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# An antigen-specific semi-therapeutic treatment with local delivery of tolerogenic factors through a dual-sized microparticle system blocks experimental autoimmune encephalomyelitis

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ABSTRACT

Antigen-specific treatments are highly desirable for autoimmune diseases in contrast to treatments which induce systemic immunosuppression. A novel antigen-specific therapy has been developed which, when administered semi-therapeutically, is highly efficacious in the treatment of the mouse model for multiple sclerosis, experimental autoimmune encephalomyelitis (EAE). The treatment uses dual-sized, polymeric microparticles (dMPs) loaded with specific antigen and tolerizing factors for intra- and extra-cellular delivery, designed to recruit and modulate dendritic cells toward a tolerogenic phenotype without systemic release. This approach demonstrated robust efficacy and provided complete protection against disease. Therapeutic efficacy required encapsulation of the factors in controlled-release microparticles and was antigen-specific. Disease blocking was associated with a reduction of infiltrating $CD4^+$ T cells, inflammatory cytokine-producing pathogenic $CD4^+$ T cells, and activated macrophages and microglia in the central nervous system. Furthermore, $CD4^+$ T cells isolated from dMP-treated mice were anergic in response to disease-specific, antigen-loaded splenocytes. Additionally, the frequency of $CD86^{hi}MHCII^{hi}$ dendritic cells in draining lymph nodes of EAE mice treated with Ag-specific dMPs was reduced. Our findings highlight the efficacy of microparticle-based drug delivery platform to mediate antigen-specific tolerance, and suggest that such a multi-factor combinatorial approach can act to block autoimmunity.



## 1. Introduction

Multiple sclerosis (MS) is an immune-mediated neurological disease that typically affects young adults with higher prevalence in females [1–3]. MS is a complex inflammatory disease of the central nervous system (CNS) where immune cells target and destroy oligodendrocytes and myelin sheath on nerve cells causing autoimmune demyelination [4,5]. The precise instigating factor(s) that initiates MS remains unknown [4], but it is well established that proinflammatory $CD4^+$ T cells are important in mediating MS pathogenesis, as well as that of experimental autoimmune encephalomyelitis (EAE), an animal model of MS [6]. Blood circulating $CD4^+$ T cells from MS patients have been shown to recognize myelin oligodendrocyte glycoprotein (MOG) and myelin basic protein (MBP), two myelin-associated proteins shown to play a role in MS pathogenesis and used as basis for EAE induction [7–9]. Several subsets of proinflammatory $CD4^+$ T cells have been implicated as crucial drivers of EAE, namely Th17 and Th1 cells. Th17 cells are $CD4^+$ T cells that express the lineage transcription factor Rorγt and produce the proinflammatory cytokines IL-17A, IL-17F, and, in the setting of EAE, GM-CSF [10–14], while Th1 cells express the lineage transcription factor T-bet and produce the proinflammatory cytokine IFNγ, and were also demonstrated to be important in EAE disease pathogenesis [15]. Defects in Th17 and Th1 cells or GM-CSF production prevented disease in EAE, thus solidifying the central role of proinflammatory $CD4^+$ T cells and the corresponding cytokines IL-17A, IFNγ, and GM-CSF in EAE [13,15].

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MS does not have a cure and current therapeutic options are limited. In the acute setting of MS exacerbation/relapse, methylprednisolone or other corticosteroids are used to provide immunosuppression [16]. Long-term management of MS involves disease-modifying therapies that may be poorly tolerated, inadequate in controlling disease, or incur life-threatening side effects and opportunistic infections [17]. Type 1 interferon monotherapy or in combination with glatiramer acetate is generally the safest and most tolerated therapy but often with inadequate response rate [18]. Natalizumab, a monoclonal antibody targeting the α4 integrin, thereby blocking leukocyte trafficking into the CNS, is highly effective in relapsing-remitting MS treatment, but it may cause progressive multifocal leukoencephalopathy (PML), a life-threatening opportunistic viral infection of the CNS [19]. Fingolimod, a sphingosine-1-phosphate receptor modulator, has been approved for oral treatment of MS, but it has multiple severe side effects, among which are PML and other infections [20]. Immunosuppressive regimens have also been used in MS treatment, including the purine analogue Cladribine, the immune modulator Laquinimod, anti-CD52 monoclonal antibody Alemtuzumab, anti-CD25 monoclonal antibody Daclizumab, and anti-CD20 monoclonal antibody Rituximab, but all have side effects related to immune suppression [16,21,22].

The goal in MS treatment is to develop therapies that induce tolerization of antigen-specific $CD4^+$ T cells, without generalized non-specific immune suppression. Such a goal can be achieved by generation of tolerogenic dendritic cells (DCs), known to exhibit 'semi-mature' profiles and induce antigen-specific T cell anergy or generation of regulatory T ($T_{reg}$) cells [23]. Exogenous generation of autologous DCs has been a major focus of the field, yet has numerous limitations including high cost, poor yield, and inefficient DC homing to regional lymph nodes upon re-administration [24–27]. As an alternative, polymeric particle vaccines are being pursued for the targeted delivery of conditioning factors to DCs *in vivo* [28–30]. Desirable key features of particle vaccines for immunotherapy include: control over phagocytosability, delivery of antigen to DCs, and local release of desired agents. Numerous materials have been investigated in particle-based biodegradable drug delivery, with a number of groups investigating vaccines consisting of antigen-loaded particles to target DCs [31–36]. Microparticles (MPs) fabricated from poly (lactic-*co*-glycolic acid) (PLGA) have been the most investigated vehicles for delivering immunotherapeutics. PLGA is approved by the U.S. Food and Drug Administration for use in numerous applications such as biodegradable surgical sutures and drug delivery products, which makes it extremely attractive for development of products quickly translatable to clinical use. PLGA is degraded in the body via bulk erosion and hydrolysis. PLGA MPs of ~1 μm are phagocytosed efficiently by antigen presenting cells (APCs), providing directed delivery of antigens for immune recognition [37]. Following phagocytosis by APCs, sufficient levels of endosomal release of encapsulated antigens from PLGA microparticles have been demonstrated to generate both MHC II-directed immune responses, as well as MHC I-directed immune responses through cross-presentation [38–40]. Given this capability along with a long-standing safety history, PLGA particles are therefore an excellent candidate as a carrier vehicle for vaccines utilizing encapsulated antigenic proteins or peptides along with immunomodulatory agents with intracellular targets.

We have developed a novel combinatorial dual-sized MP system (dMP), encapsulating, in addition to specific antigens, agents selected for their capacity to modulate DC function: both through intracellular delivery of agents encapsulated in small (~1 μm) MPs, and subcutaneous local deposition of agents for controlled release in MPs too large to phagocytose (~50 μm) [41,42]. Our dMP system combines the attractive notion of antigen-specificity and combination therapy with our dual-sized controlled release scheme to provide immune modulation without systemic delivery. Specifically two sizes of MPs are used in the dual MP (dMP) system (Fig. 1): (1) phagocytosable ~1 μm MP for delivery of antigen (Ag) and drugs to intracellular targets within phagocytes, and (2) non-phagocytosable ~50 μm MP for controlled release of factors targeted to cell surface receptors in a localized microenvironment. Two phagocytosable MPs are used: (*a*) MPs encapsulating MS-specific antigens, myelin oligodendrocyte glycoprotein peptide ($MOG_{35-55}$), or as a control, the irrelevant $OVA_{323-339}$ peptide, and (*b*) MPs loaded with vitamin D3 (VD3). Two non-phagocytosable MPs are used: (*c*) MPs encapsulating TGF-β1, and (*d*) MPs encapsulating GM-CSF. These four MPs are mixed in equal mass and administered subcutaneously.

The active metabolite of vitamin $D_3$ ($VD_3$), $1\alpha,25(OH)_2D_3$, has been shown to induce tolerogenic DCs through oxidative and glycolytic metabolic pathways, where glucose, glycolysis, and PI3K/Akt/mTOR are essential, and is intracellularly delivered to its nuclear receptor [43,44]. Transforming growth factor-beta 1 (TGF-β1) is loaded into the 50 μm MP to provide extracellular release to target its receptor on the DC surface. TGF-β1 treated-DCs show reduced expression of MHC II, costimulatory molecules and inflammatory cytokines, as well as increased production of IDO [45]. Exposure of T cells to TGF-β1 treated-DCs results in the induction of antigen-specific $T_{reg}$ cells, as well as deletion of antigen-reactive effector T cells [46]. Granulocyte-macrophage colony-stimulating factor (GM-CSF) is also separately loaded into a 50 μm MP for extracellular release, to locally attract and sustain DCs, as has been reported in a cancer vaccine approach [47]. Although it is the case that in EAE GM-CSF is produced by pathogenic $CD4^+$ T cells localized in the CNS [13,14] and can potentiate activation of innate leukocytes [48], in low doses and provided locally, GM-CSF is a critical mediator in the differentiation and development of tolerogenic DCs with low expression of MHC-II/CD80/CD86, as well as low inflammatory cytokines, and increased PD-L1 expression [48–50]. In sum, the four factors are loaded into separate MPs, with those targeting intracellular pathways in phagocytosable MPs and those targeting surface receptors in non-phagocytosable MPs, hence, dual-sized. We have recently demonstrated the utility of a similar formulation of this multi-factor combinatorial dMP system in the early prevention of autoimmune type 1 diabetes in NOD mice, where a diabetes disease-relevant antigen insulin was incorporated [41].

Myelin antigen-coupled PLG sub-micron particles have previously been used in preventive and therapeutic regimens for relapsing/remitting EAE by intravenous administration, but was inefficient when administered subcutaneously [51]. Alternatively, intraperitoneal administration of nanoparticles delivering myelin antigen and aryl hydrocarbon receptor ligands promoted tolerogenic DCs, expanded $T_{reg}$ cells, and mitigated EAE [52]. Treatment approaches for EAE are defined as follows: (*a*) preventative/prophylactic treatment is when factors are administered before EAE disease induction, (*b*) therapeutic treatment is applied when agents are delivered after appearance of clinical EAE disease signs, and (*c*) semi-therapeutic regimen is used when agents are administered after EAE disease induction but before clinical disease signs [51,53]. The multi-factor dMP treatment investigated here offers the advantage of a subcutaneous localized administration, as opposed to systemic administration, with low-dose, localized, controlled release of specific factors designed to be retained at the injection site. We show that this dMP approach does not result in an increase of the tolerogenic factors systemically, efficiently treats EAE in a semi-therapeutic regimen, and is antigen-specific.