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Anti-Amyloid Chimeric Antigen Receptor Macrophages for Alzheimer's Disease Immunotherapy

Qiuyun Pan

Washington University in St. Louis

McKelvey School of Engineering

Department of Biomedical Engineering

Thesis Examination Committee:

Carl DeSelm, Chair

Hong Chen

Michael Vahey

Abhinav Diwan

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By

Qiuyun Pan

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Abstract

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Research Advisor: Professor Carl DeSelm

Alzheimer’s disease is the most common cause of dementia. None of the available drugs can cure the disease. Chimeric Antigen Receptor (CAR) macrophages, because of their phagocytic activity, have potential as a cellular treatment for amyloid aggregation. In this study, we generated an anti-amyloid CAR hematopoietic progenitor cell line. By inducing the progenitor cell line to differentiate into macrophages, we show that the anti-amyloid CAR-Macrophage has enhanced specific phagocytic activity towards amyloid in in vitro experiments. In addition, in ex vivo experiments, anti-amyloid CAR significantly reduces the plaque load on brain slice from APP/PS1 mice when compared to a non-targeted CAR. We then transduce the progenitor cell line with GFP-luciferase and found that transduction of GFP-luciferase did not have a significant effect on the phagocytosis of amyloid. By tracking the luciferase activity in brain, we found that these CAR-macrophages can live for more than 10 days after intracranial injection.

Chapter 1: Introduction

Alzheimer's Disease (AD), a disease discovered more than 100 years ago, is a neurodegenerative disease whose pathological features include amyloid plaques and neurofibrillary tangles[1,2]. AD is a common cause of dementia, and usually comes with the symptom of loss of memory, cognitive recession and will eventually influence individual behaviors in visionary, speech, and physical aspect[3]. Another characteristic of AD is that it frequently has a long preclinical phase that can hardly be discovered so that normal individuals might also have this disease[2,3]. According to estimation, 6.2 million of the 65 or older Americans are living with dementia caused by AD, and more than 1 trillion dollars will be spent as the cost of these diseases annually[3]. The prevalence of AD is estimated to reach 13.8 million at 2060, and the estimated prevalence for people over 65 is 10% and 40% for people over 80[3]. With a huge increment of personal and financial cost, the importance of treatment helps to halt disease before the symptomatic occurrence comes into a much higher level.

By 2021, FDA has approved five drugs for treatment of AD, including rivastigmine, galantamine, donepezil, memantine, and memantine combined with donepezil[4]. None of these drugs could slow down the progression of AD[5]. In 2021, a new monoclonal antibody drug – aducanumab was approved by FDA[6]. However, the effectiveness of aducanumab is controversial[5,7]. The drug failed to prove its clinical effectiveness to improve cognitive disorder and memory impairment[8]. Other monoclonal antibodies under research including anti-tau antibodies, and anti-amyloid beta antibodies which targets other epitopes[4].

Chimeric Antigen Receptor (CAR) immune cell therapy is a promising treatment for cancer[9]. A CAR construct is usually composed by an antigen specific scFv, an extracellular domain, a transmembrane domain and an intracellular signaling domain[10,11]. By engineering

immune cells with recombinant receptors target diverse antigens, CAR helps to redirect immune cells and focuses on binding specific antigen and induce anti-tumor response.[12] CAR-T therapy has been approval by US Food and Drug Administration (FDA) for lymphoma and multiple myeloma[13,14]. However, the poor filtration of T cells restricts the application of CAR-T treatment on solid tumors, some problems like exhausted CAR-T related toxicities, cytokine release syndrome, and a lack of efficacy on healing solid tumor or resistance in B cell malignancies require a powerful solution[12,15]. Generating CAR on other immune cells is under research, for example, CAR-NK cells and CAR-macrophages[16]. Inside of these novel CAR cells, CAR-macrophage is thought to be potential because of its powerful phagocytosis ability[11]. While CAR-NK and CAR-T cells can only target cells, CAR-macrophages can be engineered to target small molecules.[16,17] Previous studies have shown the promising application of CAR-Macrophage of elimination tumor cells by antigen-specific phagocytosis in vitro[18–20]. CAR-Macrophages were also proved to have ability to induce pro-inflammatory response and recruit immune cells in tumor microenvironment in vivo[18].

Hoxb8-FL cell line is an immortalized hematopoietic progenitor cell line that has myeloid and lymphoid potential[21]. To generate Hoxb8-FL cells, bone marrow cells were transduced with a retrovirus that expresses fused hoxb8 and estrogen receptor[21,22]. By doing this, the hoxb8 gene transcription could be controlled with flt3 ligand and estrogen. These hoxb8-FL cells showed myeloid differentiation potential in macrophage colony-stimulating factor (M-CSF)[21,22]. In addition, the differentiated cells and primary cells share a similar phenotype and behavior[21]. Thus, Hoxb8-FL is a promising model for studying macrophage functions.

In this study, we generated anti-amyloid CAR-Macrophage to target amyloid protein in AD. CAR with an anti-amyloid aducanumab receptor was transduced into Hoxb8-FL cells. Then

the Hoxb8-FL was induced to differentiate into macrophages in M-CSF. We hypothesized that these CAR-Macrophages could have specific bind with amyloid and enhanced phagocytic activity towards amyloid. By eliminating amyloid in AD brain, we expected the anti-amyloid CAR-Macrophage could alleviate symptoms and slow down progression of AD.

Chapter 2: Methods and materials

Plasmid construction and bacterial transformation

The Z117 construct was generated by adding aducanumab scFv, FLAG, mFcr-γ extracellular domain, transmembrane domain, and intracellular domain to retroviral backbone. For Z105, the construct was generated by adding anti-EphA2 scFv, CD8 extracellular domain and transmembrane domain to retroviral backbone.

The recombinant DNA was added to NEB Stable Competent *E. coli* (New England BioLabs) and was incubated for 5 minutes on ice. Then it was heat shocked at 42°C for 30 seconds and incubated for another 5 minutes on ice. The bacteria were then plated to obtain plasmids. Glycerol stock was then made from the bacteria for storing.

Cell transfection

RD114 retroviral packaging cell line was seeded to 6-well plate at a concentration of 500,000 cells/well[23]. 2.5 µg of plasmid was mixed with 5 µL PEI. Let the mixture sit for 15 minutes at room temperature and then add the mixture into RD114 cells. Change media on the next day and collect media 2 days later for transduction.

Cell line

RD114 and plat-e cells were cultured in DMEM media with 10% FBS, 1% penicillin and streptomycin. Hoxb8 cells were maintained in RPMI media with 10% FBS, 1% penicillin and streptomycin, sodium pyruvate, non-essential amino acids, HEPES, L-glutamine, beta-mercaptoethanol, flt3 ligand and estrogen. All the hoxb8-FL cells were transduced with retroviral vectors obtained from RD114 cell line. After transduction, FACS sorting is performed to obtain

100% positive hoxb8-FL population. To induce differentiation, sorted hoxb8-FL cells is incubated in RPMI media with 10% M-CSF for 5 days. All the cells were tested for mycoplasma before the usage.

Flow cytometry

Hoxb8-FL cells were checked for CAR and FLAG expressions. The cells were stained with Goat anti-human IgG Alexa Fluor 647 (156339, Jackson ImmunoResearch) and anti-FLAG PE (B322356, BioLegend). M-CSF induced differentiated macrophages were tested for CAR and FLAG expression and macrophage markers. The macrophages were lifted from plat-e with accutase (Innovative Cell Technologies) before staining. Macrophage was tested according to the following panel: Goat anti-human IgG Alexa Fluor 647 (156339, Jackson ImmunoResearch), anti-F4/80 BV421 (0351659, BD BioScience), anti-CD64 BV786 (0328308, BD BioScience), anti-MHCII FITC (B261237, BioLegend), anti-CD11c BV650 (B213051, BioLegend), anti-CD86 BV605 (B294306, BioLegend), anti-CD206 BV711(B341077, BioLegend), anti-FLAG PE (B322356, BioLegend), and anti-CD45.1 Pe-Vio770 (5191108468, Miltenyi Biotec). Flow cytometry data is acquired on a Cytek NL-3000. The data is analyzed with SpectroFlow.

In vitro co-incubation phagocytosis assay

Day 4 CAR-macrophages were prepared and seeded in 96-well plate at a concentration of 20,000/well in 200 µL serum free RPMI. 0.1mg of HiLyte™ Fluor 488-labeled Amyloid-beta (1-42) (Anaspec) was reconstituted in 50 µL of 1% NH_4OH. Add 1 µL of Amyloid-beta peptide into each well. Incubate the peptide with CAR-macrophages for 2 or 4 hours. After co-incubation,

wash the wells with PBS, then pictures of the cells were taken by Cytation 5 (BioTek). The cells were then collected for flow cytometry.

Mouse brain samples

Brain slices were obtained from Jin-Moo Lee Lab. Perfusion was performed on 12-month female APP/PS1 mice and the brain was collected from the mice. After dissection, the brain was fresh frozen on dry ice and then stored in -80°C. Before using it, obtain the brain from -80°C and cut the brain into 10 µm slices with CryoStat. Attach the brain PDL coating coverslips and store the slice with the coverslip in -80°C.

Ex vivo phagocytosis assay

CAR Hoxb8 cells was induced to differentiate in RPMI media with M-CSF for 6 days. On day 6, washed away floating cells with PBS and lifted adherent CAR-Macrophages with accutase (Innovative Cell Technologies). Incubate thawed APP/PS1 mice brain slices with $2x10^5$ Z105 or Z117 CAR-Macrophages in 1mL of differentiation media for 44 hours (n=6). After incubation, fix the slices in 4% PFA for 20 minutes. Then, the slice is stained with X-34 dye and HJ3.4 antibody. High resolution image of the slice was taken by NanoZoomer Digital Scanner (Hamamatsu Photonics). The total area of plaque coverage was measured with NIH ImageJ software and was expressed as percentage total area. The frequency of size classified plaques was analyzed with ImageJ software.

Intracranial injection and BLI imaging

Preconditioning is performed 2 days and 1 day before intracranial injection. Wildtype 3-months female mice was used in this assay. Each mouse was given PBS or Busulfan () at 30 mg/kg by IP injection for preconditioning. Busulfan was dissolved in DMSO at a concentration of 30 mg/mL. Before injection, the Busulfan solution was diluted to 3mg/mL in PBS.

Cells used in intracranial injection was prepared by incubating CAR hoxb8-FL cells in 5% MCSF for differentiation. On Day 6 post differentiation, the macrophages were lifted by accutase. We then washed the cells with PBS for 3 times. Then, load the 5 µL Hamilton syringe with the macrophages at a concentration of $2x10^5$ cells/µL. Injection was performed by WUSTL Animal Surgery Core. 1 µL Cells was injected to two sites in anterior hippocampus in each side of the brain.

Chapter 3: Results

Generation of CAR hoxb8-FL cell line

To generate CAR hoxb8-FL cell line, we transduced murine hematopoietic progenitor hoxb8 cells with a retroviral vector that expresses anti-amyloid CAR (Z117). After transducing the hoxb8-FL cell for 3 times, CAR transduction rate of hoxb8-FL cells reached over 87 percent (Fig. 1B). The FLAG tag for this contract could not be detected by anti-FLAG antibody. To obtain a population with high expression of CAR, fluorescence-activated cell sorting (FACS) was performed to isolate the population with higher mean fluorescent intensity (Fig. 1C). In the same way, we generated a control CAR macrophage cell line that has anti-EphA2 scFv and no intracellular domain (Z105).

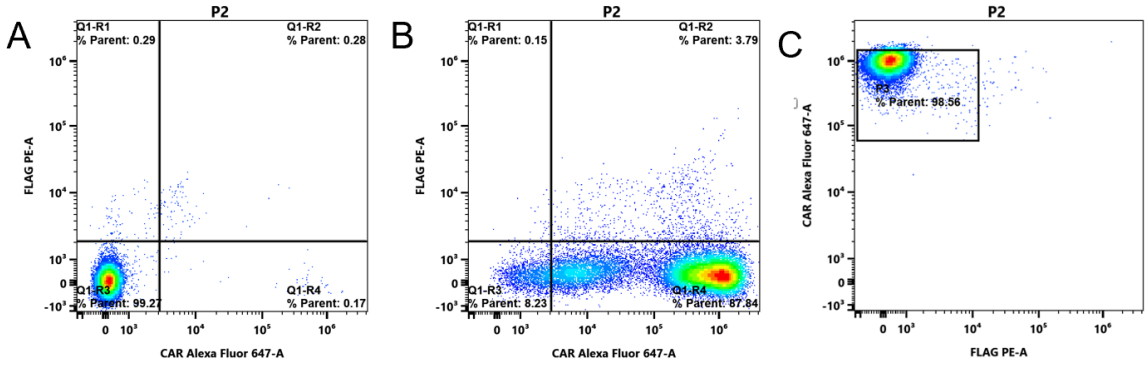

Figure 1. Flow cytometry plots of transduction of hoxb8-FL cells. A). Flow cytometry plot of untransduced control hoxb8-FL cells. B). Flow cytometry plot of anti-amyloid CAR transduced hoxb8-FL cells (Z117). C). Flow cytometry plot of FACS sorted anti-amyloid CAR transduced hoxb8-FL cells. For all the panels, the cells were stained with Goat anti-human IgG Alexa Fluor 647, and anti-FLAG PE.

Phenotype of M-CSF induced differentiation of hoxb9-FL cells

In order to obtain mature macrophages, sorted hoxb8-FL cells that express anti-amyloid CAR was cultured with M-CSF to induce differentiation. Day 6 post differentiation, the macrophages express high level of F4/80 and lower level of CD64 receptor, which are both macrophage markers (Fig. 2). After differentiation, CAR-macrophages maintained the high level of CAR expression. Z117 has higher expression of CD206, indicating they are more M2 like macrophages. Z105 has a population with higher expression of CD86, indicating its phenotype is closer to the M1 macrophages.

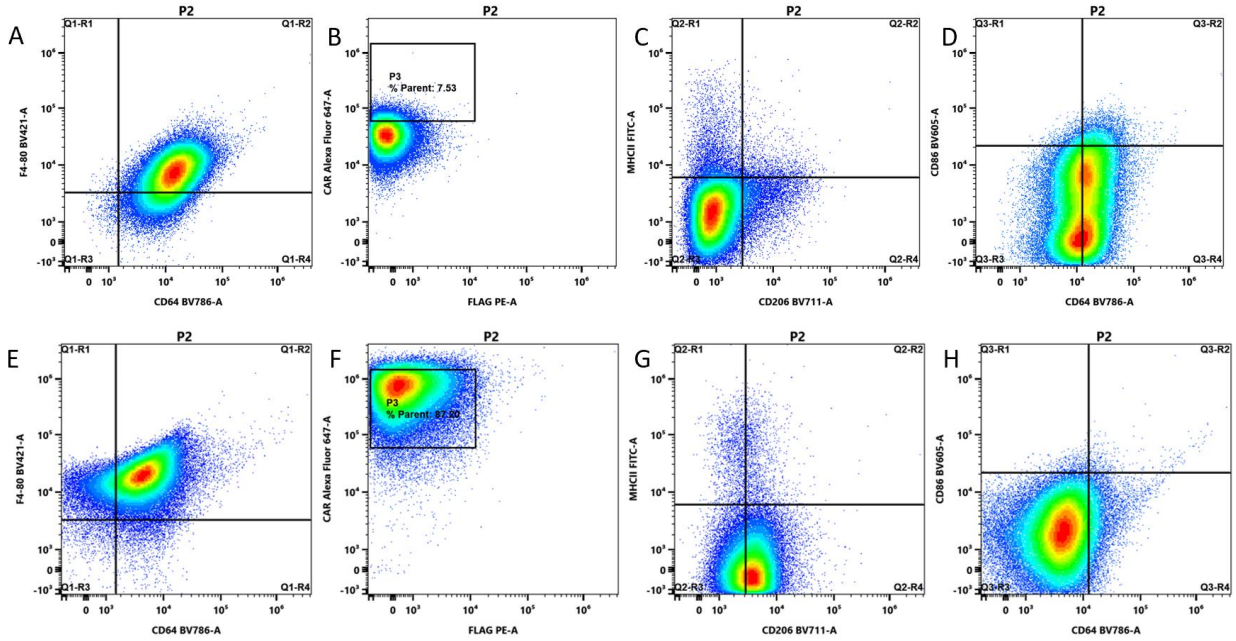

Figure 2. Flow cytometry plot of Day 6 macrophage differentiated from Z117 hoxb8-FL cells. A-D). flow cytometry plot of Z105 CAR-macrophages. E-H). flow cytometry plot of Z117 CAR-macrophages. Expression of A and E). CD64 vs. F4/80. B and F). FLAG vs. CAR. C and G). CD206 vs. MHCII. D and H). CD64 vs. CD86.

Anti-amyloid CAR enhances internalization of Amyloid-beta peptides in vitro

We next tested anti-amyloid specific phagocytosis of Z117 CAR-macrophages and Z105 CAR-macrophages. After co-incubation with fluorescence labelled amyloid-beta(1-42) peptides for 4 hours, both cells have GFP signals under microscope (Fig. 3 A-B). However, fluorescent signal of Z117 is brighter than signals of Z105, indicating a more active phagocytic behavior of Z117. We then collected the cells at 2 hours and 4 hours post co-incubation for flow cytometry (Fig. 3 C-F). Percentage of high GFP intensity cells according to the gating in panel C-F is calculated. According to the quantification plot, Z117 CAR-macrophages have a higher percentage of high GFP intensity cells (Fig 3. G) and higher mean GFP intensity across all the single cells (Fig. 3 H). Therefore, Z117 CAR-Macrophages have an enhanced internalization activity towards amyloid peptide.

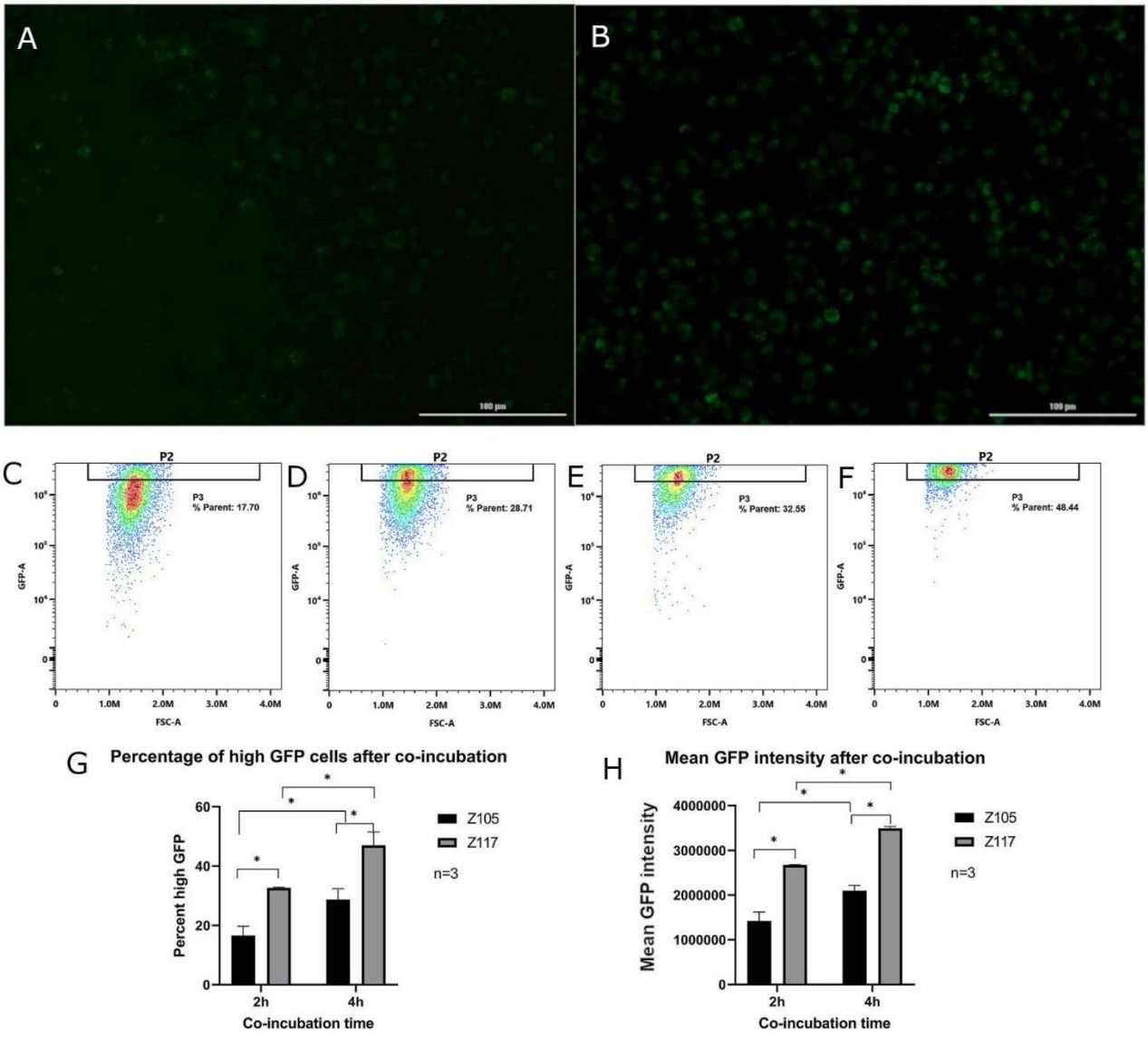

Figure 3. Results of in vitro co-incubation assay. A-B). Microscopy images of CAR-macrophages after co-incubation. A). Z105 CAR-macrophages. B). Z117 CAR-macrophages. C-F). Flow cytometry plot of CAR-macrophages after co-incubation with HiLyte™ Fluor 488-labeled Amyloid-beta peptides. C). Z105 CAR-macrophages 2 hours post co-incubation. D). Z105 CAR-macrophages 4 hours post co-incubation. E). Z117 CAR-macrophages 2 hours post co-incubation. F). Z117 CAR-macrophages 4 hours post co-incubation. G-H). Quantification of flow plot. G). Percentage of high intensity GFP macrophages. High intensity is defined by gating

in panel C-F. H). Mean GFP intensity of the macrophages. For all the panels, *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. (n=3)

Ex vivo phagocytosis of anti-amyloid CAR macrophage is enhanced.

In order to investigate the phagocytosis of amyloid plaques, we performed an ex vivo phagocytosis assay (Fig 4). Adjacent brain slice at 10 μm was obtained from 12-month female APP/PS1 mice to keep the plaque load to be similar in each group ($n = 6$). After co-incubate the slice with D6 CAR-macrophages or with no cells, the slice was fixed and stained with X-34 dye and HJ3.4 antibody. Slices that were co-incubated with Z117 CAR-macrophages have lower ratio of plaque area to total area than no cells group both in whole brain and in cortex (Fig. 4 D and E) ($p < 0.01$). The Z117 CAR-macrophages also decrease the frequency of plaques on the slice when compared with no cell group and control group both in whole brain and in cortex (Fig.4 F and G) ($p < 0.05$).

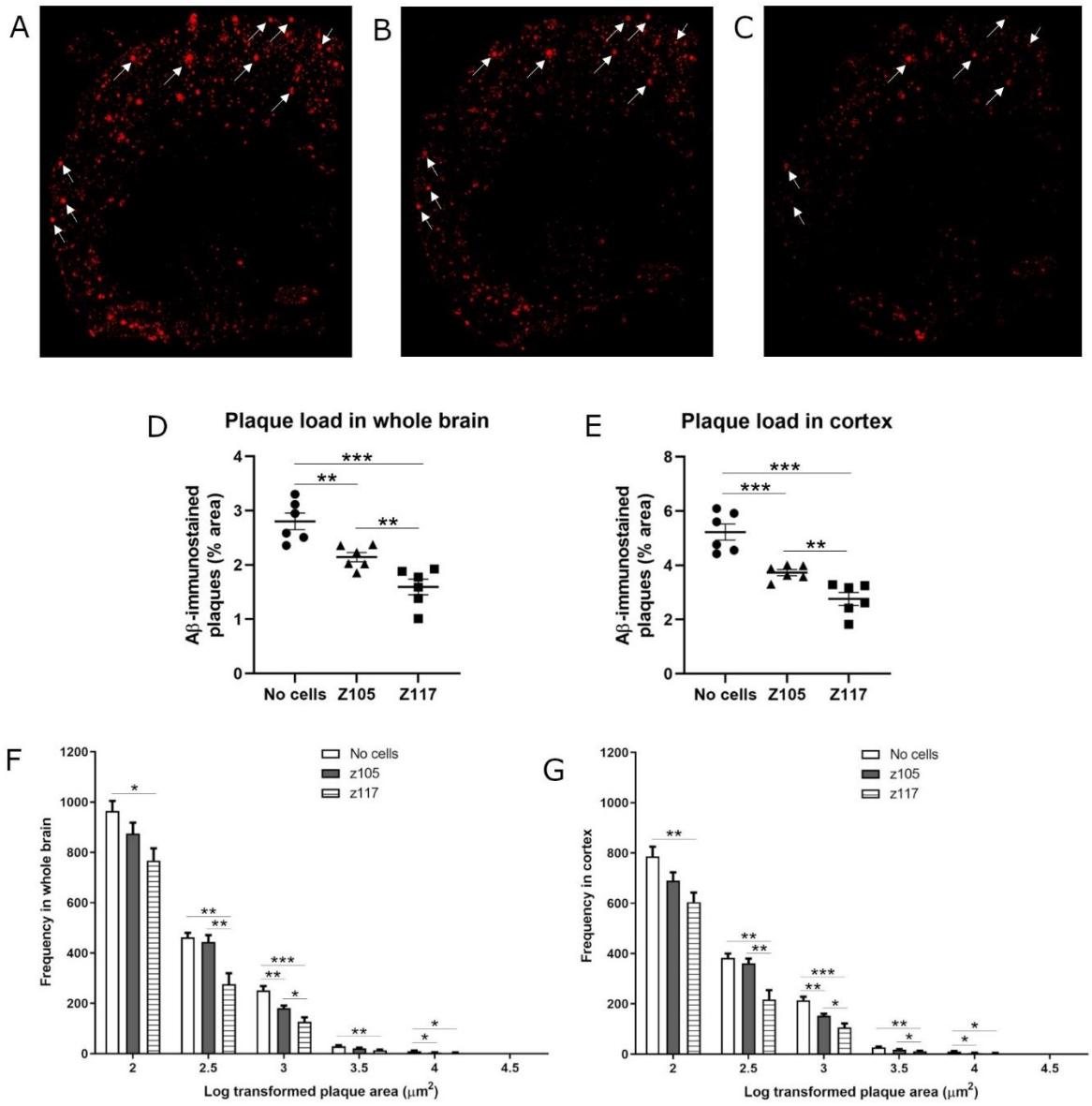

Figure 4. Ex vivo assays of phagocytosis. A-C). brain slice of APP/PS1 mice. White arrows indicate plaque on adjacent slices. The plaques were stained with X-34 dye. A). slice treated with control media. B). slice treated with control Z105 macrophages. C). slice treated with anti-amyloid Z117 CAR-Macrophages. D-E). statistical analysis of plaque load of the brain slices (n = 6). D). plaque load in the whole brain. E). plaque load in cortex. F-G). Frequency of plaques classified with log transformed area. F). Frequency of plaques in whole brain. G). Frequency of plaques in cortex. (*p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001)

GFP transduction has no impairment on phagocytic activity of Z117.

In order to track longevity and migration of CAR-Macrophages in future in vivo experiments, we transfect Z117 CAR-hoxb8-FL cells with GFP-luciferase with retroviral vector. After GFP-luciferase transduction, we tested phagocytosis of this cell line on the brain slice from 12-month female APP/PS1 mice (Fig 5.). After incubation, the slices were stained with X-34 and HJ 3.4 antibody. Slices that were incubated with Z117 and GFP-luciferase Z117 has significantly decreased percentage of amyloid load (Fig 5. E-H) compared with slices that were incubated with media only and with Z105 macrophages. Z105 CAR-macrophages this time did not show a significant non-specific phagocytic activity. There is no remarkable difference in function of Z117 and GFP-luciferase Z117.

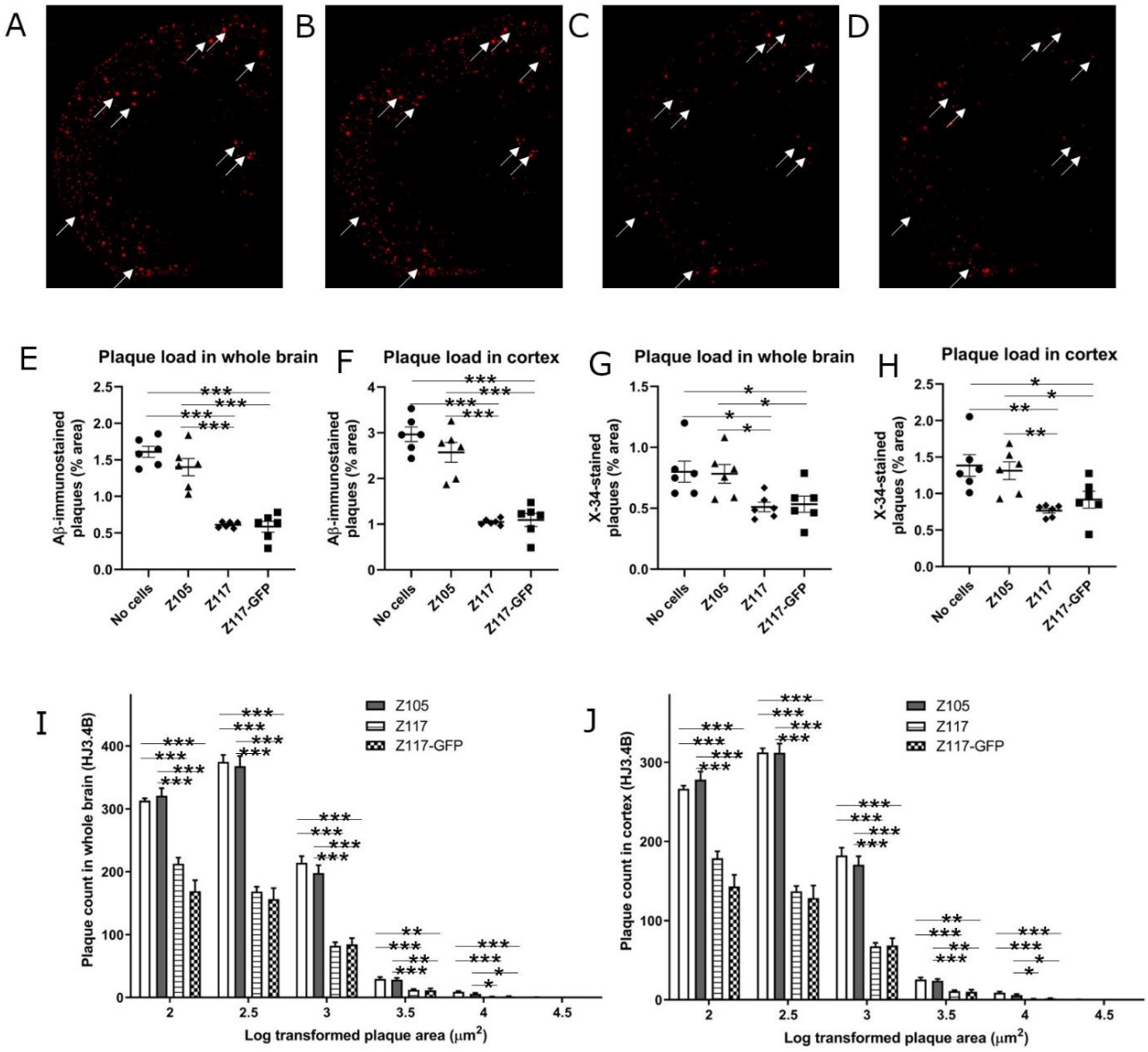

Figure 5. Ex vivo assays of phagocytosis of GFP-luc Z117 CAR-macrophages. A-D). brain slice of APP/PS1 mice. White arrows indicate plaques on adjacent slices. The plaques were stained with both X-34 dye and HJ3.4 antibody. A). slice treated with control media. B). slice treated with control Z105 macrophages. C). slice treated with Z117 CAR-Macrophages. D). slice treated with GFP-luc Z117 CAR-Macrophages. E-H). statistical analysis of percentage of plaque on the brain slices (n = 6). E). HJ3.4 antibody stained plaque load in the whole brain. F). HJ3.4

antibody stained plaque load in cortex. G). X-34 stained plaque load in the whole brain. H). X-34 stained plaque load in cortex. I-J). Frequency of plaques classified with log transformed area. I). in whole brain. J). in cortex. (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$)

Chapter 4: Discussion

Aducanumab was approved by FDA in June 2021[6]. The monoclonal antibody significantly reduces amyloid load in AD brain[8]. In this work, we generated an anti-amyloid CAR progenitor cell line with the aducanumab scFv. The progenitor cell line has similar behavior with primary cells and can be induced to differentiate in M-CSF. We obtained the CAR-macrophages from the progenitor by culture the cells in RPMI media with M-CSF. We showed that these anti-amyloid CAR expressing cells have higher potential to phagocytosis amyloid than CAR cells that don't have anti-amyloid scFv both in vitro and ex vivo.

Transduction of hoxb8-FL cells with retrovirus are effective and we are able to make purified stable CAR cell line from this immortalized progenitor cell line by FACS. After differentiation, the macrophages share similar phenotype with primary macrophages[21]. These macrophages also conserved the function of phagocytosis. However, primary cells are still a better choice for in vivo experiments.

Previous work had shown that CAR-macrophages are potential to treat solid tumors[17–19]. Macrophages, compared with microglia, has a stronger ability to phagocytosis. This feature of macrophage makes it a prospective cell to be engineered to target amyloid protein. Compared with CAR-T therapy, CAR-macrophages are expected to have more functions including recruiting other immune cells[11,18]. However, it also shares the same risk with CAR-T therapy – the cytokine release syndrome[15]. In the contrast of targeting tumor cells, induction of pro-inflammatory response can be harmful for the patient. Further in vivo studies to investigate the potential risk of cytokine and chemokine releasing in the future will be needed.

Our in vitro and ex vivo data shows that anti-amyloid CAR macrophages have enhanced phagocytic activity towards amyloid. We quantified the phagocytosis by fluorescent signaling.

Although Z105, which is the non-targeting CAR macrophages, has some internalization activities, Z117 has a better performance. This can be explained by the anti-amyloid CAR that induces specific binding and phagocytosis of amyloid protein. After internalized by the macrophages, the fluorescent labelled protein is present in the cytoplasm. We have not observed the process of degradation. In future, we will do in vitro kinetics studies to investigate the mechanism of degradation after phagocytosis.

In addition, we tried to inject the macrophages directly into hippocampus on both sides of the brain to explore the longevity of CAR-macrophages in brain. We injected the mice with PBS or Busulfan as a preconditioning prior to the intracranial injection. We expected the Busulfan can eliminate brain specific immune cells and therefore improve infiltration into brain. After injection, we detected luminescent signals from the cells by BLI (Fig. S1). However, the injection was inconsistent. No signal could be detected by BLI in some of the injected regions. There is no remarkable difference between busulfan group and PBS group. After the in vivo intracranial injection, GFP signals of the CAR-macrophages can exist for 10 days. In future works, we plan to perform more trials to improve the consistency of this technique.

Chapter 5: Conclusion

CAR-Macrophage, because of its phagocytic activity, can be potential as a cellular treatment for amyloid aggregation. In this study, we show that anti-amyloid CAR-Macrophage has enhanced phagocytic activity towards amyloid in ex vivo experiment. After co-incubation with anti-amyloid CAR-Macrophages, the plaque load of slice significantly decreased when compared to non-targeted CAR. However, lacking in vivo data, the effectiveness and risk of CAR-M as a cellular therapy for AD still need to be further studied.

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Appendix: Supplementary material

Intracranial injection can be an effective way to deliver the CAR-macrophage into brain.

To test the longevity of CAR-macrophages in vivo, we performed an intracranial injection on both sides of the brain in wild type mice. Two days before the injection, we performed preconditioning by injection Busulfan or PBS. Starting on Day 3 post injection, we tracked the signaling of the CAR-macrophages by Bioluminescent Imaging (BLI) (Fig S1. A). The signal is inconsistent with injections and cells in some injections cannot be detected. In the mice that have detectable signals, the cells can be tracked until Day 10. Quantification of signal showed that the signaling decrease overtime (Fig S1 B.). Drop on Day 7 may be caused by manipulation error considering the signaling level on Day 3 and Day 10.

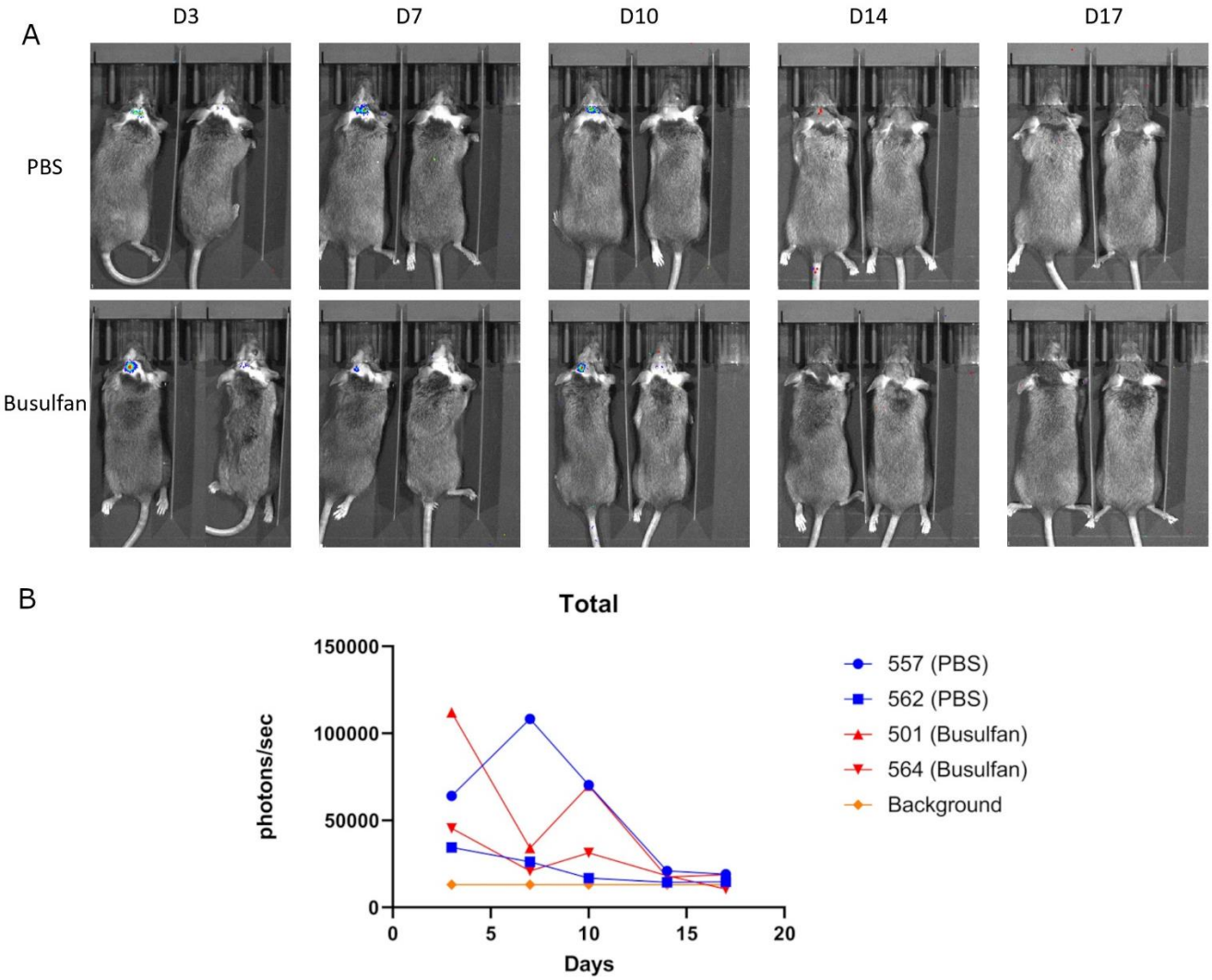

Figure S1. Bioluminescent Imaging post intracranial injection. A). Time coursed BLI photo of mice. B). Quantification of bioluminescent signal. The background level is determined by the signal at the chest of the mice.