Modulating lung immune cells by pulmonary delivery of antigen-specific nanoparticles to treat autoimmune disease

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Antigen-specific particles can treat autoimmunity, and pulmonary delivery may provide for easier delivery than intravenous or subcutaneous routes. The lung is a hub for autoimmunity where autoreactive T cells pass before arriving at disease sites. Here, we report that targeting lung antigen-presenting cells (APCs) via antigen-loaded poly(lactide-co-glycolide) particles modulates lung CD4+ T cells to tolerate murine experimental autoimmune encephalomyelitis (EAE), a mouse model of multiple sclerosis. Particles directly delivered to the lung via intratracheal administration demonstrated more substantial reduction in EAE severity when compared with particles delivered to the liver and spleen via intravenous administration. Intratracheally delivered particles were associated with lung APCs and decreased costimulatory molecule expression on the APCs, which inhibited CD4+ T cell proliferation and reduced their population in the central nervous system while increasing them in the lung. This study supports noninvasive pulmonary particle delivery, such as inhalable administration, to treat autoimmune disease.

INTRODUCTION

Autoimmune disorders, which number in the hundreds, include diseases such as multiple sclerosis (MS), lupus, type 1 diabetes, and rheumatoid arthritis and lead to chronic inflammation and potentially fatal conditions. Particularly, MS is a neurological disease estimated to affect over 1 million people globally (1). Current treatments for MS, including fingolimod, cyclophosphamide, and natalizumab, have efficacy against relapse of MS; however, these treatments lead to adverse effects, such as nonspecific immunosuppression, progressive multifocal leukoencephalopathy, and cardiac complications, limiting their utilization (2, 3). Alternatively, antigen-specific immunotherapies are emerging to induce tolerance against disease-specific autoantigens without suppressing the global immune system (4). Soluble antigens and autologous cells loaded with disease-specific antigens have been investigated, which then led to antigen-specific treatment using micro- and nanoparticles for treatment of autoimmune disease with minimal side effects (4). Various engineered nanoparticles loaded with disease-specific antigens were applied to EAE and type 1 diabetes models to induce tolerance (4, 5). Antigen-loaded nanoparticles are primarily delivered in invasive manners via intravenous (6–9), subcutaneous (7, 10–12), or intraperitoneal (13, 14) injections to target antigen-presenting cells (APCs) to modulate lymphocytes in the spleen, lymph nodes, or liver, which are thought to be sites for tolerance induction.

The lung can readily be targeted for noninvasive pulmonary delivery and is a hub for autoimmunity through which autoreactive myelin-specific T cells first pass before arriving at disease sites, such as the central nervous system (CNS) for MS (15). While the lung has not generally been considered as a tolerogenic organ to treat autoimmune disease, recent studies have demonstrated the success of approaches that deliver disease-specific soluble antigens via the pulmonary route for treatment of EAE; however, antigen needs to be codelivered with an immunomodulatory cytokine or requires daily administration for efficacy (16, 17). This requirement may be explained by the poor stability of the free peptide and rapid clearance from the lung, resulting in limited therapeutic results (18). Particle delivery to the lung results in internalization by a large number of cells, with controlled release of the peptide and enhanced biological activity, which has been applied for treatments of lung infections and diseases such as tuberculosis, cancer, and fibrosis (18–20), yet not been established for treatment of MS. In addition to the noninvasive manner of particle delivery, the lung has lower levels of drug metabolism than the liver and allows for efficient delivery of proteins and peptides into the body (21). The large adsorptive surface area of alveoli in the lungs compared with the oral or intravenous routes provides potential advantages for pulmonary administration by reducing drug dose and improving the local therapeutic efficacy while decreasing systemic side effects (22).

Here, we investigated the capacity of lung immune cells to support tolerance induction and whether lung-specific delivery of antigen-engineered particles could treat autoimmune disease. We hypothesized that antigen-loaded biodegradable particles would be phagocytosed by lung APCs, with subsequent tolerance induced in disease-specific CD4+ T cells and amelioration of EAE disease. We examined two delivery pathways, intravenous (IV) and intratracheal (IT) pathways, to target lung APCs, including alveolar macrophages (AMs), interstitial macrophages (IMs), and lung dendritic cells (DCs). Conventional intravenous delivery was initially performed using two poly(lactide-co-glycolide) (PLG) particle sizes containing disease-specific proteolipid protein (PLP139–151), 400 nm and 15 μm (termed as Nano-PLP and Micron-PLP, respectively), to investigate the lung as a tolerogenic organ via altering the biodistribution of particles. Subsequently, intratracheal Nano-PLP particle delivery was performed to investigate direct particle delivery to the lung on amelioration of EAE disease. The interplay between particles and
immune cells was examined by the biodistribution among the organs, the interaction with specific APCs, and the resulting cell phenotypes. Furthermore, we investigated whether antigen-loaded PLG particles would still circulating CD4+ T cells in the lung, limiting their migration to the CNS. Our results suggest that intratracheally delivered antigen-loaded PLG particles inhibit lung APC costimulatory molecule expression, limit antigen-specific CD4+ T cell proliferation, and induce accumulation of CD4+ T cells in the lung rather than the CNS to prevent EAE pathology.

RESULTS

The lung can be a tolerogenic organ

Targeting the lung to induce immunological tolerance was initially investigated by the intravenous delivery of two sizes of PLG particles containing PLP139-151: Nano-PLP (targeted diameter of 400 nm) and Micron-PLP (targeted diameter of 15 μm), based on the data showing that cyanine 5.5 dye (Cy5.5)–conjugated PLG particles with 400-nm diameter accumulated in the liver, while particles with 15-μm diameter accumulated in the lung (Fig. S1A). The data agree that intravenously delivered particles around 500 nm in diameter typically accumulate in the reticuloendothelial system, especially the liver and spleen, and experience phagocytosis by APCs (5, 9, 23). Scanning electron microscopy (SEM) images confirmed the spherical shapes of Nano-PLP and Micron-PLP particles as well as control particles containing ovalbumin (OVA323–339) (Nano-OVA particles) (Fig. 1A). The average diameters of the particles were approximately 400 nm for Nano-PLP and Nano-OVA particles and approximately 15 μm for Micron-PLP particles, and the zeta potentials were ~40 mV or lower for all particles (Fig. 1B). The PLP139-151 loading and the in vitro release of the antigen from Nano-PLP and Micron-PLP particles were similar and ultimately reached approximately 80% of total encapsulated PLP139-151 by day 19 (Fig. 1B and fig. S1B). A single dose of particles (1.5 mg per mouse) was intravenously injected into EAE mice at day 7 postimmunization (p.i.). Intravenously injected Cy5.5+ Micron-PLP particles (with Cy5.5) accumulated in the lung in much greater quantities compared with intravenously injected Cy5.5+ Nano-PLP particles (with Cy5.5) or Cy5.5+ Nano-OVA particles (without Cy5.5, control) (Fig. 1, C to E, and fig. S1C).

EAE clinical scores and cumulative EAE clinical scores indicate that Nano-PLP particles significantly reduced EAE clinical scores compared with Nano-PLP and Nano-OVA particles (Fig. 1, F and G, and fig. S1, D and E). Nano-OVA particles were chosen as a control to show the antigen specificity of Nano-PLP and Micron-PLP particles since the previous studies demonstrated Nano-OVA particles had similar EAE clinical behavior as no treatment and micrometer-sized particles (6, 7). Mice treated with Micron-PLP particles had minor first disease peak at day 17 p.i., which is significantly better than mice treated with Nano-OVA (control) and Nano-PLP particles with the first disease peak at 13 and 16 days p.i., respectively (Fig. 1F). Fluorescence-activated cell sorting (FACS) analysis revealed that Micro-PLP particles significantly reduced the numbers of myeloid cells (CD45+/Ly6G+/CD11b+), CD4+ T cells (CD45+/CD11b+/CD3+/CD4+), and B cells (CD45+/CD11b+/B220+) in the CNS at day 14 p.i. (first peak of disease), relative to mice treated with Nano-PLP or Nano-OVA particles (Fig. 1, H to J, and fig. S1, F and G). This decrease in these cell populations is consistent with the decreased EAE score for the Micro-PLP particles relative to the Nano-PLP and Nano-OVA particles. The data suggest that targeting antigen-loaded particles to the lung over the liver improved tolerance in EAE.

Pulmonary particle delivery promotes tolerance

We next investigated the efficacy of antigen-loaded particles delivered directly to the lung by intratracheal administration. Nano-PLP and Nano-OVA particles were used instead of micrometer-sized particles to target lung APCs in alveolar since particle sizes in the range of 0.5 to 1 μm have been reported to be deposited in lung alveolae following intratracheal delivery, where they are phagocytosed by APCs, such as AMs (18, 20, 24). Intratracheal administration of a single dose (1.5 mg per mouse) of Cy5.5+ Nano-PLP or Cy5.5+ Nano-OVA particles confirmed greater accumulation of the particles in the lung compared with the liver, although some accumulation in the liver was observed (Fig. 2, A to C). Negligible signals of particles or particle-associated cells were detected in the CNS and spleen (fig. S2, A and B).

Both particle types decreased their intensity by day 14, indicating particle internalization or degradation. Mice with intratracheally delivered Nano-PLP particles (Nano-PLP-IT) maintained EAE clinical disease scores of zero until day 18 p.i., whereas the intravenously delivered Nano-PLP (Nano-PLP-IV) group showed disease activity starting at day 11 p.i. (Fig. 2D). Nano-PLP-IT significantly decreased the clinical scores compared with the other groups until day 20 p.i., indicating the potential for intratracheal delivery to ameliorate EAE disease. Reduced doses of Nano-PLP by intratracheal particle administration (1.5, 1.0, and 0.5 mg per mouse) indicated that a dose of 1.0 mg per mouse reduced EAE clinical scores similar to the dose of 1.5 mg per mouse (Fig. 2E and fig. S2C).

The mechanism of intratracheally delivered Nano-PLP or Nano-OVA particles (1.5 mg per mouse dose) was investigated by analyzing the accumulation of inflammatory cells, macrophages (CD45+/CD11b+/Ly6G+/CD11c+/F4/80+), DCs (CD45+/CD11b−/Ly6G−/CD11c+/F4/80+), neutrophils (CD45+/CD11b+/Ly6G+), and monocytes (CD45+/CD11b−/Ly6G−/CD11c−/CD6+) in the CNS of EAE mice at day 14 p.i. Mice treated with Nano-PLP particles had more macrophages and their associated cytokines (CCL3, CXCL1, CXCL2, and CCR2), yet fewer DCs, neutrophils, and monocytes, than those of the mice treated with Nano-OVA particles, in the CNS (Fig. 2F and fig. S2D), correlating with their reduced EAE clinical score. Nano-PLP particles also reduced the expression of costimulatory molecules, CD86, and major histocompatibility complex class II (MHCII) on both macrophages and DCs (Fig. 2, G and H). Collectively, intratracheally delivered Nano-PLP particles reduced the number of activated immune cells in the CNS, which coincided with diminished EAE clinical scores.

The potential to extend the tolerizing period was tested through multiple administrations of particles by intratracheal delivery. Weekly intratracheal administration of Nano-PLP particle (1.0 mg per mouse) for 3 weeks had an extended duration with zero clinical disease score until day 42 p.i. and significantly reduced clinical scores relative to weekly intratracheally delivered Nano-OVA particle group (Fig. 2I). These data demonstrated that intratracheal delivery of Nano-PLP particles was able to transiently prevent EAE clinical manifestations, and that efficacy can be extended via weekly intratracheal administration of particles.

Intratracheally delivered Nano-PLP particles reduce CD86 expression on lung APCs

Lung immune cells were assessed following intratracheally delivered Nano-PLP particles for their association with particles and their
phenotypes. Within the lung, eight kinds of myeloid cells were investigated, including AMs, CD11b+ IMs, CD11c+ IMs, conventional DCs (cDCs), monocyte-derived DCs (Mo DCs), inflammatory macrophages (Inf. Macs), inflammatory monocytes (Inf. Monos), and neutrophils, as shown in the gating scheme (fig. S3). The lung immune cell signatures were monitored using t-distributed stochastic neighbor embedding (tSNE) analysis to provide quantification of AMs, CD11b+ IMs, CD11c+ IMs, Mo DCs, cDCs, Inf. Macs, Inf. Monos, and neutrophils (Fig. 3A).

Nano-PLP particle intratracheal injection was associated with increased numbers of AMs, CD11c+ IMs, Mo DCs, cDCs, Inf. Macs, and Inf. Monos compared with Nano-OVA particles (Fig. 3B), consistent with greater numbers of lung immune cells with intravenously delivered Micron-PLP particles that accumulated in the lung (Fig. 1E and fig. S4). The tSNE analysis and FACS data showed that Nano-OVA particles were associated at higher levels with lung immune cells compared with Nano-PLP particles (Fig. 3C and fig. S5A). However, Nano-PLP particles had greater numbers of particle-positive AMs, cDCs, and Mo DCs compared with Nano-OVA particles (Fig. 3D). Considering that Nano-PLP particle intratracheal treatment increased the number of immune cells in the lung, the increase in Nano-PLP particle–positive APCs is not unexpected.

Intratracheally delivered Nano-PLP particles increased MHCII expression on AMs, CD11c+ IMs, Mo DCs, cDCs, and Inf. Macs.
compared with Nano-OVA particles (Fig. 3E); however, little difference was observed on CD86 expression (fig. S5B), suggesting that neither Nano-PLP nor Nano-OVA particle treatment up-regulated the costimulatory molecule CD86 in the lung. The Nano-PLP particle treatment group showed greater numbers of AMs, CD11c+ IMs, cDCs, Mo DCs, and Inf. Macs compared with Nano-OVA particle–treated mice that expressed MHCII (MHCII+), but were CD86 negative (CD86−), consistent with the capacity for induction of T cell anergy (Fig. 3F and fig. S5C) (25). Thus, Nano-PLP particle intratracheal treatment led to the accumulation of APCs in the lung that are MHCII+, without induction of the costimulatory molecule, CD86.

Intratracheally delivered particles modulate APCs in the mediastinal lymph nodes
APCs in the mediastinal lymph nodes (Med LNs) were investigated since particles internalized by lung APCs migrate and accumulate in the lung draining Med LNs (26–28). Intratracheally delivered Nano-PLP particles were associated with increased accumulation of activated macrophages (CD45+/Ly6G−/CD11c+/CD11b−/MHCII+) and activated DCs (CD45+/Ly6G−/CD11c+/MHCII+) and cytokines, especially CCL3 and CCR2 in the Med LNs compared with Nano-OVA particles (Fig. 4A and fig. S6). Expression of CD86 was similar between particle treatment groups (Fig. 4B). Nano-PLP particles...
were associated with DCs more than Nano-OVA particles (Fig. 4C), and these Nano-PLP particle–positive (Nano-PLP⁺) cells had less CD86 expression than Nano-PLP particle–negative (Nano-PLP⁻) cells (Fig. 4D). Collectively, these data suggest that intratracheally delivered Nano-PLP particles were associated with DCs in the lung and then migrated to the Med LNs, yet these cells did not express costimulatory molecule, CD86.

**Intratracheally delivered particles increase CD4⁺ T cells in the lung and reduces them in the CNS**

The hypothesis that intratracheally delivered Nano-PLP particles would increase migration of lymphocytes to the lung, corresponding to a decrease in the lymphocyte numbers in the CNS, was examined, as previous studies showed that T cells are licensed in the lung and maintained during lung inflammation (15, 29). At the first EAE peak, intratracheal Nano-PLP particle treatment reduced the number of CD4⁺ T and B cells in the CNS compared with intratracheal Nano-OVA particle treatment, while these cells increased in the lung following intratracheal Nano-PLP particle treatment (Fig. 5, A and B, and fig. S7A). In addition, Nano-PLP particles decreased the number of CD4⁺ T cells expressing interferon-γ (IFN-γ) and interleukin-17 (IL-17) in the CNS, whereas this treatment increased numbers of CD4⁺ T cells expressing those factors in the lung (Fig. 5, C and D). CD4⁺ T cells expressing forkhead box P3 (Foxp3) and IL-4 were also reduced in the CNS of mice treated with intratracheal Nano-PLP particles more than in those receiving intratracheal Nano-OVA particles (Fig. 5E and fig. S7B). For comparison, intravenously delivered Micron-PLP particles, which also reduced EAE clinical scores, similarly increased the numbers of CD4⁺ T cells and IFN-γ–, IL-17–, and Foxp3–expressing CD4⁺ T cells in the lung, but not B cells (fig. S8). The populations of CD4⁺ T cells were similar in the Med LNs between the treatment groups. Together, the data suggest that intratracheally delivered Nano-PLP particles increased CD4⁺ T cell accumulation in the lung, a site where the self-antigen (PLP) is not normally expressed, and reduced CD4⁺ T cell accumulation and activation in the CNS.

The hypothesis that intratracheal delivery of Nano-PLP particles alters chemokine expression that maintains circulating T cells in the lung, thereby limiting EAE pathogenesis, was tested. Reverse transcription polymerase chain reaction (RT-PCR) data further revealed that Nano-PLP particle intratracheal instillation resulted in lower
Fig. 4. Intratracheally delivered Nano-PLP particles influence the population and phenotypes of APCs in the Med LNs. FACS analysis of APCs in the Med LNs of EAE mice at day 14 p.i. after intratracheal delivery of 1.5 mg of particles at day 7 p.i. (A) Number of macrophages and DCs in the Med LNs. (B) Population of CD86+ macrophage and DCs in the Med LNs. (C) Population of particle-associated macrophages and DCs in the Med LNs. (D) CD86 expression of macrophages and DCs with or without Nano-PLP particles (Nano-PLP\(^+\), Nano-PLP\(^-\)) in the Med LNs. (A to D) Unpaired two-tailed \(t\) test was performed (\(n=5\) mice per group). \(* P < 0.05\) and \(** * P < 0.001\).

Fig. 5. Intratracheally delivered Nano-PLP particles influence the population of CD4\(^+\) T and B cells, expression of IFN-\(\gamma\), IL-17, Foxp3, and gene expressions in the CNS, lung, and Med LNs. The CNS, lung, and Med LNs were harvested from EAE mice at day 14 p.i. after intratracheal delivery of 1.5 mg of particles at day 7 p.i. Numbers of (A) CD4\(^+\) T cells and (B) B cells. Numbers of (C) IFN-\(\gamma\), (D) IL-17, and (E) Foxp3 expressing CD4\(^+\) T cells. (A to E) Unpaired two-tailed \(t\) test (\(n=5\) mice per group). (F to I) Real-time polymerase chain reaction (PCR) quantification of relative expression of chemokine ([F] CCL5, [G] CXCL1, [H] CCL21B, and [I] CCL19) genes. Expression of each gene was calculated relative to the expression of housekeeping gene, GAPDH. Unpaired \(t\) test (\(n=5\) mice per group). \(* P < 0.05\) and \(** * P < 0.001\).
gene expression of CCL5 and CXCL11 in the CNS and higher expression in the lung and Med LNs (Fig. 5, F and G), supporting that the migration of T cells would be favored into the lung or Med LN rather than the CNS. The chemokines typically associated with the recruitment of T cells to the lymph nodes (CCL19 and CCL21B) did not explain the localization for T cells (Fig. 5, H and I). In contrast to the differences in chemokines themselves associated with T cells, most chemokine receptors did not show differences between the treatment groups (fig. S9). Collectively, the data suggest that intratracheally delivered Nano-PLP particles maintain circulating T cells in the lung, thus limiting EAE pathogenesis.

**Lung MHCII+ cells associated with intratracheally delivered Nano-PLP particles reduce CD4+ T cell proliferation**

Last, the capacity of lung MHCII-expressing cells with intratracheally delivered Nano-PLP particles for inducing CD4+ T cell anergy was investigated on the basis of the data of increased MHCII expression delivered Nano-PLP particles for inducing CD4+ T cell anergy was investigated on the basis of the data of increased MHCII expression without increasing their CD86 expression on lung APCs. CD4+ T cells were isolated from the spleen and non-lung draining lymph nodes (inguinal, axillary, and brachial lymph nodes) of EAE mice without particle treatment and then cultured with lung MHCII+ cells with or without Nano-PLP particles (Fig. 6A). Lung MHCII+ cells containing Nano-PLP particles (Nano-PLP+) failed to stimulate the proliferation of CD4+ T cells, resulting in proliferation profiles similar to CD4+ T cells alone (negative control) (Fig. 6B, a and b). In contrast, CD4+ T cells proliferated well when cultured with lung MHCII+ cells that were Nano-PLP-, and these responses were similar to CD4+ T cell proliferation when cultured with lung MHCII+ cells from EAE mice without Nano-PLP particle treatment (positive control) (Fig. 6B, c and d). Collectively, the data indicate that lung MHCII+ cells associated with Nano-PLP particles failed to induce CD4+ T cell antigen-specific proliferation, and this is likely one mechanism that contributes to reduced EAE scores.

**DISCUSSION**

We demonstrated that modulation of lung immune cells by disease antigen-loaded PLG particles resulted in the amelioration of EAE. PLG particles were chosen because of their minimal toxicity for pulmonary administration (19, 30, 31), controllability of drug release for treatment of inflammatory disease (32), as well as use for treatment of MS (8, 10). Intravenous delivery of antigen-loaded PLG particles demonstrated that targeting the lung with the particles resulted in decreased EAE clinical scores and disease-associated immune cells in the CNS compared with delivery of the particles to the liver and the spleen (Fig. 1 and fig. S1). Furthermore, direct delivery of Nano-PLP particles to the lung via the intratracheal route had a more profound effect on reducing EAE symptoms as compared with intravenous administration of Nano-PLP particles and exhibited increased particle association with lung macrophages (Fig. 2). Intratracheal particle delivery resulted in greater particle accumulation in the lung than the liver, consistent with previous reports (33). Intratracheal Nano-PLP particles were more potent than previous reports that explored nasal delivery of soluble disease-specific antigens and required frequent administration (16, 17). Pulmonary-delivered soluble antigen was rapidly cleared from the lung after 1 day of delivery, while about 75% of antigen delivered by nanoparticles remained, suggesting that antigen delivery using the particle system supported sustainable delivery of antigen (26). One possible explanation is that soluble antigens may not be efficiently phagocytosed by lung APCs including AMs (31, 33, 34). Furthermore, antigen-loaded PLP particles can extend antigen presentation by APCs compared with soluble antigen and PLG particles themselves (35).

Intratracheally delivered particles were highly associated with macrophages, and DCs phagocytosed fewer particles than AMs or IMs (Fig. 3 and fig. S5) (36). AMs are numerous in populating the alveolar space, the first line of defense within the lung to inhaled antigens and likely the first cells to directly contact a drug administered via the intratracheal route, and may be the cell most likely to suppress T cell proliferation in our model (21, 37). In addition, the particles are associated with other macrophages and DCs in the lung, agreeing with the previous report that pulmonary-delivered nanoparticles are phagocytosed by AMs, IMs, and DCs since nanoparticles can translocate beyond the epithelial barrier into the interstitium (38). Single intratracheal administration of Nano-PLP particles tolerized EAE for relatively short times (10 days), although the period was extended via weekly intratracheal administration of the particles (Fig. 2). Lung APCs, especially AMs, are highly phagocytic and clear the particles soon after administration (39). This clearance was corroborated by in vivo imaging system (IVIS), which indicated that

**Fig. 6. Reduction in CD4+ T cell proliferation by lung MHCII+ cells associated with intratracheally delivered Nano-PLP particles.** (A) Schematic of experiment showing Nano-PLP particles delivered intratracheally at day 7 p.i. (no particle control), with isolation and sorting of lung MHCII+ cells with or without Nano-PLP particles (Nano-PLP+) by FACS at day 13 p.i. These isolated Nano-PLP+/− MHCII+ cells were cultured with CD4+ T cells from non-lung draining lymph nodes and spleen of EAE mice that had not been treated with Nano-PLP particles. (B) CD4+ T cell proliferation data including (a) T cell only (from EAE mice without particle treatments), (b) MHCII+ cells with Nano-PLP particles (Nano-PLP+), (c) MHCII+ cells without Nano-PLP particles (Nano-PLP−), and (d) MHCII+ cells (from EAE mice without particle treatments).
the signal in the lung following particle administration was significantly reduced by day 7 after administration (Fig. 2). Lung APCs may internalize and process nanoparticles more quickly than APCs in the liver and spleen, which would be consistent with our observation that pulmonary delivery was able to tolerate EAE with a smaller dose of particles relative to the intravenous route.

Lung APCs associated with PLP-loaded particles showed a higher expression of MHCII yet did not have a corresponding elevation in costimulatory CD86 (Fig. 3). In addition, the lung APCs associated with the particles reduced antigen-specific CD4+ T cell proliferation, consistent with previous studies showing the capability of lung APCs to induce T cell anergy (Fig. 6) (40, 41). AMs and IMs internalized both intratracheally delivered Nano-PLP and Nano-OVA particles; however, administration of Nano-PLP particles resulted in a greater accumulation of MHCII+CD86−-expressing cells (lacking costimulatory molecule expression) in comparison with Nano-OVA particles. Nano-PLP particle treatment also resulted in greater numbers of MHCII+CD86−DCs and MHCII+CD86− Mo DCs in the lung compared with Nano-OVA particles, suggesting that DCs may also contribute to reducing CD4+ T cell responses. The fact that Nano-PLP particles were more effective than Nano-OVA particles in reducing costimulation highlights the importance of antigen specificity in this therapeutic regimen.

Although we did not examine the trafficking of lung APCs, the data demonstrated that macrophages and DCs were both associated with Nano-PLP and Nano-OVA particles in the Med LNs (Fig. 4). These data are consistent with reports that lung macrophages and DCs phagocytose particles and migrate to draining lymph nodes (26–28). Macrophages are preferably associated with Nano-OVA particles, whereas DCs are preferentially associated with Nano-PLP, suggesting that cell association and migration to lymph nodes may be dependent on particle type. However, the features that dictate such preferential association are unclear. Our data did not directly compare the stimulatory capacity of particle-associated DCs with that of macrophages, yet one cell type may be better able to limit T cell activation.

We examined CD4+ T cell deactivation by particle-associated lung APCs, as a previous study reported that CD4+ T cells circulate through and are licensed in the lung before they enter the CNS (15). Both intravenously delivered Micron-PLP and intratracheally delivered Nano-PLP particles showed greater numbers of CD4+ T cells including IFN-γ+, IL-17+, and Foxp3+ CD4+ T cells, in the lung, while the numbers of those cells were reduced in the CNS (Fig. 5 and fig. S8). IFN-γ−, IL-17−, and Foxp3+ double-secreting CD4+ T cells have previously been implicated in the pathogenesis of EAE, and these lymphocytes are primed in the periphery and migrate into the CNS and correlate with the onset of EAE symptoms (42). We noticed a concomitant increase in IFN-γ+ and IL-17+ secreting CD4+ T cells in the lungs of particle-treated mice, suggesting that inflammatory responses in the lung sequestered pathogenic T cells and inhibited their migration to the CNS. In addition, increased gene expressions of CCL5 and CXCL11 were observed in the lung after the intratracheal delivery of Nano-PLP particles, which is consistent with T cells being preferentially maintained in the lung (Fig. 5). In addition, although approximately 20% of DCs in the Med LNs were positive with Nano-PLP particles and reduced their costimulatory molecule expression (Fig. 4, C and D), the number of the CD4+ T cells in the lymph node was not significantly different between Nano-PLP and Nano-OVA particle treatments (Fig. 5), suggesting that greater tolerance was induced in the lung rather than the lymph nodes. Collectively, our data support a hypothesis whereby Nano-PLP particle delivery to the lung alters the migration and activation patterns of CD4+ T cells to maintain them in the lung and, thus, prevents them from encountering antigen in CNS tissue.

Although our data demonstrated that antigen-specific nanoparticle delivery to lung APCs was able to tolerate disease-specific CD4+ T cells, APC phagocytosis pathways and the fate of nanoparticles and antigen remain to be investigated. In addition, the trafficking and ultimate destination of CD4+ T cells before and after interacting with lung APCs are opportunities for further study. Furthermore, the activation of CD4+ T cells may change during the relapse and remission phases of MS that are associated with epitope spreading, which affects their interaction with lung APCs. Characterization of these dynamics will also contribute in understanding the dosing schedule that may be needed for pulmonary delivery, which may differ from the intravenous delivery currently in clinical trials for similar particles.

PLG particles have been extensively studied for their safety in pulmonary delivery, and previous in vitro and in vivo studies indicate minimal toxicity of PLG particles regardless of their surface charges (30, 31, 43). A similar PLG nanoparticle platform had no toxicity noted in completed phase 1 (NCT03486990) and phase 2 (NCT03738475) clinical trials conducted to treat the autoimmune condition celiac disease. Collectively, these observations support the safety of PLG particles for pulmonary application (19, 30, 31). However, a potential cause of toxicity in the lung may be due to the slow degradation of PLG particles (22). Therefore, the properties of PLG particles may need to be fine-tuned to limit unwanted toxicity without losing the induction of tolerance.

In conclusion, the present study demonstrated that direct delivery of antigen-specific nanoparticles to the lung is a promising avenue to treat autoimmune disease. Our study revealed that intratracheally administered nanoparticles increased the number of lung APCs, including AMs, IMs, and DCs, all of which can internalize the administered disease-specific nanoparticles. Last, APCs associated with disease-specific nanoparticles reduced the proliferation of autoreactive CD4+ T cells. In addition, a consequence of intratracheal delivery was to maintain CD4+ T cells in the lung via altered chemokine responses, which impaired the trafficking of antigen-specific CD4+ T cell to the CNS. Pulmonary delivery of antigen-specific nanoparticles can be achieved by noninvasive or needle-free methods, with a lower effective dose of nanoparticles needed relative to intravenous administration, and may provide an alternative avenue to treat autoimmune disease.

MATERIALS AND METHODS
Materials
Poly(DL-lactide-co-glycolide) (50:50) (IV = 0.17 dl g) with carboxylic acid end groups were purchased from DURECT Corporation (Cupertino, CA). Poly(ethylene-alt-maleic anhydride) (PEMA) was purchased from Polysciences Inc. (Warrington, PA). Proteolipid protein (HSLGKWLGHDPKF) (PLP) (139–151) and ovalbumin protein (ISQAVHAAHAEINEAGR) (OVA) (323–339) were purchased from GenScript USA Inc. (Piscataway, NJ). Cy5.5 amine dye was purchased from Lumiprobe (Florida, USA). Dichloromethane (DCM) and N-(3-dimethylaminopropyl)-N’-ethylcarbodiimide hydrochloride (EDC) were purchased from Sigma-Aldrich (St. Louis, MO).
N-hydroxysuccinimide (NHS) and phosphate-buffered saline (PBS) were purchased from Thermo Fisher Scientific (Waltham, MA).

Fluorochrome-conjugated antibodies and Fc block were purchased from BioLegend (San Diego, CA), as shown by their names and catalog numbers here: TrueStain FcX (catalog no. 101320, anti-mouse CD16/32 antibody), anti-mouse/human CD11b (catalog no. 101216, PE/Cy7), anti-mouse CD45 (catalog no. 103136, PerCP (peridinin-chlorophyll-protein complex)), anti-mouse CD4 (catalog no. 100510, FITC (fluorescein isothiocyanate)), anti-mouse/human CD45R/B220 (catalog no. 103244, Brilliant Violet 605), anti-mouse CD3 (catalog no. 100341, Brilliant Violet 421), anti-mouse I-A^k (A^B) (catalog no. 109905, FITC), anti-mouse CD86 (catalog no. 105105, PE), anti-mouse Ly-6G (catalog no. 127628, Brilliant Violet 421), anti-mouse F4/80 (catalog no. 123137, Brilliant Violet 421), anti-mouse/human CD11b (catalog no. 101263, Brilliant Violet 510), anti-mouse Ly-6-G (catalog no. 127639, Brilliant Violet 605), anti-mouse CD11c (catalog no. 117339, Brilliant Violet 650), anti-mouse Ly-6C (catalog no. 128043, PE/Dazzle 594), anti-mouse CD4 (catalog no. 100555, Brilliant Violet 650), anti-mouse CD3 (catalog no. 100219, PE/Cy7), anti-mouse IL-17A (catalog no. 506926, Brilliant Violet 421), anti-mouse IFN-γ (catalog no. 505815, Alexa Fluor 488), anti-mouse/rat/human Foxp3 (catalog no. 320008, PE), and Zombie NIB Fixable Viability kit (catalog no. 423105) and Zombie Yellow Fixable Viability kit (catalog no. 423103). In contrast, anti-mouse Siglec F (catalog no. 562068, PE) was purchased from BD Biosciences (San Jose, CA). Dilution followed the manufacturers’ instructions.

**Particle fabrication and characterization**

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

Nano-PLP particles containing Polymer-Cy5.5 were fabricated using the water-in-oil-in-water (w/o/w) double emulsion solvent evaporation method. Briefly, PLP139–151 or OVA323–339 in PBS solution (150 µl) was added dropwise to 50:50 poly(DL-lactide-co-glycolide) (200 mg) water-in-oil (w/o) containing Polymer-Cy5.5 [1% (w/v)] dissolved in DCM 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 w/o primary emulsion was mixed with the PEMA solution [1% (w/v)] and then sonicated. Micron-PLP particles were fabricated in the same manner as Nano-PLP particles except that the secondary emulsion was formed using a Kinematica Polytren PT 3100D homogenizer (Kinematica Inc., Bohemia, NY) at 7500 rpm for 3 min. The secondary emulsions were poured into PEMA [0.5% (w/v)] and stirred on a Bellstir Multistir magnetic stirrer (Bellco Glass Inc., Vinland, NJ) overnight to remove the organic phase. The resulting polymeric particles were washed three times and lyophilized with sucrose [2% (w/v)] and D-mannitol [3% (w/v)].

Fabricated particle sizes and surface ζ potential distributions were obtained using dynamic light scattering on a Zetasizer Nano ZSP (Malvern Instruments Ltd., Malvern, UK). The size of the Micron-PLP particles was measured using a Mastersizer 2000 (Malvern Instruments Ltd., Malvern, UK). The amount of encapsulated peptide was determined using the Micro BCA Protein Assay (Pierce Biotechnology, Rockford, IL) after dissolving particles in NaOH (0.1 M) overnight.

**Peptide release from the particles**

The amount of peptide released from the peptide-encapsulated particles was investigated, with PLG particles (10 mg) washed, resuspended in PBS (1 ml), and incubated at 37°C for 456 hours (19 days). At the 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. Cumulative release and release at each time point were calculated as percent of the amount of peptide detected divided by total amount of peptide released plus the remaining peptide in the particles.

**Induction and clinical evaluation of peptide-induced EAE**

Female SJL/J mice at 8 to 10 weeks old purchased from Envigo were immunized subcutaneously at three spots on the flank with 100 µl of an emulsion of PLP peptide in Adjuvant Incomplete Freund (BD, Sparks, MD) containing Mycobacterium tuberculosis H37Ra (200 µg) (Difco, Detroit, MI). For the intravenous injection model, 1.5 mg per mouse of Nano-PLP, Micron-PLP, and PLG particles containing ovalbumin (OVA323–339)–encapsulated (named OVA) particles suspended in PBS were intravenously injected through the tail at day 7 p.i. For the intratracheal administration model, various doses of particles (0.5, 1.0, or 1.5 mg per mouse) or PBS were administered through the intratracheal route at day 7 p.i. Weekly administration of particles was performed, administering 1.0 mg per mouse of particles every week for 3 weeks. Individual mice were observed daily, and clinical scores were assessed on a 0 to 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; and 5, moribund state. The data are reported as the mean daily clinical score. Paralyzed mice were given easier access to food and water. All animals were housed under specific pathogen-free conditions and maintained in the University of Michigan in compliance with the Institutional Animal Care and Use Committee regulations.

**Evaluation of in vivo particle destination**

Optical images of brain, spinal cord, liver, and spleen were taken using an IVIS Lumina LET camera system (Caliper Life Sciences, Hopkinton, MA). Near-infrared fluorescence images were obtained with Cy5.5 filter channel (emission, 720 nm; excitation, 675 nm).

**Cell isolation and flow cytometry of CNS, lung, liver, and Med LNs**

Cells from Med LNs and spleen were passed through a 70-µm nylon and 40-µm nylon mesh filter and then were treated with ACK Lysing Buffer (Thermo Fisher Scientific, Waltham, MA) and washed with PBS. The CNS (brain and spinal cord), lung, and liver were harvested and ground, and single cells were isolated on a discontinuous Percoll gradient. Isolated cells were suspended in PBS and Fc block and incubated for 15 min. Myeloid cells were identified with fluorochrome-conjugated antibodies (anti-mouse F4/80, anti-mouse Ly-6C, anti-mouse Ly-6G, anti-mouse CD11b, anti-mouse CD11c, anti-mouse...
MHCII, anti-mouse CD86, anti-mouse Siglec F, and anti-mouse CD45) besides Live/Dead staining. For intracellular cytokine staining, cells were diluted to 1 x 10^6/ml and stimulated for 4 hours with phorbol 12-myristate 13-acetate (10 ng/ml), ionomycin (10 μM), and GolgiStop reagent (BD Bioscience, San Jose, CA). The cells were stained with fluorochrome-conjugated antibodies (anti-mouse IL-17A, anti-mouse CD11b, anti-mouse/human CD45R/B220, anti-mouse CD45, anti-mouse CD3, anti-mouse CD4, anti-mouse IFN-γ, and anti-mouse/rat/human Foxp3). Data were acquired with a MoFlo Astrios (Beckman Coulter Inc., Brea, CA) or LSRFortessa (BD Biosciences, San Jose, CA) and analyzed with FlowJo software (FlowJo LLC, Williamay Way, OR).

Nonlinear dimensionality reduction using tSNE was also performed in FlowJo to visualize changes in numerous cell populations simultaneously. In short, after initial quality control to remove dead cells and doublers, flow cytometry data for all samples were first downsampled and concatenated into a single file. Dimensional reduction was then applied using the fSNE plugin with the following setting, perplexity 50, learning rate 1000, for 1000 iterations.

**CD4⁺ T cell proliferation study**

Lung cells from EAE mice treated with or without Nano-PLP particles were isolated, as previously described. The cells were stained with PE anti-mouse I-κB (Abkö) (BioLegend, catalog no. 109908) along with LIVE/DEAD Fixable Violet Dead Cell Stain Kit (Life Technologies, M), and GolgiStop setting, perplexity 50, learning rate 1000, for 1000 iterations.

Gene expression assay using RT-PCR

Gene expression was detected using TaqMan Array Plates Fast 96-well plates with designed genes (Life Technologies). The panel of genes in this plate contains 16 TaqMan assays for T cells, macrophages, endogenous control genes, and manufacturing genes (table S1). After cell isolation, total RNA was isolated using the TRIzol reagent (Invitrogen, catalog no. 15596026). cDNA was synthesized using SuperScript IV VILO Master Mix (Invitrogen). Real-time PCR was performed on an ABI PRISM 7000 thermocycler (Applied Biosystems, Waltham, MA). The average cycle threshold (Ct) was determined for each sample from a given experiment. Relative gene expression was calculated using the comparative Ct method, which assesses the difference in gene expression between the gene of interest and an internal standard gene (GAPDH).

**Statistical analysis**

All experiments were performed with at least three replications, as detailed in the figure legends. One-way analysis of variance (ANOVA), two-way ANOVA, unpaired t test, or multiple t test was performed. \*P < 0.05, \**P < 0.01, and \***P < 0.001 show significant difference between the groups unless otherwise noted. Values for n, P, and the specific statistical test performed for each experiment are described in the appropriate figure legends. Quantitative analyses as scatter or bar graphs are presented as the means ± SD. EAE clinical data are presented as the means ± SEM and were collected data from at least two independent experiments. The GraphPad Prism software (San Diego, CA) was used for all statistical analysis.

**SUPPLEMENTARY MATERIALS**

Supplemental material for this article is available at http://advances.sciencemag.org/cgi/content/full/6/42/eabc9317/DC1

View/request a protocol for this paper from Bio-protocol.

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Acknowledgments: We thank the Flow Cytometry Core at the University of Michigan, Biomedical Research Core Facilities, and the Michigan Center for Materials Characterization.

Funding: This work was supported by the NIH (R01EB013198 to L.D.S. and S.D.M., and R35HL144481 to B.B.M.).

Author contributions: E.S. and L.D.S. conceptualized and designed the research. E.S., S.J.G., K.R.K., C.A.W., and B.B.M. performed the experiments and/or analyzed the data. E.S. and L.D.S. wrote the manuscript. S.J.G., B.B.M., and S.D.M. edited the manuscript.

Competing interests: L.D.S. and S.D.M. are cofounders, paid consultants, members of the Scientific Advisory Board, and grantee of and hold stock options in Cour Pharmaceutical Development Company Inc. S.D.M. is a paid consultant for Takeda Pharmaceuticals International Co., a paid consultant and member of the Scientific Advisory Board of NextCure Inc., a paid consultant for Kite Pharmaceuticals, and a paid consultant and member of the Scientific Advisory Board of Myeloid Therapeutics.

Data and materials availability: All data needed to evaluate the conclusions in the paper are present in the paper and/or the Supplementary Materials. Additional data related to this paper may be requested from the authors.

Submitted 25 May 2020
Accepted 31 August 2020
Published 16 October 2020
10.1126/sciadv.abc9317

Citation: E. Saito, S. J. Gurczynski, K. R. Kramer, C. A. Wilke, S. D. Miller, B. B. Moore, L. D. Shea, Modulating lung immune cells by pulmonary delivery of antigen-specific nanoparticles to treat autoimmune disease. Sci. Adv. 6, eabc9317 (2020).
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Sci Adv 6 (42), eabc9317
DOI: 10.1126/sciadv.eabc9317