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An antigen-encapsulating nanoparticle platform for T_H1/17 immune tolerance therapy

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Abstract

Tolerogenic nanoparticles (NPs) are rapidly being developed as specific immunotherapies to treat autoimmune disease. However, many NP-based therapies conjugate antigen (Ag) directly to the NP posing safety concerns due to antibody binding or require the co-delivery of immunosuppressants to induce tolerance. Here, we developed Ag encapsulated NPs comprised of poly(lactide-co-glycolide) [PLG(Ag)] and investigated the mechanism of action for Ag-specific tolerance induction in an autoimmune model of T helper type 1/17 dysfunction – relapse-remitting experimental autoimmune encephalomyelitis (R-EAE). PLG(Ag) completely abrogated disease

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <http://dx.doi.org/XXXXX>

induction in an organ specific manner, where the spleen was dispensable for tolerance induction. PLG(Ag) delivered intravenously distributed to the liver, associated with macrophages, and recruited Ag-specific T cells. Furthermore, programmed death ligand 1 (PD-L1) was increased on Ag presenting cells and PD-1 blockade lessened tolerance induction. The robust promotion of tolerance by PLG(Ag) without co-delivery of immunosuppressive drugs, suggests that these NPs effectively deliver antigen to endogenous tolerogenic pathways.

Graphical abstract

Keywords

Nanoparticle; Immune tolerance; Autoimmune disease; Drug delivery

Background

Undesired immune system-mediated destruction of healthy endogenous cells results in loss of tissue function and limits strategies to restore or replace the damaged tissue. In the context of autoimmune disease, activated T and B cells respond inappropriately against self-antigens (Ags).^{1, 2} The current standards of care for autoimmune disease primarily address disease symptoms or rely upon non-specific suppression of the immune system.³ Immunosuppression non-specifically curbs aberrant immune responses by deleting or inactivating entire T or B cell subsets or non-selectively inhibiting Ag presentation, pro-inflammatory cytokine production, or lymphocyte trafficking. Therefore, immunosuppression is not completely efficacious and carries inherent risks of patient susceptibility to opportunistic infections, viral reactivation, and neoplasia.

In healthy individuals, tissue integrity and immune homeostasis are normally maintained through Ag-specific immune tolerance, which is driven by immunological ignorance, clonal energy, and modulation by regulatory immune cells that limits the proliferation and activation of pathogenic immune cell clones.^{1, 2, 4} The dysregulated immune function present in autoimmune disease is typically addressed through non-specific immunosuppression that can have deleterious side effects. Recently, Ag-specific immunotherapies have emerged with the goal of re-establishing tolerance through delivery

of proteins⁵ or peptides.^{6, 7} Immune tolerance for the treatment of tissue-specific inflammation has been induced through cell-based immunotherapies for over 30 years, with an initial trial with Ag-coupled leukocytes in patients with multiple sclerosis (MS) demonstrating promise.⁸ Nevertheless, autologous leukocyte isolation and manipulation in a clinical setting is challenging, complex, and expensive, and has motivated the development of alternative Ag delivery strategies for immune tolerance.^{9–11}

The induction of Ag-specific tolerance using cells as Ag carriers has recently been extended to using nanoparticles (NPs). Immune tolerance for the treatment of autoimmune diseases such as MS and type-1 diabetes (T1D) has been induced by the delivery of Ags coupled to NPs.^{12–14} Ag-coupled NPs comprised of either polystyrene (PS) or poly(lactide-co-glycolide) (PLG) have demonstrated the complete abrogation of relapse-remitting experimental autoimmune encephalomyelitis (R-EAE) induced by proteolipid protein (PLP), a major myelin protein in the CNS, without the need of immunosuppressive agents.^{15, 16} Alternative formulations have been developed that require co-delivery of immune modifying agents such as rapamycin, IL-10, and others to achieve tolerance induction.^{9–11} These unique findings established the precedence that tolerogenic NPs could be developed for the induction of Ag-specific tolerance.

Failure to delete self-reactive T cells in the thymus can lead to their escape into the periphery and cause autoimmunity. To limit the development of autoimmunity, peripheral tolerance mechanisms exist such as anergy, ignorance, deletion, induction of regulatory T cells (Tregs), and co-inhibitory molecule expression.¹⁷ Tregs and co-inhibitory signaling through the programmed death 1 (PD-1)/programmed death ligand (PD-L) pathway are crucial to mitigate self-reactive immune responses. Tregs are important for the maintenance of immune self-tolerance and homeostasis through the suppression of aberrant and excessive immune responses.¹⁸ PD-1 is a co-inhibitory molecule that plays an important role in reducing immune activation. Prior studies using antigen-coupled splenocytes (SP-Ag) have implicated these mechanisms to promote tolerogenic responses in vivo.¹⁹ Importantly, the spleen has been implicated as a critical organ for tolerance induction using SP-Ag^{19, 20} but has not been investigated for its role in tolerance induced by synthetic NPs.¹⁶

In this report, we investigated the design of Ag-encapsulating NPs and their mechanisms of action for inhibiting undesired immune responses in autoimmunity without affecting critical protective elements of the immune response. A previous report with Ag-coupled NPs has demonstrated efficacy at promoting tolerance in a T_H1/17 mouse model of MS,¹⁵ yet recent reports have indicated that Ag coupling to the NP surface allows for binding of antibodies that has the potential to alter trafficking and may induce an anaphylactic responses.²¹ Thus, this manuscript focuses on the encapsulation of Ag within biodegradable NPs comprised of PLG(Ag), and we investigate the mechanism of action of these tolerogenic NPs. Specifically, we investigate the organ distribution and the cell types that internalize the particles, the role of the spleen in tolerance induction, and the contribution of signaling through PD-1 to prevent disease progression. Taken together, these NPs provide a safe and effective platform for Ag-specific tolerance induction in a T_H1/17 model of MS, which can also be employed to investigate the mechanisms of action and may extend to other applications of immune dysregulation.

Methods

Nanoparticle production

PLG NPs with surface carboxylate groups and a negative ζ -potential were prepared using an emulsion-solvent evaporation method as previously described.¹⁵ A double emulsion process was employed for Ag encapsulation, whereas a single emulsion process was used for Ag conjugation. Proteolipid peptide or ovalbumin peptide was surface-coupled to PLG NPs using carbodiimide chemistry as previously described.¹⁵ NP size and ζ -potential were measured by dynamic light scattering (DLS) and zeta potential analysis using a Zetasizer Nano ZS (Malvern Instruments Inc., Westborough, MA). PLG NP-encapsulated or -conjugated Ag was quantified using the CBQCA assay as previously described.²² The release of antigen from the particles was determined by suspending particles with radiolabeled peptide in 7.5 mL D-PBS containing 1% w/v SDS and incubated at 37°C with shaking at 200 RPM, with protein amounts in release solution determined using a scintillation counter. Biodistribution data for PLG NPs was obtained by loading NPs with silver and upon injection, the amount of silver present in each organ was determined by inductively coupled plasma mass spectrometry (ICP-MS). More detailed methods for particles and other procedures can be found online.

R-EAE disease induction and measurement

R-EAE was induced by immunization with encephalitogenic peptides as previously described.²³ To induce R-EAE with PLP_{139–151} or PLP_{178–191}, mice were immunized by S.C. administration of 100 μ L of 0.5 mg/mL PLP_{139–151}/complete Freund's adjuvant (CFA) or 2 mg/mL of PLP_{178–191}/CFA, respectively, distributed over 3 spots on the flanks of SJL/J mice. Disease severity in individual mice following I.V. administration of particles was assessed using a 0 to 5 point scale: 0 = no disease, 1 = limp tail or hind limb weakness, 2 = limp tail and hind limb weakness, 3 = hind limb paralysis, 4 = hind limb paralysis and forelimb weakness, 5 = moribund or dead. All protocols were approved by the Institutional Animal Care and Use Committee of Northwestern University.

R-EAE ex vivo recall response measurements

Ex vivo recall responses were elicited from lymphocytes prepared from inguinal lymph nodes (iLNs) of mice treated with PLG(PLP_{139–151}), PLG(OVA_{323–339}), and Naïve controls collected 14–43 days after R-EAE induction by immunization with PLP_{139–151}/CFA. Lymphocytes were cultured with peptide (PLP_{139–151} or OVA_{323–339}) at concentrations ranging from 0.1 to 100 μ g/mL.¹⁶ Lymphocyte incorporation of ³H-TdR measured as counts per minute (CPM) at 72 hours was quantified using a Scintillation Counter. Cytokines secreted by lymphocytes were quantified using ELISA and by analysing cell-free supernatants collected from identical replicate cultures for IL-17A, IFN- γ and GM-CSF.

Effects of splenectomy or PD-1 blockade on PLG(Ag) tolerance induction

To investigate the necessity of the spleen in tolerance induction, PLG(Ag) were intravenously administered to SJL/J mice that had undergone prior splenectomies or control surgeries. To investigate the necessity of PD-1 in tolerance induction, PLG(Ag) were

intravenously administered to SJL/J mice that had I.P. received anti-mouse PD-1 antibody or control. R-EAE was then induced by immunization with PLP_{139–151}/CFA 7 days after PLG(Ag) administration and disease severity in individual mice was assessed as described above.

Results

Nanoparticle efficacy of Ag-encapsulated PLG particles in R-EAE

NPs were synthesized from PLG using a double emulsion process with the anionic surfactant poly(ethylene-*alt*-maleic anhydride) (PEMA). Ag-encapsulated particles [PLG(Ag)] had mean diameters of 450 nm to 850 nm and mean surface ζ -potentials of approximately -50 mV, with similar loading and release profiles (Table 1, Figures S1 and S2). I.V. administration of PLG(PLP_{139–151}) prevented the development of PLP_{139–151}-induced R-EAE (Figure 1A). Furthermore, the maintenance of tolerance induced by PLG(Ag) was observed to 400 days, the time frame of the study (Figure S3). The tolerance induction obtained with encapsulated Ag was similar to that obtained with Ag-coupled PLG NPs (Figure 1B).¹⁵ We note that the Ag-encapsulated NPs contained less than half of the dose of Ag ($2.4 \mu\text{g PLP}_{139–151}/\text{mg NPs}$) relative to the Ag-coupled NPs ($5.8 \mu\text{g PLP}_{139–151}/\text{mg NPs}$). Specific regulation is demonstrated in that mice receiving NPs that were coupled or encapsulated with the non-encephalitogenic peptide, OVA_{323–339}, developed fulminant R-EAE (Figures 1A, B). These experiments demonstrated the efficacy of Ag-encapsulated PLG NPs to prophylactically inhibit the induction of R-EAE without the co-delivery of immunosuppressives.

We next investigated the capacity of the PLG(Ag) platform to effectively treat mice with established disease. In the acute phase of PLP_{178–191}-induced R-EAE, inflammation and myelin damage leads to release and presentation of endogenous myelin epitopes within the CNS.^{24, 25} Initiation of disease with PLP_{178–191} results in a first relapse driven mainly by PLP_{139–151}-specific CD4⁺ T cells as PLP_{139–151} is immunodominant. Mice were immunized with PLP_{178–191} and administered NPs during disease remission at 18 days post-immunization. Approximately 60% of the mice treated with PLG(OVA_{323–339}) relapsed within 3 days of treatment and all mice relapsed within 7 days, *i.e.*, 25 days post-immunization (Figure 1C). The arrow indicates the day on which various particles were administered to mice previously immunized with PLP_{178–191}/CFA. In contrast, mice treated with PLG(PLP_{139–151}) had a modest, but significant delay in onset of primary relapse with only 50% of the mice relapsing 7 days after PLG(PLP_{139–151}) administration. Mice treated with NPs containing both the initiating (PLP_{178–191}) and spread (PLP_{139–151}) epitopes, [PLG(PLP_{139–151}+PLP_{178–191})] had almost complete amelioration of the primary relapse (Figure 1C). Delivery of PLP_{178–191} was likely necessary to induce effective tolerance due to the continued priming of PLP_{178–191}-specific T cells from the Ag/CFA bolus that remained in the periphery.

Examination of *ex vivo* T cell recall responses from draining iLNs revealed that tolerance was associated with a significant defect in T cell priming. Compared to cultures from PLG(OVA_{323–339}) treated mice, cultures from PLG(PLP_{139–151}) treated mice exhibited a significant decrease in PLP_{139–151}-specific proliferation (Figure 1D) concomitant with

significantly diminished production of the signature T_H17 cell pro-inflammatory cytokines (IL-17 and GM-CSF), although T_H1-driven IFN- γ was only marginally reduced (Figures 1E–G).

Functional role of the spleen in Ag-encapsulated PLG particle-induced tolerance

Previous reports with SP-Ag indicated that the spleen was necessary for inducing prophylactic tolerance in R-EAE,²⁶ and we subsequently investigated whether the spleen was required for PLG(Ag)-induced tolerance. Relative to control mice, splenectomized mice had no significant difference in R-EAE disease course (Figures 2A and B) with delivery of PLG(PLP_{139–151}), indicating the spleen was not required to attenuate clinical disease scores. Interestingly, the recall proliferation (Figures 2C and D) was increased for splenectomized mice. Conversely, T_H1/17 pro-inflammatory cytokine secretion (Figures 2E–H) was decreased in splenectomized mice compared to control mice. Nevertheless, the impact on disease remains Ag-specific as R-EAE disease course, recall proliferation, and cytokine secretion were significantly reduced with administration of PLG(PLP_{139–151}) compared to PLG(OVA_{323–339}). In line with our observation, the spleen was similarly demonstrated to be dispensable in studies of allogeneic tolerance.²⁷ These results demonstrated the potential for differences in mechanism of tolerance induction between PLG(Ag) NPs and SP-Ag.

Biodistribution and organ-specific T cell localization following intravenous PLG nanoparticle delivery

The mechanisms by which the innate and adaptive immune systems interact with and respond to the PLG(Ag) were investigated. NPs accumulated largely within the liver (up to 30% of injected dose (I.D.)) and almost negligibly within the spleen and lungs, (< 0.5% of I.D.) (Figure 3A). NP accumulation in the liver increased through the initial 6 hours, with a subsequent decline at 12 hours and similar levels through 24 hours. The heart, kidney, stomach, brain and spinal cord were also evaluated and displayed low levels of NP accumulation (Figure S4A). The biodistribution of PLG(Ag) particles was also confirmed using IVIS imaging of Cy5.5-labeled PLG particles (Figure S4B). Immunohistochemical staining of liver sections indicate FITC-labeled PLG NPs were co-localized with F4/80⁺ macrophages (Figure 3B). Histological analysis of spleen, lungs, and inguinal lymph nodes also confirmed NP accumulation (Figure S5). Contrary to the large numbers of FITC-labeled NPs observed throughout the liver at 18 hours, NPs were present at much lower levels within the spleen, lungs, and inguinal lymph nodes. These results indicate that the liver was a major site of NP accumulation of the organs evaluated and that it may play a role in NP-induced immune tolerance.

We next examined the biodistribution of adoptively transferred transgenic T cells specific for the PLP_{139–151} as a means to further elucidate the role antigen presentation in the various organs in tolerance induction. Naïve CD90.1⁺ PLP_{139–151}-specific (5B6) CD4⁺ transgenic T cells were adoptively transferred into naïve CD90.2⁺ SJL/J mice on day -1, followed by administration of PLG(PLP_{139–151}) on day 0. At 18 hours post administration of PLG(PLP_{139–151}), CD90.1⁺ T cells accumulated to a more significant extent within the liver (Figure 3C) and spleen (Figure 3D) but not the lungs (Figure 3E) or iLNs (Figure 3F) relative to mice that received PLG(OVA_{323–339}) or untreated mice. It has been reported that

the retention efficiency of transferred naïve T cells within the secondary lymphoid organs is only 10–15% of the transferred population after 24 hours.²⁸ As we did not evaluate the entire secondary lymphoid system in these experiments, we did not expect to recover the majority of transferred cells. Consistent with the large number of NPs present in the liver from the biodistribution studies, the liver had the greatest fold enhancement in T cell accumulation (> 5-fold) compared to the spleen (< 2-fold) suggesting that the Ag presenting cells (APCs) in the liver were efficiently presenting NP-delivered Ag.

PD-1 signaling in liver contributes to PLG(Ag)-induced particle tolerance induction

Given that the accumulation of NPs and adoptively transferred T cells in the liver was significant (Figures 3A and C), the response of APCs to PLG(Ag) within the liver was subsequently investigated. The expression of PD-L1, a known contributor to the induction of tolerance, was significantly increased on F4/80⁺MHCII^{low}CD11b^{low} Kupffer cells (KC; macrophages) (Figures 4A, S6B, and S6D) and MHCII^{high}CD11c⁺CD11b^{low}CD103⁺ dendritic cells (DC) (Figures 4B, S6A and S6C) in mice that had received an prior adoptive transfer of naïve CD90.1⁺ PLP_{139–151}-specific (5B6) CD4⁺ transgenic T cells) treated with PLG(PLP_{139–151}) relative to PLG(OVA_{323–339}). Specific lineage markers were used to identify KCs.²⁹ CD103⁺ DCs were evaluated due to their associated role with the induction of Tregs.^{30, 31} Blockade of PD-1 signaling appeared to negatively affect Ag-specific tolerance as demonstrated by increased R-EAE relapse between days 25 to 33 (Figure 4C) and the elimination of Ag-specific recall responses (Figure 4D) in mice treated prophylactically with PLG(PLP_{139–151}). Additionally, PD-1 blockade further reduced tolerance-associated reductions in IL-17A (Figure 4E), but had no effect on reductions in IFN- γ (Figure 4F) upon *in vitro* recall stimulation with PLP_{139–151}. These results indicate that upregulation of the co-inhibitory molecule PD-L1 by APCs partially contributes to the PLG(Ag) tolerance induction and maintenance, especially during relapse.

Discussion

The restoration or induction of Ag-specific immune tolerance while maintaining the integrity of protective immune responses is crucial for the development of novel Ag-specific therapies. Our results support the use of Ag-encapsulated PLG NPs for tolerance, i.e., non-immunosuppressive Ag-specific strategies for the treatment of autoimmunity (*e.g.* MS, T1D), with the potential for use in a range of other applications such as allergy, and cell transplantation,³² and augmentation of gene and protein replacement therapies. The ability of Ag-encapsulated NPs, in the absence of any immunosuppressive drug and unlike other studies in which the inclusion of immune modifying agents into the NP formulations was necessary to promote tolerance, may result from delivery of antigens into endogenous peripheral tolerance pathways that can lead to anergy, depletion of Ag-specific T cells and induction of Ag-specific Tregs.^{9–11} Intravenously delivered peptide has previously been reported capable of inducing tolerance; however, this pathway is also associated with the risk of an anaphylactic response or exacerbation of disease.¹ More recently, the delivery of a cocktail of peptide Ags that mimic the naturally processed T-cell epitope demonstrated efficacy in a preclinical model of EAE and were safe and well-tolerated at high doses in patients with secondary progressive MS.⁷ Association of the Ag with a NP avoids high

concentrations of free peptide in the blood that may lead to anaphylaxis, and targets Ag delivery to APCs. The versatility and safety of the PLG(Ag)-induced tolerance procedure is illustrated by our demonstration that PLG(OVA_{323–339}) particles can safely and specifically tolerate T_H2 responses in both naïve and primed (IgE-expressing) recipients without inducing anaphylaxis.²¹ Additionally, the same study found OVA-specific IgG1 binding to Ag-coupled particles and not to Ag-encapsulated particles confirming the enhanced safety of Ag-encapsulation compared to coupling approaches.

We demonstrated that the spleen is dispensable in promoting a tolerogenic response with PLG(Ag), while previous reports have indicated that SP-Ag accumulated within the splenic marginal zone, and that tolerance was not established in a splenectomized mouse.²⁶ This may be due to the size difference between SP-Ag (8–10 µm) vs, PLG(Ag) (400–800 nm) at infusion which may cause retention of SP-Ag in the spleen. PLG(Ag) NPs accumulated most significantly within the liver (Figure 3A), which also increased the percentage of antigen-specific T cells recruited (Figure 3C). APCs in the liver including KCs/macrophages and liver sinusoidal endothelial cells (LSECs) have been reported to associate with I.V. administered NPs.^{13, 14} While macrophages have been implicated in tolerance by SP-Ag, DCs, LSECs, and hepatic stellate cells are potent enhancers of peripheral Tregs *in vivo*, which may be an additional mechanism by which the NPs are working.^{33–35} Heymann et al. used Ag-coupled latex particles and indicated tolerance induction by KCs.¹³ Interestingly, the work by Carambia et al. has suggested that poly(maleic anhydride-alt-1-octadecene)-coated particles are inducing tolerance through LSECs.¹⁴ The liver has been suggested to be more tolerogenic than the spleen due to differences in cell composition.³⁶ The liver must balance immunity and tolerance³⁷ and thus, many cells with immune functions in the liver have a low abundance of MHC and co-stimulatory molecules, and can thus dampen an immune response. Furthermore, the expression of anti-inflammatory molecules (e.g., IL-10) can reduce MHC expression and support a state of immune non-responsiveness, or anergy.^{38, 39} Taken together, the properties of the particles and their biodistribution must be investigated for each particle, as there are multiple pathways by which tolerance can be induced.

NPs target Ag delivery to APCs that are associated with clearing NPs, such as the F4/80⁺MHCII^{low}CD11b^{low} KCs/macrophages and MHCII^{high}CD11c⁺CD11b^{low}CD103⁺ DCs identified herein. PLG(Ag) administration led to upregulation of the co-inhibitory molecule PD-L1 by APCs (Figures 4A and B), which is crucial for tolerance induction and maintenance as illustrated by the ability of anti-PD-1 to attenuate tolerance induction during relapse (Figure 4C). Because the increase in PD-L1 expression was only observed in PLG(PLP_{139–151}) treated mice, we hypothesize that NP induced increase in PD-L1 expression is dependent both on APC interactions with NP as well as Ag specific T-cells. These studies demonstrate a role of the PD-1/PD-L co-inhibitory signalling pathway in tolerance induction by particles, though other mechanisms are expected to be involved.

Encapsulation of Ag within the NPs induced tolerance similarly to Ag-coupled NPs. The I.V. infusion of SP-Ag^{40–42} or NPs^{15, 16} has previously been reported to promote tolerance in multiple models of immune dysfunction. This surface coupling has been achieved through carbodiimide coupling of peptides or proteins to the NP surface. Due to the presence of

multiple amines on these peptides or proteins, this coupling likely leads to a complex network on the surface that could lead to non-homogeneous sizes of particles. Additionally, the presence of proteins on the surface determines the physical properties of the NP that can influence their biodistribution and function,^{43, 44} and may provide binding sites for antibodies that could trigger immune responses compared to Ag-encapsulated NPs.²¹ In contrast, the surface properties of NP with encapsulated Ag can be controlled through the fabrication process, such as the choice of polymer or the emulsifying agent. The negatively charged NPs used herein are opsonized within the serum following injection, with opsonization thus facilitating internalization by and delivering Ag to APCs.^{43, 44}

The relative amounts of Ag delivered by NPs are marginally different (Tables 1). PLG(Ag) NPs delivered a lower amount of Ag per injection (6 µg) with a significant alleviation of R-EAE induction compared to Ag-coupled NPs. Variations in Ag coupling efficiency and loading likely contributed to the observed differences in NP efficacy. Future studies are aimed at determining the impact of Ag-loading and NP dose required to induce tolerance.

The versatility of NPs such as PLG(Ag) with respect to surface chemistries, particle tracking, quantification, and functionalization (loading with multiple Ags to cover multiple disease-relevant epitopes; co-delivery of tolerogenic drugs or cytokines to enhance efficacy; surface display of cellular targeting ligands, *etc.*) enables studies to identify mechanisms underlying establishment of peripheral tolerance. Previous reports had identified the spleen and marginal zone macrophages as involved in tolerance induction; however, this report demonstrated tolerance in a splenectomized mouse and identified a significant role for APCs in the liver, which was a significant site for the NP biodistribution, led to the accumulation of Ag-specific T cells, and influenced cytokine production. A better understanding of how the primary interaction between APCs and the Ag-loaded NPs influences the adaptive response will help to inform the rational design of successive particle formulations, and potentially broaden the versatility of this application. Ongoing studies, which are enabled in part through modulating the particle design, will further contribute to the understating of the mechanisms of tolerance induction.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Treatment with PLG(Ag) prevents and treats the progression of R-EAE

(A) Clinical scores for SJL/J mice treated with PLG(OVA₃₂₃₋₃₃₉) (red) or PLG(PLP₁₃₉₋₁₅₁) (black) and immunized with PLP₁₃₉₋₁₅₁ 7 days later. (B) Clinical scores for mice were treated with PLG-OVA₃₂₃₋₃₃₉ (red) or PLG-PLP₁₃₉₋₁₅₁ (black) and immunized with PLP₁₃₉₋₁₅₁ 7 days later. (C) Clinical scores for SJL/J mice immunized with PLP₁₇₈₋₁₉₁ and treated with PLG(OVA₃₂₃₋₃₃₉) (red), PLG(PLP₁₃₉₋₁₅₁) (black), or PLG(PLP₁₃₉₋₁₅₁+PLP₁₇₈₋₁₉₁) (green) on day 18 (black arrow). Groups were examined for clinical paralysis for an additional 8 days. The arrow indicates the day on which various particles were administered to mice previously immunized with PLP₁₇₈₋₁₉₁/CFA. (D) iLNs harvested from PLG(PLP₁₃₉₋₁₅₁) or PLG(OVA₃₂₃₋₃₃₉) treated or naïve mice and *ex vivo* proliferation of lymphocytes in response to 10 μ M PLP₁₃₉₋₁₅₁ (black) or OVA₃₂₃₋₃₃₉ (red). Each data point represents the mean counts per minute (³H-TdR incorporation) for each mouse. Supernatants from the cultures were examined for (E) IL-17A, (F) GM-CSF, and (G) IFN- γ . Data are representative of at least 2–3 independent experiments. Differences between disease courses of different treatment groups were analysed for statistical significance using the Mann-Whitney U test (Figure 1A–C). Differences in proliferation and cytokine production were analysed for statistical significance using a 2-way ANOVA followed by the Sidak test for multiple comparisons (Figure 1D–G). Error bars represent statistical variation between individual animals within each group, 3 mice per group. An asterisk is indicative of statistical significance where * indicates $p < 0.05$, ** indicates $p < 0.01$, and *** indicates $p < 0.001$.

Figure 2. Tolerance induced by PLG(Ag) treatment does not require the spleen
 Splenectomy does not abrogate prophylactic tolerance induction with PLG(PLP₁₃₉₋₁₅₁) in R-EAE *in vivo* (A,B), recall proliferation (C,D), IFN-γ (E,F), or IL-17A (G,H) secretion *ex vivo*. Differences between disease courses of different treatment groups were analysed for statistical significance using the Mann-Whitney U test (Figure 2A–B). Differences in proliferation and cytokine production upon restimulation with PLP₁₃₉₋₁₅₁ were analysed for statistical significance using a 2-way ANOVA followed by the Sidak test for multiple comparisons (Figure 2C–H). Error bars represent assay standard deviation of pooled samples

(**C–H**) and standard error of the mean (**A–B**). N=3–5 mice per group (**A–B**). An asterisk is indicative of statistical significance where * indicates $p < 0.05$, ** indicates $p < 0.01$, and *** indicates $p < 0.001$.

Figure 3. PLG(Ag) distribute predominantly to the liver

(A) PLG-silver(PLP₁₃₉₋₁₅₁) biodistribution to the liver, lungs, and spleen at multiple times after I.V. administration as measured by ICP-MS and expressed as a percentage of the total mass of silver administered per mouse. (B) PLG_{PEMA} particles (green) co-localized primarily with F4/80⁺ cells (red) in the liver (white arrows), counter-stained with DAPI (blue), 18 hours following I.V. infusion. Accumulation of PLP₁₃₉₋₁₅₁-specific CD90.1⁺ CD4⁺ transgenic T cells in liver (C), spleen (D), lung (E), and inguinal lymph nodes (iLN) (F) was quantified by flow cytometry 18 hours post administration. Differences in biodistribution of PLG(Ag) particles were analysed for statistical significance using a 2-way ANOVA followed by the Sidak test for multiple comparisons (Figure 3A). Differences in T

cell accumulation in various organs were analysed for statistical significance using a one-tailed t test (Figure 3C–F). Error bars represent standard error of the mean (A–F). N=3 mice per group (A–F). An asterisk is indicative of statistical significance where * indicates $p < 0.05$.

Figure 4. Tolerance induced by PLG(Ag) treatment is abrogated by blockade of PD-1
MFI for (A) F4/80⁺MHCII^{low}CD11b^{low} Kupffer cells and (B) MHCII^{high}CD11c⁺CD11b^{low}CD103⁺ dendritic cells in the liver 18 hours post administration in mice that had received adoptive transfer of PLP₁₃₉₋₁₅₁-specific CD90.1⁺ CD4⁺ transgenic T cells. (C) Clinical scores for mice treated with mouse anti-PD-1 monoclonal antibody (αPD-1, clone RMP1-14) 24 h prior to particle administration (day -7 relative to disease induction). (D) Restored recall PLP₁₃₉₋₁₅₁ specific proliferation and (E) IL-17A and (F) IFN-γ secretion. (D–F) follow the same legend as in (C). Differences in MFI of PD-L1 on Kupffer cells and dendritic cells were analysed for statistical significance using the Kruskal-Wallis test (Figure 4A–B). Differences between disease courses of different treatment groups were analysed for statistical significance using the Mann-Whitney U test (Figure 4C). Differences in proliferation and cytokine production were analysed for statistical significance using a 2-way ANOVA followed by the Sidak test for multiple comparisons (Figure 4D–F). Error bars represent standard error of the mean (A–F). N=2–4 mice per group (A–B), and N=5–8 mice per group (C–F). An asterisk is indicative of statistical significance where * indicates $p < 0.05$, ** indicates $p < 0.01$, and *** indicates $p < 0.001$.

Table 1

Characterization of various nanoparticle formulations utilized in this study.

Nanoparticle formulation	Size (nm)	Zeta potential (mV)	Encapsulation or coupling efficiency (%)	Loading (µg/mg)
PLG _{PEMA} (OVA _{323–339})	448.0 ± 2.7	−53.1 ± 1.0	4.6 ± 0.5	2.7 ± 0.3
PLG _{PEMA} (PLP _{139–151})	621.5 ± 12.6	−43.7 ± 4.1	4.4 ± 0.2	2.4 ± 0.1
PLG _{PEMA} (PLP _{139–151} + PLP _{178–191})	834.8 ± 114.5	−50.0 ± 6.7	16.5 ± 4.3	8.3 ± 2.2
PLG _{PEMA} –OVA _{323–339}	358.6 ± 11.0	−74.6 ± 1.4	9.1 ± 1.4	7.2 ± 1.1
PLG _{PEMA} –PLP _{139–151}	377.9 ± 4.3	−72.8 ± 1.6	7.2 ± 0.2	5.8 ± 0.1