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Engineering chimeric autoantibody receptor T cells for targeted B cell depletion in multiple sclerosis model: An *in-vitro* study

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ABSTRACT

Background: Recent evidence suggests that B cells and autoantibodies have a substantial role in the pathogenesis of Multiple sclerosis. T cells could be engineered to express chimeric autoantibody receptors (CAARs), which have an epitope of autoantigens in their extracellular domain acting as bait for trapping autoreactive B cells. This study aims to assess the function of designed CAAR T cells against B cell clones reactive to the myelin basic protein (MBP) autoantigen.
Methods: T cells were transduced to express a CAAR consisting of MBP as the extracellular domain. experimental autoimmune encephalomyelitis (EAE) was induced by injecting MBP into mice. The cytotoxicity, proliferation, and cytokine production of the MBP-CAAR T cells were investigated in co-culture with B cells.
Results: MBP-CAAR T cells showed higher cytotoxic activity against autoreactive B cells in all effector-to-target ratios compared to Mock T cell (empty vector-transduced T cell) and Un-T cells (un-transduced T cell). In co-cultures containing CAAR T cells, there was more proliferation and inflammatory cytokine release as compared to Un-T and Mock T cell groups.
Conclusion: Based on these findings, CAAR T cells are promising for curing or modulating autoimmunity and can be served as a new approach for clone-specific B cell depletion therapy in multiple sclerosis.

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1. Introduction

Multiple sclerosis (MS) is a complex disease mediated by cascades of immune responses initiated in the peripheral immune system. It causes inflammation, loss of myelin sheaths, and degeneration of axons in the white matter of the central nervous system (CNS) [1]. Recent studies have fundamentally broadened the knowledge on the immunopathology of MS. While earlier concepts focused mainly on T lymphocytes; the evidence suggests that B cells and antibodies have essential roles in MS pathogenesis. Evidence of B cell accumulation in CNS lesions and ectopic B cell follicles in the meninges of patients with MS further suggests B cell roles in the etiopathology of the disease [2,3]. In addition to the secretion of antibodies by plasmablasts and plasma cells, B-cell functions implicated in the pathogenesis of the disease include the following: (i) Antigen-presenting and stimulation of self-proliferation of T cells and differentiation of autoreactive T Helper 1 and 17 cells in the central and peripheral nervous system (ii) Production of pro-inflammatory cytokines and chemokines that propagate inflammation, such as IFNγ, the IL-6, and granulocyte-macrophage colony-stimulating factor (GM-CSF) [4]. (iii) Production of soluble toxic factors such as death-inducing exosomes and antibodies that promote T cell activation or complement deposition in the myelin and contribute to damage in oligodendrocytes and neurons. (iv) Contribution to the formation of abnormal lymphoid aggregates in the meninges. (v) Acting as a reservoir for Epstein-Barr (EBV) virus infection. These actions of B cells may contribute to both the occurrence of relapses in multiple sclerosis and the progression of the disease [5].

Previously, studies had shown that B cell depletion would halt the progression of autoimmune diseases including systemic lupus erythematosus [6], multiple sclerosis [7], and arthritis rheumatoid [8]. B cell depletion therapies such as targeting CD20, CD19, and B-cell activating factor (BAFF) with monoclonal antibodies had also favorable treatment outcomes by stopping new lesions in CNS and exerting long-lasting effects on T cells [7,9,10]. However, there are beneficial B cell lineages producing IL-10 and IL-35 anti-inflammatory cytokines that regress the disease pathogenesis [11]. Therefore autoreactive B-cell destruction without harming the beneficial B cell population has become an interesting research topic for immune therapy in autoimmune diseases [12]. Moreover, due to the beneficial B cell lineages in the disease pathogenesis and the preliminary role of B cells in the immune system, patients must be carefully evaluated to ensure that beneficial subsets of B cells are not eliminated. Depleting B cell repertoire reduces serum antibody levels and predisposes the body to hypogammaglobulinemia and multiple infections [13]. As another problem, applying Daratumumab (anti-CD-38) and rituximab (anti-CD-20) as a pan-clonal B cell depletion therapy for treating autoimmune disease has shown limited response durability associated with a re-expansion of the same autoantibody-producing B-cell clones present before treatment [14]. The result is consistent with the incomplete elimination of B memory cells and/or plasma cells due to poor mAb distribution into the secondary lymphoid tissue sites [15].

On the other side, engineered T cells are shown to traffic efficiently to various immune-related anatomic compartments (i.e., blood, bone marrow, secondary lymphoid tissues, CNS, and pleural cavity). Indeed, therapeutic T cells have yielded responses in cancer patients who have previously failed therapy with anti-CD20 or anti-CD38 [14]. The promising outcomes of using chimeric antigen receptors (CARs) in directing the cytotoxic activity of T cells against hematologic malignancies have attracted attention to implementing such chimeric receptors against autoreactive B cells [16,17]. The extracellular domain of chimeric autoantibody receptors (CAAR) consists of conformational epitopes of autoantigens acting as bait for trapping autoreactive B cells [18]. The intracellular domain consists of one or more T-cell stimulatory domains to induce cytotoxic reactions such as CD137 and CD3zeta. This strategy has successfully been tested in eliminating autoreactive B cells responsible for the blistering autoimmune disease in pemphigus Vulgaris using CAAR T cells recognizing Desmoglein 3 in the mice model [19]. Preclinical studies also demonstrated satisfactory results in treating pemphigus Vulgaris and Myasthenia gravis [20,21].

Several autoantigenic targets have been proposed to contribute to MS pathophysiology, among them the myelin basic protein (MBP) and its reactive immune cell lines are shown to be sufficient to induce experimental autoimmune encephalomyelitis (EAE). MBP acts as an autoantigen against which autoantibodies are secreted and target the myelin components in CNS, and have a significant role in MS pathogenesis [22]. Based on this argument, the expression of MBP as an extracellular domain in the CAAR receptor can specifically redirect T cells' cytotoxicity against MBP-reactive B cells. The objective of this study was to investigate the proof-of-concept of the fundamental functionality of a second-generation CAAR receptor to be expressed efficiently on T cells, form an immunological synapse with MBP autoantigen-reactive B cells, and induce cytotoxicity on the target cells.

2. Results

2.1. Assessing the EAE mice development

Mice were monitored for signs of EAE disease and in most immunized mice, EAE clinical symptoms started ten days post-immunization and progressively continued for 21 days. The signs of EAE development were increased clinical scores and weight loss of more than 10%. After 21 days, the weight in the control and EAE mice was 23 ± 0.05 and 18.54 ± 0.39, respectively ($p < 0.001$). The clinical scores were 0 in the control and 2.5 ± 0.98 in the EAE group (Supplementary Fig. S1 A–B). These clinical symptoms were supported by inflammatory cells in the CNS of EAE-induced mice (Supplementary Fig. S1 C). Another finding in favor of EAE was that the anti-MBP IgG concentration, measured on serum samples one week after EAE induction, increased to 1.32 ± 0.98 ng/ml in the EAE after injection vs. 0.07 ± 0.01 ng/ml before injection; ($p = 0.049$) (Supplementary Fig. S1 D). Accordingly, EAE was successfully developed in mice.

2.2. T cells expressed MBP-CAAR after lentiviral gene transfer

Fig. 1 A represents the schematic of the vector employed in the study (Fig. 1 A). The EF1α promoter in the vector backbone was employed to express GFP reporter in transduced cells. Accordingly, microscopy and flow cytometry were implemented to approve the transduced genes expressed successfully in MBP-CAAR T cells. Four days after cell transduction, the GFP-positive cells could be detected. An average of 80–90% of T cells in each field of the microscope were GFP positive and flow cytometry approved GFP expression in 97% of the MBP-CAAR T cells. The CAAR expression on the cell surface was also examined using labeled anti-MBP antibodies and detected on 86.8% of MBP-CAAR T cells versus 2.4% of Mock T cells (Fig. 1B–D).

MBP-CAAR T cells drive cytotoxicity, proliferate, and produce inflammatory cytokines in the *in-vitro* co-culture with B cells of EAE mice.

The B cells of MBP-sensitized mice were isolated and co-cultured with the engineered T cells. To provide evidence on the functionality and specificity of the engineered CAAR T cells against autoreactive B cells, the cytotoxicity, proliferation, and cytokine production of the CAAR T cells were investigated and compared with Mock T and Un-T cells. MBP-CAAR T cells showed higher cytotoxicity in the 10:1 effector to target (E: T) ratio compared to Un-T and Mock cells (48.93 ± 26.82 in MBP-CAAR T cells vs. 19.8 ± 10.84 in Un-T cells and 13.66 ± 7.49 in Mock T cells; $p < 0.01$). The higher cytotoxicity was also observed in the 5:1 (51.31 ± 27.93 in MBP-CAAR T cells vs. 19.2 ± 10.52 in Un-T cells and 12.33 ± 6.76 in Mock T cells; $p < 0.01$) and 1:1 E: T ratios (35.53 ± 20.07 in MBP-CAAR T cells vs. 17.16 ± 9.43 in Un-T and 13.0 ± 7.12 in Mock T cells; $p < 0.05$) (Fig. 2.

A). We also investigated whether there is a difference in the cytotoxic efficiency of CAAR T cells when they encounter numerous target cells. We found that the cells are potent even in low E: T ratios and the percent of killed B cells was comparable in all ratios ($p > 0.05$), albeit highest in the 5:1 E: T ratio.

Given the success of the CAAR T cells in showing cytotoxicity against B cells, we then tested the proliferation rate of CAAR T cells in their co-culture with target cells as another manifestation of the T cell activation. It was shown that MBP-CAAR T cells co-cultured with B cells displayed a higher proliferation rate relative to Un-T and Mock cells (6.79 ± 0.1 in MBP-CAAR T cells vs. 2.68 ± 0.15 in Un-T cells and 1.92 ± 0.09 in Mock T cells; $p < 0.001$) (Fig. 2 B). In the absence of target cells, MBP-CAAR T cells showed a lower proliferation rate (4.8 ± 0.26). In contrast, Un-T and Mock cells showed no response to the target in terms of proliferation, and their proliferation rate was comparable in the co-culture with B cells and media alone (which does not have any cells). Thus, the MBP-CAAR T cells can robustly proliferate when they were exposed to B cells and are relatively inactive in the absence of their targets.

We also observed that IFN- γ, IL-2, and TNF-α cytokines were only secreted when CAAR T cells co-cultured with target cells. The IFN-γ was higher in the CAAR T: target co-culture (878.83 ± 11.11) compared to Mock T: target (53.13 ± 0.15 ng/ml), Un-T: target (326.50 ± 0.36 ng/ml), and CAAR T: media (278.4 ± 0.1 ng/ml) co-cultures; ($p < 0.001$) (Fig. 3). A similar result was observed when

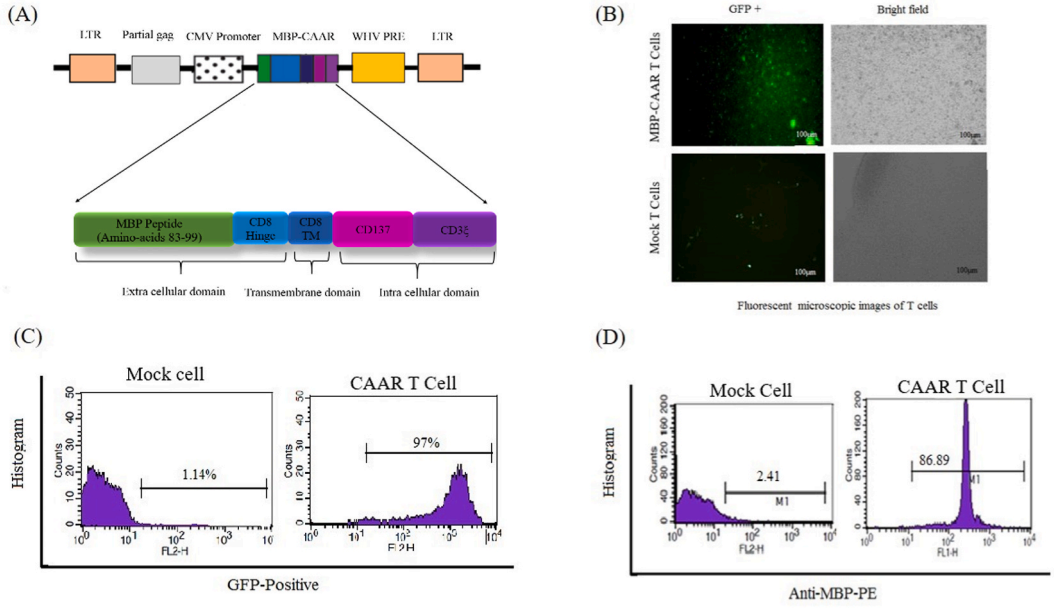

Fig. 1. **(A)** Schematic diagram of the vector used in this study is presented. **(B–D)** To confirm cells transduction, the GFP and MBP-CAAR expression in the MBP-CAAR T cells (transduced with pCDH-CMV-CAAR vector) were compared with Mock T cells (transduced with empty vector) as the control, using light microscopy and flow cytometry four days after transduction **(B)** Fluorescent and bright-field microscopic images of T cells after transduction by pCDH-CMV-CAAR vector and empty vector as the control, show that the transduced genes were expressed successfully in MBP-CAAR T cells. **(C)** The GFP expression was also quantified by flow cytometry, showing that 97% of the MBP-CAAR T cells are GFP-positive. **(D)** To detect the MBP-CAAR expressions on the T cell surface, the cells were stained using a PE-conjugated anti-MBP antibody. Flow cytometry showed that 86.89% of the pCDH-CMV-CAAR vector transduced cells are MBP-positive.

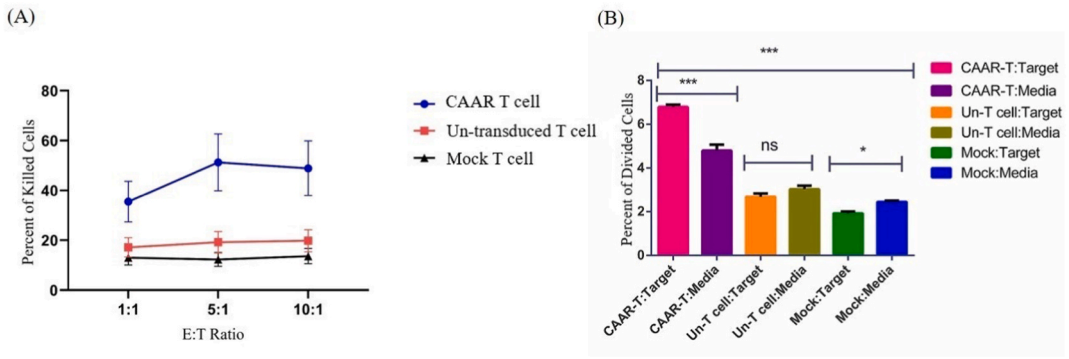

Fig. 2. **(A)** Cytotoxic activity was compared between MBP-CAAR T cells, Un-T, and Mock T cells co-cultured at 1:1, 5:1, and 10:1 E: T ratios with autoreactive B cells. After 18 h, the cytotoxicity was evaluated by using Annexin V apoptosis assay, in which the percentage of lysed target cells (V-PE$^+$/7AAD$^+$cells) was determined by flow cytometry **(B)** Antigen-dependent T cell proliferation was evaluated after co-incubating 2×10^5 of MBP-CAAR T, Un-T, or Mock T cells with B cells for 48 h (1:1 E: T ratio). The cells also co-incubated in the media alone as the control and CFSE dilution method was applied to quantify cells' proliferation. MBP-CAAR T cells co-cultured with B cells showed a significantly higher proliferation relative to Un-T and Mock cells. Data are presented as *mean* ± *SD*, and *a p-value*< 0.05 was considered statistically significant. The error bars represent SD. (***: $p < 0.001$), (*: $p < 0.05$), ns: non-significant.

comparing IL-2 and TNF-α concentrations in CAAR T: target co-culture with other co-cultures. For IL-2, the concentration was 243.0 ± 2.97 ng/ml in CAAR T: target, 44.95 ± 0.21 ng/ml in Mock T: target, 122.85 ± 0.5 ng/ml in Un-T: target, and 123.05 ± 9.55 in CAAR T: media co-cultures (Fig. 3). For TNF-α, the concentration was 263.64 ± 1.82 ng/ml in CAAR T: target, 73.48 ± 2.9 ng/ml in Mock T: target, 134.84 ± 1.25 ng/ml in Un-T: target, and 150.32 ± 6.09 ng/ml in CAAR T: media co-cultures (Fig. 3). A significantly lower amount of cytokines were produced when CAAR T cells are exposed to media alone (which does not have any cells). Therefore, assays approved that CAAR T cells could specifically recognize target B cells expressing anti-MBP receptors on their surface, which could lead to cytokine production by either of these cells.

3. Discussion

Recently, CAAR T cells have been successfully applied to target autoreactive B cells causing autoimmune disorders. Ellebrecht et al. engineered CAAR T cells against autoreactive B cells that target desmoglein 3 causing pemphigus vulgaris and reported effective and target-specific cytotoxicity [19]. Similarly, CAAR T cells were developed against autoreactive B cells producing anti-La/SSB antibodies in systemic lupus erythematosus and Sjögren's syndrome, anti-MuSK antibodies in myasthenia gravis, and anti-FVIII antibodies in hemophilia [23–25]. All the studies confirmed that CAAR T cells can kill autoreactive B cells *in vitro* and expand, persist, and specifically eliminate the target cells *in vivo*. To our knowledge, CAAR T cells have not been used against autoreactive B cell clones that may play a role in MS pathogenesis. Nevertheless, chimeric antigen receptor regulatory T cells (CAR Tregs) that were tested to encounter autoimmunity in MS showed the promise of cellular immunotherapies in autoimmune diseases [26,27]. CAR Tregs against

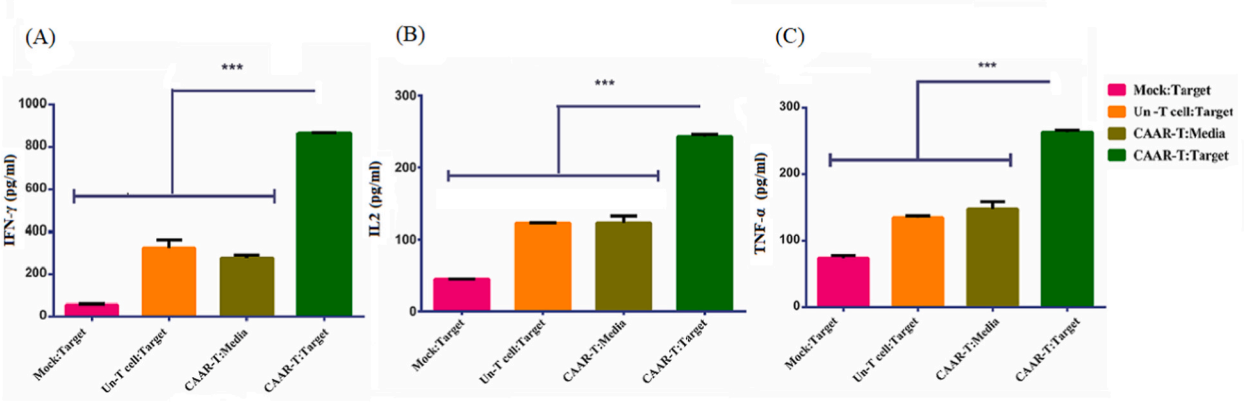

Fig. 3. 2×10^5 MBP-CAAR-T, Mock, and Un-T cells were co-incubated with an equal number of autoreactive B cells or media alone for 24 h, and the cytokine production of the cells was measured. Supernatants were stored at −20 °C until examined by the ELISA technique. Bar graphs show **(A)** IFN-γ, **(B)** IL-2, and **(C)** TNF-α cytokine levels in Mock, Un-T, and MBP-CAAR T cells co-cultured supernatants with B cells (in 1:1 E: T ratio) or media, respectively. All three cytokines were produced in higher titers in the CAAR-T: target group than other controls. The error bars represent SD (*** shows $p < 0.001$).

MBP and MOG autoantigens recognized their target antigens in the neural tissue and caused a site-specific and efficient suppression of ongoing inflammation in addition to improvement in the clinical score of the EAE model.

Considering the previous success of engineered T cells in controlling autoimmune diseases, we postulated whether engineered chimeric autoantibody receptor T cells can act against MS by targeting these B cells. In this regard, T cells were transfected with an engineered vector and expressed CAAR, which consisted of a part of the MBP autoantigen (aa: 83–99) fused to CD137-CD3ζ signaling domains. Therefore, the MBP-reactive B cells can recognize and attach to the CAAR receptor extracellular domain, which would activate the CAAR T cells' cytotoxicity. Several antigenic targets have been proposed to contribute to MS pathophysiology, including myelin oligodendrocyte glycoprotein (MOG), MBP, and proteolipid protein (PLP) [28]. Among these antigenic targets, it has been shown that $CD4^+$ T cells targeting MBP are sufficient to induce autoimmune encephalomyelitis. MBP and its relationship with HLA-DRB1*15:01 and HLA-DRB5*01:01, as the disease-associated haplotype, is of particular interest [29]. It is also worth mentioning that during the MS pathogenesis, multiple epitopes of the MBP could be recognized by the T cell lines. However, the 80–105 amino acid residues are among the most commonly recognized sites [30]. In fact, the immunodominant epitope of MBP is the 83–99 amino-acid residues, which showed the strongest binding affinity to the HLA-DR2 haplotype [31,32].

In this study, CAAR T cell-induced cytotoxicity was observed against the B cells isolated from EAE mice, which was significantly higher than the Mock and Un-T groups. A comparable cytotoxic activity of MBP-CAAR T cells was observed at all E: T ratios. This finding exemplifies that CAAR T cell doses greater than 1:1 are unnecessary for high cytotoxic activity. The lower CAAR T cell doses could provide more safety with lower doses and costs while maintaining desirable results. However, previous studies have shown a dose-dependent cytotoxic efficacy with higher cytotoxicity in higher E: T ratios [19,23,25]. Further studies with larger samples and wider ranges of E: T ratios are needed to ascertain the optimal effector dose for the MBP-CAAR T cells that provide both the lowest relapse probability and costs. The production of IFN-γ, IL-2, and TNF-α cytokines was also shown in response to the formation of immunological synapses between CAAR T and autoreactive B cells. This result may raise questions about the source of these cytokines in the context of CAAR T cell therapy, in which both targeting and targeted cells are immune cell populations with the ability to produce the cytokines. Thus, future studies are needed to elucidate the source of and mechanisms underlying cytokine production.

3.1. Limitations

The CAAR T cell-based approach applied in the study is not devoid of limitations and can be evolved in the future. Even though MBP is commonly considered for antigen-specific therapies for MS, therapies based on a single antigen could be challenging. There are variations in autoantigens that contribute to pathogenesis among different patients. Moreover, MS pathogenesis is dynamic in time and epitope spreading in the disease course sensitizes the immune system to new antigens [33,34]. To overcome this challenge, it has been demonstrated that strategies that target more than one antigen tend to yield a higher therapeutic efficacy [35]. CAR T cells that can be redirected to new antigens (termed universal CAR T cells) have been promising in targeting various autoimmunity effectors, and this strategy can also be implemented in MS [36]. Another limitation regarding the CAAR-based approach is that CAAR T cells may be ineffective against plasma cells, which downregulate surface immunoglobulins as they develop from B cell precursors. When long-lived plasma cells are spared, they can cause resistance to treatment [14]. In this regard, innovative approaches can be applied for selective plasma cell depletion *in vivo*. Taddeo et al. showed that a protein conjugate consisting of a target antigen and an antibody against surface markers of plasma cells can label reactive plasma cells with their own antibody. The reason is that antibodies secreted by plasma cells reactive to the target antigen are more likely to bind to their own cell surface rather than other non-reactive cells. Using this technique, autoantigen-specific plasma cells were selectively lysed *in vitro* by the complement system. Single injections of the conjugate protein depleted target plasma cells by 60%–70% but irrelevant antigen-specific plasma cells by less than 20% [37]. It can be proposed that similar protein conjugates consisting of an antibody recognizing plasma cells surface marker and antibody receptor can redirect CAAR T cells against long-lived plasma cells by the same mechanism.

4. Conclusion

CAAR T cells are an innovative therapeutic option that avoids the risks of general immunosuppression and can likely be applied to various autoantibody-mediated diseases. Here, we showed that autoantigen-based chimeric immune receptors direct T cells to kill anti-MBP autoreactive B lymphocytes. A comparable CAAR T cell-induced cytotoxicity was observed against the B cells isolated from the EAE mice in all E: T ratios, which was significantly higher than the control groups. Targeting the antibody-producing B cells may block the interaction between autoreactive B cells and active T cells; therefore, it will be exciting to study whether MBP-CAAR T cells can inhibit T cell activation *in vivo*. CAAR T therapy for autoimmunity, however, will not be subject to some of the limitations of CAR-T therapy for cancer. First, target cell escape would be impossible because the mutation should cause B cells to no longer bind auto-antigen, which will make the B cells irrelevant to the disease. Additionally, B cells that down-regulate their BCR to escape CAARs will lose survival and activation signals and are unlikely to persist or mature into antibody-secreting cells. Tumor lysis and cytokine release syndrome are also unlikely since autoreactive B cells are only a small fraction of the total B cell population, in comparison with hematologic malignancies where a large proportion of WBCs are malignant [18]. The prospering potency of CAR T cells against hematologic malignancies keeps hopes alive that the homolog design, CAAR T cell, can also cure or modulate autoimmunity by targeting autoreactive B cells. All the postulates promise the potency of CAAR T cells against MS and the *in vivo* validation of the study findings would be in our future direction.

5. Material and methods

5.1. EAE induction and autoreactive B cell isolation

Six to eight-week female mice C57BL/6 mice were obtained from the Pasteur Institute of Iran (Tehran, Iran) and maintained under 12-h light-dark cycle conditions. Mice were randomly selected for the experimental (n = 10) and control group (n = 10). Blood samples were taken from the mice tails to check the level of anti-MBP antibodies one day before MBP injection. To induce EAE in C57BL/6 mice using MBP (aa: 83–99) and a boost protocol, mice were injected subcutaneously with 100 μg of MBP emulsified in CFA on days 0 and 14. Then 1 day after the immunization, the mice were injected intraperitoneally with 200 ng of pertussis toxin in PBS. Pertussis toxin was not injected after the second immunization. Animals were observed daily and killed after 21 days [38,39]. Mice were monitored daily for weight changes and the clinical score of EAE was determined as follows: 0, healthy; 1, loss of tail tone; 2, hind limb weakness; 3, hind limb paralysis; 4, hind limb and forelimb paralysis; 5, moribund or dead. The animals were humanely euthanatized and their blood, spleen, and spinal cord were isolated. The spinal cords were fixed in 4% paraformaldehyde at 4 °C and stained by the H&E. Blood samples were centrifuged at 14,000 rpm for 30 min at 4 °C, serum was collected, and MBP-antibody was evaluated using enzyme-linked immunosorbent assay (ELISA). Spleen cells were collected and pure B-cell populations were isolated from splenocytes using Magnetic-Activated Cell Sorting (MACS) bead Isolation kits (Miltenyi Biotec, Germany). B cells were stained by PE-Anti-Mouse CD19 (0.25 μg per million cells) (BioLegend) and analyzed by BD FACS Calibur (BD Biosciences, San Jose, CA, USA) flow cytometer.

5.2. CAAR Construction

The MBP-CAAR construct consists of genes encoding a part of the myelin basic peptide (amino-acid 83–99), the CD8 signal peptide, the CD8α hinge domain, the transmembrane region of the CD8 molecule, and an intracellular signaling domain containing both CD137 and CD3ξ intracellular domains. The CAAR encoding gene was inserted into the pCDH-CMV-MCS-EF1α-GreenPuro vector downstream to the CMV promoter. The EF1α promoter in the vector backbone would express copGFP reporter in transduced cells.

5.3. Lentiviral vector production

HEK293T cells were transfected with the constructed vector (pCDH-CMV-CAAR) together with packaging plasmid psPAX2 (Addgene, 12251), and enveloping plasmid pMD2.G (Addgene, 12259), using linear-polyethyleneimine (PEI) (Mw 25,000) (Polysciences, 23966-1). Briefly, 6×10^6 HEK293T cells were plated 24hr before transfection in the T75 flask. 14 μg DNA (6.9 μg vector, 5.11 μg psPAX2, and 2 μg pMD2.G) was diluted in serum-free DMEM and mixed with 35 mg PEI. The mixture was incubated for 20 min at room temperature. After incubation, the DNA-PEI complexes were added to the cells cultured in 7 ml complete DMEM and refreshed after 4 h. Viral supernatants were collected 48 h later and purified by centrifugation and filtration using a 0.45-μm filter. Viral supernatants were concentrated using ultracentrifugation at 26,000 rpm for 2 h and 30 min. Virus-containing pellets were resuspended in complete DMEM media and stored at −80°C. Lentiviral particles were titrated by serial dilution method on HEK293T cells [40]. At 72h post-transduction, cells were harvested, GFP expression was analyzed by flow cytometry, and the titer of the viral particle was calculated.

5.4. Mice primary T cells isolation and expansion

According to the Ethical Committee of the Iran University of Medical Sciences (IUMS) approved collection protocol, the fresh spleen was collected after sacrificing healthy C57BL/6J mice. The splenocytes were obtained after cell straining and Ficoll density gradient centrifugation. Monocytes adherent to culture dishes were eliminated by the conventional plate adhesion method. Non-adherent cells were cultured in RPMI-1640 media containing penicillin-streptomycin (100 μg/mL) and IL-2 (50 IU/mL; Miltenyi Biotec). Cells were then activated and expanded for three days using CD3/CD28 mAbs (Gibco; Thermo Fisher Scientific) in the bead to cell ratio of 1:3. T cells were kept in culture for three days, resulting in an average 4.5-fold expansion rate.

5.5. T cell transduction and CAAR T cells generation

T cells were pretreated with polybrene 8 μg/mL (Santa Cruz Biotechnology, Santa Cruz, CA) and transduced by lentiviruses at a multiplicity of infection (MOI) of ~3 adopting the spinoculation method. Accordingly, T cells were mixed and incubated with the lentivirus for 30 min at room temperature and centrifuged for 30 min at 800g at 32 °C. Positive selection of cells expressing vector genes was performed using puromycin antibiotic, which its resistance gene (i.e. puromycin-N-acetyltransferase (pac) gene) was in the vector.

After 7 h, transduction media were replaced with complete RPMI1640 media supplemented with 50 IU/ml of interleukin-2 (IL-2) (MiltenyiBiotec, Germany), 10-mmol/L HEPES, 10% heat-inactivated FBS, 1% L-glutamine, and 1% penicillin/streptomycin (PAN-Biotech, Aidenbach, Germany). Four days after transduction, the expression of MBP-CAAR was investigated by indirect staining with Anti-MBP 1/100 (BioRad, Hercules, CA), and goat anti-rat antibody-PE 1/100 (BioRad, Hercules, CA, USA). Before conducting experiments, 5–6 days of cell expansion were necessary. To promote cell expansion, the cells were incubated at 37 °C, and following a 4-h incubation period, the cultures received a supplement of 700 μl of fresh media, fortified with 100 IU/ml of IL-2. The cultures were monitored on a daily basis, with half of the existing media replaced with fresh media every 48 h. Additionally, 50 IU/ml of IL-2 was

added during each media replacement event.

5.6. In vitro cytotoxicity assay

B cells were co-incubated with MBP-CAAR T, Mock T cell (empty vector transduced T cell), and Un-T cells (un-transduced T cell) at 1:1, 5:1, and 10:1 Effector: Target (E: T) ratios in 96-well U-bottomed plates in complete RPMI 1640 for 18 h. *In-vitro* cytotoxicity was measured for each group by Annexin V apoptosis kit (IQ Products) according to the manufacturer's instructions. All nine co-cultures were prepared in three replicates ($n = 3$).

5.7. Proliferation assay and cytokine measurement upon antigen stimulation

To perform the antigen-dependent proliferation assay, cells were washed and resuspended in 110 μL PBS. The MBP-CAAR T cells, Mock-T, and Un-T cells were labeled with 1.1 μL of 5, 6-carboxyfluorescein diacetate succinimidyl ester (CFSE) (Biolegend) for 5 min in dark, then washed with PBS and co-cultured at a 1:1 ratio with target cells or media alone (without any cell to control auto-proliferation) in 48-well plates at a final volume of 800 μl/well. All six co-cultures were prepared in three replicates ($n = 3$). Flow cytometry analysis was performed 48 h after the beginning of cell culture using BD FACS Calibur (BD Biosciences, San Jose, CA, USA). To measure the concentration of the secreted IFN-γ, TNF-α, and IL-2 by effector cells, supernatants were harvested 24 h after co-culture and stored at $-20\ ^{\circ}C$ until examined by the ELISA technique.

5.8. Statistical analysis

Statistical analysis was performed using IBM SPSS Statistics 20.0. Independent samples T-test was used to compare the clinical scores and anti-MBP IgG concentration between the EAE-induced mice and the control group. The one-way ANOVA test was performed with Tukey's post-hoc test to compare different co-culture groups. Data are summarized as mean and standard deviation [Mean ± SD], or median and quartiles [median (Q1-Q3)]. P values < 0.05 were considered statistically significant.

Ethics approval

This study and handling of mice, in general, was conducted in strict accordance with the principles outlined in the Iran University of Medical Sciences, Tehran, Iran animal Protection Law (Number: IR.IUMS.REC 1395.9323513001). This study was financially supported by the Iran National Science Foundation (INSF), Tehran, Iran (grant number 69000120).

Author contribution statement

Maryam Sahlolbei: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Mohammadreza Azangou-Khyavy and Javad Khanali: Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Babak Khorsand, Aref Shiralipour, Naser Ahmadbeigi, Zahra Madjd, Hosein Ghanbarian and Alireza Ardjmand: Contributed reagents, materials, analysis tools or data; Wrote the paper.

Seyed Mahmoud Hashemi and Jafar Kiani: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

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Data availability statement

Data will be made available on request.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2023.e19763.

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