocytosis of Fibrillar -Amyloid through a 1 Integrin-Dependent Mechanism. *Journal of Neuroscience*. **24** (44), 9838–9846, doi: 10.1523/JNEUROSCI.2557-04.2004 (2004).
18. Kopec, K.K., Carroll, R.T. Alzheimer's β-Amyloid Peptide 1-42 Induces a Phagocytic Response in Murine Microglia. *Journal of Neurochemistry*. **71** (5), 2123–2131, doi: 10.1046/j.1471-4159.1998.71052123.x (2002).
19. Marsh, S.E. *et al.* The adaptive immune system restrains Alzheimer's disease pathogenesis by modulating microglial function. *Proceedings of the National Academy of Sciences of the United States of America*. **113** (9), E1316-1325, doi: 10.1073/pnas.1525466113 (2016).

Figure

Click here to download Figure Figure 1_R.tif



Figure



Figure

Click here to download Figure Figure 3_R.tif

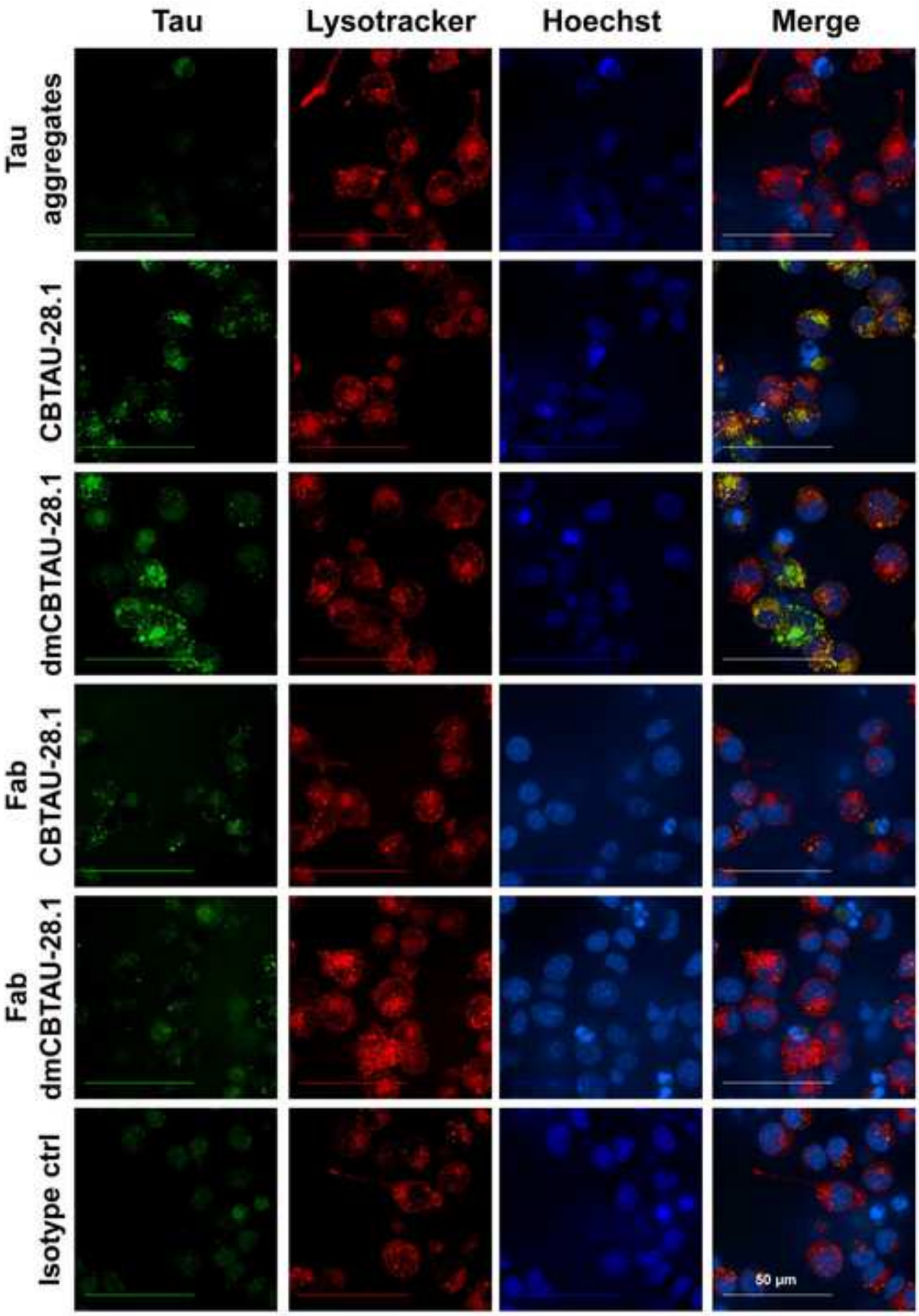


Table of Materials

Click here to download Table of Materials JoVE_Materials_MG assay_R.xlsx

Name of Material/ Equipment	Company
BV-2 cells	ICLC Interlab Cell Line Collection
Phosphate Buffered Saline (PBS) (1X)	Gibco
Trypsin-EDTA 0.05%	Gibco
DMEM 4.5 g/dl glucose	Gibco
Fetal Bovine Serum	Gibco
Penicillin-Streptomycin (10,000 U/mL)	Gibco
L-Glutamine 200mM	Lonza
EasYFlask	Nunc
pHrodo Green STP ester	Life Technologies
Sodium Bicarbonate pH 8.5 100 mM	
Sodium Bicarbonate pH 5 100 mM	
DMSO	Sigma
PD10 columns	GE Healthcare
BCA Protein Assay Kit	Thermo Fisher Scientific
Greiner CELLSTAR multiwell culture plates	Greiner
Falcon 96-Well Assay Plates	Falcon
Heparin	Sigma
Trypsin-EDTA 0.25%	Sigma
BSA	Sigma
EDTA 0.5M, pH8	
FACS Canto II	BD
FlowJo	
Hoechst 33342 Solution (20 mM)	Thermo Fisher Scientific
LysoTracker Deep Red	Thermo Fisher Scientific
Opera Phenix	Perkin Helmer

Catalog Number	Comments/Description
ATL03001	
10010-015	
25300-054	
41966-029	
10091-148	
15140122	
17-605E	
156499 / 159910	
P35369	
D2650-100ml	
17-0851-01	
23225	
665180	
353910	
H3393-50KU	
T4049-100ml	
A7030-100G	
	for flow cytometry data analysis
62249	
L12492	
HH14000000	

jove JOURNAL OF VISUALIZED EXPERIMENTS
1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

ARTICLE AND VIDEO LICENSE AGREEMENT

Title of Article:	A cell-based assay to study antibody-mediated tau clearance by microglia
Author(s):	Donata De Marco, Renske Taggenbrock, Rosa Crespo, Wouter Koudstaal, Elizabeth Ramsburg and Adrian C. Apetri

Item 1: The Author elects to have the Materials be made available (as described at http://www.jove.com/publish) via:

☐ Standard Access ☑ Open Access

Item 2: Please select one of the following items:

☐ The Author is **NOT** a Janssen Vaccines employee.

☑ The Author is a Janssen Vaccines employee and the Materials were prepared in the course of his or her duties as a Janssen Vaccines employee.

☐ The Author is a Janssen Vaccines employee but the Materials were NOT prepared in the course of his or her duties as a Janssen Vaccines employee.

ARTICLE AND VIDEO LICENSE AGREEMENT

1. **Defined Terms.** As used in this Article and Video License Agreement, the following terms shall have the following meanings: "**Agreement**" means this Article and Video License Agreement; "**Article**" means the article specified on the last page of this Agreement, including any associated materials such as texts, figures, tables, artwork, abstracts, or summaries contained therein; "**Author**" means the author who is a signatory to this Agreement; "**Collective Work**" means a work, such as a periodical issue, anthology or encyclopedia, in which the Materials in their entirety in unmodified form, along with a number of other contributions, constituting separate and independent works in themselves, are assembled into a collective whole; "**CRC License**" means the Creative Commons Attribution-Non Commercial-No Derivs 3.0 Unported Agreement, the terms and conditions of which can be found at: http://creativecommons.org/licenses/by-nc-nd/3.0/legalcode; "**Derivative Work**" means a work based upon the Materials or upon the Materials and other pre-existing works, such as a translation, musical arrangement, dramatization, fictionalization, motion picture version, sound recording, art reproduction, abridgment, condensation, or any other form in which the Materials may be recast, transformed, or adapted; "**Institution**" means the institution, listed on the last page of this Agreement, by which the Author was employed at the time of the creation of the Materials; "**JoVE**" means MyJove Corporation, a Massachusetts corporation and the publisher of The Journal of Visualized Experiments; "**Materials**" means the Article and / or the Video; "**Parties**" means the Author and JoVE; "**Video**" means any video(s) made by the Author, alone or in conjunction with any other parties, or by JoVE or its affiliates or agents, individually or in collaboration with the Author or any other parties, incorporating all or any portion of the Article, and in which the Author may or may not appear.

2. **Background.** The Author, who is the author of the Article, in order to ensure the dissemination and protection of the Article, desires to have the JoVE publish the Article and create and transmit videos based on the Article. In furtherance of such goals, the Parties desire to memorialize in this Agreement the respective rights of each Party in and to the Article and the Video.

3. **Grant of Rights in Article.** In consideration of JoVE agreeing to publish the Article, the Author hereby grants to JoVE, subject to **Sections 4** and **7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Article in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Article into other languages, create adaptations, summaries or extracts of the Article or other Derivative Works (including, without limitation, the Video) or Collective Works based on all or any portion of the Article and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and(c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. If the "Open Access" box has been checked in **Item 1** above, JoVE and the Author hereby grant to the public all such rights in the Article as provided in, but subject to all limitations and requirements set forth in, the CRC License.

4. **Retention of Rights in Article.** Notwithstanding the exclusive license granted to JoVE in **Section 3** above, the Author shall, with respect to the Article, retain the non-exclusive right to use all or part of the Article for the non-commercial purpose of giving lectures, presentations or teaching classes, and to post a copy of the Article on the Institution's website or the Author's personal website, in each case provided that a link to the Article on the JoVE website is provided and notice of JoVE's copyright in the Article is included. All non-copyright intellectual property rights in and to the Article, such as patent rights, shall remain with the Author.

5. **Grant of Rights in Video – Standard Access.** This **Section 5** applies if the "Standard Access" box has been checked in **Item 1** above or if no box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby acknowledges and agrees that, Subject to **Section 7** below, JoVE is and shall be the sole and exclusive owner of all rights of any nature, including, without limitation, all copyrights, in and to the Video. To the extent that, by law, the Author is deemed, now or at any time in the future, to have any rights of any nature in or to the Video, the Author hereby disclaims all such rights and transfers all such rights to JoVE.

6. **Grant of Rights in Video – Open Access.** This **Section 6** applies only if the "Open Access" box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby grants to JoVE, subject to **Section 7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Video in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Video into other languages, create adaptations, summaries or extracts of the Video or other Derivative Works or Collective Works based on all or any portion of the Video and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and (c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. For any Video to which this **Section 6** is applicable, JoVE and the Author hereby grant to the public all such rights in the Video as provided in, but subject to all limitations and requirements set forth in, the CRC License.

7. **Government Employees.** If the Author is a Janssen Vaccines employee and the Article was prepared in the course of his or her duties as a Janssen Vaccines employee, as indicated in **Item 2** above, and any of the licenses or grants granted by the Author hereunder exceed the scope of the 17 U.S.C. 403, then the rights granted hereunder shall be limited to the maximum rights permitted under such statute. In such case, all provisions contained herein that are not in conflict with such statute shall remain in full force and effect, and all provisions contained herein that do so conflict shall be deemed to be amended so as to provide to JoVE the maximum rights permissible within such statute.

8. **Protection of the Work.** The Author(s) authorize JoVE to take steps in the Author(s) name and on their behalf if JoVE believes some third party could be infringing or might infringe the copyright of either the Author's Article and/or Video.

9. **Likeness, Privacy, Personality.** The Author hereby grants JoVE the right to use the Author's name, voice, likeness, picture, photograph, image, biography and performance in any way, commercial or otherwise, in connection with the Materials and the sale, promotion and distribution thereof. The Author hereby waives any and all rights he or she may have, relating to his or her appearance in the Video or otherwise relating to the Materials, under all applicable privacy, likeness, personality or similar laws.

10. **Author Warranties.** The Author represents and warrants that the Article is original, that it has not been published, that the copyright interest is owned by the Author (or, if more than one author is listed at the beginning of this Agreement, by such authors collectively) and has not been assigned, licensed, or otherwise transferred to any other party. The Author represents and warrants that the author(s) listed at the top of this Agreement are the only authors of the Materials. If more than one author is listed at the top of this Agreement and if any such author has not entered into a separate Article and Video License Agreement with JoVE relating to the Materials, the Author represents and warrants that the Author has been authorized by each of the other such authors to execute this Agreement on his or her behalf and to bind him or her with respect to the terms of this Agreement as if each of them had been a party hereto as an Author. The Author warrants that the use, reproduction, distribution, public or private performance or display, and/or modification of all or any portion of the Materials does not and will not violate, infringe and/or misappropriate the patent, trademark, intellectual property or other rights of any third party. The Author represents and warrants that it has and will continue to comply with all government, institutional and other regulations, including, without limitation all institutional, laboratory, hospital, ethical, human and animal treatment, privacy, and all other rules, regulations, laws, procedures or guidelines, applicable to the Materials, and that all research involving human and animal subjects has been approved by the Author's relevant institutional review board.

11. **JoVE Discretion.** If the Author requests the assistance of JoVE in producing the Video in the Author's facility, the Author shall ensure that the presence of JoVE employees, agents or independent contractors is in accordance with the relevant regulations of the Author's institution. If more than one author is listed at the beginning of this Agreement, JoVE may, in its sole

jove JOURNAL OF VISUALIZED EXPERIMENTS
1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

ARTICLE AND VIDEO LICENSE AGREEMENT

discretion, elect not take any action with respect to the Article until such time as it has received complete, executed Article and Video License Agreements from each such author. JoVE reserves the right, in its absolute and sole discretion and without giving any reason therefore, to accept or decline any work submitted to JoVE. JoVE and its employees, agents and independent contractors shall have full, unfettered access to the facilities of the Author or of the Author's institution as necessary to make the Video, whether actually published or not. JoVE has sole discretion as to the method of making and publishing the Materials, including, without limitation, to all decisions regarding editing, lighting, filming, timing of publication, if any, length, quality, content and the like.

12. **Indemnification.** The Author agrees to indemnify JoVE and/or its successors and assigns from and against any and all claims, costs, and expenses, including attorney's fees, arising out of any breach of any warranty or other representations contained herein. The Author further agrees to indemnify and hold harmless JoVE from and against any and all claims, costs, and expenses, including attorney's fees, resulting from the breach by the Author of any representation or warranty contained herein or from allegations or instances of violation of intellectual property rights, damage to the Author's or the Author's institution's facilities, fraud, libel, defamation, research, equipment, experiments, property damage, personal injury, violations of institutional, laboratory, hospital, ethical, human and animal treatment, privacy or other rules, regulations, laws, procedures or guidelines, liabilities and other losses or damages related in any way to the submission of work to JoVE, making of videos by JoVE, or publication in JoVE or elsewhere by JoVE. The Author shall be responsible for, and shall hold JoVE harmless from, damages caused by lack of sterilization, lack of cleanliness or by contamination due to the making of a video by JoVE its employees, agents or independent contractors. All sterilization, cleanliness or decontamination procedures shall be solely the responsibility of the Author and shall be undertaken at the Author's expense. All indemnifications provided herein shall include JoVE's attorney's fees and costs related to said losses or damages. Such indemnification and holding harmless shall include such losses or damages incurred by, or in connection with, acts or omissions of JoVE, its employees, agents or independent contractors.

13. **Fees.** To cover the cost incurred for publication, JoVE must receive payment before production and publication the Materials. Payment is due in 21 days of invoice. Should the Materials not be published due to an editorial or production decision, these funds will be returned to the Author. Withdrawal by the Author of any submitted Materials after final peer review approval will result in a US$1,200 fee to cover pre-production expenses incurred by JoVE. If payment is not received by the completion of filming, production and publication of the Materials will be suspended until payment is received.

14. **Transfer, Governing Law.** This Agreement may be assigned by JoVE and shall inure to the benefits of any of JoVE's successors and assignees. This Agreement shall be governed and construed by the internal laws of the Commonwealth of Massachusetts without giving effect to any conflict of law provision thereunder. This Agreement may be executed in counterparts, each of which shall be deemed an original, but all of which together shall be deemed to me one and the same agreement. A signed copy of this Agreement delivered by facsimile, e-mail or other means of electronic transmission shall be deemed to have the same legal effect as delivery of an original signed copy of this Agreement.

A signed copy of this document must be sent with all new submissions. Only one Agreement is required per submission.

CORRESPONDING AUTHOR OR LEGAL REPRESENTATIVE

Name:	
Department:	
Institution:	Maarten Santman
Title:	Senior Legal Counsel & Corporate Secretary
Signature:	
Date:	29 May 2018

Please submit a **signed** and **dated** copy of this license by one of the following three methods:

1. Upload an electronic version on the JoVE submission site
2. Fax the document to +1.866.381.2236
3. Mail the document to JoVE / Attn: JoVE Editorial / 1 Alewife Center #200 / Cambridge, MA 02140

Janssen Vaccines & Prevention B.V.
Archimedesweg 6
2333CN Leiden
The Netherlands

July 12th, 2018

Dear Dr. Nguyen,

Please find our revised manuscript entitled **"**A cell-based assay to study antibody-mediated tau clearance by microglia" [JoVE58576] that we hope is now acceptable for publication in Jove. We have taken into account the reviewers' comments and have added the details requested for the protocol and provided clarifications in the text where requested.

A point-by-point response to the specific editorial comments and comments of the reviewers follows.

Sincerely Yours,

Donata de Marco, PhD
Adrian Apetri, PhD
Janssen Prevention Center, Janssen Pharmaceutical Companies of Johnson & Johnson

Response to comments

Editorial comments:
Changes to be made by the Author(s):

1. *Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.*

We proofread the manuscript.

2. *Please remove trademark (™) and registered (®) symbols from the Table of Equipment and Materials.*

We removed all trademark (™) and registered (®) symbols from the Table of Equipment and Materials.

3. *Please add more details to your protocol steps. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action.*

We improved the protocol by including more experimental details.

4. *3.1.1: How much is used to wash?*

We added the volume (10 ml) used to wash as example since it depends on the size of the cell flask used.

5. *3.1.2/5.1.2: What volume of trypsin is used? Reaction conditions?*

We added details on the volume of trypsin used (given as example, since it depends on the size of the cell flask used) and the exact reaction conditions.

6. *Please provide RRIDs or lot numbers for all antibodies.*

All antibodies used are Janssen proprietary antibodies that are not commercially available. Therefore, lot numbers are not relevant here.

7. *Please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader.*

We highlighted the essential steps of the protocol in the revised manuscript.

8. Please ensure that the highlighted steps form a cohesive narrative with a logical flow from one highlighted step to the next. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted part of the step includes at least one action that is written in imperative tense.

We highlighted the essential steps of the protocol in the revised manuscript.

9. *Please do not abbreviate journal titles.*

We used the JoVE Endnote template to format the bibliography.

Reviewers' comments:

Reviewer #1:
Manuscript Summary:
This manuscript provided a very detailed protocol about a cell-based assay to study antibody-mediated tau clearance by microglia. It can be very helpful for therapeutic development of tau antibody based immunotherapy for AD treatment.

Major Concerns:
-None

We thank the reviewer for his/her enthusiasm for our assay.

Minor Concerns:

-line 56, what does "non-productive" mean here for neuroinflammation?

During a productive inflammation, the proliferative phase predominates causing an infiltrate to appear in the tissue. Such an infiltrate doesn’t predominate in course of AD. This is common knowledge in the field.

-It will be helpful to include the procedures for preparation of tau aggregation, a key experimental reagent in this assay, instead of Citation of the previous publication.

We respectfully disagree with the reviewer. This protocol describes an assay in which tau aggregates are a reagent. Preparation of this reagent is described in detail in the referenced publication.

-line 100, spread out ThT

We spelled out Thioflavin T in the revised protocol

-*line 108, concentration of (~20?M)*

We added the right unit of measurement.

-*line 111, please specify the power in addition to 65%*

We have specified the sonicator power.

-*line120, please specify the column as PD10?*

JoVe guidelines indicate to avoid using commercial names.

-*line 197, explain why using a different sanitation protocol*

We assumed that the reviewer meant “sonication protocol”. We explained that a mild sonication improves microscopy results.

-*Figure 3, it will greatly beneficial if there will be imaging quantification method or figure provided.*

Microscopy is not used as a quantitative assay, but merely as a means to support observations from FACS analysis.

-*In discussion, it will be very helpful for others if the potential influencing factors that are key for the success of this assay. Also discuss the most important step(s) that controls the variation of the data. For example, how much will the semi-attachment of BV2 impact on the results; is any concern associated with the sickness of tau aggregates to pipets or tubes?*

We added extra discussion on role of cell density in assay reproducibility together with the importance of using stable and well characterized tau aggregates.

Reviewer #2:
Manuscript Summary:
Dear Authors you paper is very interesting, some small clarification are necessary before final full acceptation.

Mayor/Minor concerns:
-You should add "TM" or "(R)" when the name of the product is a commercial one

The editorial policy of JoVE actually prevents use of trademark (™) and registered (®) symbols.

-You should add full name of the products then put their acronyms between parenthesis and after that you can use the acronyms. For example "EDTA", "PBS" etc etc

We spelled out the names the first time used in the text and then replaced with acronyms.

-In the sentence "2.1. Resuspend tau aggregates in 0.1 M sodium bicarbonate buffer (NaHCO3), pH 8.5 to a 108 concentration of 1 mg/mL as assessed by initial absorption of tau monomers at 280 109 nm using an extinction coefficient of 0.31 mL mg-1cm-1" is not clear. Is there a temperature that should be maintained during this procedure? If yes, could you please explain how you preserve your cold temperature.

We re-wrote this sentence to me make it clearer. Concerning the temperature, we don't control it, but we avoid over-heating during sonication keeping the aggregates on ice.

-Line 119:"RT", you mean room temperature? Follow my suggestion below. Furthermore later in your paper you write "room temperature". Please try to be consistent with you nomenclature, it is important for well understanding your paper.

We replaced "RT" with "room temperature".

-Line 125: remove "then", please

We have removed the word "then".

-Line 131: "Store the labeled protein at -20°C" Please say clearly were you store your product, frigo? refrigerator?

We specified that both -20 and -80 are freezers.

-Line 145: please add between parenthesis "SFM", I think that this is the acronymous of serum-free medium

We put "SFM" in parenthesis here and used it as acronym later on.

-Line 162: "rcf": You should write it in extend and then cite by using the acronymous ref

We spelled out "rcf" the first time used in the text and then used it as acronym.

-Line 217:"For high quality images, use a 63x water immersion objective and acquire 0.5 µm 217 planes (20 per Z-stack) per imaged field" Which type microscope do you use? I think that you use confocal but it better to clarify.

Point 5.3.6 specifies that we used a confocal microscopy. The materials table gives also further info on vendor name and catalog number of the microscopy used.

-Lines 224 to 226:"In particular, we used a chimeric version (mouse IgG1 Fc region) of CBTAU-28.1. This human antibody binds to the N-terminal insert region of tau and is able to bind in vitro generated tau fibrils" Sorry but i did not find this antibody cited in your experiment...Why you are talking about something that you did not use ?

The protocol refers in a general way to the incubation of antibody and tau aggregates. In the representative results, we give an example of the kind of results obtainable using the described assay and in particular we described results for one of our ant-tau antibodies: CBTAU-28.1.

Reviewer #3:

Manuscript Summary:
The manuscript by De Marco et al., presents a detailed description of an in vitro based assay to examine the BV2 clearance of tau through antibody dependent mechanisms. They provide good descriptions of their assay with two different readouts (FACS vs. microscopy). While the majority of methodology employed is appropriate there are some methodological concerns. Finally, the use of BV2 cell line alone is inappropriate to achieve the goals stated in the abstract and discussion sections. If concerns are addressed I believe this manuscript will significantly add research and be of great interest to the Alzheimer's field.

Major Concerns:

1. *-The use of BV2 cells as the only cells in the screening is significantly problematic. BV2 cells represented a significant advance in creating an easy to use microglia cell line resource when they were created in 1990. However in the nearly 3 decades since substantial technical and methodological innovations have made the isolation and culture of primary mouse cells significantly easier and new innovations have created protocols for human iPSC derived microglia like cells. Two recent studies (PMID: 29788964, PMID: 27400875) have shown what many in the field have known for years that BV2 baseline transcriptional signature and activation signature is dramatically different from primary microglia in vitro. No in vitro assay will be analogous to in vivo function but the microglial field in particular has realized the need to move towards better practices in order to get as close as possible to in vivo signatures. BV2 cells are sufficient for first pass analyses before proceeding to primary cells but not as sole analysis. The authors need to perform their assays in primary cells as well to recapitulate the results. If the results do not recapitulate in primary cells then the usefulness of this assay and the results obtained for development of future therapies is questionable. Finally validation of results is not sufficient to justify use of BV2 cells. The field needs to move beyond models simply because of ease of use when they do not significantly resemble their in vivo counterparts. Particularly concerning for this paper and the focus on Fc receptor dependent functions is that quick glance at the supplemental table in PMID 29788964 reveals large differences in the expression of Fc receptors between BV2 and primary cells.*

2. *The authors claims of learning from BV2 mouse cells about development of human therapies is also problematic because of the significant difference between human and mouse Fc receptors and their function. Several protocols have emerged in the past year for differentiation of human iPSC to microglia-like cells. Of these protocols the manuscript from Albud et al., 2017 (PMID 28426964) produces large numbers of cells from minimal starting cells. The protocols are quick and highly reproducible and avoid the cross-species comparison and allow for use of patient specific lines to test differential therapeutic efficacies.*

   We thank the reviewer for these insightful comments. The reviewer's request to use alternatives for BV-2 cells is essentially a request to develop a different assay. Like any in vitro assay, it is a model of a more complex in vivo situation that will always require further study (ultimately in humans). As with any model, there is a balance between representativeness and practical applicability. While the relevance of any model system to answer a specific scientific question can be debated, the robustness of the current assay cannot be questioned. Nevertheless, in recognition of the limitations of the BV2 cells, we have revised the intro and discussion to narrow our claims.

3. *The FACS analysis either needs better description of controls and added controls.*
   - *Creating histogram gate directly off the singlet gate is problematic. It means that even cells that failed to uptake tau are included and fails to gate out autofluorescence prior to analyzing fluorescent intensity.*

     In our analysis we don't create gates on the fluorescence histogram plot, but we show results as MFI of the singlet gate. This because we don't observe a two picks/populations histogram for partially positive samples, but rather a shift of the pick which varies based on level of uptake. Comparison to BV-2 cells alone allows exclusion of cell autofluorescence.

   - *Analysis of percentage of fluorescent cells is important characteristic that should also be examined.*

     We added an example of analysis showing the percentage of fluorescent cells. We updated figure 1and 2 and corresponding legends to describe this analysis.

   - *Following controls should be shown in methods and used to accurate quantify the internalize pHRodo signal:*
     - *BV2 cells without any immune complexes (elim autoflurescence)*

       We added this control in the revised figure 2.

     - *BV2 cells fed unlabeled tau immune complexes (elim autofluorescence)*

While this is a useful suggestion, we don't consider this a crucial control given our assay's good experimental window that allows a strong separation between positive and negative samples.

- *Figure 1 also fails to state what the difference is between the two sets of panels (different antibodies, different replicates of same thing, etc)*

  We amended legend to figure 1 to provide the requested information.

- *Singlet gate looks little strange (should be pure rectangular and narrow gate along the diagonal and not odd shape it currently is).*

  A rectangular gate rather than a freely shaped gate doesn't change the analysis as long as it excludes cells which follow outside the main population give their increased FSC area.

4. *The microscopy based assay has several issues. First and foremost, there is no quantification of the microscopy. This needs to be presented as comparison to the FACS based assay. Second, there is clearly significant autofluorescence in the microscopy assay (tau-Hoechst double labels with different pattern than tau that colocalizes with lysosomes).*

We don't use the microscopy in a quantitative way, but merely to support observations from FACS analysis. Using the microscopy for quantitative analyses would require development of a separate assay altogether.

Additionally, the authors do not present the same crucial controls on Fab antibody fragments that they use with the FACS assay. These are important controls and need to be included.

We added the requested additional controls to the revised version of our manuscript.

Minor Concerns:

1. *Cells should ideally be serum starved overnight or 24 hours before incubation with tau-immune complexes to avoid problems with serum presence in phagocytosis assays and aid cells normalization to serum-free conditions.*

   Based on our experience, an overnight/24hrs serum starvation drastically affect BV-2 adherence to the plate, which is crucial for assay success especially in the microscopy read-out.

2. *Use of word proved should be avoided in scientific publications. Please replace with more appropriate word in abstract.*

   We replaced the word "proved" with "shown"