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Design of biodegradable nanoparticles to modulate phenotypes of antigen-presenting cells for antigen-specific treatment of autoimmune disease

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Abstract

Current therapeutic options for autoimmune diseases, such as multiple sclerosis (MS), often require lifelong treatment with immunosuppressive drugs, yet strategies for antigen-specific immunomodulation are emerging. Biodegradable particles loaded with disease-specific antigen, either alone or with immunomodulators, have been reported to ameliorate disease. Herein, we hypothesized that the carrier could impact polarization of the immune cells that associate with particles and the subsequent disease progression. Single injection of three polymeric carriers, 50:50 poly (DL-lactide-co-glycolide) (PLG) with two molecular weights (Low, High) and poly (DL-lactide) (PLA), loaded with the disease-specific antigen, proteolipid protein (PLP_{139–151}), were investigated for the ability to attenuate clinical scores in experimental autoimmune encephalomyelitis (EAE), a mouse model of MS. At a low particle dose, mice treated with PLA-based particles had significantly lower clinical scores at the chronic stage of the disease over 200 days post immunization, while neither PLG-based particles nor OVA control particles reduced the clinical scores. Compared to PLG-based particles, PLA-based particles were largely associated with Kupffer cells and liver sinusoidal endothelial cells, which had a reduced co-stimulatory

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Conflicts of interest

L.D.S., and S.D.M have financial interests in Cour Pharmaceutical Development Company.

Appendix A. Supplementary data

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molecule expression that correlated with a reduction of CD4⁺ T-cell populations in the central nervous system. Delivery of PLA-based particles encapsulated with higher levels of PLP_{139–151} at a reduced dose were able to completely ameliorate EAE over 200 days along with inhibition of Th1 and Th17 polarization. Collectively, our study demonstrates that the carrier properties and antigen loading determine phenotypes of immune cells in the peripheral organs, influencing the amelioration of both acute and chronic stages of autoimmunity.

Keywords

Nanoparticles; Antigen-specific treatment; Drug delivery; Autoimmune disease; Experimental autoimmune encephalomyelitis (EAE); Immune tolerance

1. Introduction

Autoimmune diseases, such as multiple sclerosis (MS), lupus and rheumatoid arthritis, require lifelong treatment. MS is a neurological disease estimated to affect over one million people in the world [1]. MS is classified into subtypes including relapsing-remitting (RR-MS) and secondary progressive (SP-MS) disease course. RR-MS is seen in 85% of MS patients, showing defined clinical exacerbations of neurologic symptoms, while SP-MS is seen in about 15% of MS patients with disease progression without remission [1,2]. The mouse model of MS, experimental autoimmune encephalomyelitis (EAE), has been shown to represent the MS disease course and used to identify that myelin-specific CD4⁺ T-cells are activated by antigen-presenting cells (APCs) in the peripheral draining lymph nodes [3]. These T-cells cross the blood brain barrier (BBB) and infiltrate the central nervous system (CNS). Entering the brain or spinal cord, CD4⁺ T-cells are then re-stimulated by local APCs, and the resulting inflammation leads to myelin destruction, astrogliosis and the production of chemotactic cytokines [3].

The desired therapy would involve the induction of long-term, durable antigen-specific T-cell tolerance. Current treatments for MS, such as cyclophosphamide and natalizumab, have partial efficacy against relapse of MS; however, these treatments are performed weekly, and the adverse effects such as nonspecific immunosuppression and potential worsening of MS limit their utilization [4–7]. Antigen-specific immunotherapies are emerging to suppress targeted immune responses without suppressing the global immune system for treatment of auto-immune diseases and allergies [8,9]. Antigen-loaded autologous cells that are systemically delivered have been shown to induce tolerance in both EAE and an animal model of Type 1 diabetes [10–12]. The delivery of autologous cells conjugated with specific antigens underwent a Phase I clinical trial, which showed good safety and efficacy [13]. However, harvesting autologous cells from patients with subsequent conjugation of antigens has challenges to translation.

Nanoparticles loaded with self-antigen, with or without a bioactive payload, have been developed for immune tolerance using the EAE model [14–19] and a type I diabetes model [20]. Particles around 500 nm size are thought to accumulate in the reticuloendothelial system, especially the liver and the spleen, and experience phagocytosis by APCs [21–23]. Poly (lactide-co-glycolide) (PLG) and Poly (lactide) (PLA) are FDA approved

biodegradable materials and have been used as carriers for drug delivery and treatment of inflammatory disease [24–26]. The degradation of these polymers is determined in part by the molar ratio of lactide to glycolide, and molecular weight of the polymer [27–29]. PLG particles have been mostly investigated in antigen-specific treatment for MS and have shown the capability to modulate immune cells for EAE disease amelioration [18,19,30]. An additional methyl group in lactide makes PLA more hydrophobic than PLG, which was reported to facilitate association with APCs [24,31]. Although previous studies have identified that the polymeric carrier may influence uptake and polarization of immune cells [27,32,33], their effects on autoimmune disease have not been well characterized.

We investigated the polymeric carrier for modulation of immune cells for tolerance induction in EAE. We characterized the disease course in response to multiple nanoparticle formulations and extent of antigen loading, and subsequently investigated immune cell internalization and polarization in response to particle delivery. Three types of PLP_{139–151}-encapsulated particles were fabricated using 50:50 poly (DL-lactide-co-glycolide) with inherent viscosity (IV) = 0.17 dL/g and 0.66 dL/g (termed agPLG-L and agPLG-H, respectively), and Poly (D,L-lactide) (PLA) particles with IV = 0.21 dL/g (termed agPLA). Fluorescently labeled particles were employed to examine the biodistributions among the organs, interaction with specific APCs within the organs, and the resulting phenotypes. This study highlights the contribution of the carrier properties along with antigen loadings towards modulation of immune cells for treatment of EAE.

2. Materials and methods

2.1. Materials

50:50 Poly (DL-lactide-co-glycolide) (IV = 0.17 dL/g in chloroform, Mw = 4.6 kDa, Mn = 2.5 kDa in tetrahydrofuran), 50:50 Poly (DL-lactide-co-glycolide) (IV = 0.66 dL/g in chloroform, Mw = 47.5 kDa, Mn = 28.3 kDa in tetrahydrofuran), Poly (DL-lactide) (IV = 0.21 dL/g in chloroform, Mw = 16.1 kDa, Mn = 8.9 kDa in tetrahydrofuran) with carboxylic acid end groups were purchased from DURECT corporation (Cupertino, CA). Poly (ethylene-alt-maleic anhydride) (PEMA) was purchased from Polyscience, Inc. (Warrington, PA). Proteolipid protein (HSLGKWLGHDPKF) (PLP_{139–151}) and ovalbumin protein (ISQAVHAAHAEINEAGR) (OVA_{323–339}) were purchased from GenScript USA Inc. (Piscataway, NJ). Cyanine 5.5 amine dye was purchased from Lumiprobe (Florida, USA). Dichloromethane (DCM) and N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC) was purchased from Sigma-Aldrich (St. Louis, MO). N-hydroxysuccinimide (NHS) was purchased from Thermo Fisher Scientific (Waltham, MA).

All fluorochrome-conjugated antibodies and Fc block were purchased from BioLegend (San Diego, CA) and their names and catalog numbers are listed here: TruStain FcX™ (Cat#101320, anti-mouse CD16/32) antibody, anti-mouse/human CD11b (Cat#101216, PE/Cy7), anti-mouse DC45 (Cat#103136, PerCP), anti-mouse CD4 (Cat#100510, FITC), anti-mouse/human CD45R/B220 (Cat#103244, Brilliant Violet 605™), anti-mouse CD3 (Cat#100341, Brilliant Violet 421™), anti-mouse I-A^k (Aβ^k) (Cat#109905, FITC), anti-mouse CD86 (Cat#105105, PE), anti-mouse Ly-6G (Cat# 127628, Brilliant Violet 421™), Zombie NIR™ Fixable Viability Kit (Cat#423105), anti-mouse F4/80 (Cat#123137,

Brilliant Violet 421TM), anti-mouse/human CD11b (Cat#101263, Brilliant Violet 510TM), anti-mouse Ly-6G (Cat#127639, Brilliant Violet 605TM), anti-mouse CD11c (Cat#117339, Brilliant Violet 650TM), anti-mouse CD146 (Cat#134713, PE/Cy7), anti-mouse CD4 (Cat#100555, Brilliant Violet 650TM), anti-mouse CD3 (Cat#100219, PE/Cy7), anti-mouse IL-17A (Cat#506926, Brilliant Violet 421TM), anti-mouse IFN- γ (Cat#505815, Alexa Fluor® 488) and Zombie Yellow Live Dead (Cat# 423103). Dilution was performed following the manufacturers' instructions.

2.2. Mice

Female SJL/J mice were purchased from Envigo. All mice were housed under specific pathogen-free conditions and maintained in the University of Michigan in compliance with Institutional Animal Care and Use Committee (IACUC) regulations.

2.3. Particle fabrication and characterization

Conjugates of polymer and cyanine 5.5 amine dyes (Polymer-Cy5.5) were fabricated using a EDC/NHS chemistry [34]. Polymer (0.002 mmol) was dissolved in 5 mL of DCM in a 20-mL scintillation vial and mixed with 1.7 mg of EDC in 1 mL DCM for 5 min. NHS (1.0 mg) in 0.5 mL of DCM was added dropwise to the mixture and allowed to stir for 10 min. Finally, the 1.5 mg of cyanine 5.5 amine dye in 1 mL DCM was added to the mixture and stirred overnight. The solution was purified using 3500 molecular weight cut-off dialysis membrane in 4 L of distilled water over 3 days.

Particles containing Polymer-Cy5.5 were fabricated using the w/o/w double emulsion solvent evaporation method [35]. Briefly, 150 μ L of PLP_{139–151} or OVA_{323–339} in PBS solution was added dropwise to 20% (w/v) (agPLG-L) or 10% (w/v) (agPLG-H and agPLA) of polymer dissolved in dichloromethane containing 1% (w/v) of Polymer-Cy5.5 and emulsified using a Cole-Parmer CPX130 Ultrasonic Processor with a Cole-Parmer 3 mm probe with stepped tip (Cole-Parmer Inc., Vernon Hills, IL). The resulting water-in-oil (w/o) primary emulsion was mixed with 1% (w/v) PEMA solution (agPLG-L) or 2% (w/v) PEMA solution (agPLG-H and agPLA) and then sonicated. The secondary emulsion was poured into 0.5% (w/v) PEMA and stirred on a Bellstir Multistir magnetic stirrer (Bellco Glass Inc., Vinland, NJ) overnight to remove organic phase. The resulting polymeric particles were washed three times and lyophilized with 2% (w/v) sucrose and 3% (w/v) D-mannitol.

Nanoparticle morphology was analyzed using Tescan RISE Scanning Electron Microscope (SEM) after particles were coated with carbon. The particle sizes were measured using ImageJ/FIJI (National Institute of Health, MD), with at least 50 particles analyzed per condition. In addition, the particle size and surface ζ -potential distributions were obtained using dynamic light scattering (DLS) on a Zetasizer Nano ZSP (Malvern Instruments Ltd., Malvern, United Kingdom). The amount of encapsulated peptide was determined using the Micro BCA Protein Assay Kit (Pierce Biotechnology, Rockford, IL) after dissolving particles in 0.1 M NaOH overnight.

2.4. Peptide release from the particles

The amount of peptide released from peptide-encapsulated particles was investigated, with 10 mg of PLG particles washed, resuspended in 1 ml PBS, and incubated at 37 °C with agitation. The *in vitro* release of PLP_{139–151} from agPLG-L, agPLA and agPLG-H particles was measured at several time points during incubation in PBS at 37 °C for 456 h (19 days). At indicated time points, particles were centrifuged, and the supernatant was collected and replaced with fresh PBS. Peptide in the supernatant was measured using the Micro BCA Protein Assay Kit. Cumulative release and release at each time point was calculated as percent of the amount of peptide detected divided by total amount of encapsulated peptide.

2.5. Induction and clinical evaluation of peptide-induced EAE

Peptide-induced EAE was induced in SJL/J mice as previously reported [36]. 8–10 weeks old female mice were immunized subcutaneously at three spots on the flank with 100 µL of an emulsion of PLP peptide in CFA containing 200 µg *Mycobacterium tuberculosis* H37Ra (Difco, Detroit, MI). After 7 days of the disease immunization, particles (2.5, 2.0, 1.5 or 1.0 mg/mouse) suspended in PBS were intravenously injected through the tail. Ovalbumin (OVA_{323–339}) encapsulated PLG-L particles (named OVA) were also injected as a control. Individual mice were observed daily, and clinical scores were assessed on a 0–5 scale as follows: 0, no overt signs of disease; 1, limp tail or hind limb weakness but not both; 2, limp tail and hind limb weakness; 3, partial limb paralysis; 4, complete limb paralysis; 5, moribund state. The data are reported as the mean daily clinical score. Paralyzed mice were given easier access to food and water.

2.6. Evaluation of in vivo particle biodistribution

agPLG-L, agPLA, and OVA particles (1.5 mg) were injected at 7 days post immunization (p.i.). Animals were euthanized at 14 days (p.i.), and optical images of the brain, spinal cord, liver and spleen were taken using an IVIS Lumina LET camera system (Caliper Life Sciences, Hopkinton, MA). Near-infrared fluorescence (NIR) images were obtained with cyanine 5.5 filter channel (emission: 720 nm, excitation: 675 nm).

2.7. Cell isolation and staining for flow cytometry analysis of CNS, spleen and liver

Single cells from the blood and spleen were obtained as follows: blood was collected in EDTA-coated tubes and lysed using ACK buffer, and then the rest of the cells were passed through 40-µm nylon mesh: Splenocytes were passed through a 70-µm nylon and 40-µm nylon mesh filter. All cells were treated with ACK lysed buffer and washed with PBS. The CNS (brain and spinal cord) and liver were harvested and ground, and single cells were isolated on a discontinuous percoll gradient.

Isolated cells from the CNS were suspended in PBS and stained with Fc Block and live dead staining for 15 min and subsequently incubated with fluorochrome-conjugated antibodies (anti-mouse Ly-6G, anti-mouse CD11b, anti-mouse CD45, anti-mouse CD3, and anti-mouse CD4) besides live dead staining. Single stain, fluorescence-minus-one (FMO) stain and full panel stains were performed. Data was acquired with a Moflo Astrios (Beckman Coulter, Inc. Brea, CA) and analyzed with FlowJo software (FlowJo, LLC, Williamson Way, OR).

Isolated cells from the liver and spleen were suspended in PBS and incubated with Fc Block and live dead staining for 15 min. Myeloid cells were identified with fluorochrome-conjugated antibodies (anti-mouse F4/80, anti-mouse Ly-6G, anti-mouse CD11b, anti-mouse CD11c, anti-mouse I-A^k (A β ^k), anti-mouse CD86, anti-mouse CD146, anti-mouse CD45) besides live dead staining. Additionally, T and B lymphocytes were stimulated using cell activation cocktail (Cat#423303, Biolegend) for 4 h, permeabilized using True-Nuclear Transcription Factor Buffer Set (Cat#424401, Biolegend) and then stained with fluorochrome-conjugated antibodies (anti-mouse IL-17A, anti-mouse CD11b, anti-mouse B220, anti-mouse CD45, anti-mouse CD3, anti-mouse CD4, and anti-mouse IFN- γ). Single stain, fluorescence-minus-one (FMO) stain and full panel stains were performed. Data was acquired with a LSRFortessa (BD Biosciences, San Jose, CA) and analyzed with FlowJo software.

2.8. Ex vivo recall study

The spleen cells were isolated from EAE mice on day 10 and 14 p.i. and cultured following Hooke Laboratory protocol (Hooke Laboratories, Inc., Lawrence, MA). Splenocytes were passed through a 70- μ m nylon and 40- μ m nylon mesh filter. All cells were treated with ACK lysed buffer and washed with PBS. 8×10^5 cells/well were cultured with 200 μ L of RPMI 1640 Gultamax supplemented with 10% fetal bovine serum (FBS) and 1% Penicillin-Streptomycin in 96-well cell culture plate. The cells were immediately rechallenged with 40 μ g/mL of PLP_{139–151} and incubated for 72 h at 37 °C in a CO₂ (5%) incubator. The supernatant was collected, and cytokines (IL-17A, IFN- γ , MG-CSF, TNF- α , IL-2, and IL-10) were measured using ELISA assay.

2.9. Statistical analysis

GraphPad Prism software (San Diego, CA) was employed for all statistical analysis. Two-way analysis of variance (ANOVA) followed by Tukey was used for *in vivo* EAE studies over the time. One-way ANOVA followed by Tukey or nonparametric analysis followed by Dunn's test for comparison of three or more groups or t-test using Holm-Sidak method for comparison of two groups was performed in the other *in vivo* and *in vitro* studies. Data represented as mean $\pm$ standard deviation (SD). Asterisks indicate *p* values of * < 0.05, ** < 0.01, and *** < 0.001 to show significant difference between the groups unless noted otherwise.

3. Results

3.1. agPLA and agPLG-H had sustained PLP_{139–151} release in vitro compared to agPLG-L particles

We have fabricated particles with similar diameter and PLP_{139–151} loading to evaluate the impact of the polymer composition. SEM images show the spherical shapes of fabricated agPLG-L, agPLA, and agPLG-H particles (Fig. 1A), and the measured diameters of agPLG-L, agPLG-H and agPLA particles are 356.3 ± 78.7 , 427.8 ± 85.6 , 401.4 ± 62.4 (nm), respectively. In addition, DLS showed that the particles had diameters of approximately 400 nm, and zeta-potentials of approximately -40 mV (Fig. 1B). We also confirmed that the loadings of the fabricated particles were within the range of 2–3 μ g of PLP_{139–151}/mg of

particles (Fig. 1B), a loading that has previously been effective for reducing disease scores [15]. *In vitro* release studies were performed through 3 weeks, at which time the agPLG-L particles had completely degraded, yet the agPLG-H and agPLA particles could be visualized. agPLG-L particles had an initial plateau at 20% peptide release compared to a plateau at 40% that was observed among agPLA and agPLG-H particles at 6 h (Fig. 1C). The release from agPLG-L particles was reduced prior to 120 h and higher at 456 h relative to agPLA and agPLG-H particles (Fig. 1C). For agPLG-L particles, the release rate between 24 and 456 h increased and ultimately 80% of total PLP_{139–151} was released compared to approximately 60% in agPLG-H and agPLA particles (Fig. 1C). The release at each time point further indicated that the PLP_{139–151} release from agPLG-L particles was initially slower than those of agPLA and agPLG-H particles up to 6 h, and substantially faster after 120 h of incubation (Fig. 1D). No difference was observed in PLP_{139–151} release profiles between agPLA and agPLG-H particle groups at any time point. The data demonstrated that particle composition determined the release profiles of PLP_{139–151}.

3.2. agPLA particles were most effective for ameliorating the chronic stage of EAE disease

We subsequently investigated the ability of these particle formulations to reduce the clinical scores during EAE disease. All *in vivo* studies were performed with a single dose of particles at day 7 post immunization (p.i). Initial studies employed agPLG-L, agPLA particles and control PLG-L particles loaded with an irrelevant antigen from ovalbumin (OVA_{323–339}) (these particles are hereafter called OVA) were intravenously injected (2.5 mg/mouse) into EAE mice. agPLA particles maintained lower EAE clinical scores for up to 200 days p.i. relative to agPLG-L particles (Fig. 2A). Interestingly, agPLG-L particles had lower disease scores in the acute phase, yet these mice appeared to relapse after 100 days p.i. (Fig. 2A). Decreasing the dose of agPLG-L particles to 1.5 and 1.0 mg/mouse (3.75 and 2.5 µg of PLP_{139–151}/mouse, respectively) was not as effective at reducing the clinical score as a dose of 2.5 and 2.0 mg/mouse (6.25 and 5.0 µg of PLP_{139–151}/mouse, respectively), indicating that tolerance induction by particles depends on their dose (Fig. S1A).

The ability to attenuate EAE disease by agPLG-L, agPLG-H and agPLA particles was subsequently investigated at a dose of 1.5 mg/mouse, with significant yet not complete reduction in the clinical score, in order to identify differences in disease course between the particle formulations. EAE clinical scores showed that agPLG-L, agPLA and agPLG-H particle groups (3.75, 3.75 and 4.2 µg of PLP_{139–151}/mouse, respectively) had the first peak of disease around 14 days p.i (Fig. 2B and C) followed by remission. Subsequently, agPLA particles had a negligible second peak and did not have a significant relapse (Fig. 2B), while agPLG-L, agPLG-H and OVA particles showed multiple peaks, reflective of ongoing relapse and remission, throughout the chronic stage of the disease (Fig. 2C). agPLA particles had more days of significantly lower clinical scores than agPLG-L and OVA particles compared to agPLG-H particles (Fig. S1B).

3.3. agPLA particle treatment reduced inflammatory immune cell populations in the CNS

We next examined the disease-associated immune cell populations and their phenotypes in the CNS for agPLG-L and agPLA particles (Fig. 3A), which differentially reduced clinical

scores. CD4⁺ T-cells are well known for induction of disease, and B-cells can promote the reactivation of CNS-infiltrating myelin specific T-cells [3,37]. Therefore, the populations of CD4⁺ T-cells (CD45⁺/CD11b⁻/CD3⁺/CD4⁺) and B-cells (CD45⁺/CD11b⁻/B220⁺) in the CNS of EAE mice were determined using flow cytometry. Flow cytometry revealed that particle composition affected the population of CD4⁺ T-cells (Fig. 3B and C). Mice treated with agPLA and agPLG-L particles had lower CD4⁺ T-cell populations at 14 days p.i., compared to that of OVA particle treatment. Delivery of agPLA particles decreased the percentage of CD4⁺ T-cells relative to agPLG-L particles at day 42 p.i. Additionally, investigating CD4⁺ T-cell populations across the time revealed that agPLG-L particles had increased CD4⁺ T-cell populations at day 42 compared with days 14 and 28. Simultaneously, agPLA particles maintained lower CD4⁺ T-cell populations across the time points. Collectively, agPLA particles decreased infiltration of CD4⁺ T-cells into the CNS, while agPLG-L particles had increased CD4⁺ T-cell infiltration at later time points, consistent with the EAE data. For delivery of agPLA particles, a higher percentage of Foxp3⁺ CD4⁺ T-cells were observed in the CNS at day 14 p.i. relative to the other groups, indicating potential upregulation of regulatory T-cells (Fig. S2) [16,38]. Delivery of agPLA resulted in fewer B-cells in the CNS than with that of agPLG-L and OVA particles at day 28 and agPLG-L particles at day 42 (Fig. 3D, Fig. S3A). Additionally, the numbers of CD4⁺ T-cells and B-cells showed the same trend (Figs. S3B and C). Although the reduction of CD4⁺ T-cell and B-cell populations by agPLA treatment at day 200 p.i. was not significant compared to the other groups, the number of B-cells for the agPLA treatment group at day 200 was significantly reduced (Fig. 3C, D, Figs. S3B and C). This data suggests that agPLA particles were effective in tolerizing the chronic immunity of EAE compared with agPLG-L and OVA particles.

The number and population of myeloid cells (CD45⁺/Ly-6G⁻/CD11b⁺) in the CNS of EAE mice had similar trends as CD4⁺ T-cells and B-Cells, with agPLA particles being associated with lower myeloid cell numbers and percentages than OVA particles at day 28 and agPLG-L particles at day 42 (Fig. 3E, Figs. S4A and B). Myeloid cells, including macrophages and dendritic cells, activate myelin-specific CD4⁺ T-cells and increase their proinflammatory signals at the onset and relapse stages of EAE [39–41]. The flow cytometry data suggests that agPLA particles reduced the percentage and numbers of CD4⁺ T-cells, B-cells and myeloid cells starting at day 28 p.i., which was associated with reduced EAE clinical scores after the initial peaks (Fig. 2B). The phenotype of the myeloid cells was also characterized by the expression of co-stimulatory molecules, CD86, and major histocompatibility complex class II (MHCII). MHCII⁺ myeloid cells were increased in the mice treated with agPLA particles relative to agPLG-L particles at day 14 p.i., while fewer MHCII⁺ myeloid cells were observed for agPLA compared to OVA particle treatment at day 28 p.i. and agPLG-L particle treatment at day 42 and 200 p.i. (Fig. 3F, Fig. S4C). CD86⁺ expression on myeloid cells was also reduced in agPLA treated mice at later time points in the disease compared to mice treated with agPLG-L or OVA particles (Fig. 3G, Fig. S4D). Collectively, the data indicates that agPLA particles had a greater reduction at chronic time points relative to agPLG-L in the population of CD4⁺ T-cells, B-cells and myeloid cells and the expression of MHCII⁺ and CD86⁺ on these myeloid cells in the CNS, which was consistent with the reduced EAE clinical scores at chronic time points (Fig. 2B).

3.4. agPLA particles persisted longer than agPLG-L particles in the liver and spleen

Next, the biodistribution of the particles within organs associated with nanoparticle formulations, as well as the particles' association with specific immune cell populations within those organs was investigated. Our previous studies demonstrated that the intravenously injected PLG and PLA particles were accumulated in the liver and spleen at 24 h after the particle injection [34,42]. Based on the EAE data indicating similar responses at day 14 for the particles, and differential responses at day 28, we analyzed this biodistribution at the time points used for the CNS flow cytometry analysis (Fig. 4A). Although EAE disease occurs in the CNS, systemically injected antigen-loaded nanoparticles accumulate in other organs, such as the liver, spleen and lung, to induce peripheral tolerance. To detect particles, agPLG-L and agPLA particles were loaded with cyanine 5.5 dye (cy5.5). IVIS data revealed that particles accumulated in the liver in larger quantities compared to the spleen, CNS and lung (Fig. 4B, C, Figs. S5A and B). A stronger signal was identified for agPLA particles relative to agPLG-L particles, which likely results from the slower degradation rate of the particles (Fig. 4B and C). Similarly, flow cytometry data for the liver and spleen revealed greater association of agPLA particles with the major APCs that present antigen to CD4⁺ T-cells over 42 days p.i. compared to that of agPLG-L particles (Figs. S6A and B).

Flow cytometry was employed to analyze the population of APCs and their association with particles. The cell types analyzed include Kupffer cells (CD45⁺/CD11b⁺/F4/80⁺), liver dendritic cells (liver DCs) (CD45⁺/CD11c⁺/F4/80⁻), liver sinusoidal endothelial cells (LSECs) (CD45⁻/CD146⁺), splenic macrophages (CD45⁺/CD11b⁺/F4/80⁺) and splenic dendritic cells (splenic DCs) (CD45⁺/CD11c⁺/F4/80⁻), all of which have been associated with tolerogenic antigen presentation. agPLA particle delivery resulted in decreased Kupffer cell percentages at days 14 and 42 p.i. compared with agPLG-L and OVA particles (Figs. S7A and B), and decreased percentages of liver DCs at days 14 and 42 p.i. compared with agPLG-L particles (Figs. S7C and D). Interestingly, the population of LSECs with agPLA particles was significantly lower relative to agPLG-L and OVA particles at all time points (Figs. S7E and F). Only minor differences were observed between the particle formulations for the percentage of splenic macrophages and DCs (Figs. S8A–D). The agPLA particles associated with a greater percentage of Kupffer cells, liver DCs and LSECs compared with agPLG-L particles (Fig. 4D) suggesting that agPLA particles were phagocytosed by liver APCs more than agPLG-L particles. In the spleen, agPLA particles associated with splenic macrophages more than agPLG-L particles, of which the populations of cy5.5 positive cells were similar to background (OVA particles) at all time points (Fig. 4E). agPLA particles associated with splenic DCs more than agPLG-L particles only at day 14 p.i. (Fig. 4E). Although some differences were observed between agPLA and agPLG-L in splenic macrophages and DCs, less than 1% of each cell type phagocytosed the particles indicating that the majority of particles accumulated in the liver.

3.5. agPLA particles reduced expression of co-stimulatory molecule (CD86) by liver cells

We next examined the phenotype of the APCs within the tolerogenic organs, specifically focusing on expression of MHCII⁺ and the co-stimulatory molecule (CD86), as decreased expression of the co-stimulatory molecule has been associated with the potential to induce T-

cell anergy or apoptosis rather than T-cell activation [43,44]. agPLA particles consistently increased the percentage of MHCII⁺/CD86⁻ Kupffer cells compared with agPLG-L and OVA particles (Fig. 5A). In addition, agPLA particle delivery decreased the percentage of MHCII⁺/CD86⁺ Kupffer cells at day 42 p.i. compared with the other groups (Fig. 5B). Conversely, agPLA particle delivery had a greater percentage of MHCII⁺/CD86⁻ liver DCs at day 42 p.i. and MHCII⁺/CD86⁺ liver DCs at all time points (Fig. 5C and D) which suggests that liver DCs may not induce anergy of CD4⁺ T-cells. Interestingly, for agPLA particle, a higher percentage of MHCII⁺/CD86⁻ LSECs was observed (Fig. 5E), though the percentage of MHCII⁺/CD86⁺ LSECs was also higher at day 14 p.i. (Fig. 5F). A similar analysis was performed in the spleen for macrophages and dendritic cells. No difference between agPLA and agPLG-L particle delivery was observed in MHCII⁺/CD86⁻ macrophages (Fig. S9A), while higher population of MHCII⁺/CD86⁺ splenic macrophages were observed in the group treated with agPLA particles (Fig. S9B). No correlation was observed between MHCII⁺/CD86⁻ splenic DCs and MHCII⁺/CD86⁺ splenic DCs for the two particle types (Figs. S9C and D). Collectively, these data suggest that Kupffer cells and LSECs have reduced CD86 expression, which has been associated with induced anergy or inactivation of CD4⁺ T-cells and may contribute to the reduced disease severity of EAE mice treated with agPLA particles.

3.6. agPLA particles reduced population of CD4⁺ T-cells and B-cell in the liver and spleen

We next investigated the CD4⁺ T-cells and B-cells in the liver and spleen, as the T- and B-cells can interact with the APCs in these organs and influence their subsequent response. In previous studies, we reported that antigen-loaded particles reduced the population of CD4⁺ T-cell and their subtypes, Th1 (IFN- γ ⁺) and Th17 (IL-17⁺) CD4⁺ T-cells, in the CNS at the peak of EAE disease [14,15]. We also demonstrated that antigen-loaded particles induce greater accumulation of T-cells in the liver and spleen compared with disease-irrelevant peptide (OVA₃₂₃₋₃₃₉) loaded particles, suggesting that APCs in the liver and spleen present particle-delivered antigen to T-cells [34]. In the liver, agPLA particle delivery corresponded with a lower percentage of CD4⁺ T-cells compared to agPLG-L particles at day 42 p.i. (Fig. 6A, Fig. S10A) and a lower percentage of B-cells compared to OVA at day 28 p.i. and agPLG-L particles at day 42 p.i. (Fig. 6B, Fig. S10B). Interestingly, agPLA particle delivery resulted in an enhanced population of IFN- γ ⁺ CD4⁺ T-cells at days 28 and 42 p.i. and IL-17⁺ CD4⁺ T-cells at day 14 p.i. compared with agPLG-L and OVA particles (Fig. 6C, Figs. S10C and D). In the spleen, agPLA particle delivery also corresponded with a reduced population of CD4⁺ T-cells at day 28 p.i. compared to agPLG-L particles (Fig. 6D, Fig. S11A) and a reduced population of B-cells at day 28 p.i. compared to OVA (Fig. 6E, Fig. S11B). No difference in the population of IFN- γ ⁺ CD4⁺ T-cells and IL-17⁺ CD4⁺ T-cells was observed in the spleen for the particle groups (Fig. 6F, Figs. S11C and D). Collectively, these studies suggest that agPLA particles increased the IFN- γ ⁺ CD4⁺ T-cells in the liver, yet not the spleen, which coincided with a decreased presence in the CNS (Fig. 3B and C).

3.7. Higher PLP antigen loading in PLA ameliorated the disease with a lower dose of particles

PLA-based particles (agPLA particles) were identified to more efficiently induce EAE tolerance compared with the other particle groups. However, the particles were not able to

tolerize acute disease with 1.5 mg of particle dose. We tested the hypothesis that the efficacy of the particles on EAE could be improved through a higher loading of PLP₁₃₉₋₁₅₁. PLA-based particles with a high antigen loading, named agH-PLA, were delivered at a dose of 1.5 mg per mouse or lower. The fabricated agH-PLA particles maintained spherical shapes with the measured diameter 515.8 ± 98.6 nm as shown in the SEM image (Fig. 7A). The DLS analysis revealed the diameter (496.0 ± 56.3 nm), and charge (-40.0 ± 6.1 mV) similar to the particles used above, though the loading of PLP₁₃₉₋₁₅₁ was increased to 8.3 ± 0.4 $\mu\text{g}/(\text{mg of particles})$ ($N = 3$). The *in vitro* release demonstrated that the release profile from agH-PLA particles was similar or less to that of agPLA particles (Fig. 7B). Injecting 1.5 mg/mouse of agH-PLA particles (12.5 μg of PLP₁₃₉₋₁₅₁/mouse) reduced the clinical scores compared to OVA particles, and the EAE scores of agH-PLA particle group were lower than those of agPLA particles at the acute stages (days 14–18 and 22 p.i.) (Fig. 7C). Furthermore, a dose of 1.0 mg/mouse (8.3 μg of PLP₁₃₉₋₁₅₁/mouse) for agH-PLA particles similarly reduced disease score compared to the OVA particle control (Fig. 7C).

We next investigated the antigen-specificity of tolerance induced by agH-PLA particles for the disease using *ex vivo* recall studies. *Ex vivo* recall studies typically use cells from lymphoid organs, such as the spleen [16,30,34,45]. We performed these studies using cells from the spleen based on the particle biodistribution and the role of the spleen in tolerance induction, as well as the ability to easily isolate T-cells. Inflammatory cytokines were assayed before disease onset (at day 10 p.i.) and at the peak of the disease (at day 14 p.i.) via challenging with PLP₁₃₉₋₁₅₁ after injecting 1.5 mg of agH-PLA or OVA particles as shown in the experimental scheme (Fig. 7D). A reduction of IFN- γ at 10 days p.i (Fig. 7E) was observed, as was IL-17 at 14 days p.i (Fig. 7F) and GM-CSF at 10 and 14 days p.i (Fig. 7G) in agH-PLA particle treatment indicating the ability to down-regulate the polarization of T-helper 1 (Th1) (IFN- γ producing) and T-helper 17 (Th17) (IL-17 and GM-CSF producing) cells [46,47]. Reduced levels of TNF- α were observed with agH-PLA particle treatment relative to OVA particle treatment (Fig. 7H). Upregulation of IL-10 and downregulation of IFN- γ and IL-2 at 10 days p.i. indicates suppression of Th1/Th17 responses and upregulation of anti-inflammatory IL-10 in EAE mice treated with agH-PLA particles (Fig. 7E, I, J) [48]. Collectively, the reduced IFN- γ , IL-17 and GM-CSF along with higher IL-10 secretion from the recall study suggests efficient downregulation of Th1 and Th17 polarization.

4. Discussion

Recent studies show the efficacy of particle-based antigen-specific treatment for autoimmune disease, such as multiple sclerosis and type 1 diabetes. The particles were developed with conjugation of disease-specific antigens on the surface [14,15,17], which may result in the associate with pre-existing antibodies that could induce adverse events [49]. Encapsulating antigens in particles with or without immunomodulator (e.g. vitamin D3, IL-10, rapamycin) ameliorated the disease with decreased antibody association [19,30,34,50,51]. Our lab previously has demonstrated that intravenously injected PLG particles loaded with disease-specific antigen (without any immunomodulator) trafficked primarily to the liver and spleen to induce tolerance in an antigen-specific manner [15,34]. The previous studies investigated the polymer primarily as a carrier for antigens, and PLG

has been most widely used as a carrier due to its biocompatibility and flexibility to form particles. However, the polymer platform itself influences immune responses [27,32], which may subsequently affect tolerance induction. Herein, we demonstrated the polymer compositions, especially PLA, have ability to influence the APC phenotypes in an effort to improve efficacy of tolerance induction. Furthermore, disease-specific antigen-loaded PLA-based particles without immunomodulator are potent enough to extend amelioration of EAE.

PLA-based particles were more effective at reducing disease scores at chronic time points, and antigen loading was related to the extent of acute disease, which also enabled a reduction in the particle dose. At the reduced dose, agPLA particles had lower EAE scores and less CNS-infiltrating immune cell populations in the chronic stage of the disease compared with agPLG-L and agPLG-H particles (Fig. 2). Reduced doses of particles (1.5 mg/mouse) did not completely ameliorate immune cell influx to the CNS to reduce the initial peak of EAE clinical scores, however, slower degrading PLA-based particles with lower amounts of antigen ameliorated the chronic stage of the disease. Further examination of PLA-based particles with higher loading showed reduction of the acute stage of the inflammation with further reduced particle dose (1.0 mg/mouse). 1.0 mg dose of agH-PLA particles performed better than our previous study in which 1.25 mg of PLP_{139–151} was conjugated on the surface of PLG particles [44] suggesting that antigen loading and particle material play important roles for tolerizing in EAE.

The liver has been reported to be a more tolerogenic organ compared to the spleen [52]. Kupffer cells, LSECs and liver DCs are major APCs in the liver which endocytose antigens as well as particles from sinusoidal blood [53]. Kupffer cells and LSECs phagocytosed particles relatively more efficiently than liver DCs (Fig. 4), consistent with previous reports that Kupffer cells and LSECs efficiently phagocytose negatively-charged nanoparticles through scavenger receptors [54]. Particles have been reported to accumulate in the sinusoids where Kupffer cells and LSECs are able to access and co-localize with the particles one day after particle injection [55,56]. In contrast, liver DCs had relatively low capability of endocytosis as well as T-cell stimulation [56]. Furthermore, DCs reduce their uptake of particles with size greater than around 100 nm, while macrophages preferably phagocytose particles around 500 nm [57–59] suggesting that particles in this study were phagocytosed less by liver DCs. Collectively, Kupffer cells and LSECs may preferably phagocytose both agPLA and agPLG-L particles with diameters around 400 nm. Interestingly, agPLA particle treatment decreased the percentage of Kupffer cells and LSECs, yet only the number of LSECs were decreased compared with the other treatments, which may suggest that agPLA may decrease proliferation or viability of LSECs (Fig. S7).

Investigating the nanoparticle interaction with specific immune cells in the liver revealed greater association with Kupffer cells and LSECs, which presented the antigen with reduced co-stimulatory molecule expression. This likely reduced CD4⁺ T-cells activation or induced anergy. MHCII and CD86 are cell surface molecules expressed by APCs, such as macrophages and dendritic cells, that are critical for the activation of CD4⁺ T-cells. APCs present antigens on MHCII molecules to be recognized by the T-cell receptor on T-cells in an antigen-specific manner. At this point, the fate of the T-cell depends heavily on the presence or absence of co-stimulatory molecules, a second signal from the APC. Some of

the best characterized co-stimulatory molecules are CD80 and CD86, which bind to CD28 on the T-cell surface to stimulate activation and proliferation. Without these co-stimulatory molecules, engagement of the T-cell receptor complex can result in anergy of the T-cell [43,44].

PLG particles endocytosed by APCs, such as macrophages, follow the endosomal/lysosomal pathway and peptide/MHCII complexes are presented to CD4⁺ T-cells [60,61]. Antigen-loaded PLG particles have been shown to extend antigen presentation compared to soluble antigen and PLG particles themselves [62] suggesting that released antigen from degraded particles into extracellular space did not contribute to antigen presentation. Therefore, slower degrading agPLA particles in sinusoid were eventually phagocytosed by APCs and extended their antigen presentation to T-cells compared to agPLG-L particles. In addition, PLA particles with higher loading of PLP₁₃₉₋₁₅₁ (agH-PLA particles) may reduce co-stimulatory molecule expression more than agPLA and induced greater tolerance (Fig. 7) [44]. The previous reports demonstrate that hydrophobicity of PLA-based particles are preferably phagocytosed by APCs and improve their immune responses [31,32] supporting that agPLA particles were preferentially associated with liver APCs which had prolonged antigen presentation. In addition, our previous report demonstrated that higher particle uptake by macrophages resulted in reduced co-stimulatory molecule expression [44]. Kupffer cells and LSECs of agPLA particle group showed lower co-stimulatory molecule expression compared to the cells of agPLG-L particle group indicating that the cells may phagocytose more agPLA particles and induce T-cell anergy.

The mechanism behind tolerance involves multiple steps, i) intravenously injected particles were sequestered and accumulated in the liver and spleen, ii) the particles were associated with APCs in these organs, which can present disease-specific antigen [44], and iii) these APCs can be recognized by circulating T-cells. As shown in our data (Fig. 6), an increased population of IFN- γ ⁺ CD4⁺ T-cells in the liver of the mice treated with agPLA day 28 and 42 p.i. suggested that accumulation of antigen-loaded nanoparticles in the liver leads to accumulation of antigen-specific CD4⁺ T-cells, specifically Th1 cells [34]. T-cells and B-cells systemically circulate through both the liver and the spleen, and memory T-cells migrate from the lymph nodes and the spleen into the CNS before the onset of the disease [63]. Adoptive transfer of antigen-specific T-cells has shown these T-cells accumulate in the liver following nanoparticle administration [34]. Thus, we speculate that the APCs within the liver are interacting with auto-reactive CD4⁺ T-cells and possibly down-regulate activation of the T-cells [64,65]. In addition, higher population of IFN- γ ⁺ CD4⁺ T-cells in the liver of the agPLA group indicated that activated CD4⁺ T-cells may be held within the liver thus inhibiting their migration to the CNS, thereby reducing the disease clinical score. Besides tolerance induced by Kupffer cells and LSECs, generation of regulatory T-cells is another mechanism for tolerance induction in the spleen [66], which is consistent with our finding of IL-10 upregulation and reduced IFN- γ and IL-17/GM-CSF via *ex vivo* recall with agH-PLA particles (Fig. 7) [50]. In addition, Kupffer cells and LSECs have the capability to stimulate regulatory T-cells [66]. Collectively, the higher loading of PLP₁₃₉₋₁₅₁ is associated with an inhibition of Th1 and Th17 polarization of CD4⁺ T-cells and upregulation of regulatory T-cells, with a corresponding decrease in the clinical scores during the acute stage of disease in antigen-specific manner.

The efficacy of the particles in reducing EAE scores may also be influenced by their degradation and antigen release. These polymers degrade hydrolytically and enzymatically in the body, and the degradation rate depends on the composition of glycolide and lactide, and polymer molecular weight which has been extensively studied using gel permeation chromatography [67]. An *in vitro* release study demonstrated that agPLG-H and agPLA particles degrade slower than agPLG-L particles due to either higher molecular weight or higher lactide to glycolide ratio generally agreeing with previous reports [68,69] (Fig. 1). While, agPLA and agPLG-H particles showed greater initial burst release than agPLG-L particles at the earlier time points. We note that agPLG-L particles were formed from a 20% (w/v) polymer solution, while the others were formed with 10% (w/v) polymer solution. This greater polymer concentration would be expected to impact the distribution within the particle and particle morphologies resulting in the observed release profile [70,71]. The biodistribution for all particles agrees with previous reports that particles of this diameter accumulate mostly in the liver, lung and spleen [19,22,72]. While agPLG-H and agPLA particles have a slower antigen release than agPLA-L *in vitro*, agPLA maximally reduced clinical disease scores. agPLA particles persisted longer in the liver and the spleen than agPLG-L, which likely reflects a slower degradation of PLA (Figs. 4 and S4). PLA may have a distinct degradation profile *in vivo* relative to PLG based on enzymatic activity and the acidic environment of endosomes and lysosomes which may affect intracellular and extracellular degradation of particles, antigen release, and APC phenotypes [33,73,74].

5. Conclusion

We investigated the particle platform for antigen-specific immunotherapy for the efficient induction of tolerance in the EAE disease model of MS. The study demonstrated that the polymer composition of the particle, without any immunomodulatory factor, can modulate phenotypes of immune cells that leads to the amelioration of disease. Our findings demonstrate that PLA, which was correlated with a more tolerogenic polarization of the APCs, is a more efficacious platform relative to PLG. In addition, the accumulation of particles in the liver may play an important role for induction of long-lasting tolerance. The investigation of particle interaction with APCs demonstrated that Kupffer cells and LSECs internalize particles and then may deactivate CD4⁺ T-cells, while liver DCs may act as adjuvant to hold activated CD4⁺ T-cells locally rather than allowing them to traffic to the CNS. In addition to particle composition, higher antigen loading helped to reduce inflammation during the acute stage of the disease and should allow for reduced particle dosage that would minimize potential adverse effects during tolerogenic treatment.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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HIGHLIGHTS

- Nanoparticle composition influences tolerance of experimental autoimmune disease.
- Poly-lactide (PLA) particles persist within APCs of liver and spleen.
- PLA particles reduce co-stimulatory molecule expression of APCs to T-cells.
- Loading of antigen in particles improves tolerance during acute disease.

Fig. 1.

Characterization of fabricated particles and PLP₁₃₉₋₁₅₁ released from particles. (A) SEM images of agPLG-L (left), agPLA (middle), and agPLG-H (right) particles show spherical shapes of the fabricated particles. Scale bar indicates 2 μ m. (B) Size, charge and loading of PLP₁₃₉₋₁₅₁ of fabricated particles were measured using at least 3 batches of each particle group. (C) Cumulative release of PLP₁₃₉₋₁₅₁ from the particles (# indicates significance in release from agPLG-L particles compared with agPLG and agPLG-H particles), and (D) PLP₁₃₉₋₁₅₁ release at each time point is shown (N = 3).

Fig. 2.

EAE clinical score of mice treated with agPLG-L, agPLG-H or agPLA particles. 8–10 weeks old SJL/J mice were immunized with PLP_{139–151} and particles were injected at 7 days post immunization (p.i.). (A) EAE clinical scores of 2.5 mg/mouse injection of agPLG-L, agPLA and ovalbumin (OVA_{323–339}) encapsulated particles (named OVA) (N = 5). (B, C) EAE clinical scores of 1.5 mg/mouse injection of agPLG-L, agPLA, agPLG-H and OVA particles. (B) agPLA particles were compared with agPLG-L particles and OVA particles (N = 10–12). (C) agPLG-H particles were compared with agPLG-L and OVA particles (N = 5–6).

Fig. 3.

Frequency of immune cells in the CNS after treatment of particles. (A) Schematic illustration of the experiment shows time points of particle injection and flow cytometry analysis. (B) Representative images of flow cytometry data of CD4⁺ T-cells in the CNS at 14, 28 and 42 days p.i. show decrease of CD4⁺ T-cells in agPLA particle treatment compared with agPLG-L and OVA particle treatment group. The frequency of (C) CD4⁺ T-cells, (D) B-cells and (E) myeloid cells as a percent of live cells in the CNS was determined at 14, 28, 42 and 200 days p.i. (N = 4). Frequency of expression of (F) MHCII and (G) CD86 as a percent of APCs at 14, 28, 42 and 200 days p.i. (N = 4).

Fig. 4. Particle biodistribution in the liver and spleen. (A) Schematic illustration of the experimental time points of particle injection, IVIS and flow cytometry analysis. The representative images of agPLG-L and agPLA particle biodistribution in the (B) liver and (C) spleen were performed at 14, 28, 42 days p.i. (7, 21, 35 days after particle administration), using an IVIS Lumina LET camera system. OVA particles without cy 5.5 dye were used as a control. Blue color indicates greater accumulation of particles. Flow cytometry analysis determined the proportion of association of particles with (D) Kupffer cells, liver DCs and LSECs, and (E) splenic macrophages and DCs (N = 5). Frequency is calculated as a percent of each cell type.

Fig. 5. MHCII and CD86 expression on liver cell populations. Percentage of Kupffer cells with expression patterns of (A) MHCII⁺/CD86⁻ or (B) MHCII⁺/CD86⁺ (N = 5). Percentage of liver DCs with expression patterns of (C) MHCII⁺/CD86⁻ or (D) MHCII⁺/CD86⁺ (N = 5). Percentage of LSECs with expression patterns of (E) MHCII⁺/CD86⁻ or (F) MHCII⁺/CD86⁺ (N = 5).

Fig. 6.

Population of CD4⁺ T-cells and B-cells and expression of IFN- γ and IL-17 in the liver and spleen. Frequency of (A) CD4⁺ T-cells and (B) B-cells was determined as a percent of live cells in the liver (N = 5). (C) Expression of IFN- γ and IL-17 was determined as a percent of CD4⁺ T-cells in the liver (N = 5). Frequency of (D) CD4⁺ T-cells and (E) B-cells was determined as a percent of live cells in the spleen (N = 5). (F) Expression of IFN- γ and IL-17 was determined as a percent of CD4⁺ T-cells in the spleen (N = 5).

Fig. 7.

Release, EAE clinical scores and *ex vivo* recall study of agH-PLA particles. agH-PLA indicates an antigen loading of 8.3 $\mu\text{g}/(\text{mg}$ of particles), where agPLA represents a loading of 2.5 $\mu\text{g}/(\text{mg}$ of particles). (A) SEM images of agH-PLA. Scale bar indicates 2 μm . (B) Particles were incubated in 37 $^{\circ}\text{C}$ and release of PLP_{139–151} was measured (N = 3). (C) 1.5 mg or 1.0 mg of agH-PLA particles, and 1.5 mg of agPLA and OVA particles were intravenously injected at 7 days p.i. and EAE clinical scores were examined (N = 6). Injection of 1.5 or 1.0 mg/mouse of agH-PLA particles significantly reduced EAE clinical scores compared with OVA particles (indicated by *). EAE mice were treated with agH-PLA and OVA particles, then splenocytes were harvested from EAE mice at 10 and 14 days p.i. and incubated for 72 h with 40 $\mu\text{g}/\text{mL}$ of PLP_{139–151} as shown in the experimental scheme (Fig. 7D). Supernatant was collected and cytokine levels, (E) IFN- γ , (F) IL-17, (G) GM-CSF, (H) TNF- α , (I) IL-2 and (J) IL-10 were determined (N = 4).