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TITLE: Amyloid Fibril-Specific Chimeric Antigen Receptor Macrophages (CAR-M)-Based Immunotherapy for Amyloid Clearance in Light Chain Amyloidosis

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14. ABSTRACT

This proposal is focused on the development of a novel immunotherapy using amyloid fibril specific chimeric antigen receptor engineered macrophages (CAR-M) to treat amyloid light-chain (AL) amyloidosis. AL amyloidosis remains incurable with the lack of effective therapy to clear the insoluble amyloid fibrils. Inspired by the revolutionary advances of using synthetic transmembrane CAR receptors to reprogram T cells towards antibody directed killing of cancer cells, and given the fact that macrophage dependent phagocytosis is the key mechanism in amyloid fibril clearance, we hereby designed a novel AL amyloid fibril specific CAR engineered macrophage therapy to target the insoluble amyloid deposits and achieve effective fibril clearance through phagocytosis. Our initial effort showed that transduction and expansion of the primary mouse macrophages are technically impractical to generate enough cells for the proposed in vivo study. Therefore, we developed and optimized the production protocol of generating primary human CAR macrophages. The AL amyloid phagocytosis activity of the human CAR macrophages was validated by in vitro phagocytosis assay. Next, the in vivo activity of the human CAR macrophages was tested on a novel animal model to enable the real-time in vivo monitoring of amyloid targeting and clearance by CAR macrophages. Patient derived AL amyloid fibril extract was conjugated by Dylight fluorescence dye and then *s.c.* injected into NSG-SGM3 mice, which support the human macrophages expansion in mice. Our result showed that AL amyloid burden could be quantitatively monitored by this novel model. Our results also suggested that CAR macrophages inoculation route needs to be further explored to facilitate their targeting to the subcutaneously injected amyloidoma.

15. SUBJECT TERMS

AL amyloidosis, light chain, fibril, CAR, macrophage, immunotherapy, cell therapy, imaging, humanized AL amyloidosis mice model.

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1. INTRODUCTION:

Amyloid light chain (AL) amyloidosis is an incurable rare monoclonal plasma cell dyscrasia closely related to multiple myeloma. The clonal plasma cells produce excessive misfolded monoclonal immunoglobulin light chains which form extracellular insoluble fibrils, destroy tissue architecture, cause functional damage in organs, and eventually lead to death. Current chemotherapies only target clonal plasma cells to suppress the synthesis of new amyloidogenic light chains, however the already formed fibrillar deposits are not affected and keep causing persistent organ damages. Therefore, direct fibrillar targeting therapy is urgently needed to resolve the amyloid deposit and cure this ultimately fatal disease. Recently, CAR macrophages (CAR-M) emerged as a novel immunotherapeutic approach *via* triggering tumor cell specific phagocytosis by macrophages and achieved promising tumor clearance in animal studies. Since phagocytosis is the major mechanism of AL amyloid fibrillar clearance *in vivo*, we developed a first-in-class AL amyloid fibril specific CAR macrophage immunotherapy to induce effective fibril deposit specific phagocytosis thereby achieve efficient amyloid fibril clearance. We have successfully built a first-generation AL amyloid specific CAR construct. AL amyloid specific CAR transduced macrophages demonstrated promising AL amyloid fibrils engulfment and clearance efficiency in *in vitro* phagocytosis assay. We hereby propose to validate the in vivo AL amyloid targeting and clearance effects of the amyloid specific CAR macrophages in a novel humanized AL amyloidosis mice model. This project will provide the proof-of-concept data demonstrating the feasibility of using amyloid fibril specific CAR macrophages to treat AL amyloidosis at the preclinical level.

2. KEYWORDS:

AL amyloidosis, light chain, fibril, CAR, macrophage, immunotherapy, cell therapy, imaging, humanized AL amyloidosis mice model.

3. ACCOMPLISHMENTS:

What were the major goals of the project?

The major goals were to evaluate the AL amyloid fibril specific targeting/recruiting of 11-1F4-FcRr CAR macrophage *in vivo*.

We proposed to transduce Nude mice bone marrow derived macrophage with 11-F4-CAR-FcRr-GFP luciferase lentivirus and generate 11-1F4 CAR macrophages for mice injection (proposed date: 10/1/21). Following, we will inject the AL amyloid extract and CAR macrophage into Nude mice to investigate the *in vivo* amyloid targeting of 11-1F4 CAR macrophages (proposed date: 2/1/22).

However, our efforts to produce mouse primary CAR macrophages showed that mouse bone marrow derived macrophages are resistant to lentiviral infection. Although >90% macrophages were GFP positive on the second day after infection, most of the cells lost the GFP signal starting from day 3 following infection, probably because inflammasome pathways or phagocytosis were induced in the mature macrophages in response to viral infection. Therefore, we modified our SOW (submitted on 03/10/22) to prepare CAR transduced human macrophages in the immunodeficient NSG-SGM3 mice for the *in vivo* study (with 1 year of No-Cost-Extension approved on 7/21/22). The new major goals and target dates were the following:

Specific Aim 1: Evaluate the AL amyloid fibril specific targeting/recruiting of human 11-1F4-FcRγ CAR macrophage in vivo.

Subtask 1: Institutional IACUC and ACURO approval of the animal protocol.

Subtask 2: HRPO approval for the use of human cells from patient blood samples.

Subtask 3: Transduction and expansion of human control or CAR macrophages.

Specific Aim 2: Investigate the in vivo immunotherapeutic efficacy of human CAR macrophages using a novel in vivo model allowing real-time monitoring of the CAR macrophage recruitment and amyloid clearance.

Subtask 1 - Injection of human 11-1F4 CAR-M or control macrophages into DL-labeled amyloidoma bearing NSG-SGM3 mice (N=5 each group. Groups: 1) saline injection; 2) control macrophage injection; 3) CAR macrophage injection), and monitoring the real-time amyloid clearance by IVIS fluorescence/BLI imaging.

Subtask 2 - Validation of the CAR-M recruiting to amyloidoma by immunohistology staining.

What was accomplished under these goals?

1. Generation of mouse CAR macrophages for *in vivo* assay.

To generate the 11-1F4 CAR macrophages, we first packaged the lentivirus: pCDH-GFP-Luciferase (EV) and pCDH-11-1F4-FcRr-GFP luciferase (CAR) from HEK293T cells and concentrated the lentivirus to $2x10^7$/ml titer by ultracentrifugation. Mouse bone marrow derived macrophage (BMDM) was generated from nude mice bone marrow mononuclear cells after 5 days culture with 25 ng/ml M-CSF. The lentivirus and BMDMs (MOI=10) were coated onto Retronectin coated plates followed by centrifugation at 800g for 90 minutes. On day 2, the cells were examined under fluorescence microscope for GFP signal. Results showed that >90% cells were GFP^+. However, by day 3, green fluorescence signal almost completely disappeared (Fig. 1). It is recognized that primary macrophages are resistant to gene transduction (Sci Rep 10, 4181). We posited that the GFP^+ signal on day 2 came from the engulfed GFP+ lentiviral particles in macrophages, which was degraded by day 3.

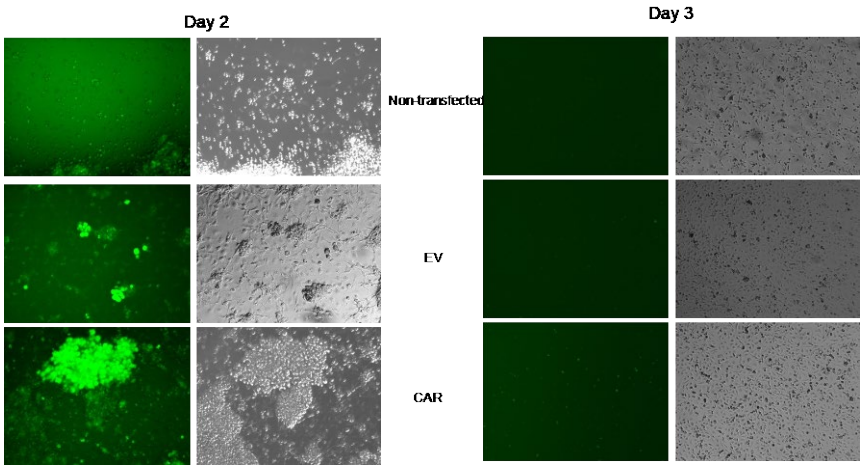

Fig. 1. Transduction of mouse bone marrow derived CAR macrophages.

To improve the infection rate, we then tested the following conditions: 1) increasing the MOI from 10 to 100; and 2) using different transduction reagents (Polybrene, LentiBOOST, and TransDux). However, none of the methods significantly improved the yield of the transduced cells. We posited that inflammasome pathways or phagocytosis were activated in the mature macrophage in response to viral infection. We also tried to use mouse bone marrow $CD11b^+$ cells, however similar phagocytosis was induced by lentiviral infection. To avoid phagocytosis in the target cells, we switched to the infection of mouse bone marrow mononuclear cells, followed by the induction of macrophages. To do this, we produced $1x10^8$ lentivirus and infected $1x10^7$ mouse bone marrow mononuclear cells (MOI=10) in the presence of LentiBOOST. ~35% transduction rate was achieved in the mononuclear cells. GFP^+ cells were sorted by flow cytometry and induced for macrophages using M-CSF culture. However, BM mononuclear cells and macrophages did not expand *in vitro*, therefore after 7 days of macrophage differentiation culture, only $1x10^5$ CAR transduced mouse bone marrow derived macrophages were harvested. This low yield rate makes the *in vivo* experiment impractical. We therefore decided to work with human macrophages and test the amyloidosis clearance activities on the humanized AL amyloidoma mice model, which is also more clinically relevant.

2. Generation of human CAR macrophages.

Previously, our lab has successfully established the protocol to generate primary human CAR macrophages. EV transduced (GFP) and 11-1F4 CAR transduced macrophages were confirmed by flow cytometry staining of the cell surface markers including CD33, CD45, CD64, CD14 & CD172a (Fig. 2).

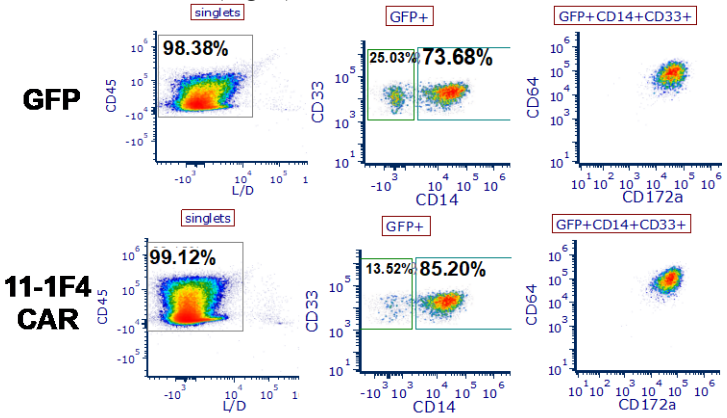

Fig. 2 Transduction of human CAR Macrophages.

We then confirmed the AL amyloid targeting/clearance function of CAR transduced human macrophages by *in vitro* amyloid phagocytosis assay. For this purpose, we covalently labeled AL amyloid fibril with PH sensitive fluorescence dye pHrdo-Red. As shown in the cartoon below, labeled amyloid fibril will only show red fluorescence under acidic PH environment (PH<4) when it is engulfed into the phagosome for clearance. We incubated the labeled amyloid fibril with EV or 11-1F4 CAR macrophages for 3 hours and the phagocytosis of AL amyloid was detected under fluorescence microscope (Red channel). As shown in Fig.3, CAR macrophages showed significantly enhanced phagocytosis activity against AL amyloid fibril.

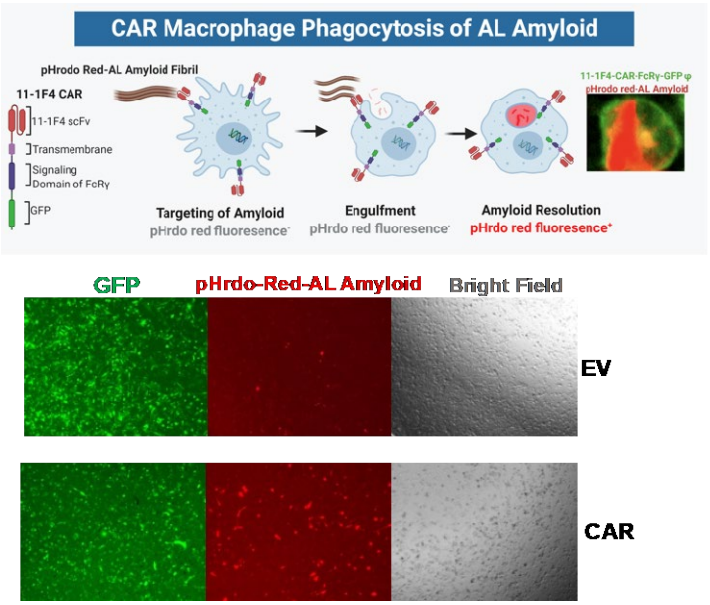

Fig.3 Phagocytosis of AL amyloid by human CAR macrophages.

3. AL amyloid fibril specific targeting/recruiting of 11-1F4 tansduced human CAR macrophages *in vivo*.

Next, we expanded the human EV or 11-1F4 CAR macrophages for mice injection. NSG-SGM3 mice (n=5/group) were sub-lethally irradiated followed by *s.c.* injection of lambda patient liver amyloid extract to establish the amyloidomas, followed by macrophages injection. Contrary to the earlier publication that assessed amyloidoma burden through daily palpation (*American Journal of Pathology, Vol 157, No.4, p1239*), we found that the subcutaneously injected amyloidomas could not form solid lumps, preventing reliable measurements through palpation. The majority of amyloidomas tended to form semi-solid flat masses within the cavity between the spine and loose skin, making accurate measurement through palpation impossible. Wet weight of the amyloidomas was then assessed upon euthanizing the mice; however, the weight showed a wide range of values with significant variations. We posit that this was due to the massive infiltration of mouse immune cells, fluid, and possibly the human macrophages we injected into the amyloid tissues. Therefore, the wet weight does not accurately reflect the actual amyloid burden. Direct labeling of the amyloid is necessary to enable precise quantification.

For the real-time monitoring and quantification of amyloid burden/clearance *in vivo*, we next tested the fluorescence labeling of amyloid fibril using Dylight 555. As shown in Fig. 4A, lambda patient liver amyloid extract was labeled with Dylight 555, and the fluorescence signal was confirmed by fluorescence microscope using the cy3 channel. Next, NSG-SGM3 mice (n=5/group, female, 6-8 wks) were sub-lethally irradiated followed by *s.c.* injection of Dylight labeled lambda patient liver extract to establish the amyloidomas, followed by macrophages injection. Upon injection, amyloid burden was examined immediately by IVIS imaging (Fig. 4B), then followed up to 8 days. As shown in Fig. 4C, amyloid burden could be successfully quantified and monitored based on ROI reading of the Dylight dye. However, there were no significant acceleration of the amyloid clearance observed after injecting 11-1F4 CAR macrophages in comparison to saline control or the empty vector transduced macrophage cells (Fig. 4C).

We next euthanized the mice and harvested the remaining amyloidoma together with the connected skin tissues for IHC staining. However, only massive mouse macrophages instead of human macrophages were detected in the tissue, suggesting that the human CAR macrophages failed to circulate or penetrate to the amyloidoma. NSG-SGM3 mice express human GM-CSF and IL3, both of which also activate mouse macrophages (Res Vet Sci 2021 134:137-146). This explained the high baseline mouse macrophage infiltration to the amyloidoma, but also suggested that it would be challenging to observe the effects of human CAR macrophages in this model. In the previous CAR macrophage study (*Nature Biotechnology* volume 38, pages947–953 (2020)), the CAR macrophages were given to the mice by either intratumorally, or *i.p.* or *i.v.* together with the target tumor cells. CAR macrophages circulation or recruitment to the target has never been addressed systematically, given that in all the tests so far CAR macrophages were directly administrated to the tumor. Therefore, CAR macrophage injection route in this s.c. amyloidoma model needs to be further explored to facilitate their circulation to the amyloidomas.

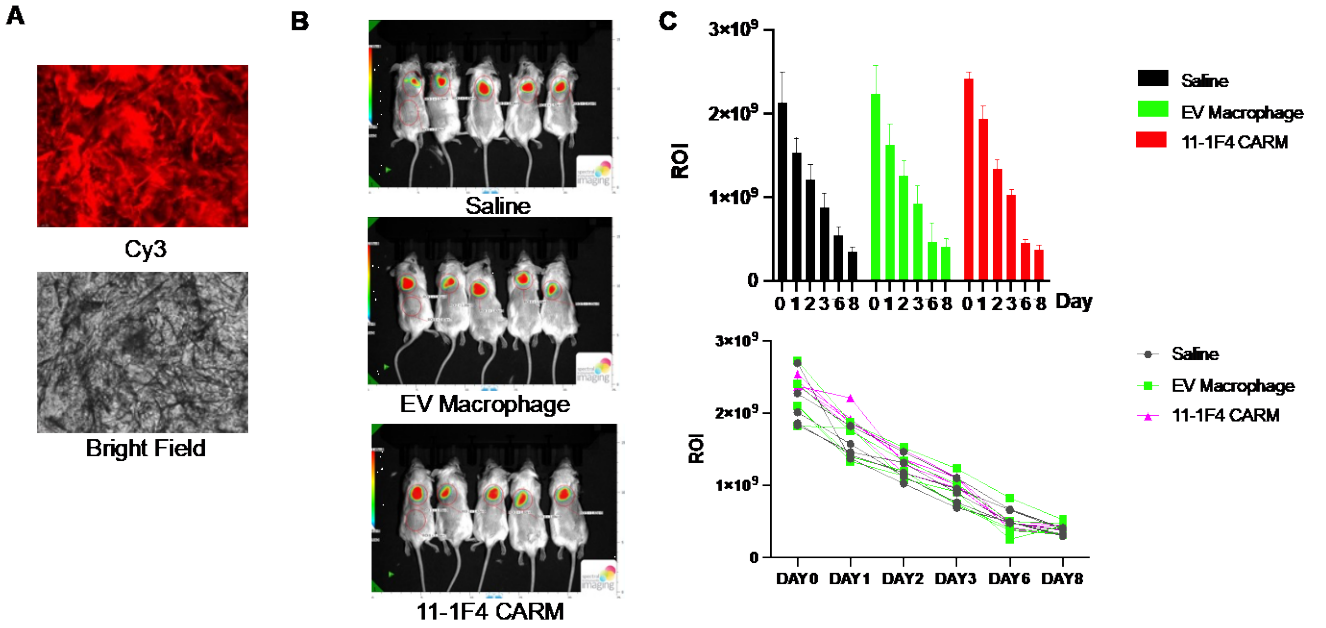

Fig.4 Real-time AL amyloidoma mice model*. **(A)** Lambda patient liver amyloid extract was labeled with Dylight 555, and the fluorescence signal was confirmed by fluorescence microscope using the Cy3 channel. **(B)** NSG-SGM3 mice received s.c. injection of Dylight labeled amyloid extract, and then imaged by IVIS. **(C)** Dylight signal was monitored at the indicated timeline.*

In summary, in this project, we established the novel approach to produce human anti-AL amyloid CAR macrophages and verified their amyloid clearance function *in vitro*. We also established the novel human AL Amyloidoma mice model enabling the human macrophages *in vivo* activities study and the real-time quantification of amyloid burden in NSG-SGM3 mice. We tested the *in vivo* amyloid clearance/targeting of human CAR macrophages on this model but did not observe the accelerated amyloid clearance by CAR macrophages. Further study demonstrated that the lack of sufficient recruitment of human macrophages to the amyloidoma was responsible for this outcome. Given that mouse macrophages are activated in this NSG-SGM3 mice, and the unknown CAR macrophages circulation/fate in mice, it is concluded that the macrophage injection route needs to be further explored to facilitate their targeting to the s.c. amyloidoma.

What opportunities for training and professional development has the project provided?

Nothing to Report

How were the results disseminated to communities of interest?

Nothing to Report

What do you plan to do during the next reporting period to accomplish the goals?

Nothing to Report

4. IMPACT:

What was the impact on the development of the principal discipline(s) of the project?

In this project, we established the novel approach to produce human CAR macrophages and verified their amyloid clearance function *in vitro*. We also established the novel human AL Amyloidoma mice model which enables the human macrophages in vivo activities study and the real-time quantification of amyloid burden in NSG-SGM3 mice. We tested the *in vivo* amyloid clearance/targeting of CAR macrophages on this model but did not observe the accelerated amyloid clearance by CAR macrophages. Further study demonstrated that the lack of sufficient recruitment of human macrophages to the amyloidoma was responsible for this outcome. Given that mouse macrophages are activated in this NSG-SGM3 mice, and the unknown CAR macrophages circulation/fate in mice, it is concluded that the macrophage injection route needs to be further explored to facilitate their targeting to the subcutaneously inoculated amyloidoma.

What was the impact on other disciplines?

Nothing to Report

What was the impact on technology transfer?

Nothing to Report

What was the impact on society beyond science and technology?

Nothing to Report

5. CHANGES/PROBLEMS:

Nothing to Report

Changes in approach and reasons for change

Nothing to Report

Actual or anticipated problems or delays and actions or plans to resolve them

Nothing to Report

Changes that had a significant impact on expenditures

Nothing to Report

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

Nothing to Report

Significant changes in use or care of human subjects

Nothing to Report

Significant changes in use or care of vertebrate animals

Nothing to Report

Significant changes in use of biohazards and/or select agents

Nothing to Report

6. PRODUCTS:

- **Publications, conference papers, and presentations**

Journal publications

Nothing to Report

Books or other non-periodical, one-time publications.

Nothing to Report

Other publications, conference papers and presentations.

Nothing to Report

- **Website(s) or other Internet site(s)**

Nothing to Report

- **Technologies or techniques**

Nothing to Report

- **Inventions, patent applications, and/or licenses**

Patent application CU22146 “Development of Amyloid Fibril Specific Chimeric Antigen Receptor Macrophages for Light Chain Amyloidosis Immunotherapy” was submitted on 12/6/21.

- **Other Products**

Nothing to Report

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name:	*Jing Fu*
Project Role:	*Principal Investigator*
	No change
Name:	*Shirong Li*
Project Role:	*Key Investigator*
	No change

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Nothing to Report

What other organizations were involved as partners?

Nothing to Report

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS:

Nothing to Report

QUAD CHARTS:

Nothing to Report

9. APPENDICES:

Nothing to Report