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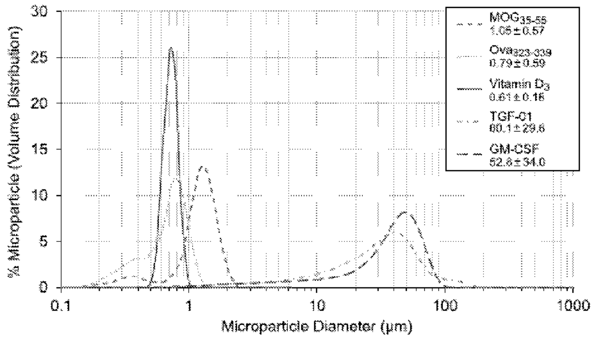

FIG. 1A

Biological/Pharmacologic Agent	Mass Loaded/PLGA (μg/500 mg)	Encapsulation Efficiency ± SD (%)	Mass Injected per 2.5 mg PLGA ± SD (ng)
MOG_{35-55}	4000	48.6 ± 9.0	9,711± 1,809
$OVA_{323-339}$	4000	49.9 ± 2.8	9,988± 556
Vitamin D_3	50	65.5 ± 3.0	164 ± 8
TGF-01	25	44.2 ± 12.1	55 ± 15
GM-CSF	40	59.6 ± 6.8	119 ± 14

FIG. 1B

(57) **Abstract:** Provided are a dual microparticle system to treat Multiple Sclerosis, the system comprising phagocytosable and non-phagocytosable microparticles for delivery of at least one antigen, at least one immunomodulatory agent, at least one immunosuppressive agent and at least one chemoattractant to a subject suffering from Multiple Sclerosis to generate tolerogenic dendritic cells in the subject and treat the Multiple Sclerosis.

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MICROPARTICLE SYSTEMS AND THEIR USE FOR THE TREATMENT OF MULTIPLE SCLEROSIS

GOVERNMENT SUPPORT

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BACKGROUND OF THE INVENTION

Autoimmune diseases are frequently associated with a reduction in the number and function of regulatory T cells. These cells are known to suppress the levels of physiologic auto-reactive T cells. When the levels of auto-reactive T cells are elevated pathological destruction of desirable cells can result.

Dendritic cells (DCs) play a critical role in the maintenance of peripheral tolerance. DCs promote T_{reg} induction, thereby suppressing excessive immune responses. Dendritic cell-based vaccines have been demonstrated to promote tolerance through antigen-presenting cells (APCs). APCs process and present self-peptides in a tolerogenic manner to T-cells, and induce T_{reg} proliferation. Apoptotic cells express surface ligands recognized by APCs via surface molecules such as the phosphatidyl serine (PS) receptor, CD 47, CD 36, and $\alpha v\beta_3$. Recent studies imply that these receptors could inhibit DC maturation and induce tolerance.

The present use of dendritic cell-based vaccines, however, suffers from several limitations. For instance, the present approach typically requires ex-vivo manipulation of patients' cells, which can adversely affect patient safety, and is associated with high cost.

The incidence rates of immune-mediated diseases are reaching epidemic proportions in the US, with millions of individuals suffering from autoimmune disorders such as multiple sclerosis (MS), type 1 diabetes, rheumatoid arthritis, and systemic lupus erythematosus, amongst others. No true cure exists for these conditions, posing a significant long-term risk to the affected individual and increasing our society’s healthcare burden.

While non-specific immune-suppressive agents remain the current standard-of-care for many autoimmune disorders, such therapies are often associated with significant off-target actions as well as side-effects resulting from their targeting complex pathways. As a result, the biomedical research community has increasingly sought to identify an improved means for inducing specific "immune tolerance" (i.e., failure to respond to self) as an approach to overcome autoimmune disease.

Recently, it has become increasingly clear that changes in the approach to autoimmune disease therapy need to be developed, capitalizing on how the body normally maintains self-tolerance, by harnessing an individual's own immune system. Key to implementing this paradigm shift will be to take advantage of the immune system's reliance on DCs, the professional antigen presenting cell and master-regulator of the immune response.

Tolerogenic DCs maintain antigen-specific T-cell tolerance either directly by inducing anergy, apoptosis, or phenotype skewing or indirectly by induction of regulatory T cells (Tregs). Therefore, therapeutic vaccination approaches for MS utilizing DCs holds great promise to correct antigen-specific autoimmune responses; yet therapies involving exogenous generation and manipulation of DCs possess numerous shortcomings including unsustainable antigen presentation, inefficient homing of DCs to the lymphatic system, and critically high treatment costs from the isolation and storage of DCs.

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 auto-immune 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].

MS does not have a cure and current therapeutic options are limited. In the acute setting of MS exacerbation/relapse, methyl- prednisolone 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].

Thus, there is a need for developing alternative vaccine compositions that are effectively delivered to target cells to treat diseases like MS.

SUMMARY

The present invention provides antigen-specific, tolerance-inducing microparticle systems for targeted delivery to immune system cells in order to treat multiple sclerosis (MS).

Antigen-specific treatments are highly desirable for autoimmune diseases in contrast to treatments that induce systemic immunosuppression. The subject invention provides an antigen-specific therapy for the treatment of multiple sclerosis.

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 demonstrates robust efficacy and provides protection against disease.

In one embodiment, the invention provides a method of inducing antigen-specific immune tolerance in a subject who has MS. The method involves administering a dual microparticle system that targets antigen-presenting immune cells in the subject. One set of microparticles is phagocytosable by the antigen-presenting immune cell of interest, and the other set of microparticles is non-phagocytosable by the antigen-presenting immune cell.

The phagocytosable microparticles together comprise at least one antigen and at least one immunomodulatory agent. The non-phagocytosable microparticles together comprise at least one immunosuppressive tolerogenic agent and at least one agent that recruits the antigen-presenting immune cell of interest.

The immunosuppressive tolerogenic agent can be for example, IL-10, TGF-β, or a nonsteroidal anti-inflammatory drug (NSAID). The recruiting agent can be, for example, GM-CSF, G-CFS, M-CSF, CCL19, CCL20, CCL 21 or VEGF-C.

The composition of the subject invention can further comprise a remyelinating agent selected from clemastine, clobetasol, digoxin, miconazole, phenytoin, and quetiapine; wherein the remyelinating agent is administered in soluble form by intravenous injection or is incorporated into the non-phagocytosable microparticles.

The phagocytosable microparticle and the non-phagocytosable microparticles are made of a biodegradable material.

The therapeutic efficacy of the compositions of the subject invention is improved by encapsulation of the factors in controlled-release microparticles.

BRIEF DESCRIPTION OF THE DRAWINGS

Figures 1A-1B show dual microparticle (dMP) fabrication and characterization. **Figure 1A** shows the quantification of microparticle sizes by dynamic light scattering analyses with average sizes of ~0.8 μm for vitamin D3, MOG_{35-55}, and $OVA_{323-339}$ MPs, and average ~55 μm for TGF-β1 and GM-CSF MPs. **Figure 1B** shows the loading amount, encapsulation efficiency, and dose per injection for MPs containing each biological/pharmacological agent.

Figures 2A-2M show subcutaneous DC recruitment and tolerization, and microparticle-associated cell trafficking to local lymph nodes *in vivo*. **Figure 2A** shows the subcutaneous dMP injection sites (nodules) in B6 mice excised 8 days after administration and the evaluation for cellular infiltration by hematoxylin and eosin staining; scale bar represents 400 μm. **Figure 2B** shows representative flow cytometry analysis of DC recruitment in dMP or unloaded MP nodules. A fluorescence minus one (FMO) control, containing all antibodies except CD11c, was assessed to determine gating of DCs. **Figure 2C** shows the total frequency of DCs in respective nodules as a percent of live $CD45^+$ cells. **Figure 2D** shows a representative flow cytometry analysis for DC surface expression of co-stimulatory molecule CD86. **Figure 2E** shows the total frequency of $CD86^+$ expression on DCs isolated from respective nodules. **Figure 2F** shows DC ($Lyg6\text{-}CD11c^+$), macrophage ($Lyg6\text{-}CD11c\text{-}CD11b^+$), and neutrophil ($Lyg6^+$) MP uptake in dMP or unloaded MP nodules as a percentage of total phagocytosed MPs ($CD45^+DiO^+$). **Figure 2G** shows inguinal lymph nodes (ILNs) that were excised at 24 and 48 h after dMP injection and analyzed for dMP^+ phagocyte populations. **Figure 2H** shows PD-L1 expression of DCs isolated from ILNs 24 h after MP injection and analyzed according to dMP or unloaded MP phagocytosis. **Figure 2I** shows MHC-II expression of DCs isolated from ILNs 48 h after MP injection and analyzed according to dMP or unloaded MP phagocytosis. **Figure 2J** shows the proximal draining lymph nodes (axillary [ALN] and inguinal [ILN]) and distal lymphoid organs (mesenteric lymph nodes [MLN] and spleen) that were excised eight days after dMP injection and analyzed via flow cytometry for MP trafficking. The frequency of dMP^+ DCs as a percent of total DCs is characterized in mice that received either no MPs (No Treatment) or the dMP. **Figure 2K** shows dMP distribution across phagocyte populations at the eight day time point in proximal draining lymph nodes as a percent of total dMP^+ cells. **Figure 2L** shows serum TGF-β1 and **Figure 2M** shows GM-CSF levels (pg/mL) measured by ELISA from mice without treatment, mice days 2, 4, and 7 after subcutaneous dMP treatment, and mice with intravenous injection of TGF-β1 and GM-CSF immediately prior to blood collection. n = 3-5 per group. p values were obtained from Student's t tests (C, E, F, G, and J) or one-way

ANOVA with Tukey's post-hoc analysis (H, I, K, L, and M), * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, n.s. = $p > 0.05$ (not significant), ND (not detectable).

Figures 3A-3B show that the dMP-MOG$_{35-55}$ formulation blocks experimental autoimmune encephalomyelitis in a treatment setting. **Figure 3A** shows the EAE disease score (mean ± SEM) of B6 mice treated with either dMP MOG$_{35-55}$ (filled circle) or dMP formulation without MOG$_{35-55}$ (unloaded 0.8 μm MP were substituted) (open square) on days 4, 7, and 10 (filled arrows) following EAE induction; n = 10 per group. p value was obtained from Student's *t*-test. **Figure 3B** shows the EAE disease score trend of B6 mice treated with either dMP MOG$_{35-55}$ formulation (filled circle) or dMP formulation without MOG$_{35-55}$ (open square) on days 4, 7, and 10 following EAE induction; n = 10 per group. p value was obtained from ANOVA.

Figures 4A-4C show that EAE mice treated with dMP-MOG$_{35-55}$ have reduced leukocytes and CD4$^+$ T cells infiltrating into the CNS. **Figure 4A** shows a representative hematoxylin and eosin staining of spinal cord section from B6 EAE mice treated either with dMP MOG$_{35-55}$ or soluble factors co-administered with empty MPs (S+U MPs); scale bar represents 200 μm; n = 10 per group. **Figure 4B** shows a representative flow cytometry analysis performed on day 21 following EAE induction of live CD4$^+$ and CD8$^+$ T cell frequencies from CNS of mice treated on day 4 following EAE induction either with dMP MOG$_{35-55}$ or S+U MPs; n = 5 per group. **Figure 4C** shows the absolute numbers of CD4$^+$ T cells in CNS of healthy naïve mice, or on day 21 following EAE induction of mice treated on day 4 following EAE induction either with dMP MOG$_{35-55}$ or S+U MPs. p value was obtained from Student's *t*-test.

Figures 5A-5B show that EAE mice treated with dMP-MOG$_{35-55}$ have reduced CD4$^+$ T cells producing IL-17A, GM-CSF and IFNγ in the CNS. **Figure 5A** shows the representative frequencies of CD4$^+$ T cells positive for IL-17A, GM-CSF, IFNγ, and dual cytokines analyzed by intracellular cytokine staining and flow cytometry on day 21 following EAE induction in the brain of EAE mice treated on day 4 following EAE induction either with dMP MOG$_{35-55}$ or S+U MPs; n = 5 per group. **Figure 5B** shows the absolute numbers of the CNS-infiltrating CD4$^+$ T cells producing the indicated cytokines on day 21 following

EAE induction in the brain of EAE mice treated on day 4 following EAE induction either with dMP $MOG_{35\text{-}55}$ or S+U MPs; n = 5 per group. p value was obtained from Student's *t*-test.

Figures 6A-6B show that EAE mice treated with dMP-$MOG_{35\text{-}55}$ have decreased pathogenic $CD4^+$ T cells expressing the transcription factors Rorγt and T-bet in the CNS. **Figure 6A** shows a representative intranuclear flow cytometry analysis of frequencies of $Ror\gamma t^+$, $T\text{-}bet^+$, and $Ror\gamma t^+T\text{-}bet^+$ CD4+ T cells on day 21 following EAE induction in the brain of EAE mice treated on day 4 following EAE induction either with dMP $MOG_{35\text{-}55}$ or S+U MPs; n = 5 per group. **Figure 6B** shows the absolute numbers of the CNS-infiltrating $Ror\gamma t^+$, $T\text{-}bet^+$, and $Ror\gamma t^+T\text{-}bet^+$ $CD4^+$ T cells on day 21 following EAE induction of EAE mice treated on day 4 following EAE induction either with dMP $MOG_{35\text{-}55}$ or S+U MPs; n = 5 per group. p value was obtained from Student's *t*-test.

Figures 7A-7B show that activated macrophages/microglial cells are reduced in the CNS of mice treated with dMP-$MOG_{35\text{-}55}$. **Figure 7A** shows a representative flow cytometry analysis performed on day 21 following EAE induction of leukocytes isolated from CNS of mice treated on day 4 following EAE induction either with dMP $MOG_{35\text{-}55}$ or S+U MPs; n = 5 per group. **Figure 7B** shows the absolute numbers of activated microglia/macrophages on day 21 following EAE induction in CNS of mice treated on day 4 following EAE induction either with dMP $MOG_{35\text{-}55}$ or S+U MPs; n = 5 per group. Live leukocytes were gated on $CD11b^+CD11c^+CD68^+CD45^+$ and further on F4/80 and CD80 high. p value was obtained from Student's *t*-test.

Figures 8A-8B show that the efficacy of dMP treatment is dependent on antigen specificity. **Figure 8A** shows the EAE disease score (mean ± SEM) of B6 mice treated with either dMP $MOG_{35\text{-}55}$ (filled circle) or dMP $Ova_{323\text{-}339}$ (open square) on days 4 and 7 (filled arrows) following EAE induction; n =10 per group. p value was obtained from Student's *t*-test. **Figure 8B** shows the EAE disease score trend of B6 mice treated with either dMP $MOG_{35\text{-}55}$ (filled circle) or dMP $Ova_{323\text{-}339}$ (open square) on days 4 and 7 following EAE induction; n = 10 per group. p value was obtained from ANOVA.

Figures 9A-9B show that T cells from EAE mice treated with dMP-MOG$_{35-55}$, but not with dMP-Ova$_{323-339}$, failed to expand in response to MOG$_{35-55}$-dependent stimulation. **Figure 9A** shows a representative flow cytometry analysis of CFSE level in CD4$^+$ T cells isolated on day 21 following EAE induction from draining lymph nodes of B6 mice treated with either dMP MOG$_{35-55}$ (dash line) or dMP Ova$_{35-55}$ (solid line) on days 4 and 7 (filled arrows) following EAE induction after 72 h co-culture with T-cell depleted splenocytes loaded with MOG$_{35-55}$. **Figure 9B** shows the rrequencies of CFSE negative CD4$^+$ T cells isolated on day 21 following EAE induction from draining lymph nodes of B6 mice treated with either dMP MOG$_{35-55}$ (dash line) or dMP Ova$_{323-339}$ (solid line) on days 4 and 7 (filled arrows) following EAE induction after 72 h co-culture with T-cell depleted splenocytes loaded with MOG$_{35-55}$. p value was obtained from Student's t-test.

Figures 10A-10C show that dendritic cells from draining lymph nodes of EAE mice treated with dMP-MOG$_{35-55}$ displayed a tolerized phenotype. **Figure 10A** shows a representative flow cytometry analysis of CD86 and MHCII expression on CD11b$^+$CD11c$^+$ dendritic cells isolated on days 13 and 25 from draining lymph nodes of mice treated on day 4 and 7 following EAE induction either with dMP MOG$_{35-55}$ or dMP Ova$_{323-339}$; n = 4 per group. **Figure 10B** shows the frequency of CD86^{hi}MHCII^{hi}CD11b$^+$CD11c$^+$ dendritic cells isolated on days 13 and 25 from draining lymph nodes of mice treated on day 4 and 7 following EAE induction either with dMP MOG$_{35-55}$ or dMP Ova$_{323-339}$; n = 4 per group. **Figure 10C** shows the mean fluorescence intensity of CD86 in CD11b$^+$CD11c$^+$ dendritic cells isolated on days 13 and 25 from draining lymph nodes of mice treated on day 4 and 7 following EAE induction either with dMP MOG$_{35-55}$ or dMP Ova$_{323-339}$; n = 4 per group. p value was obtained from Student's *t*-test.

Figure 11 shows sequences of antigens included in the microparticles of the subject invention.

Figure 12 shows that dMP MOG$_{35-55}$ treatment halts EAE when initiated at the onset of disease. EAE disease score (mean±SEM) of B6 mice treated with dMP MOG$_{35-55}$ (blue square) or dMP OVA$_{323-339}$ (black circle). Mice were induced with EAE and treated when they reached a score of one (onset) (first dose) and after three additional days (second dose).

The range of days is due to rolling admission. n=5-7 per group. * denotes $p<0.05$ using Mann-Whitney U Test. Clinical scoring: score 1: flaccid tail, score 2: weak hind limbs, score 3: hind limb paralysis, score 4: quadriplegia.

Figure 13 shows that dMP MOG_{35-55} treatment at the peak of disease majorly reduced the scores and improves mobility of EAE mice. EAE disease score (mean±SEM) of B6 mice treated with dMP MOG_{35-55} (blue square) or dMP $OVA_{323-339}$ (black circle). Mice were induced with EAE and treated with two doses of dMP MOG_{35-55} or dMP $OVA_{323-339}$, first when they reached a score of 3 (peak) (first dose) and after 3 additional days (second dose). The range of days is due to rolling admission. n=5-7 per group. * denotes $p<0.05$ using Mann-Whitney U Test. Clinical scoring: score 1: flaccid tail, score 2: weak hind limbs, score 3: hind limb paralysis, score 4: quadriplegia.

BRIEF DESCRIPTION OF SEQUENCES

SEQ ID NO: 1 shows the amino acid sequence of human myelin oligodendrocyte glycoprotein (MOG) protein (GenBank: AQY76934.1)

SEQ ID NO: 2 shows the amino acid sequence of human myelin basic protein (MBP) (GenBank: NP_001020252.1)

SEQ ID NO: 3 shows the amino acid sequence of human proteolipid protein (PLP) (GenBank: P60201)

SEQ ID NO: 4 shows the amino acid sequence of human 2’,3’-cyclic-nucleotide 3’-phosphodiesterase (CNP) (GenBank: NP_149124.3)

SEQ ID NO: 5 shows the amino acids sequence of human myelin-associated glycoprotein (MAG) (GenBank: NP_002352)

SEQ ID NO: 6 shows the amino acid sequence of human myelin-associated oligodendrocyte basic protein (MOBP) (GenBank: Q13875.2.1)

SEQ ID NO: 7 shows the amino acid sequence of human S100 calcium binding protein B (S100β) (GenBank: AAH01766.1)

SEQ ID NO: 8 shows the amino acid sequence of human transaldolase (GenBank: NP_006746.1)

SEQ ID NO: 9 shows the amino acids sequence of human neurofascin (GenBank: O94856.4)

SEQ ID NO: 10 shows the amino acids sequence of human contactin (GenBank: CAA79696.1)

SEQ ID NO: 11 shows the amino acids sequence of human potassium-dependent channel KIR4.1 (GenBank: AAB07046.1)

SEQ ID NO: 12 shows the amino acid sequence portion of MBP pertaining to MBP_{13-32}

SEQ ID NO: 13 shows the amino acid sequence portion of MBP pertaining to MBP_{83-99}

SEQ ID NO: 14 shows the amino acid sequence portion of MBP pertaining to $MBP_{131-155}$

SEQ ID NO: 15 shows the amino acid sequence portion of MBP pertaining to $MBP_{146-170}$

SEQ ID NO: 16 shows the amino acid sequence portion of PLP pertaining to PLP_{40-60}

SEQ ID NO: 17 shows the amino acid sequence portion of PLP pertaining to PLP_{89-106}

SEQ ID NO: 18 shows the amino acid sequence portion of PLP pertaining to $PLP_{139-154}$

SEQ ID NO: 19 shows the amino acid sequence portion of PLP pertaining to $PLP_{178-197}$

SEQ ID NO: 20 shows the amino acid sequence portion of PLP pertaining to $PLP_{190-208}$

SEQ ID NO: 21 shows the amino acid sequence portion of MOG pertaining to MOG_{1-20}

SEQ ID NO: 22 shows the amino acid sequence portion of MOG pertaining to MOG_{11-30}

SEQ ID NO: 23 shows the amino acid sequence portion of MOG pertaining to MOG_{35-55}

SEQ ID NO: 24 shows the amino acid sequence portion of CNP pertaining to $CNP_{343-373}$

SEQ ID NO: 25 shows the amino acid sequence portion of CNP pertaining to $CNP_{356-388}$

DETAILED DESCRIPTION OF THE INVENTION

The present disclosure provides antigen-specific, tolerance-inducing microparticles and therapeutic compositions comprising the microparticles. Advantageously, the present disclosure allows for the targeted delivery of therapeutic agents to immune cells. In addition, certain embodimentsfacilitate sustained release of therapeutic agents. Advantageously, the microparticle delivery system of the present disclosure has antigen-specificity and *ex vivo* stability.

In one embodiment, provided is a microparticle for targeted delivery of one or more antigens along with immunomodulatory molecules to antigen-presenting cells (*e.g.*, dendritic cells or macrophages).

Delivered immunomodulatory molecules (*e.g.*, transforming growth factor beta 1 (TGF-β1), rapamycin, vitamin D and retinoic acid) can provide immunosuppressant/tolerogenic conditioning of antigen-presenting cells along with delivering an antigen depot for antigen-presenting cells to internalize and present to lymphocytes. Vaccine particles thus modulate antigen-presenting cell function to effect specific tolerance, suitable for the treatment of MS.

Also provided are therapeutic uses of composition embodiments for the prevention and/or treatment of MS.

Multiple sclerosis-like encephalomyelitis can be blocked using disclosed compositions by inducing tolerogenic dendritic cells, reducing infiltrating $CD4^+$ T cells, inflammatory cytokine-producing pathogenic $CD4^+$ T cells, and reducing macrophage and microglia activation in the central nervous system. Furthermore, $CD4^+$ T cells isolated from dMP-treated mice were anergic in response to disease-specific, antigen-loaded splenocytes.

The choice of TGF-β1, GM-CSF, and vitamin D3 for the current dMP system was based on the immunomodulatory and tolerogenic profiles of these compounds, which minimize the risk for global immunosuppression, unlike potent immunosuppressive agents like IL-10 or rapamycin.

Subcutaneously administered dMP-$MOG_{35\text{-}55}$ treatment suppressed EAE through reduction of total leukocytes, $CD4^+$ T cells, including those expressing inflammatory cytokine, and activated macrophages/microglia in the CNS. The relative strength of tolerance achieved according to the current invention is superior to the preventative/prophylactic and therapeutic regimen utilized for nanoparticle platforms described previously [18,19], in that the subject invention achieved total suppression of EAE clinical disease and drastic reduction of $CD4^+$ T cell infiltration into CNS.

The quality and timing of tolerance achieved herein with the dMP administered in semi-therapeutic regimen is superior compared to the preventative/prophylactic and therapeutic regimen reported previously for nanoparticle platforms, as it can be used after disease initiation, which has clinical implication for further development into a therapeutic to be administered after disease clinical sign onset.

Controlled-release platforms for immunomodulatory applications are advantageous versus soluble administration. In addition to mitigating the risk for systemic immunosuppression, soluble drugs are rapidly cleared from the body. Thus, pharmacokinetics may prevent soluble administration to effectively restore homeostatic immunity or require more frequent or higher dosing. The impact of biomaterial encapsulation in the subject invention was demonstrated by the requirement for encapsulation of drugs in MPs, as soluble injections of the factors along with unloaded MPs did not prevent T cell infiltration in the CNS. Importantly, the encapsulated drugs could not be detected in the blood, thus preventing global immune suppression following dMP administration.

Induction of antigen-specific tolerance is crucial to developing a safe, translatable therapy for EAE/MS, with the goal to achieve therapeutic efficacy without inducing broad immunosuppression.

By utilizing multiple control groups, it was demonstrated that the dMP-MOG_{35-55} treatment specifically suppressed EAE in an antigen-dependent manner. Comparing the dMP-MOG_{35-55} to a similar formulation without antigen-loaded MPs, it was demonstrated that omission of the antigen resulted in mice developing disease. Similarly, disease was only blocked with dMP-MOG_{35-55} treatment, but not by treatment with an irrelevant antigen, dMP-$Ova_{323-339}$.

The antigen-specificity in the newly developed microparticle-based immunotherapy is especially important because, *e.g.*, the safety profile of an MS antigen-specific tolerogenic regimen is significantly superior to that of other immunotherapies that do not rely on antigen-specificity [20-23]. The observation that $CD4^+$ T cells isolated from EAE mice treated with dMP-MOG_{35-55} were not responsive to stimulation by MOG_{35-55}-loaded splenocytes whereas EAE mice treated with dMP $Ova_{323-339}$ proliferated, showed that T cell anergy is effectively induced by the dMP-MOG_{35-55} therapy in an antigen-specific manner.

Together, these results highlight an exciting combinatorial, controlled release, and immunologically-driven approach that operates through a dMP system that delivers local sustained release of multiple immunomodulatory factors, and targets both intra- and extracellular tolerogenic receptors. Using the dMP system of the subject invention, robust and durable antigen-specific autoimmune protection was achieved, which protection was superior to soluble factors or irrelevant antigen formulations. Additionally, the dMP system of the subject invention is versatile because substitutions of antigen and/or factors have the potential to elicit tolerogenic or immunogenic responses in a tailored, disease-specific fashion.

Antigen-Specific Tolerogenic Compositions

Preferably, the microparticle matrix is made of, primarily, substantially biologically inert or biologically compatible materials. The terms "inert," "biologically inert" or "biologically compatible," as used herein, refer to a substance or material that, after the normal healing period when administered into living tissues, does not elicit substantially adverse biochemical, allergic, or unwanted immune responses.

Preferably, the present microparticle matrix is biodegradable. The term "biodegradable," as used herein, refers to the ability of materials to be broken down by normal chemical, biochemical and/or physical processes such as erosion, dissolution, corrosion, degradation, hydrolysis, abrasion, etc, and their combinations.

Biologically compatible materials useful for making the microparticles include, but are not limited to, bio-degradable polymeric materials including, but not limited to, hydrogels, collagen, alginate, poly(glycolic acid) (PGA), poly(L-lactic acid) (PLA), poly(DL-lactic-co-glycolic acid) (PLGA), polyethylene glycol (PEG), polyesters, polyanhydrides, polyorthoesters, polyamides; non-polymeric biodegradable ceramic materials including, but not limited to, calcium phosphate, hydroxyapatite, tricalcium phosphate; and combinations thereof. In preferred embodiments, microparticles are fabricated from poly(lactic-co-glycolic acid) (PLGA), which is FDA approved for delivery of therapeutics. Low molecular weight oligomeric forms of lactide and/or glycolide polymers have several advantages such as good mechanical properties, low immunogenicity and toxicity, excellent biocompatibility, and predictable biodegradation kinetics. Lactide/glycolide polymers are widely accepted for biomedical applications. The mechanical strength, swelling behavior, capacity to undergo hydrolysis, and subsequently the biodegradation rate are directly influenced by the crystallinity of the PLGA polymer and the crystallinity of the PLGA copolymer is dependent on the type and the molar ratio of the individual monomer components (lactide and glycolide) in the copolymer chain. For example, PLGA polymers containing a 50:50 ratio of lactic and glycolic acids are hydrolyzed much faster than those containing higher proportion of either of the two monomers.

The PLGA copolymers of the microparticles of the subject invention are designed in such a ratio that they allow the microparticle degradation to procced over a period of at least 15 days to about 80 days. In some embodiments, the polymers degrade within 16 days to 79 days; 18 days to 77 days; 20 days to 75 days; 22 days to 73 days; 24 days to 71 days; 26 days to 69 days; 28 days to 67 days; 30 days to 65 days; 32 days to 67 days; 34 days to 65 days; 36 days to 63 days; 38 days to 61 days; 40 days to 59 days; 42 days to 57 days; 44 days to 55 days; 46 days to 53 days; or 48 days to 51 days.

In specific embodiments, the non-phagocytosable microparticles are fabricated such that the PGLA degrades at between 20 days and 60 days. In other specific embodiments, the phagocytosable microparticles are fabricated such that they degrade readily in the endocytic compartment of a phagocyte.

In some embodiments, the subject invention provides an injectable hydrogel composition, wherein the hydrogel comprises or encapsulates therein non-phagocytosable microparticles comprising at least one agent for recruiting the immune cell of interest (*e.g.*, GM-CSF); and at least one immunosuppressive agent (*e.g.*, TGF-β1) and phagocytosable microparticles comprising at least one antigen (which can be an auto-antigen and/or allergen) and at least one immunomodulatory agent (*e.g.*, vitamin D3). In specific embodiments, the injectable, biodegradable hydrogel is fabricated via *in situ* gelling, and facilitates sustained-release of its ingredients for a prolong period of time (*e.g.*, several days).

In specific embodiments, the microparticles are fabricated of PGLA using single or double emulsions. In certain embodiments, the different factors including at least one antigen, at least one immunoregulatory agent, at least one immunosuppressive agent and at least one chemoattractant are encapsulated into PGLA microparticles separately to control encapsulation efficiency. In other embodiments, the several components are encapsulated together or in different mixtures into the microparticles. For example, in some microparticles at least one antigen and at least one immunomodulatory agent are encapsulated in the same microparticles. In other microparticles, at least one immunosuppressive agent and at least one chemoattractant are encapsulated in the same microparticles.

In some embodiments, the at least one antigen and the at least one immunomodulatory agent encapsulated into the same microparticles are present within the microparticles at a specified ratio to ensure optimal antigen stimulation and immune modulation of the antigen presenting cells. In other embodiments, the at least one antigen and the at least one immunomodulatory agent are encapsulated into separate phagocytosable microparticles and are administered at a ratio that ensures optimal antigen stimulation and immune modulation of antigen presenting cells.

In specific embodiments, at least one antigen is encapsulated with at least one immunomodulatory agent into the same microparticle or at least one antigen is encapsulated in one microparticle and at least one immunomodulatory agent is encapsulated in a separate microparticle and both microparticles are administered such that the at least one antigen and the at least one immunomodulatory agent are administered at a ratio of a low of 1:20 to a high of 1:1; and any ratio therebetween, such as about 1:19; about 1:18; about 1:17; about 1:16; about 1:15; about 1:14; about 1:13; about 1:12; about 1:11; about 1:10; about 1:9; about 1:8; about 1:7; about 1:6; about 1:5; about 1:4; about 1:3; and about 1:2.

In some embodiments, at least one immune suppressive agent and at least one chemoattractant are encapsulated into the same non-phagocytosable microparticles at a specified ratio to facilitate immunosuppression and chemoattraction of immune cells. In other embodiments, at least one immune suppressive agent and at least one chemoattractant are encapsulated into separate non-phagocytosable microparticles and are administered at a specified ratio to ensure optimal immunosuppression and chemoattraction of immune cells.

In specific embodiments, at least one immune suppressive agent is encapsulated with at least one chemoattractant into the same microparticles or at least one immune suppressive agent is encapsulated in one microparticle and at least one chemoattractant is encapsulated in a separate microparticle and both microparticles are administered such that the at least one immunosuppressive agent and the at least one chemoattractant are administered at a ratio of a low of 1:5 to a high of 5:1; and any ratio therebetween, such as about 1:4; about 1:3; about 1:2; about 1:1; about 2:1; about 3:1; and about 4:1.

For example, in a specific embodiment, phagocytosable microparticles comprising a myelin-derived antigen and phagocytosable microparticles comprising the immunomodulatory agent vitamin D3 are injected together subcutaneously into a subject such that about 30 to 50 mcg of the myelin-derived antigen per mg PGLA are administered together with about 60 to 70 ng of the immunomodulatory vitamin D3 per mg PGLA.

Further, together with the above phagocytosable microparticles are injected non-phagocytosable microparticles comprising the imumunosuppressive agent TGF-β1 and non-phagocytosable microparticles comprising the chemoattractant agent GM-CSF subcutaneously into the subject together with the phagocytosable microparticles described above such that about 20 to 25 ng of the immunosuppressive agent TGF-β1 per mg PGLA of the non-phagocytosable microparticles are administered together with about 40 to 55 ng of the chemoattractant GM-CSF per mg PGLA of the other non-phagocytosable microparticles.

Advantageously, the co-administration of the two types of phagocytosable microparticles and the two types of non-phagocytosable microparticles together subcutaneously into a subject in the described ratios of myelin-derived antigen, immunomodulatory agent, immunosuppressive agent and chemoattractant allows the optimized stimulation and priming of immune cells in the subject to induce an efficient amount of antigen-specific tolerogenic dendritic cells to treat, for example, MS in the subject.

In a preferred embodiment, the antigen is a myelin-derived antigen. The at least one myelin-derived antigen of the subject invention can be derived from a myelin-specific protein including, but not limited to, a myelin oligodendrocyte glycoprotein (MOG), a proteolipid protein (PLP), a myelin basic protein (MBP), a myelin-associated oligodendrocyte basic protein (MOBP), myelin-associated glycoprotein (MAG), a glatiramer acetate (a random polymer of L-alanine, L-glutamic acid, L-lysine, and L-tyrpsine), a 2′,3′-cyclic-nucleotide 3′-phosphodiesterase (CNP), a S100β protein, a transaldolase H, a neurofascin, a contactin, a potassium-dependent channel KIR4.1 or any protein linked to the pathogenesis of multiple sclerosis in humans [25, 25]. In preferred embodiments, the myelin-derived antigen is an antigen involved in the pathogenesis of multiple sclerosis. In further preferred embodiments, the myelin-derived antigen is MOG_{35-55}.

Other examples of antigens include, but are not limited to, MOG_{1-20}, MOG_{11-30}, MOG_{35-55}, MBP_{13-32}, MBP_{83-99}, $MBP_{111-129}$, $MBP_{146-170}$, PLP_{40-60}, peptide PLP_{89-106}, $PLP_{139-154}$, $PLP_{178-197}$, $PLP_{190-208}$, $CNP_{343-373}$, and $CNP_{356-388}$.

For example, a composition of the subject invention can comprise phagocytosable microparticles comprising 400-700 mcg/kg MOG and/or 8-12 mcg/kg Vitamin D3 and non-phagocytosable microparticles comprising 2-5 mcg/kg TGF-β1 and/or 5-9 mcg/kg GM-CSF.

The at least one antigen to be used in the composition of the subject invention can be a peptide of any length comprising a low of 5 amino acids (aa) to a high of 100 aa of a myelin-related protein and any length in between, such as about 5 aa to about 95 aa; about 5 aa to about 90 aa; about 5 aa to about 85 aa; about 5 aa to about 80 aa; about 5aa to about 75 aa; about 5 aa to about 70 aa; about 5 aa to about 65 aa; about 5 aa to about 60 aa; about 5 aa to about 55 aa; about 5 aa to about 50 ; about 5 aa to about 45 aa; about 5 aa to about 40; about 5 aa to about 35aa ; about 5 a to about 30 aa; about 5 aa to about 25 aa; about 5 aa to about 20 aa; about 5aa to about 15 aa; about 5aa to about 14 aa; about 5 aa to about 13 aa; about 5 aa to about 12 aa; about 5aa to about 11 aa; about 5 aa to about 10 aa; about 5 aa to about 9 aa; about 5 aa to about 8; about 5 aa to about 7 aa; and about 5 aa to about 6 aa.

In preferred embodiments, the antigen is a peptide of about 5 aa to about 12 aa. In more preferred embodiment, the antigen is a peptide of about 5 aa to about 10 aa. In most preferred embodiments, the antigen is a peptide of about 5 aa to about 9 aa.

In some embodiments, the immunomodulatory agent used in the composition of the subject invention is selected from vitamin D3, vitamin D3 analogs, glucocorticoids, estrogens, rapamycin, and retinoic acid. In preferred embodiments, the immunomodulatory agent is vitamin D3 or a vitamin D3 analog, a glucocorticoid, an estrogen, or retinoic acid. In more preferred embodiments, the immunomodulatory agent is vitamin D3 or a vitamin D3 analog.

Anti-inflammatory or immunosuppressive agents useful according to the present invention include TGF-β1, IL-10, INF-γ and INF-λ and nonsteroidal anti-inflammatory drugs (NSAIDs) such as aspirin and ibuprofen; naproxen; and triterpinoids such as betulinic acid, bardoxolone methyl, and triterpenoid saponins.

In some embodiments, the immunosuppressive agent used in the composition of the subject invention is selected from TGF-β1, IL-10, INF-γ, INF-λ, and nonsteroidal anti-inflammatory drugs. In specific embodiments, the immunosuppressive agent is TGF-β1, IL-10 or IFN-γ. In more specific embodiments, the immunosuppressive agent is TGF-β1.

A variety of agents that recruit or attract immune cells are also known. For example, chemoattractants that recruit dendritic cells include granulocyte macrophage colony stimulating factor (GM-CSF), granulocyte-colony stimulating factor (G-CSF), macrophage colony-stimulating factor (M-CSF), C-C motif chemokine ligand 19 (CCL19), C-C motif chemokine ligand 20 (CCL 20), C-C motif chemokine ligand 21 (CCL21) and vascular endothelial growth factor C (VEGF-C). Preferably, GM-CSF, which selectively attracts immature dendritic cells, is used in the present invention.

In some embodiments, the composition of the subject invention comprises a remyelinating agent selected from clemastine, clobetasol, digoxin, miconazole, phenytoin, and quetiapine. Said remyelinating agent can be administered in soluble form by intravenous injection or can be incorporated into the non-phagocytosable microparticles.

Advantageously, the compositions disclosed herein comprising a dual microparticle system in a liquid formulation provide spatial and temporal control of the release of the cargo, *e.g.*, antigen, immunomodulatory, immunosuppressive, and chemoattractive agents after administration to a subject in such a manner that tolerogenic dendritic cells are generated in the subject. Importantly, the composition ensures that the respective components are provided in the amounts and/or ratios such that antigen presenting cells are recruited within the subject from a large area surrounding the administration site and stimulated with antigen and immunomodulatory agents to ensure the induction of a tolerogenic phenotype in antigen presenting cells. Antigen presenting cells primed by the phagocytosed microparticles are then attracted to the site close to the administration site by chemoattractants released by the non-phagocytosable microparticles and the attracted cells are exposed to sufficient amounts of immune suppressive agents to ensure the generation of tolergenic dendritic cells. Such induced tolerogenic dendritic cells, in turn, promote the induction of regulatory T cells and suppress auto-reactive T-cells.

Advantageously, certain embodiments provide a means to improve the generation of antigen-specific tolerogenic dendritic cells. In specific embodiments, an immunological pathomechanism in the brain, *e.g.*, in MS, can be effectively treated using the compositions of the subject invention.

The phagocytosable microparticles disclosed herein are designed to deliver their cargo into antigen presenting cells. Therefore, the phagocytosable microparticles have a size that avoids pinocytosis, *i.e.*, uptake into fluid vesicles of non-antigen presenting cells because such pinocytotic uptake reduces the amount of microparticles administered that can be taken up by antigen presenting cells. Phagocytosis allows for endosomal release of encapsulated antigens and therapeutic agents from a polymeric matrix such as PLGA to intracellular targets. In specific embodiments, the microparticles disclosed herein generate both MHC-II-directed and MHC-I-directed immune responses through cross-presentation.

The phagocytosable microparticles are designed to be of a size that facilitates farther distribution of these particles from the site of administration in a subject allowing for a larger area to receive phagocytosable microparticles delivering at least one antigen and at least one immunomodulatory agent.

The non-phagocytosable microparticles are designed to avoid any cellular uptake and allow extracellular release of their cargo over a desired period of time. Furthermore, the non-phagocytosable microparticles are designed in such a size that the particles are prevented from moving over a far distance from the administration site in the subject to allow a localized release and, thus, high concentration of the at least one immunosuppressive agent and the at least one chemoattractant encapsulated in the non-phagocytosable microparticles.

In specific embodiments, the microparticles phagocytosable by dendritic cells have a diameter in the range of 0.1 µm to 10.0 µm, or any range therebetween, such as 0.2 µm to 8.0 µm; 0.3 µm to 5.0 µm; 0.4 µm to 3.0 µm; 0.5 µm to 2.0 µm; 0.6 µm to 1.0 µm. In certain embodiments, the microparticle has a diameter of about 0.2 µm to 3.0 µm. In preferred embodiments, the phagocytosable microparticles are 0.2 µm to 5.0 µm or 0.3 µm to 2.0 µm. In more specific embodiments, the phagocytosable microparticles are 0.6 µm to 1.0 µm in diameter.

In certain embodiments, the microparticles are non-phagocytosable by dendritic cells and have a diameter in the range of about 15 µm to 200 µm; or any range therebetween, such as 15 µm to 180 µm; 15 µm to 150 µm; 15 µm to 120 µm; 15 µm to 100 µm; 15 µm to 80 µm; 15 µm to 60 µm; 15 µm to 50 µm; 15 µm to 40 µm; 15 µm to 30 µm; 15 µm to 20 µm; 20 µm to 200 µm; 20 µm to 180 µm; 20 µm to 150 µm; 20 µm to 120 µm; 20 µm to 100 µm; 20 µm to 80 µm; 20 µm to 60 µm; 20 µm to 50 µm; 20 µm to 40 µm; 20 µm to 30 µm; 30 µm to 200 µm; 30 µm to 180 µm; 30 µm to 150 µm; 30 µm to 120 µm; 30 µm to 100 µm; 30 µm to 80 µm; 30 µm to 60 µm; 30 µm to 40 µm; 40 µm to 180 µm; 40 µm to 150 µm; 40 µm to 120 µm; 40 µm to 100 µm; 40 µm to 80 µm; 40 µm to 70 µm; 40 µm to 60 µm; 40 µm to 50 µm; 50 µm to 200 µm; 50 µm to 180 µm; 50 µm to 150 µm; 50 µm to 120 µm; 50 µm to 100 µm; 50 µm to 80 µm; 50 µm to 70 µm; 50 µm to 60 µm; 60 µm to 180 µm; 60 µm to 150 µm; 60 µm to 120 µm; 60 µm to 100 µm; 60 µm to 80 µm; 60 µm to 70 µm; 70 µm to 180 µm; 70 µm to 150 µm; 70 µm to 120 µm; 70 µm to 100 µm; 70 µm to 80 µm; 80 µm to 200 µm; 80 µm to 180 µm; 80 µm to 150 µm; 80 µm to 120 µm; 80 µm to 100 µm; 80 µm to 90 µm; 90 µm to 200 µm; 90 µm to 180 µm; 90 µm to 150 µm; 90 µm to 120 µm; 90 µm to 100 µm; 100 µm to 200 µm; 100 µm to 180 µm; 100 µm to 150 µm; 100 µm to 120 µm; 100 µm to 110 µm; 120 µm to 200 µm; 120 µm to 180 µm; 120 µm to 150 µm; 120 µm to 140 µm; 120 µm to 130 µm.

The size of the microparticles can be optimized by those skilled in the art having the benefit of the subject disclosure to achieve optimal delivery effects, depending on various parameters, such as for example, the cell type, the amount of therapeutics encapsulated, the site of delivery, and the host species.

Immune cells that can be targeted according to the present invention include, but are not limited to, dendritic cells, macrophages, lymphocytes, monocytes, neutrophils, mast cells, B cells, T cells, and T helper cells. In certain embodiments, professional antigen-presenting cells, such as dendritic cells, macrophages, T cells, and B cells, are targeted. In certain embodiments, dendritic cell and/or Treg cells are targeted.

In some embodiments, the outer surface of the microparticles comprise one or more surface ligands, such as antibodies, that target specific immune cells. In certain embodiments, the surface ligands are chemically fixed, or covalently linked, to the microparticles. In specific embodiments, the microparticles target dendritic cells. In specific embodiments, the microparticles specifically and selectively target immature dendritic cells, when compared to mature dendritic cells. Preferably, the surface ligands or antibodies also induce apoptotic and/or tolerance-inducing pathways in immune cells.

Exemplified surface ligands for dendritic cells include, but are not limited to, antibodies, aptamers and binding partners that bind specifically to cell surface ligands/receptors of dendritic cells, such as anti-CD 11 antibodies and anti-Dec205 antibodies; phosphatidyl serine (PS){PS receptor}; 4N1K{CD36/CD47}; PD2{CD11c}; P2 {CD11b}; RGD{$\alpha_v\beta_3$}; and CS1{$\alpha_4\beta_7$}. In a specific embodiment, the surface antibody is an anti-DEC-205 antibody, which recognizes dendritic cells. In a further specific embodiment, the microparticle matrix is surface modified with PD2 for targeting dendritic cells. Preferably, the therapeutic compositions do not contain any maturation stimuli such as prostaglandin E2.

Adjuvants useful in accordance with the subject invention include, but are not limited to, CpG, poly I:C, and mPLA.

In certain specific embodiments, further therapeutic agents useful according to the teachings herein include, but are not limited to, T cell inhibitory agents such as cytotoxic T-lymphocyte antigen 4 (CTLA-4) and indoleamine 2,3 dioxygenase (IDO); Treg selective growth factors, such as IL-2, rapamycin, or a phosphodiesterase 3B (PDE3b) inhibitor, such as cilostamide; and agents that inhibit maturation of dendritic cells, such as vascular endothelial growth factor (VEGF) and transcription factor E2F1.

Induction of Antigen-Specific Immune Tolerance

In particular aspects, provided are therapeutic methods for inducing antigen-specific immune tolerance for the treatment of MS. Preferably, the methods comprise administering, to a subject to which the induction of antigen-specific immune tolerance is needed, an effective amount of the microparticles and therapeutic compositions of the subject invention. In specific embodiments, the therapeutic compositions specifically target dendritic cells, induce dendritic cells with a tolerogenic phenotype, promote induction of Treg cells, and/or suppress T cell proliferation.

The term "tolerance," as used herein, refers to a failure to respond, or a reduced response, to an antigen, including auto-antigens.

The term "tolerogenic" or "tolerance-inducing," as used herein, refers to a phenotype that induces tolerance to an antigen directly or indirectly, or is capable of silencing or down-regulating an adaptive immunological response to an antigen. Tolerogenic dendritic cells have a low ability to activate effector T cells, but have a high ability to induce and activate regulatory T cells. In some embodiments, tolerogenic dendritic cells typically have reduced MHCII, CD80, CD86 levels and express tolerogenic markers such as CD103 and indoleamine 2,3 dioxygenase.

Preferably, the microparticles of the subject invention target immature dendritic cells, and do not target mature dendritic cells. Immature dendritic cells have a very dendritic morphology and have a low T cell activation potential. Immature dendritic cells undergo an irreversible maturation process upon activation of maturation stimuli. Mature dendritic cells have an enhanced ability to process antigens and activate T cells.

As demonstrated in the examples, the microparticles disclosed herein can have improved dendritic cell-targeting specificity and increased uptake by dendritic cells; result in functional antigen processing and presentation in dendritic cells; facilitate the maintenance of immature dendritic cell phenotype, and prevent or delay the maturation and expression of tolerogenic dendritic cell markers (e.g., indoleamine 2,3 dioxygenase) following particle uptake; facilitate the suppression of allogeneic mixed lymphocyte reactions; and induce $FoxP3^+$ Treg cells.

In some embodiments, the administration of the microparticle composition results in downregulation of MHC-II, CD 86, and CD 80. In addition, microparticles surface modified with ligands 4N1K, RGD and/or CS1 show reduced T-cell proliferation in mixed-lymphocyte reaction tests compared to immature dendritic cell controls. In addition, the microparticles suppress auto-reactive T-cells through the induction of regulatory T-cells.

Advantageously, the present microparticle-encapsulated vaccine can be easily administered with simultaneous delivery of both prime and boost doses using time-release materials (e.g., poly lactide-co-glycolide).

Treatment of Multiple Sclerosis (MS)

According to certain embodiments, methods are provided for the prevention and/or treatment of MS. Preferably, the methods comprise administering, to a subject, who has been diagnosed to be in need of such treatment, an effective amount of microparticles or a composition of the present invention. The disease and disease state are typically diagnosed based on MS symptoms.

The term "treatment" or any grammatical variation thereof (e.g., treat, treating, and treatment etc.), as used herein, includes but is not limited to, ameliorating or alleviating a symptom of a disease or condition, reducing, suppressing, inhibiting, lessening, or affecting the progression, severity, and/or scope of a condition.

The term "prevention" or any grammatical variation thereof (e.g., prevent, preventing, and prevention etc.), as used herein, includes but is not limited to, delaying the onset of symptoms, preventing relapse to a disease, decreasing the number or frequency of relapse episodes, increasing latency between symptomatic episodes, or a combination thereof.

Prevention, as used herein, does not require complete inhibition or elimination of the condition or its symptoms. Symptoms of multiple sclerosis include the following.

- vision problems (included blurred vision, double vision or loss of vision);
- tingling and numbness (common sites of numbness include the face, arms, legs and fingers);
- pains and spasms (commonly observed in back and legs);
- weakness or fatigue (typically first observed in legs);
- balance problems or dizziness;
- bladder issues (including frequent urination, strong urges to urinate or incontinence);
- sexual dysfunction; or
- cognitive problems (including memory problems, shortened attention span, and language problems).

The term "effective amount," as used herein, refers to an amount that is capable of treating or preventing a disease or condition or otherwise capable of producing an intended therapeutic effect. In one embodiment, an effective amount is a tolerogenic amount. For instance, an effective amount of an antigen is capable of inducing antigen-specific immune tolerance, but is incapable of generating an immunogenic reaction.

In some embodiments, the therapeutically effective amount can be an amount of a composition of the subject invention that is effective in inducing a regulatory immune response including, but is not limited to, reducing levels of pro-inflammatory cytokines including, but not limited to, IL-1β, TNF-α, IL-6, INF-γ, Cxcl2, GM-CSF and IL-17A; decreasing the frequency of IL-1β$^+$, CD86$^+$ and MHC II expressing dendritic cells; increasing the frequency of regulatory dendritic cells such as IL-10$^+$ dendritic cells; increasing the frequency of dendritic cells expressing programmed death-ligand1 (PD-L1); decreasing the frequency of CD4$^+$ and/or CD8$^+$ T cells expressing IFNγ; decreasing the frequency of pathogenic CD4$^+$ T cells expressing the transcription factors Rorγt and T-bet; increasing the frequency of regulatory T cells (Tregs); increasing FoxP3$^+$ Tregs; and decreasing the frequency of activated macrophages and microglia in the CNS.

The term “administering” as used herein, describes the delivery of a composition of the subject invention comprising a dual microparticle system to tissues, *e.g.*, skin, muscle, an organ, etc. or other localized sites, *e.g.*, lymph nodes, Peyer’s patches etc. Administration includes, but is not limited to, subcutaneous, subdermally, intradermal, intramuscular, intravenous, intraarticular, intracranial, intracerebral, intraspinal, intravaginal, intrauterine, transdermal, transmucosal, rectal, oral or by inhalation.

The term "subject," as used herein, describes an organism, including mammals such as primates, to which treatment with the compositions according to the present invention can be provided. Mammalian species that can benefit from the disclosed methods of treatment include, but are not limited to, primates such as apes, chimpanzees, orangutans, humans, monkeys; and non-primates such as dogs, cats, horses, cattle, pigs, sheep, goats, chickens, mice, rats, guinea pigs, and hamsters.

In certain embodiments, subjects treated in accordance with the present invention have been diagnosed with MS. In other embodiments, subjects treated are diagnosed as susceptible to, or predisposed to, developing MS, where predisposition or susceptibility to MS can be determined by a combination of factors, such as presence of a personal and family history of autoimmune disease, presence of genetic markers associated with autoimmunity, and/or living and/or working in conditions with a high chance of exposure to toxin or infection. A skilled physician can readily determine whether a subject is predisposed to, susceptible to, or has, MS.

In some embodiments, the prevention and treatment methods comprise, prior to administration of the microparticles and compositions of the invention, a step of diagnosing whether the subject has, or is predisposed to, MS.

Further, the present invention can also be used to inhibit macrophage or T cell associated aspects of an immune response and inhibit macrophage or T cell activities including, but not limited to, macrophage antigen-presenting activity, macrophage-associated cytokine production, T cell cytokine production, T cell adhesion, and T cell proliferative activities. Thus, the present invention is also useful to suppress or inhibit humoral and/or cellular immune responses.

Advantageously, embodiments can be used for antigen-specific tolerizing treatments for MS. Compared to the current treatments, which induce systemic suppression, the treatment of the subject invention is antigen-specific, delivers optimized amounts and/or ratios of antigen, immmunomodulatory, immunosuppressive, and chemoattractive agents and leads to efficient induction of tolerogenic dendritic cells to treat MS, which induction of tolerogenic dendritic cells is more efficient and superior to previously used methods in the art.

Therefore, the dMP system combines the attractive notion of antigen-specificity and combination therapy with a dual-sized controlled release scheme to provide immune modulation without systemic delivery. The dMP system vaccine of the subject invention can be easily administered via subcutaneous injection and provides for sustained delivery using biodegradable, controlled-release materials. Additionally, biomaterial encapsulation provides vaccine stability and extended shelf life, thereby simplifying manufacturing, storage and shipping.

Animal models useful to test therapeutic approaches are available in the art. For example, treatment approaches for animal models of encephalomyelitis-associated diseases are defined as follows: (a) preventative/prophylactic treatment is when factors are administered before disease induction, (b) therapeutic treatment is applied when agents are delivered after appearance of clinical disease signs, and (c) semi-therapeutic regimen is used when agents are administered after disease induction but before clinical disease signs [51,53].

Formulations and Administration

According to certain embodiments, provided are therapeutic or pharmaceutical compositions. In some embodiments, the compositions comprise a therapeutically effective amount of microparticles of the present invention and, optionally, a pharmaceutically acceptable carrier.

Suitable non-toxic pharmaceutically acceptable carriers for use with the agent will be apparent to those skilled in the art of pharmaceutical formulation. See, for example, Remington's Pharmaceutical Sciences, seventeenth edition, ed. Alfonso R. Gennaro, Mack Publishing Company, Easton, Pa. (1985).

The microparticles and therapeutic compositions of the subject invention may be delivered to tissues, *e.g.*, skin, muscle, organ, etc or other localized sites, *e.g.*, lymph nodes, Peyer's patches, etc.

In some embodiments, the microparticles of the subject invention are formulated into a vaccine composition for administration to subjects having certain risks of developing inflammatory and/or autoimmune-related disorders. In addition, the compositions disclosed herein can be administered to a subject with existing symptoms of inflammatory and autoimmune-related disorders, and provides for customized vaccine schedules and compositions to prevent or minimize worsening of the diseases.

The therapeutic dosage range can be determined by one skilled in the art having the benefit of the current disclosure. Naturally, such therapeutic dosage ranges will vary with the size, species and physical condition of the patient, the severity of the patient’s medical condition, the particular dosage form employed, the route of administration and the like.

The composition can be administered in a single dose or in more than one dose over a period of time to confer the desired effect.

In preferred embodiments, the microparticles can be formulated for parenteral administration. The preparation of an aqueous composition that contains one or more agents, will be known to those of skill in the art in light of the present disclosure. Typically, such compositions can be prepared as injectables, either as liquid solutions or suspensions; solid forms suitable for using to prepare solutions or suspensions upon the addition of a liquid prior to injection can also be prepared; and the preparations can also be emulsified.

The pharmaceutical forms suitable for injectable use include sterile aqueous solutions or dispersions; formulations including sesame oil, peanut oil or aqueous propylene glycol; and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersions. In all cases the form must be sterile and must be fluid to the extent that easy syringability exists. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms, such as bacteria and fungi.

Compositions comprising the microparticles disclosed herein can be formulated into a composition in a neutral or salt form. Pharmaceutically acceptable salts include the acid addition salts and those formed with inorganic acids such as, for example, hydrochloric or phosphoric acids, or such organic acids as acetic, oxalic, tartaric, mandelic, and the like. Salts can also be derived from inorganic bases such as, for example, sodium, potassium, ammonium, calcium, or ferric hydroxides, and such organic bases as isopropylamine, trimethylamine, histidine, procaine and the like.

The carrier can also be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyethylene glycol, and the like), dimethylsulfoxide (DMSO), suitable mixtures thereof, and vegetable oils. The proper fluidity can be maintained, for example, by the use of a coating, such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. In many cases, it will be preferable to include isotonic agents, for example, sugars or sodium chloride. Prolonged absorption of the injectable compositions can be brought about by the use in the compositions of agents delaying absorption, for example, aluminum monostearate and gelatin.

Sterile injectable solutions are prepared by incorporating the active compounds in the required amount in the appropriate solvent followed by filtered sterilization. Generally, dispersions are prepared by incorporating the various sterilized active ingredients into a sterile vehicle which contains the basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum drying and freeze-drying techniques, which yield a powder of the active ingredient, plus any additional desired ingredient from a previously sterile-filtered solution thereof.

The compositions disclosed herein can be administered to the subject being treated by standard routes, including the topical, transdermal, intraarticular, parenteral (e.g., intravenous, intraperitoneal, intradermal, subcutaneous or intramuscular), intracranial, intracerebral, intraspinal, intravaginal, or intrauterine. Depending on the condition being treated, one route may be preferred over others, which can be determined by those skilled in the art. In preferred embodiments, the compositions of the present invention are formulated for parental administration. In another embodiment, the peptides and compositions of the present invention are formulated as a sustained-release formulation.

A further embodiment provides for the administration of microparticles in combination with other pharmacological therapies. Combination therapies with other medicaments targeting similar or distinct disease mechanisms have advantages of greater efficacy and safety relative to respective monotherapies with either specific medicament.

When administering more than one, the administration of the agents can occur simultaneously or sequentially in time. The agents can be administered before and after one another, or at the same time. The methods also include co-administration with other drugs that are used to treat retinopathy or other diseases described herein.

Desirable key features of particle vaccines for immunotherapy include control over phagocytosability, delivery of antigen to DCs, and local release of desired agents.

Therefore, the multi-factor dMP treatment disclosed herein 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. This dMP approach does not result in an increase of the tolerogenic factors systemically, efficiently treats EAE, and is antigen-specific.

Following are examples which illustrate procedures for practicing the invention. These examples should not be construed as limiting. All percentages are by weight and all solvent mixture proportions are by volume unless otherwise noted.

Materials and Methods

Isolation of Mononuclear Cells from CNS

Mononuclear cells were isolated from CNS as described previously [26, 27]. Briefly, cold PBS was used to perfuse CNS. Brain and spinal cord were homogenized with gentle

MACS dissociator (Miltenyi Biotec), pressed through a 70 μm mesh, then suspended in 30% isotonic Percoll (GE Healthcare). The 30% isotonic Percoll solution containing homogenized CNS was then layered on top of 70% isotonic Percoll and centrifuged for 30 min at 500 g. The 70 %-30 % interphase containing CNS mononuclear cells was collected then washed with 1x HBSS. Isolated mononuclear cells from CNS were subjected to fluorophore-conjugated antibody staining and flow cytometry analysis.

In vitro Antigen Re-Stimulation Assay

Total cells were isolated from draining lymph nodes of EAE mice treated with dMP MOG_{35-55} or dMP $Ova_{323-339}$ then loaded with CFSE as described previously [28]. CFSE-loaded cells were then co-cultured with MOG_{35-55}-loaded splenocytes isolated from congenic $Rag1^{-/-}$ mice for 72 h. After 72 h, cells were washed and surface stained with CD4, CD8, CD3, and analyzed by flow cytometry.

Antibodies

Cells were stained with the following antibodies: CD11b (APC, APC-eFluor 780, AF647, clone: M1/70), CD11c (Brilliant violet 650, PE-cyanine7, clone: N418, HL3), PD-L1 (CD274, B7-H1, Brilliant Violet 711, clone: 10F.9G2), B7-H2 (ICOS-L, CD275, eFluor 660, clone: HK5.3), CD272 (BTLA, PE, clone: 6A6), Fixable viability dye (eFluor 520, eFluor 780, FITC), IL-10 (PerCP-Cyanine5.5, clone: JES5-16E3), IL-27 p28 (PE-cyanine7, clone: MM27-7B1), Galectin-9 (Brilliant violet 421, clone: RG9-35), CD4 (Brilliant violet 711, clone: GK1.5), PD-1 (CD279, Brilliant violet 605, clone: 29F.1A12), Rorγt (APC, clone: AFKJS-9), T-bet (PE-cyanine7, clone: 4B10), IL-17a (eFluor 450, clone: eBio17B7), GM-CSF (PE, clone: MP1-22E9), IFNγ (FITC, clone: XMG1.2), Ly6C (eFluor 450, clone: HK1.4), Ly6G (GR-1, FITC, AF700, clone: 1A8-Ly6g), F4/80 (PE, clone: BM8), CD80 (Brilliant violet 605, clone: 16-10A1), CD86 (PE-cyanine7, Brilliant violet 605, clone: GL-1), MHC class II (I-A/I-E, PerCP-eFluor 710, clone: M5/ 114.15.2), CD25 (Brilliant violet 605, clone: PC61), HVEM (CD270, PE, clone: LH1), CD39 (PE-cyanine7, clone: 24DMS1), CD73 (eFluor 450, clone: TY/11.8), Foxp3 (eFluor 450, FITC, clone: FJK-16s), CTLA4

(CD152, APC, clone: UC10-4B9), GITR (CD357, PE-cyanine7, clone: DTA-1), CD103 (Integrin alpha E, APC, clone: 2E7), Lag-3 (CD223, PE, clone: eBioC9B7W), Granzyme B (FITC, clone: GB11), CD8a (PE, clone: 53e6.7), TCRb (APC, clone: H57-597), CD3e (APC-cy7, clone: 145-2C11), CD45 (PE, APC-cyanine7, clone: 30-F11), and CD16/32 (FCγ III/II receptor, clone 2.4G2).

Intracellular/Intranuclear Staining

PMA/Ionomycin stimulation with Brefeldin A and intracellular/intranuclear staining were performed as described previously [29,30]. Briefly, for detection of cytokines and transcription factors by intracellular/intranuclear flow cytometry, cells were cultured at 37°C and 5% CO2 for 4 h in IMDM media (Gibco, Life Technologies) containing PMA (20 ng/mL) (Sigma) and Ion- omycin (1 mg/mL) (Sigma). Brefeldin A (10 mg/mL) was added 1 h following PMA/Ionomycin addition. Cells were washed and stained with Fixable Viability Dye (Affymetrix, Life Technologies) and surface markers following stimulation. Surface marker stained cells were fixed and permeabilized with Foxp3 Fix/Perm Kit (Affymetrix, Life Technologies) followed by cytokine and transcription factor staining.

Flow Cytometry

Flow cytometry was performed on a BD LSR II with BD FACS DIVA software for data acquisition (BD Biosciences). All flow cytometry data were analyzed with FlowJo software (Tree Star).

Statistical Analysis

GraphPad Prism software version 5 was used for statistical analysis. Statistical significance was assessed by two-tailed un- paired Student's *t* tests for all analyses except Figures 2H, 2I, 2K, 2L, 2M, 3B, and 8B where a one-way analysis of variance (ANOVA) with Tukey's post-hoc analysis was used. Statistical significance between groups was defined at $p < 0.05$.

EXAMPLES

EXAMPLE 1: DUAL-SIZED MP SYSTEM FOR THE TREATMENT OF MULTIPLE SCLEROSIS

Microparticles (MPs) were fabricated from poly (lactic-co-glycolic acid) (PLGA) for delivering immunotherapeutics.

Specifically two sizes of MPs were 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 were 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 (VitD3). Further, two non-phagocytosable MPs were used: (c) MPs encapsulating TGF-β1, and (d) MPs encapsulating GM-CSF. These four MPs were mixed in equal mass and administered subcutaneously.

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. 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. Because the MPs of the subject invention advantageously provide GM-CSF locally for the select stimulation of APC in the context of immunosuppressive agents, the MPs of the subject invention allow the induction of tolerogenic DC in a localized environment providing exposure to antigen and immunosuppressive cytokines to treat MS while avoiding generalized effects of the cytokine.

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, which targeting is cheived through the dual size MPs.

EXAMPLE 2: MICROPARTICLE FABRICATION

Microparticles (MPs) were fabricated by standard oil-in-water single emulsion or water-in-oil-in-water double emulsion methods, as described previously [31]. All drugs were encapsulated in distinct MPs, as no two factors were loaded simultaneously. A 50:50 copolymer of poly (lactic-co-glycolic acid) (PLGA; MW ~44,000 g/mol; Corbion Purac, Gorinchem, Netherlands) in methylene chloride (Themo Fisher Scientific, NJ, USA) was used to generate MPs. Ultrapure water (Barnstead GenPure, Thermo Fisher Scientific) was used as the aqueous phase, with dissolved surfactant, poly-vinyl alcohol (PVA; MW ~15,000 g/mol; Themo Fisher Scientific), to stabilize the emulsions.

Phagocytosable MPs were fabricated by dissolving 500 mg of PLGA in methylene chloride at a 5% w/v ratio. 50 mg of vitamin D3 (Cayman Chemical) in 1 mL of methanol (Thermo Fisher Scientific) was loaded directly into the methylene chloride/PLGA solution and set to shake at 150 rpm for 10 min. This solution was then added to 50 mL of 5% w/v PVA and homogenized at 35,000 rpm for 180 s using a tissue-miser homogenizer (Thermo Fisher Scientific) to form an oil-in-water emulsion. The microparticle solution was subsequently added to a beaker of 100 mL 1% PVA and set to stir for 4e6 h for solvent evaporation and microparticle hardening to occur. For water-soluble MOG_{35-55}- (Mimotopes, Victoria, Australia) and $OVA_{323-339}$-encapsulated (Mimotopes) MPs, 4 mg of peptide in 200 mL PBS was added to the 5% methylene chloride/PLGA solution and homogenized at 35,000 rpm for 120 s to form a primary emulsion. This emulsion was added to 50 mL of 5% PVA and homogenized again at 35,000 rpm for 180 s to form the secondary emulsion, and added to 100 mL of stirring 1% PVA.

Non-phagocytosable MPs encapsulating TGF-β1 and GM-CSF were fabricated by first dissolving 500 mg of PLGA in methylene chloride at a 20% w/v ratio. Human TGF-β1 (Peprotech) was reconstituted in 10 mM hydrochloric acid and 2 mg/mL bovine serum albumin in 250 mL PBS and recombinant mouse GM-CSF (Biolegend) was reconstituted in 400 mL PBS. Protein solutions were added to the methylene chloride/PLGA solution and vortexed (Thermo Fisher Scientific) at the highest setting (~3200 rpm) for 30 s to generate the primary emulsion. This emulsion was added to 5 mL of 2.5% PVA and vortexed again at 3200 rpm for 60 s to form the secondary emulsion, and finally added to 100 mL of stirring 1% PVA. Either methanol or PBS was used to generate unloaded MPs, depending on the control group being fabricated. After 4-6 h, solutions were centrifuged at 10,000 g for 10 min to collect MPs and washed three times with ultrapure water. The resultant MPs were then flash-frozen in liquid nitrogen and lyophilized for 24 h. The MPs were stored at - 20°C until their use.

EXAMPLE 3: MICORPARTICLE CHARACTERIZATION

The size distributions of MPs were measured by the Beckman Coulter LS13320 (Beckman Coulter Inc., Brea, CA) and the Microtrac Nanotrac Dynamic Light Scattering Particle Analyzer (Microtrac, Montgomery, PA). The MP diameter is reported as mean ± standard deviation and displayed as a volume percentage.

Encapsulation efficiencies of proteins/peptides was assessed by μBCA (Thermo Fisher Scientific). Briefly, a known mass of MPs, as determined by the working range of the μBCA assay, was dissolved in a 0.2 M NaOH/5% sodium dodecyl sulfate (SDS) solution. An analogous process with unloaded MPs and soluble drug was performed. The pH of solutions was neutralized with a small volume of HCl and protein/peptide concentration measured by μBCA assay. Serial dilutions of the unloaded MP/soluble drug solution determined the encapsulation efficiency. Vitamin D3 MPs were measured by dissolving 100 mg of MPs into 2 mL MC and re-precipitating the PLGA with a known volume of methanol. The suspension was centrifuged and the supernatant removed to a new tube. Following evaporation, residue remaining in the tube was concentrated in a known, small quantity of DMSO and the solution concentration measured by spectrophotometer.

Advantageously, compared to prior formulations, the MP formulations used in the instant invention have been modified by increasing the loading of immunomodulatory factors.

Consistent sizing of phagocytosable MPs, ~0.8 μm-diameter, and non-phagocytosable MPs, ~55 μm-diameter, irrespective of the drug loaded was demonstrated, highlighting the dual-sized nature of the dMP system (Fig. 1A). The encapsulation efficiencies for the small phagocytosable MPs were 48.6± 9.0%, 65.5± 3.0%, and 49.9± 2.8% for MOG_{35-55}, vitamin D3, and $Ova_{323-339}$ MPs, respectively (Fig. 1B). Comparable encapsulation efficiencies for the large non-phagocytosable MPs were observed, with 44.2± 12.1% and 58.3± 9.4% for TGF-b1 and GM-CSF MPs, respectively (Fig. 1B).

EXAMPLE 4: SITE OF INJECTION ANALYSIS AND MICROPARTICLE TRAFFICKING

C57BL/6 mice (B6NTac) were purchased from Taconic Biosciences. All animals were housed in specific pathogen free conditions. All experiments were conducted on 8-20-week old male or female mice.

Characterization of nodules at the site of injection was carried out via flow cytometry and H&E staining. Initial studies characterizing DC recruitment and phenotype used a mixed cohort of 8-20-week-old male and female C57BL/6 mice. Animals were injected subcutaneously in the abdominal region using 20 G needles (BD Biosciences). MP injections consisted of 10 mg of MPs total (1:1:1:1 MP mass ratio) in 0.2 mL PBS. Nodules were excised 8 days after injection, enzymatically digested with 2 mg/mL collagenase type XI (Sigma-Aldrich, St. Louis, MO, USA) at 37°C for 30 min, filtered through a 30 μm filter to remove large particulates, and isolated cells stained for flow cytometry. For immunohistochemistry, nodules were fixed in 10% formalin overnight at 4°C, processed and embedded in paraffin blocks and stained.

Microparticle uptake in the nodule and trafficking to secondary lymphoid organs was assessed by loading the phagocytosable MPs concomitantly with Vybrant DiO (Invitrogen) fluorescent labelling dye and vitamin D3 or an irrelevant protein (denatured insulin). Non-drug loaded (unloaded) phagocytostable fluorescent MPs were also fabricated. Large, non-phagocytosable MPs were fabricated in the standard fashion without the addition of fluorescent dye.

At various time points (24 h, 48 h, and 8 d) after subcutaneous injection in the abdominal region mice were euthanized and cells were isolated from various secondary lymphoid organs. Cells were stained with primary conjugated antibodies and analyzed via flow cytometry.

EXAMPLE 5: EVALUATION OF TOLEROGENIC FACTORS IN THE BLOOD

A mixed cohort of 10-week old male and female C57BL/6 mice were injected subcutaneously in the mid-dorsal region with the dual-sized microparticle (dMP) formulation using 20 G needles (BD Biosciences). Blood was collected from submandibular vein of animals on days 2, 4, and 7 after subcutaneous dMP injection, processed for serum, and GM-CSF and TGF-β1 serum concentrations were measured using enzyme linked immunosorbent assay (ELISA) following the manufacturer's protocol (BD Biosciences, cat# 555167, 559119). A negative control group of C57BL/6 mice received no treatment. Conversely, a positive control group of C57BL/6 mice were injected intravenously with GM-CSF and TGF-β1 at a dose 1/10th of that delivered in the dMP immediately prior to blood collection. Absorbance was read at 450 nm using a SpectraMax M3 microplate reader (Molecular Devices) and serum concentrations of GM-CSF and TGF-β1 was calculated using a standard curve performed following manufacturer's protocol (BD Biosciences).

EXAMPLE 6: EAE INDUCTION

EAE was induced in 10-11-week old female C57BL/6 mice from Taconic Biosciences with Hooke Kit™ (Hooke Laboratories Inc., Cat# EK-2110). Briefly, 100 mL of MOG_{35-55}/CFA emulsion was injected subcutaneously in the anterior and posterior dorsal regions for a total of 200 mL emulsion per mouse, according to manufacturer's protocol. Pertussis toxin (100 mL of 4 mg/mL) was injected intraperitoneally 2 h and 24 h following MOG_{35-55}/CFA emulsion injection, according to manufacturer's protocol. Clinical scoring was established as follows: score 1: flaccid tail, score 2: weak hind limbs, score 3: hind limb paralysis, score 4: quadriplegia, score 5: moribund, euthanasia.

EXAMPLE 7: DUAL MP TREATMNET IN EAE MICE

For a total of 10 mg of dMP formulation per EAE mouse, 2.5 mg of each of the four MPs described in EXAMPLE 1 were mixed. Dual MPs were resuspended in 200 mL PBS per 10 mg of dMP. EAE mice were injected subcutaneously in the mid-dorsal region between the two MOG_{35-55}/CFA emulsion injection sites on the indicated days following EAE induction.

EXAMPLE 8: SUBCUTANEOUS DMP ADMINISTRATION CAUSES DC RECRUITMENT AND TOLERIZATION AND MICROPARTICLE-ASSOCIATED CELL TRAFFICKING TO LOCAL LYMPH NODES WITHOUT SYSTEMIC RELEASE

The capacity of the dMP formulation to recruit and tolerize DCs at the local injection site was evaluated and the ability of the cells that phagocytosed MPs to subsequently traffic to draining lymph nodes. Mice that received the dMP developed palpable nodules at the subcutaneous injection site a day after a single dMP injection. Surgical and histopathological analysis of the dMP nodules eight days after administration demonstrated high levels of proteinaceous deposition with significant nucleated cell infiltration surrounding the readily visible non-phagocytosable MPs (large white spheres) (Fig. 2A). Importantly, these nodules were resorbed within a month of injection as determined by palpation and surgical examination, approximately by the time the administered PLGA bolus completely degraded.

The composition of nodule-recruited cells was assessed by digestion of the nodule and flow cytometry analysis. DC recruitment to the local subcutaneous MP nodule was improved when MPs were loaded with bioactive factors compared to unloaded MPs, with the total frequency of infiltrating DCs rising from 11.9% to 19.2% of total CD45$^+$ cells (Fig. 2B-C). Furthermore, recruited DCs demonstrated characteristics of a non-activated phenotype, with decreased frequency of CD86$^+$ DCs in the case of loaded MPs versus unloaded (Fig. 2D-E).

Using fluorescently-loaded phagocytosable MPs in the dMP formulation, MP uptake in the nodule was assessed in phagocyte populations. Higher uptake of the phagocytosable dMP particles compared to unloaded MPs by DCs was evident, while the uptake of dMPs versus unloaded MPs was equivalent in macrophages and lower in neutrophils (Fig. 2F). Trafficking of phagocytes associated with microparticles (MP$^+$ cells) was assessed in various peripheral lymphoid organs at multiple time points (Fig. 2G-K). At 24 and 48 h post-dMP injection, MP$^+$ DCs were shown to drain to inguinal lymph nodes (ILNs) in the highest number compared to neutrophils (24 h) and both macrophages and neutrophils (48 h) (Fig. 2G). Notably, MP$^+$ DCs isolated from ILNs 24 h after MP injection demonstrated upregulation of programmed death-ligand 1 (PD-L1) expression compared to dMP$^-$ DCs or unloaded MP$^+$ DCs, while PD-L1 expression between unloaded MP$^+$ and unloaded MP$^-$ DCs remained unchanged (Fig. 2H). Similarly, dMP$^+$ DCs isolated from ILNs 48 h after MP injection maintained immature phenotypes, whereas unloaded MP$^+$ DCs significantly upregulated MHC-II expression compared to unloaded MP$^-$ DCs (Fig. 2I).

At a later time point, eight days after dMP administration, MP$^+$ DCs were present in proximal draining lymph nodes (axillary [ALNs] and ILNs), however not in distal lymphoid organs (mesenteric lymph nodes and spleen) (Fig. 2J), thus minimizing the potential for systemic immunosuppression. Upon further examination, MP$^+$ DCs had the highest frequency in ALNs, followed by MP$^+$ macrophages, while in ILNs the frequency of MP$^+$ DCs and MP$^+$ macrophages was equivalent (Fig. 2K). The frequency of MP$^+$ neutrophils was low both in ALNs and ILNs. In addition, subcutaneous injection of the dMPs did not result in serum elevation of TGF-β1 or GM-CSF at 2, 4, and 7 days compared to no treatment (Fig. 2L-M), suggesting that systemic immunosuppression is unlikely.

In sum, these proof-of-concept studies emphasize the feasibility of this platform to modulate DC recruitment and phenotype, as well as the distribution of the MP-loaded cells proximally, but not into the distal lymphoid organs or systemically.

EXAMPLE 9: DMP-MOG$_{35-55}$ FORMULATION BLOCKS EXPERIMENTAL AUTOIMMUNE ENCEPHALOMYELITIS IN A SEMI-THERAPEUTIC TREATMENT SETTING

It was examined whether the dMP system formulated with the antigenic MOG$_{35-55}$ peptide (dMP-MOG$_{35-55}$) can be used to treat EAE, the mouse model for multiple sclerosis (MS). Specifically, the dMP-MOG$_{35-55}$ formulation, consisting of non-phagocytosable TGF-β1 and GM-CSF MPs and phagocytosable vitamin D3 and MOG$_{35-55}$ MPs (dMP-MOG$_{35-55}$), was used. The dMP formulation without MOG$_{35-55}$ consisting of non-phagocytosable TGF-β1 and GM-CSF MPs, phagocytosable vitamin D3 MPs, and unloaded phagocytosable MPs was used as control (dMP No MOG). The dMP-MOG$_{35-55}$ treatment and the corresponding control were administered subcutaneously on days 4, 7, and 10 following EAE induction in

C57BL/6 mice. The results show that EAE mice treated with the dMP-MOG_{35-55} developed minimal EAE scores in significant contrast to EAE mice treated with the dMP No MOG (Fig. 3A). Linear regression analysis of EAE disease score development revealed no disease development in EAE mice treated with dMP-MOG_{35-55} compared to positive disease progression in EAE mice treated with dMP without MOG_{35-55} (Fig. 3B). Thus, these results demonstrate that early administration dMP-MOG_{35-55}, after disease induction, is highly efficacious in the prevention of EAE disease.

EXAMPLE 10: EAE MICE TREATED WITH DMP-MOG_{35-55} HAVE REDUCED LEUKOCYTES AND $CD4^+$ T CELLS INFILTRATING INTO THE CNS

The hallmark of active MS and EAE is mononuclear immune infiltration into the CNS [4,9]. Histopathological examination of spinal cord sections from EAE mice revealed perivascular cuffing with mononuclear inflammatory cells as well as extension of mononuclear inflammatory cell infiltrate into parenchyma in mice treated with the control of soluble factors (equivalent doses of TGF-β1, GM-CSF, vitamin D3 and MOG_{35-55} peptide) co-administered with empty MPs (S+U MPs) (Fig. 4A). However, spinal cord sections of EAE mice treated with dMP-MOG_{35-55} revealed an intact parenchyma with the absence of perivascular mononuclear inflammatory cells, indicating that the dMP-MOG_{35-55} treatment prevented EAE disease development through successfully blocking inflammatory cell infiltrating into the CNS. Lymphocyte infiltration into the CNS is observed in early and active MS and EAE and is considered the cause of autoimmune pathogenesis [4].

The percentages and absolute numbers of T cells in the CNS of dMP-MOG_{35-55} versus S+U MPs-treated EAE mice and naïve mice were evaluated by flow cytometry. The percentages and absolute numbers of $CD4^+$ T cells were drastically reduced in the EAE mice treated with dMP-MOG_{35-55}, but still slightly higher than that of naïve healthy mice (Fig. 4B, C). Thus, the dMP-MOG_{35-55} treatment reduced the total mononuclear inflammatory cell infiltrating into the CNS (Fig. 4A) in EAE mice, and also significantly reduced the total number of $CD4^+$ T cells infiltrating into the CNS (Fig. 4C), which indicates that dMP-

MOG_{35-55} prevents EAE disease development through impeding $CD4^+$ T cell infiltration in the CNS.

EXAMPLE 11: EAE MICE TREATED WITH DMP-MOG_{35-55} HAVE REDUCED $CD4^+$ T CELLS PRODUCING IL-17A, GM-CSF and IFNγ IN THE CNS

Given that production of proinflammatory cytokines IL-17A, GM-CSF, and IFNγ by pathogenic autoreactive $CD4^+$ T cells is critical in the EAE disease pathogenesis [14,32], it was examined whether dMP-MOG_{35-55} treatment suppressed production of these proinflammatory cytokines. Not only was the number of CNS-infiltrating $CD4^+$ T cells reduced in EAE mice treated with dMP-MOG_{35-55}, but the production of IL-17A, GM-CSF, IFNγ, and co-production of these cytokines by the few CNS-infiltrating $CD4^+$ T cells was also severely reduced in EAE mice treated with dMP-MOG_{35-55} (Fig. 5). Both the frequencies (Fig. 5A) and absolute numbers (Fig. 5B) of IL-17A, GM- CSF, IFNγ, and dual cytokine-producing $CD4^+$ T cells in the CNS were significantly reduced in EAE mice treated with dMP-MOG_{35-55}, suggesting that in addition to preventing the infiltration of $CD4^+$ T cells in the CNS, dMP-MOG_{35-55} also suppress the production of IL-17A, GM-CSF, and IFNγ by pathogenic $CD4^+$ T cells.

EXAMPLE 12: EAE MICE TREATED WITH DMP-MOG_{35-55} HAVE DECREASED PATHOGENIC $CD4^+$ T CELLS EXPRESSIN THE TRANSCRPTION FACTORS RORγT AND T-BET IN THE CNS

The Th17 transcription factor, Rorγt, and the Th1 transcription factor, T-bet, have been demonstrated to be crucial for GM-CSF production in pathogenic $CD4^+$ T cells and EAE disease pathogenesis [13,15, 33-35]. It was, therefore, examined whether dMP-MOG_{35-55} treatment suppressed Rorγt and T-bet expression in $CD4^+$ T cells in the CNS. EAE mice treated with dMP-MOG_{35-55} showed significant reduction in frequencies and absolute numbers of $Ror\gamma t^+$, $T\text{-}bet^+$, and dual Rorγt and T-bet-expressing $CD4^+$ T cells in the CNS (Fig. 6), as well as diminished Rorγt and T-bet mean fluorescence intensity as measure for the expression levels per cell. These results indicated that dMP-MOG_{35-55} treatment can block the entire transcriptional program of pathogenic $CD4^+$ T cells in EAE mice. Thus, taken together, these results indicated that the reduced EAE disease scores in the dMP-$MOG_{35\text{-}55}$-treated EAE mice were linked to reduced pathogenic $CD4^+$ T cells in the CNS.

EXAMPLE 13: ACTIVATED MACROPHAGES/MICROGLIAL CELLS ARE REDUCED IN THE CNS OF MICE TREATED WITH $MOG_{35\text{-}55}$

In EAE and MS, pathogenic effector $CD4^+$ T cells trigger activation of CNS resident microglia and the recruitment of macrophages, which are essential for inflammatory demyelinating lesions [36]. It was therefore examined whether dMP-$MOG_{35\text{-}55}$ treatment affected activated microglia/macrophage populations in the CNS of EAE mice. Both microglia and macrophages are $CD11b^+F4/80^+CD68^+$ and upregulate MHCII and CD80 following activation [37, 38]. The frequency (Fig. 7A) and absolute number (Fig. 7B) of $CD11b^+CD68^+F4/80+CD80^+$ cells, which include both activated macrophages and microglia, were significantly reduced in the CNS of EAE mice treated with dMP-$MOG_{35\text{-}55}$. These reductions are likely in relation to the decreased IL-17A, GM-CSF, and IFNγ production by CNS-infiltrating $CD4^+$ T cells (Fig. 4). Therefore, overall, the reduced EAE disease severity in mice treated with dMP-$MOG_{35\text{-}55}$ can be explained by the decreased pathogenic $CD4^+$ T cells and reduced activated macrophages/microglia in the CNS.

EXAMPLE 14: EFFICACY OF DMP-TREATMENT IS DEPENDENT ON ANTIGEN SPECIFICITY

Because MS and EAE are established with a major autoimmune component [4], it was examined whether the dMP semi-therapeutic treatment was antigen-specific. EAE mice were treated with either dMP-$MOG_{35\text{-}55}$, which includes the $MOG_{35\text{-}55}$ antigenic peptide-loaded MPs, or dMP-$Ova_{323\text{-}339}$, which includes MPs loaded with an irrelevant antigenic peptide, $Ova_{323\text{-}339}$, derived from ovalbumin. Treatment with dMP-$MOG_{35\text{-}55}$ prevented EAE disease development, but treatment with dMP-$Ova_{323\text{-}339}$ did not (Fig. 8A). Linear regression analysis of EAE disease score development revealed no disease development in EAE mice treated with dMP-$MOG_{35\text{-}55}$ compared to positive disease progression in EAE mice treated with dMP-Ova$_{323-339}$ (Fig. 8B), thus demonstrating that the success of the treatment is dependent on antigen-specificity.

EXAMPLE 15: T CELLS FROM EAE MICE TREATED WITH DMP-MOG$_{35-55}$, BUT NOT WITH DMP-Ova$_{323-339}$, FAIL TO EXPAND IN RESPONSE TO MOG$_{35-55}$-DEPENDENT STIMULATION

Based on the demonstration that dMP-MOG$_{35-55}$ treatment of EAE is antigen-specific, it was investigated whether T cells isolated from dMP-MOG$_{35-55}$ treated EAE mice can proliferate as efficiently as T cells derived from EAE mice treated with dMP- Ova$_{323-339}$, following exogenous stimulation with splenocytes loaded with MOG$_{35-55}$ peptide, in an *in vitro* antigen re-stimulation assay. The results show that CD4$^+$ T cells isolated from dMP-MOG$_{35-55}$ treated EAE mice failed to proliferate, while CD4$^+$ T cells isolated from dMP-OVA$_{323-339}$ treated EAE mice expanded robustly in response to MOG$_{35-55}$-loaded splenocyte co-culture (Fig. 9). These results demonstrate that the T cells from dMP-MOG$_{35-55}$-treated EAE mice are anergic, *i.e.*, unable to respond in an antigen-specific manner (Fig. 10).

EXAMPLE 16: DENDRITIC CELLS FROM DRAINING LYMPH NODES OF EAE MICE TREATED WITH DMP-MOG$_{35-55}$ DISPLAY A TOLERIZED PHENOTYPE

It was investigated whether the dMP-MOG$_{35-55}$ treatment of EAE mice induced a suppressive DC phenotype, as indicated by reduced expression of CD86 and MHC-II. The frequency of CD11b$^+$CD11c$^+$ DCs that highly co-express CD86 and MHC-II in draining lymph nodes was significantly reduced in EAE mice treated with dMP-MOG$_{35-55}$ compared with those treated with irrelevant antigen-loaded dMP-Ova$_{323-339}$ (Fig. 10A-B). Similarly, the mean fluorescence intensity (MFI) of CD86 expression in dMP-MOG$_{35-55}$ was reduced (Fig. 10C). Therefore, not only are the CD4$^+$ T cells anergic in the dMP-MOG$_{35-55}$-treated group (Fig. 9), but the DCs are also suppressive in an antigen-specific manner (Fig. 10).

EXAMPLE 17: MOG-SPECIFIC DUAL MICROPARTICLE SYSTEM (DMP-MOG$_{35-55}$) REVERSES THE DISEASE AND IMPROVES THE MOBILITY OF THE EAE MICE

WHEN ADMINISTERED AT THE PEAK OF DISEASE AND HALTS THE DISEASE WHEN INITIATED AT THE EAE ONSET.

dMP-MOG$_{35-55}$ therapy was tested at the onset of disease (score 1, limp tail) and at the peak of disease (score 3, hind limp paralysis). Results show that treatment with dMP MOG$_{35-55}$ at the onset, halted disease progression, as mice remained at a score of 1, while mice treated dMP OVA$_{323-339}$ showed EAE scores continuing to rise (Fig. 12).

The efficiency of dMP-MOG$_{35-55}$ therapy was also tested at the peak disease (score 3) and found that scores dropped from 3 (hind limb paralysis to a score of 1 (limp tail) in dMP MOG$_{35-55}$ treated mice, while dMP OVA$_{323-339}$ treated mice remained at a score of 3 (Fig. 13). Thus the dMP MOG$_{35-55}$ system has not only the ability to halt EAE progression in an antigen specific manner, when administered at the onset, but even reverse the disease and significantly improve the mobility of the mice induced with EAE.

REFERENCES

[1] A. Compston, A. Coles, Multiple sclerosis, Lancet 372 (9648) (2008) 1502e1517.

[2] G. Kobelt, J. Berg, D. Atherly, O. Hadjimichael, Costs and quality of life in multiple sclerosis: a cross-sectional study in the United States, Neurology 66 (11) (2006) 1696e1702.

[3] F.D. Lublin, S.C. Reingold, J.A. Cohen, G.R. Cutter, P.S. Sørensen, A.J. Thompson, J.S. Wolinsky, L.J. Balcer, B. Banwell, F. Barkhof, B. Bebo, P.A. Calabresi, M. Clanet, G. Comi, R.J. Fox, M.S. Freedman, A.D. Goodman, M. Inglese, L. Kappos, B.C. Kieseier, J.A. Lincoln, C. Lubetzki, A.E. Miller, X. Montalban, P.W. O'Connor, J. Petkau, C. Pozzilli, R.A. Rudick, M.P. Sormani, O. Stüve, E. Waubant, C.H. Polman, Defining the clinical course of multiple sclerosis: the 2013 revisions, Neurology 83 (3) (2014) 278e286.

[4] C.A. Dendrou, L. Fugger, M.A. Friese, Immunopathology of multiple sclerosis, Nat. Rev. Immunol. 15 (9) (2015) 545e558.

[5] A. Nylander, D.A. Hafler, Multiple sclerosis, J. Clin. Invest 122 (4) (2012) 1180e1188.

[6] H.L. Weiner, Multiple sclerosis is an inflammatory T-cell-mediated autoimmune disease, Arch. Neurol. 61 (10) (2004) 1613e1615.

[7] B. Bielekova, M.H. Sung, N. Kadom, R. Simon, H. McFarland, R. Martin, Expansion and functional relevance of high-avidity myelin-specific CD4 T cells in multiple sclerosis, J. Immunol. 172(6) (2004) 3893e3904.

[8] N. Hellings, M. Bare'e, C. Verhoeven, M.B. D'hooghe, R. Medaer, C.C. Bernard, J. Raus, P. Stinissen, T-cell reactivity to multiple myelin antigens in multiple sclerosis patients and healthy controls, J. Neurosci. Res. 63(3) (2001) 290e302.

[9] E. Lavi, C.S. Constantinescu, Experimental Models of Multiple Sclerosis, Springer, New York, 2005.

[10] C.L. Langrish, Y. Chen, W.M. Blumenschein, J. Mattson, B. Basham, J.D. Sedgwick, T. McClanahan, R.A. Kastelein, D.J. Cua, IL-23 drives a pathogenic T cell population that induces autoimmune inflammation, J. Exp. Med. 201(2) (2005) 233e240.

[11] H. Park, Z. Li, X.O. Yang, S.H. Chang, R. Nurieva, Y.H. Wang, Y. Wang, L. Hood, Z. Zhu, Q. Tian, C. Dong, A distinct lineage of CD4 T cells regulates tissue inflammation by producing interleukin 17, Nat. Immunol. 6(11) (2005) 1133e1141.

[12] I.I. Ivanov, B.S. McKenzie, L. Zhou, C.E. Tadokoro, A. Lepelley, J.J. Lafaille, D.J. Cua, D.R. Littman, The orphan nuclear receptor RORgammat directs the differentiation program of proinflammatory IL-17 T helper cells, Cell 126(6) (2006) 1121e1133.

[13] L. Codarri, G. Gyülve'szi, V. Tosevski, L. Hesske, A. Fontana, L. Magnenat, T. Suter, B. Becher, RORgt drives production of the cytokine GM-CSF in helper T cells, which is essential for the effector phase of autoimmune neuro- inflammation, Nat. Immunol. 12(6) (2011) 560e567.

[14] M. El-Behi, B. Ciric, H. Dai, Y. Yan, M. Cullimore, F. Safavi, G.X. Zhang, B.N. Dittel, A. Rostami, The encephalitogenicity of T(H)17 cells is dependent on IL-1- and IL-23-induced production of the cytokine GM-CSF, Nat. Immunol. 12(6) (2011) 568e575.

[15] E. Bettelli, B. Sullivan, S.J. Szabo, R.A. Sobel, L.H. Glimcher, V.K. Kuchroo, Loss of T-bet, but not STAT1, prevents the development of experimental autoimmune encephalomyelitis, J. Exp. Med. 200 (1) (2004) 79e87.

[16] D.M. Wingerchuk, J.L. Carter, Multiple sclerosis: current and emerging disease-modifying therapies and treatment strategies, Mayo Clin. Proc. 89(2) (2014) 225e240.

[17] D.S. Goodin, E.M. Frohman, G.P. Garmany, J. Halper, W.H. Likosky, F.D. Lublin, D.H. Silberberg, W.H. Stuart, S. van den Noort, T.a.T.A.S.o.t.A.A.o.N.a.t.M.C.f.C.P. Guidelines, disease modifying therapies in multiple sclerosis: report of the therapeutics and technology assessment subcommittee of the american academy of neurology and the MS council for clinical practice guidelines, Neurology 58(2) (2002) 169e178.

[18] Z. Hunter, D.P. McCarthy, W.T. Yap, C.T. Harp, D.R. Getts, L.D. Shea, S.D. Miller, A biodegradable nanoparticle platform for the induction of antigen-specific immune tolerance for treatment of autoimmune disease, ACS nano 8(3) (2014) 2148e2160.

[19] A. Yeste, M. Nadeau, E.J. Burns, H.L. Weiner, F.J. Quintana, Nanoparticle- mediated codelivery of myelin antigen and a tolerogenic small molecule suppresses experimental autoimmune encephalomyelitis, Proc. Natl. Acad. Sci. U.S.A. 109(28) (2012) 11270e11275.

[20] D.S. Goodin, B.A. Cohen, P. O'Connor, L. Kappos, J.C. Stevens, T.a.T.A.S.o.t.A.A.o. Neurology, Assessment: the use of natalizumab (Tysabri) for the treatment of multiple sclerosis (an evidence-based review): report of the Therapeutics and Technology Assessment Subcommittee of the American Academy of Neurology, Neurology 71(10) (2008) 766e773.

[21] R. Arnon, R. Aharoni, Mechanism of action of glatiramer acetate in multiple sclerosis and its potential for the development of new applications, Proc. Natl. Acad. Sci. U.S.A. 101 (Suppl 2) (2004) 14593e14598.

[22] R.J. Fox, D.H. Miller, J.T. Phillips, M. Hutchinson, E. Havrdova, M. Kita, M. Yang, K. Raghupathi, M. Novas, M.T. Sweetser, V. Viglietta, K.T. Dawson, C.S. Investigators, Placebo-controlled phase 3 study of oral BG-12 or glatir-amer in multiple sclerosis, N. Engl. J. Med. 367(12) (2012) 1087e1097.

[23] J.A. Cohen, J. Chun, Mechanisms of fingolimod's efficacy and adverse effects in multiple sclerosis, Ann. Neurol. 69(5) (2011) 759e777.

[24] A. Brickshawana et al. Investigation of the KIR4.1 potassium channel as a putative antigen in patients with multiple sclerosis: a comparative study. Lancet Neurol. 13, 795–806 (2014).

[25] C. Riedhammer, R Weissert, Antigen presentation, autoantigens, and immune regulation in multiple sclerosis and other autoimmune diseases, Front Immunol, 6: 322 (2015).

[26] P.A. Pino, A.E. Cardona, Isolation of brain and spinal cord mononuclear cells using percoll gradients, J. Vis. Exp. (48) (2011).

[27] D. Califano, K.J. Sweeney, H. Le, J. VanValkenburgh, E. Yager, W. O'Connor, J.S. Kennedy, D.M. Jones, D. Avram, Diverting T helper cell trafficking through increased plasticity attenuates autoimmune encephalomyelitis, J. Clin. Invest 124(1) (2014) 174e187.

[28] J. Vanvalkenburgh, D.I. Albu, C. Bapanpally, S. Casanova, D. Califano, D.M. Jones, L. Ignatowicz, S. Kawamoto, S. Fagarasan, N.A. Jenkins, N.G. Copeland, P. Liu, D. Avram, Critical role of Bcl11b in suppressor function of T regulatory cells and prevention of inflammatory bowel disease, J. Exp. Med. 208(10) (2011) 2069e2081.

[29] N. Mayuzumi, H. Matsushima, A. Takashima, IL-33 promotes DC development in BM culture by triggering GM-CSF production, Eur. J. Immunol. 39(12) (2009) 3331e3342.

[30] D. Califano, J.J. Cho, M.N. Uddin, K.J. Lorentsen, Q. Yang, A. Bhandoola, H. Li, D. Avram, Transcription factor Bcl11b controls identity and function of mature type 2 innate lymphoid cells, Immunity 43 (2) (2015) 354e368.

[31] J.S. Lewis, N.V. Dolgova, Y. Zhang, C.Q. Xia, C.H. Wasserfall, M.A. Atkinson, M.J. Clare-Salzler, B.G. Keselowsky, A combination dual-sized microparticle system modulates dendritic cells and prevents type 1 diabetes in prediabetic NOD mice, Clin. Immunol. 160(1) (2015) 90e102.

[32] A. Jaeger, V. Dardalhon, R.A. Sobel, E. Bettelli, V.K. Kuchroo, Th1, Th17, and Th9 effector cells induce experimental autoimmune encephalomyelitis with different pathological phenotypes, J. Immunol. 183(11) (2009) 7169e7177.

[33] K. Hirota, J.H. Duarte, M. Veldhoen, E. Hornsby, Y. Li, D.J. Cua, H. Ahlfors, C. Wilhelm, M. Tolaini, U. Menzel, A. Garefalaki, A.J. Potocnik, B. Stockinger, Fate mapping of IL-17-producing T cells in inflammatory responses, Nat. Immunol. 12(3) (2011) 255e263.

[34] Y. Yang, J. Weiner, Y. Liu, A.J. Smith, D.J. Huss, R. Winger, H. Peng, P.D. Cravens, M.K. Racke, A.E. Lovett-Racke, T-bet is essential for encephalitogenicity of both Th1 and Th17 cells, J. Exp. Med. 206(7) (2009) 1549e1564.

[35] Y. Lee, A. Awasthi, N. Yosef, F.J. Quintana, S. Xiao, A. Peters, C. Wu, M. Kleinewietfeld, S. Kunder, D.A. Hafler, R.A. Sobel, A. Regev, V.K. Kuchroo, Induction and molecular signature of pathogenic TH17 cells, Nat. Immunol. 13(10) (2012) 991e999

[36] L. Codarri, M. Greter, B. Becher, Communication between pathogenic T cells and myeloid cells in neuroinflammatory disease, Trends Immunol. 34(3) (2013) 114e119.

[37] Y. Xiao, J. Jin, M. Chang, J.H. Chang, H. Hu, X. Zhou, G.C. Brittain, C. Stansberg, Ø. Torkildsen, X. Wang, R. Brink, X. Cheng, S.C. Sun, Peli1 promotes microglia- mediated CNS inflammation by regulating Traf3 degradation, Nat. Med. 19(5) (2013) 595e602.

[38] D.M. Mosser, J.P. Edwards, Exploring the full spectrum of macrophage activation, Nat. Rev. Immunol. 8(12) (2008) 958e969.

CLAIMS

What is claimed is:

1. A dual microparticle system for targeting an antigen-presenting immune cell in a subject who is suspected of having, at risk of having or has Multiple Sclerosis, wherein the microparticle system is a composition that comprises:

microparticles that are phagocytosable by the antigen-presenting immune cell, and microparticles that are non-phagocytosable by the antigen-presenting immune cell;

wherein the phagocytosable microparticles together comprise at least one myelin-derived antigen and at least one immunomodulatory agent selected from vitamin D3, vitamin D3 analog, glucocorticoid, estrogen, rapamycin, and retinoic acid; and

wherein the non-phagocytosable microparticles comprise at least one immunosuppressive tolerogenic agent selected from IL-10, TGF-β, and nonsteroidal anti-inflammatory drugs (NSAIDs), an agent that recruits the antigen-presenting immune cell of interest selected from GM-CSF, G-CFS, M-CSF, CCL19, CCL20, CCL21, and VEGF-C.

2. The microparticle system of claim 1, further comprising a remyelinating agent selected from clemastine, clobetasol, digoxin, miconazole, phenytoin, and quetiapine.

3. The microparticle system of claim 1 or 2, wherein the remyelinating agent is administered in soluble form by intravenous injection or is incorporated into the non-phagocytosable microparticles.

4. The microparticle system of any of claims 1-3, wherein the phagocytosable microparticle has a diameter of 0.2 μm – 5.0μm and the non-phagocytosable microparticle has a diameter of 15.0μm – 50.0μm.

5. The microparticle system of any of claims 1-4, wherein the phagocytosable micropartcle or non-phagocytosable microparticle are fabricated from poly(lactic-co-glycolic acid) (PLGA).

6. The microparticle system of any of claims 1-5, wherein the at least one myelin-derived antigen comprises at least one of SEQ ID NOs 1-25 or a contiguous fragment thereof.

7. The microparticle system of claim 6, wherein the at least one myelin-derived antigen comprises at least one of SEQ ID NOs 12-25 or a fragment thereof comprising contiguous amino acids of said SEQ ID NOs.

8. The microparticle system of claim 7, wherein at least one myelin-derived antigen comprises SEQ ID NO. 23.

9. The microparticle system of any of claims 1-8, wherein the composition is in a liquid formulation further comprising a pharmaceutically acceptable carrier.

10. The microparticle system of any of claims 1-9, wherein the phagocytosable MPs comprise the amino acid sequence of SEQ ID NO. 23 and vitamin D3.

11. The microparticle system of any of claims 1-10, wherein the non-phagocytosable MPs comprise TGF-β1 and GM-CSF.

12. A method of treating a subject who is suspected of having, at risk of having or has MS comprising administering a therapeutically effective amount of a composition of any of claims 1-9.

13. The method, according to claim 12, wherein the ratio of the at least one myelin-derived antigen to the at least one immunomodulatory agent is between 1:20 and 1:1.

14. A method of treating a subject who is suspected of having, at risk of having or has MS comprising administering a therapeutically effective amount of a first composition that comprises microparticles that are phagocytosable by the antigen-presenting immune cell, and a second composition comprising microparticles that are non-phagocytosable by the antigen-presenting immune cell;

wherein the phagocytosable microparticles together comprise at least one myelin-derived antigen and at least one immunomodulatory agent selected from vitamin D3, vitamin D3 analog, glucocorticoid, estrogen, rapamycin, and retinoic acid;

wherein the non-phagocytosable microparticles comprise at least one immunosuppressive tolerogenic agent selected from IL-10, TGF-β, and nonsteroidal anti-inflammatory drugs (NSAIDs), an agent that recruits the antigen-presenting immune cell of interest selected from GM-CSF, G-CFS, M-CSF, CCL19, CCL20, CCL21, and VEGF-C; and

wherein the first composition and second composition are admixed prior administration or are administered separately.

15. The method of claim 14, wherein the first composition and second composition are administered separately.

16. The method of claim 15, wherein the first composition and second composition are administered by the same or different modes of administration.

17. The method of claim 16, wherein the modes of administration comprise subcutaneous, intradermal, intramuscular, or intravenous administration.

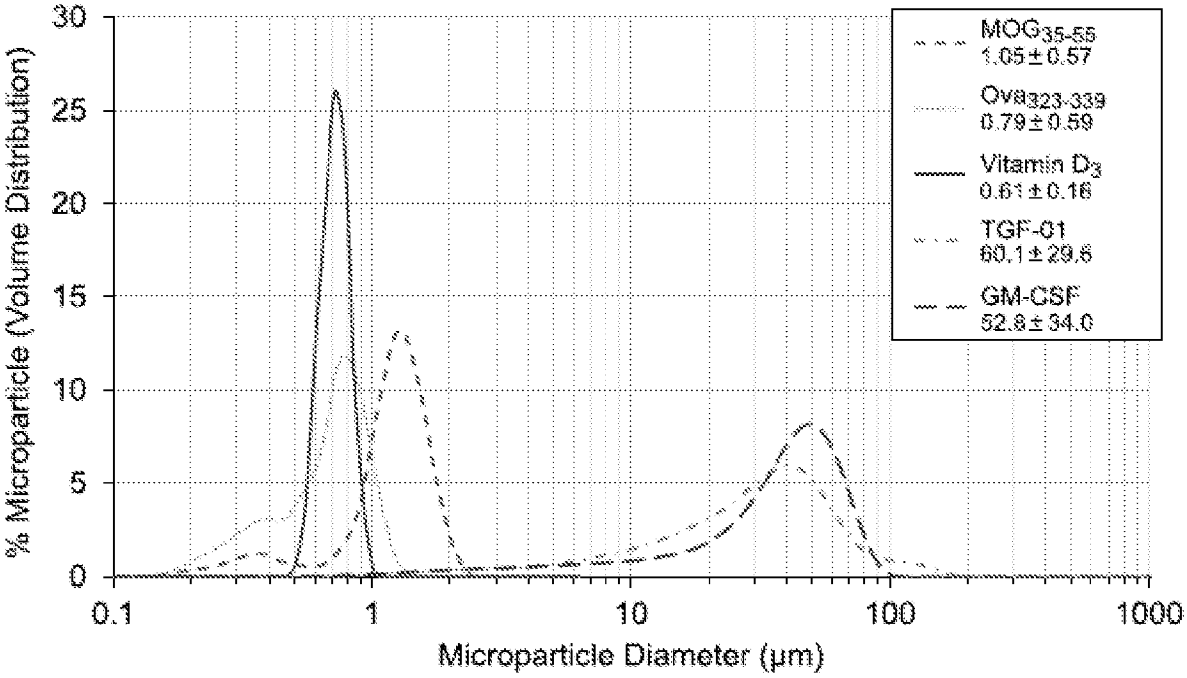

FIG. 1A

Biological/Pharmacologic Agent	Mass Loaded/PLGA (μg/500 mg)	Encapsulation Efficiency ± SD (%)	Mass Injected per 2.5 mg PLGA ± SD (ng)
MOG_{35-55}	4000	48.6 ± 9.0	9,711± 1,809
$OVA_{323-339}$	4000	49.9 ± 2.8	9,988± 556
Vitamin D_3	50	65.5 ± 3.0	164 ± 8
TGF-01	25	44.2 ± 12.1	55 ± 15
GM-CSF	40	59.6 ± 6.8	119 ± 14

FIG. 1B

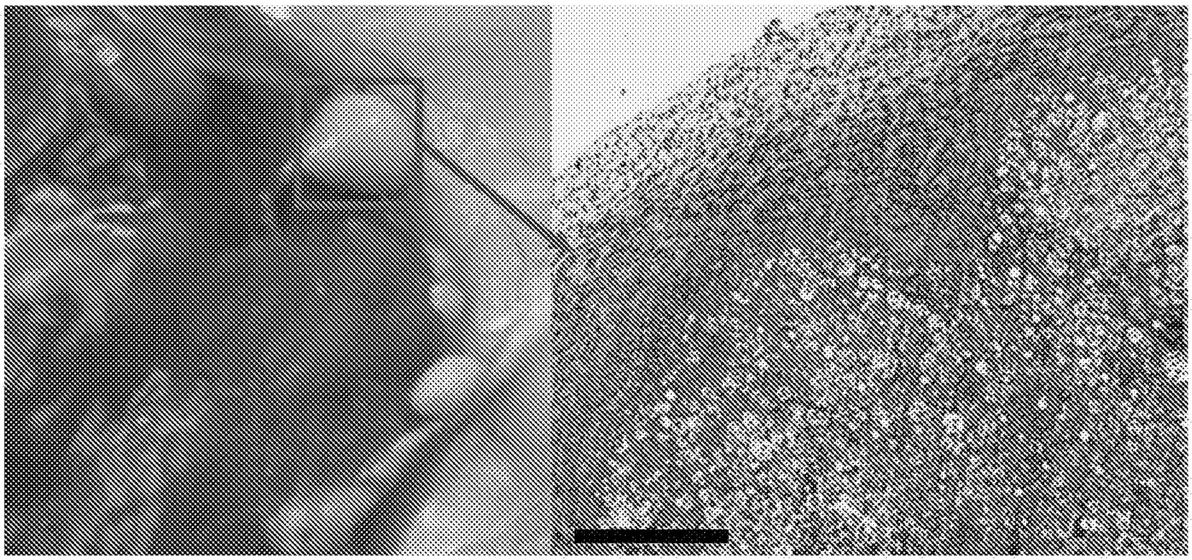

FIG. 2A

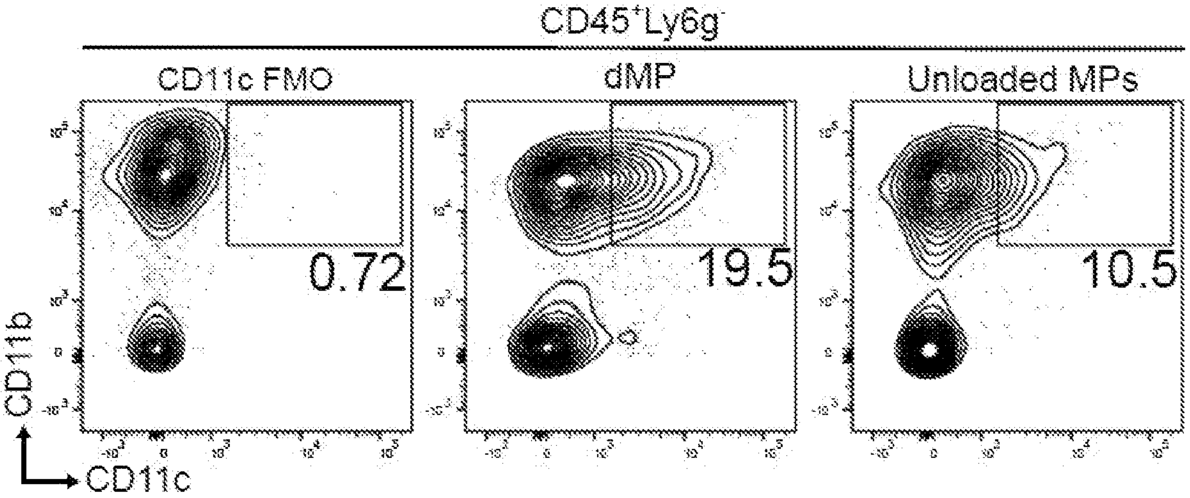
CD45+Ly6g-
CD11c FMO
dMP
Unloaded MPs
0.72
19.5
10.5
CD11b
CD11c
-10^3
0
10^3
10^4
10^5

FIG. 2B

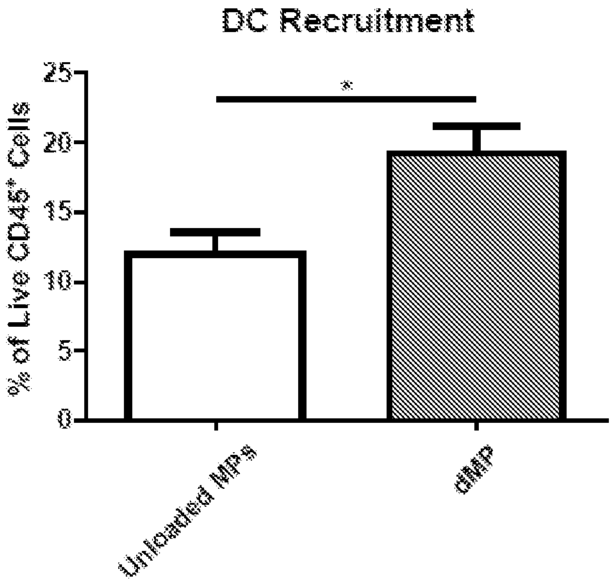
DC Recruitment
% of Live CD45+ Cells
25
20
15
10
5
0
*
Unloaded MPs
dMP

FIG. 2C

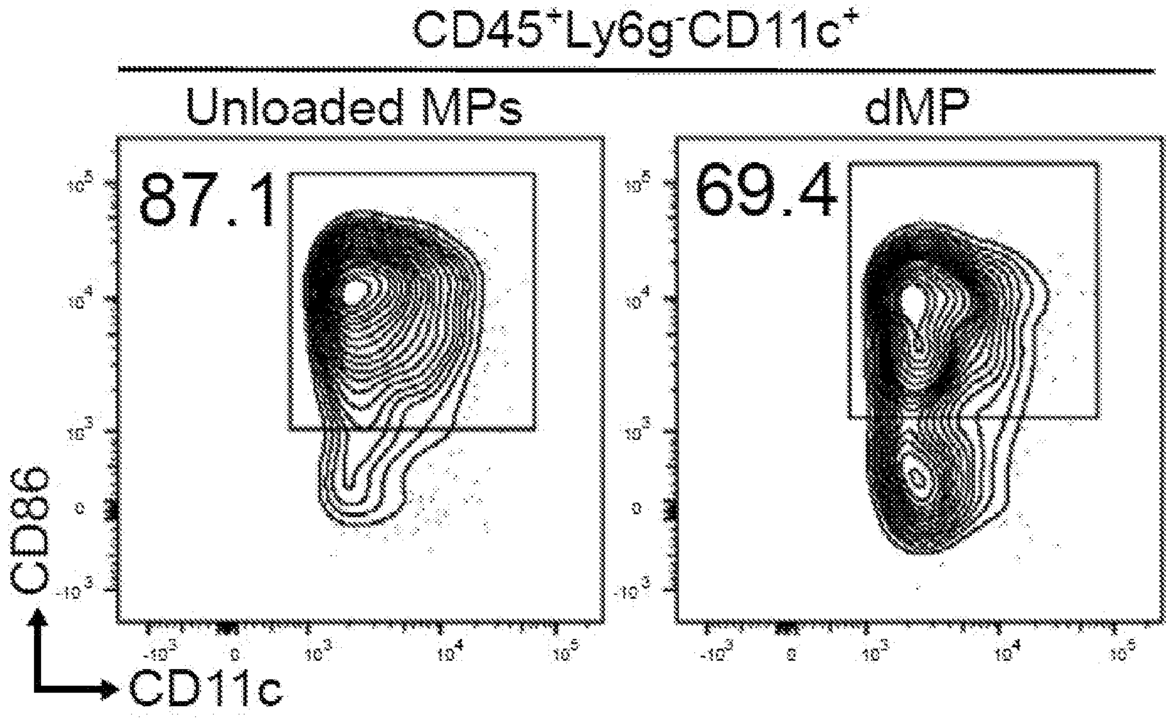
CD45+Ly6g-CD11c+
Unloaded MPs
dMP
87.1
69.4
10^5
10^4
10^3
0
-10^3
CD86
CD11c
-10^3
0
10^3
10^4
10^5

FIG. 2D

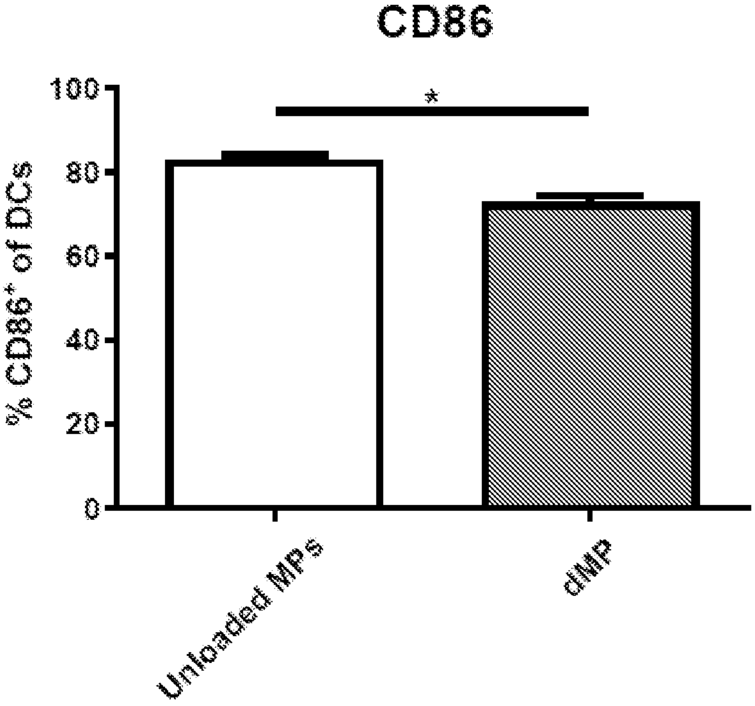
CD86
% CD86+ of DCs
100
80
60
40
20
0
*
Unloaded MPs
dMP

FIG. 2E

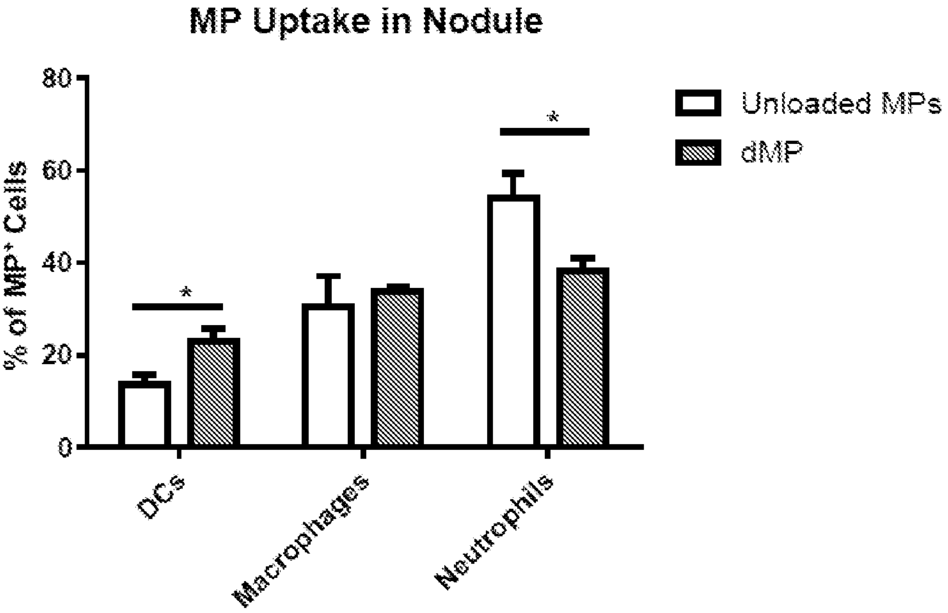
MP Uptake in Nodule
% of MP+ Cells
80
60
40
20
0
*
*
DCs
Macrophages
Neutrophils
Unloaded MPs
dMP

FIG. 2F

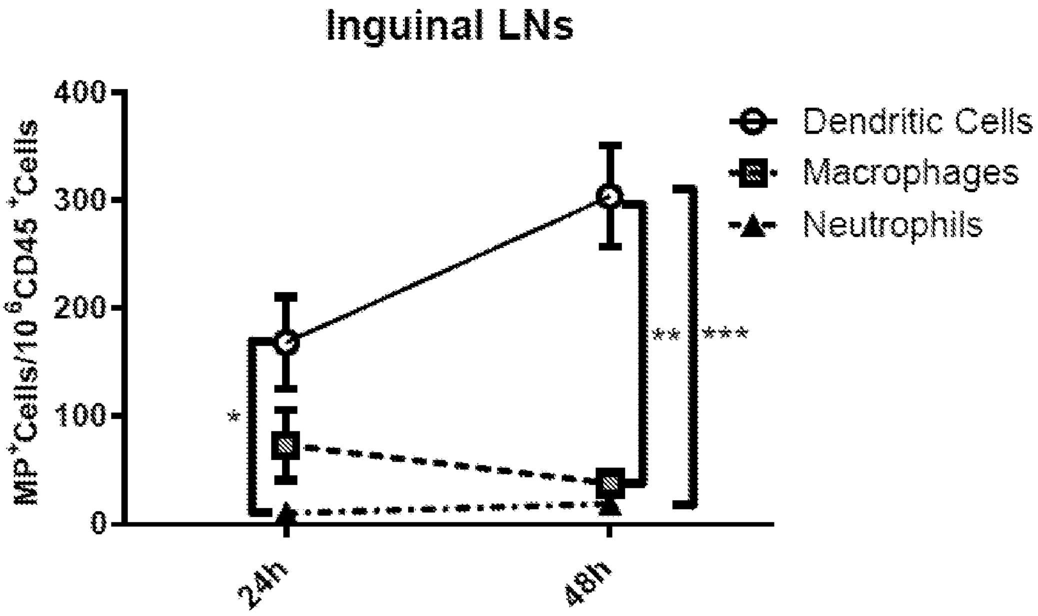
Inguinal LNs
MP⁺Cells/10⁶CD45⁺Cells
400
300
200
100
0
24h
48h
Dendritic Cells
Macrophages
Neutrophils
*
**
***

FIG. 2G

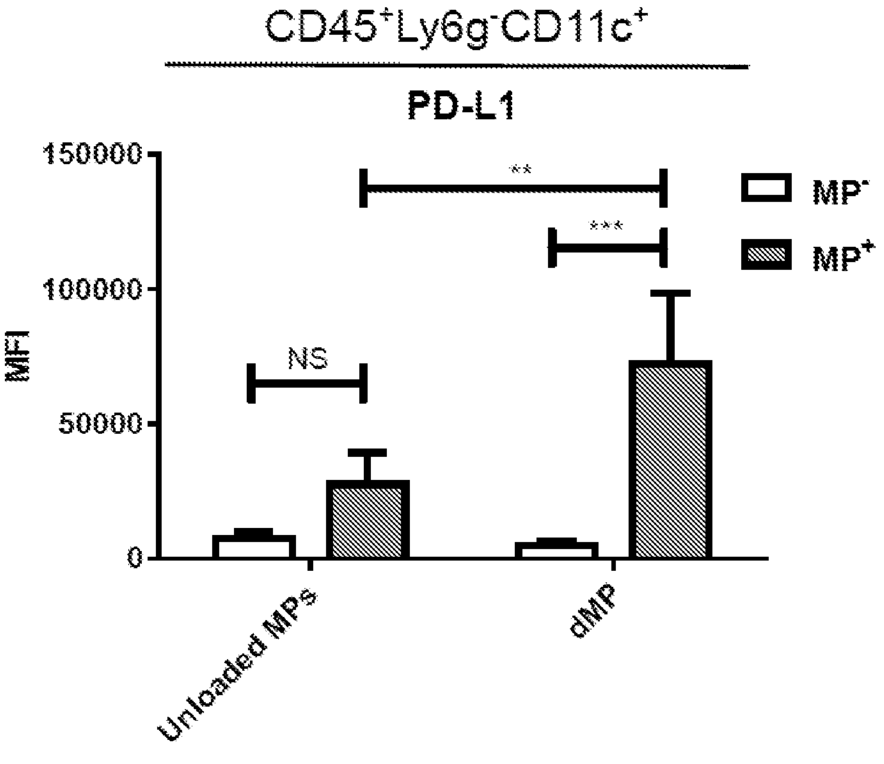
CD45⁺Ly6g⁻CD11c⁺
PD-L1
MFI
150000
100000
50000
0
Unloaded MPs
dMP
NS
**
***
MP⁻
MP⁺

FIG. 2H

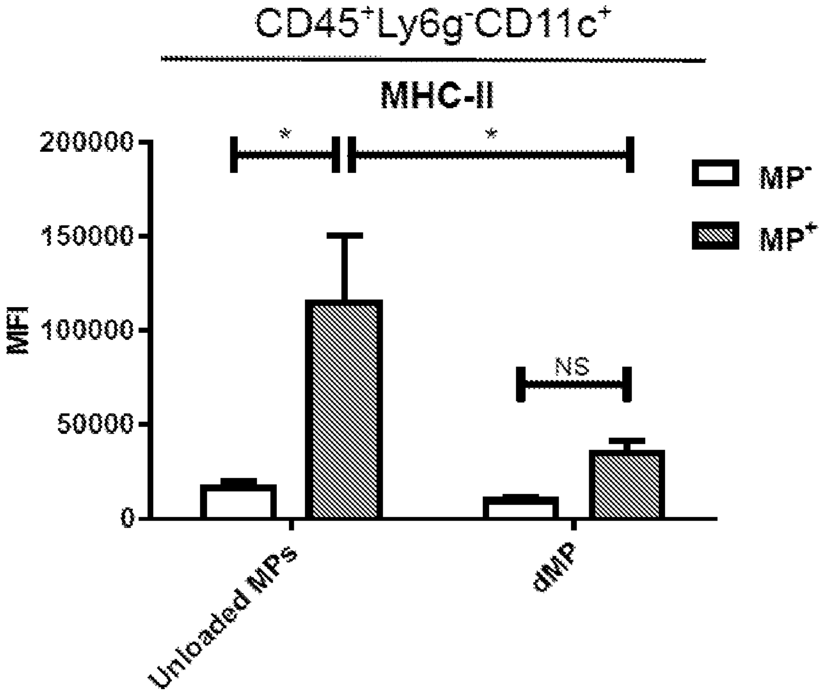

FIG. 2I

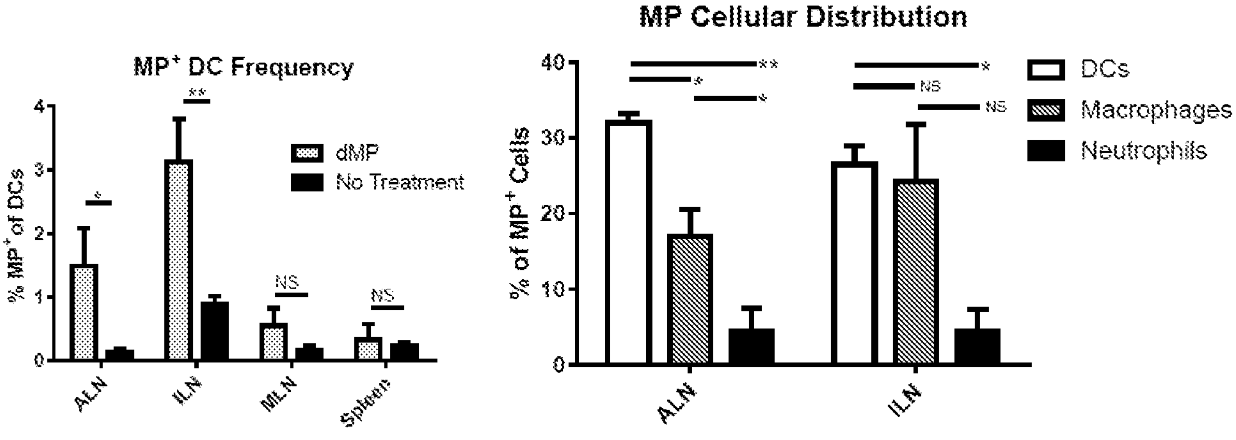

FIG. 2J

FIG. 2K

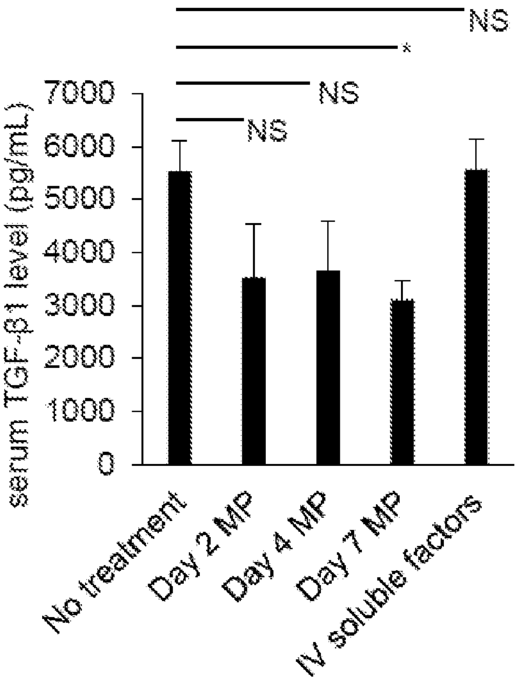

FIG. 2L

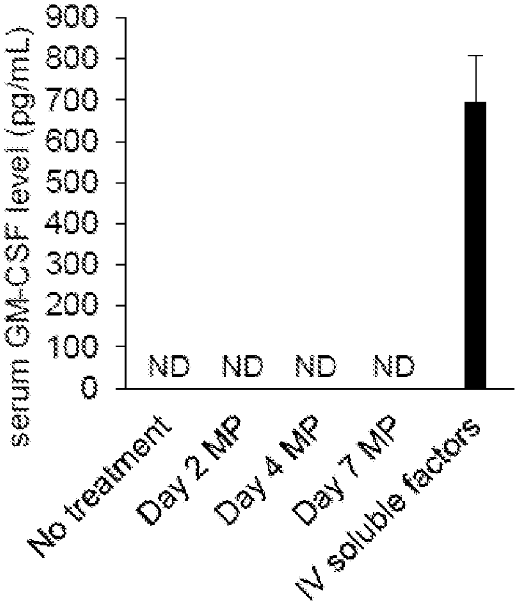

FIG. 2M

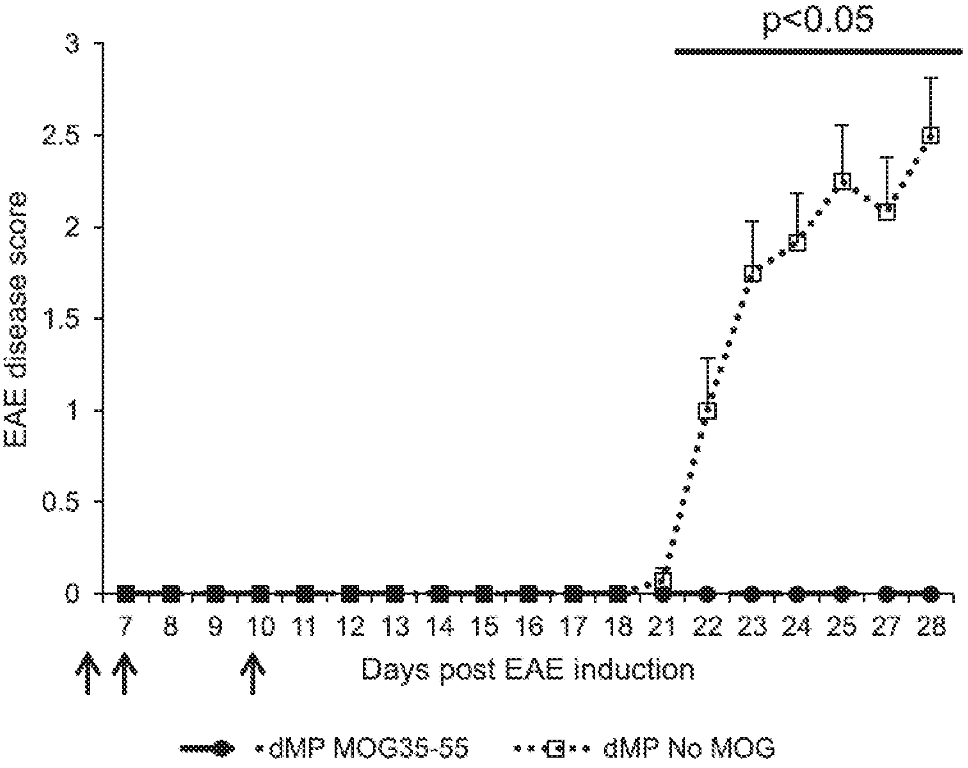

FIG. 3A

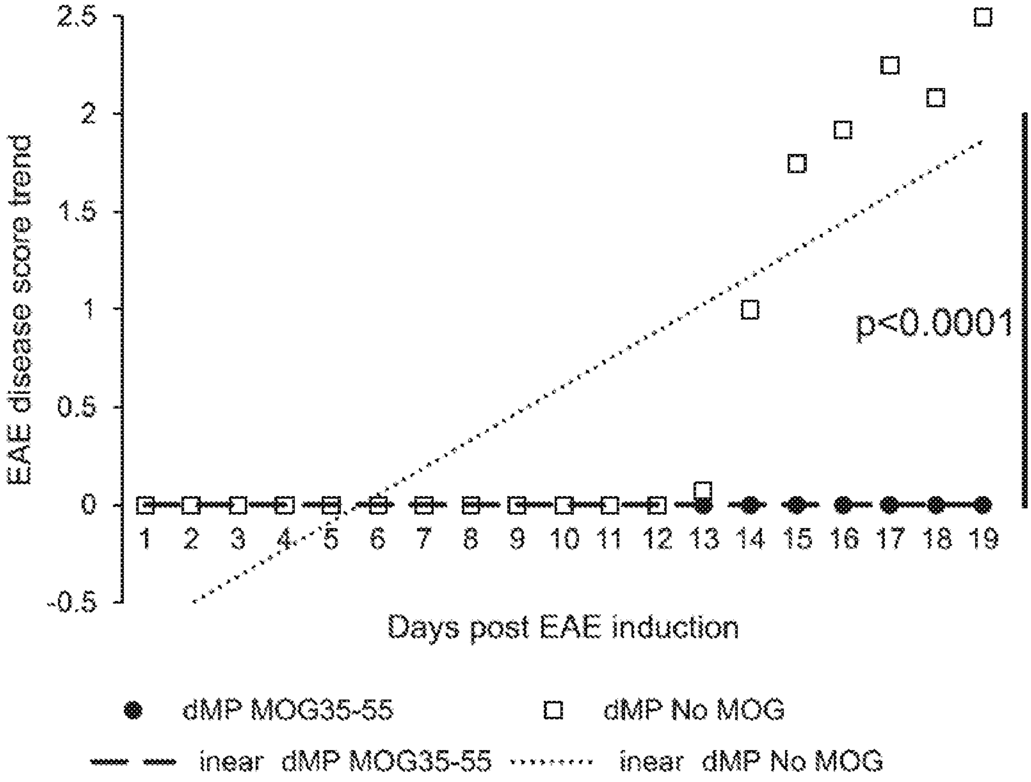
EAE disease score trend
2.5
2
1.5
1
0.5
0
-0.5
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19
Days post EAE induction
p<0.0001
● dMP MOG35-55
□ dMP No MOG
inear dMP MOG35-55
inear dMP No MOG

FIG. 3B

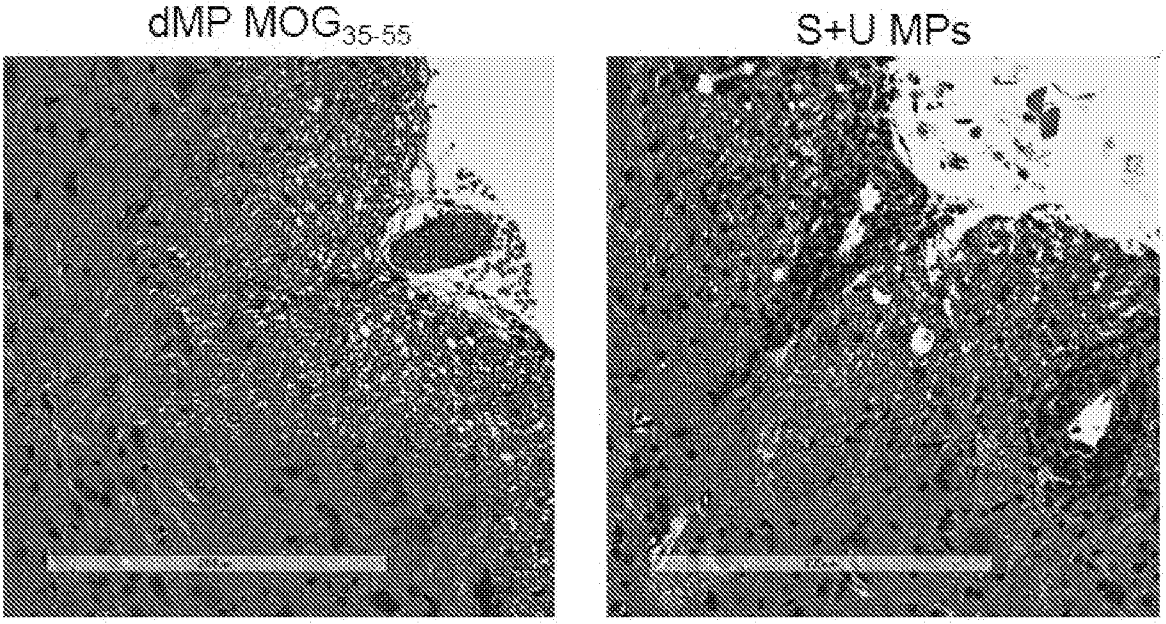
dMP MOG$_{35-55}$
S+U MPs

FIG. 4A

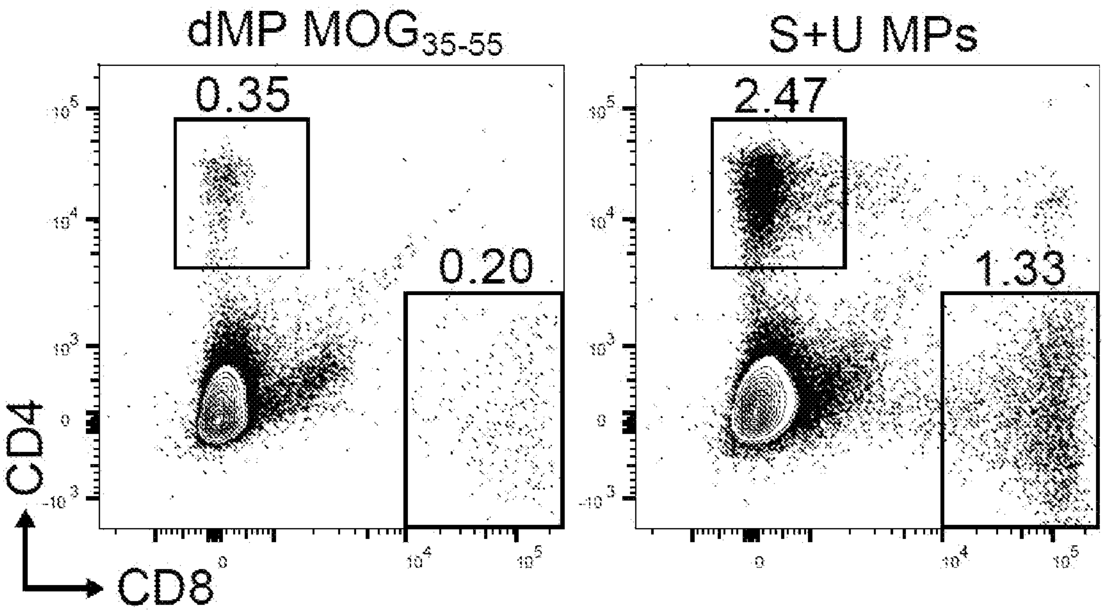
dMP MOG$_{35-55}$
S+U MPs
0.35
0.20
2.47
1.33
CD4
CD8
10^5
10^4
10^3
0
-10^3

FIG. 4B

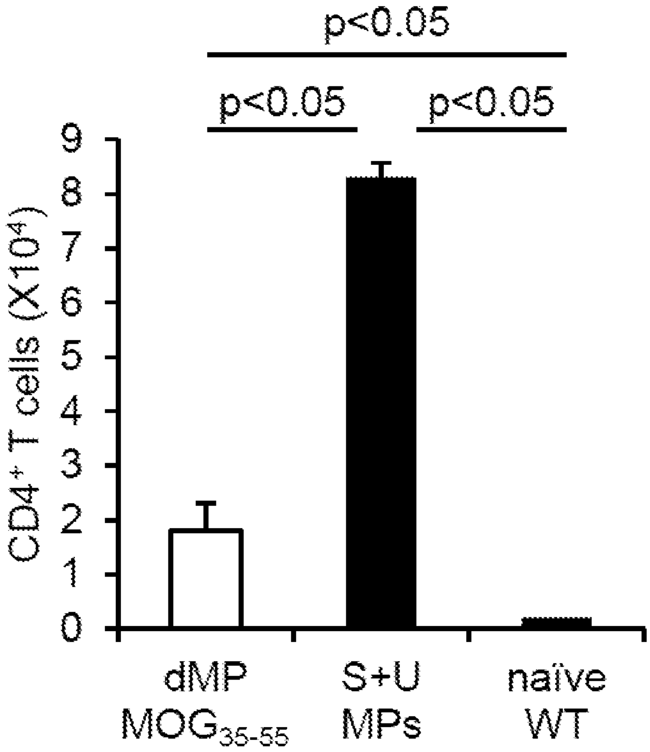
p<0.05
p<0.05
p<0.05
CD4$^+$ T cells (X10^4)
0
1
2
3
4
5
6
7
8
9
dMP MOG$_{35-55}$
S+U MPs
naïve WT

FIG. 4C

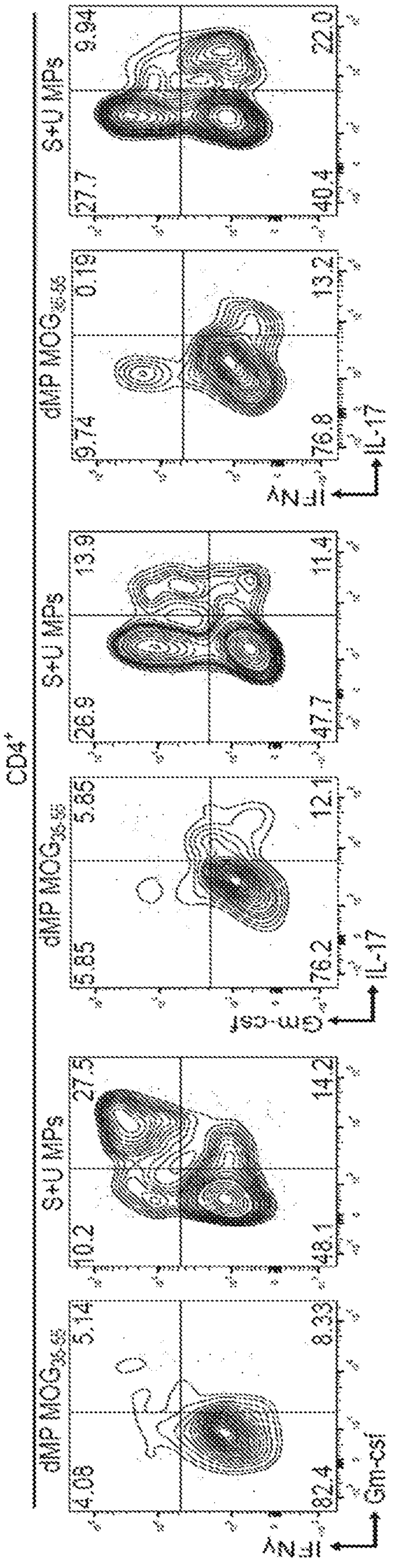
CD4$^+$
dMP MOG$_{35-55}$
S+U MPs
dMP MOG$_{35-55}$
S+U MPs
dMP MOG$_{35-55}$
S+U MPs
4.08
5.14
82.4
8.33
10.2
27.5
48.1
14.2
5.85
5.85
76.2
12.1
26.9
13.9
47.7
11.4
9.74
0.19
76.8
13.2
27.7
9.94
40.4
22.0
IFNγ
Gm-csf
Gm-csf
IL-17
IFNγ
IL-17

FIG. 5A

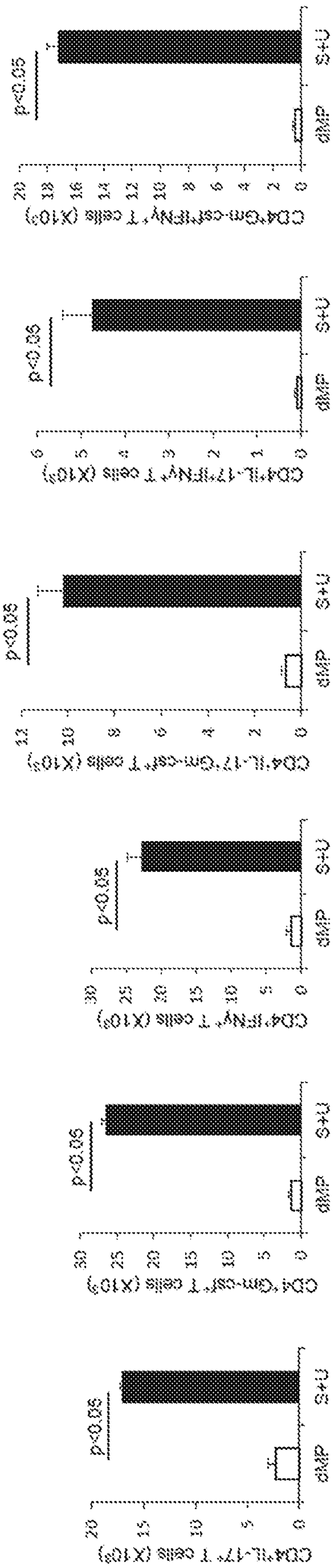
CD4$^+$IL-17$^+$ T cells (X10^3)
0
5
10
15
20
p<0.05
CD4$^+$Gm-csf$^+$ T cells (X10^3)
0
5
10
15
20
25
30
p<0.05
CD4$^+$IFNγ$^+$ T cells (X10^3)
0
5
10
15
20
25
30
p<0.05
CD4$^+$IL-17$^+$Gm-csf$^+$ T cells (X10^3)
0
2
4
6
8
10
12
p<0.05
CD4$^+$IL-17$^+$IFNγ$^+$ T cells (X10^3)
0
1
2
3
4
5
6
p<0.05
CD4$^+$Gm-csf$^+$IFNγ$^+$ T cells (X10^3)
0
2
4
6
8
10
12
14
16
18
20
p<0.05
dMP MOG$_{35-55}$
S+U MPs

FIG. 5B

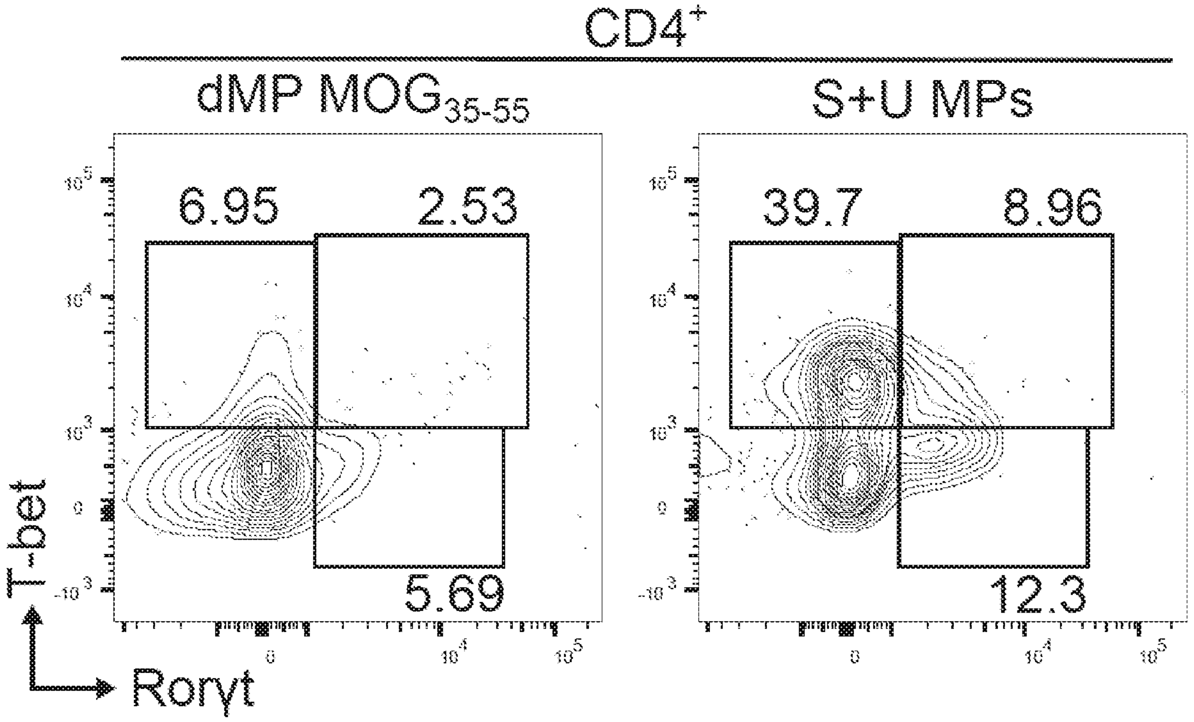
CD4+
dMP MOG35-55
S+U MPs
6.95
2.53
5.69
39.7
8.96
12.3
T-bet
Rorγt
10^5
10^4
10^3
0
-10^3

FIG. 6A

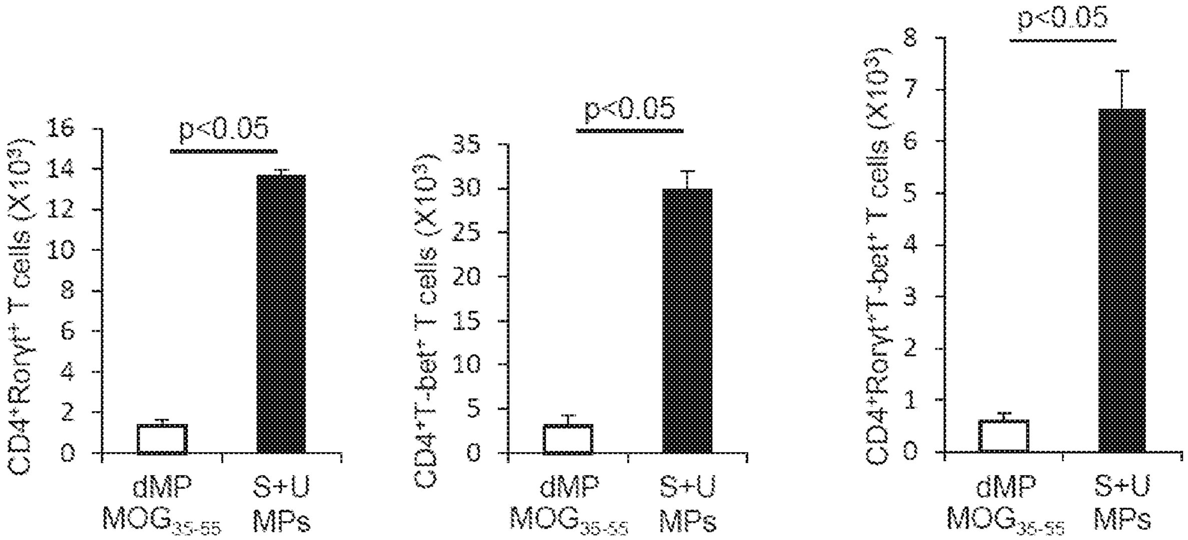
p<0.05
CD4+Rorγt+ T cells (X10³)
CD4+T-bet+ T cells (X10³)
CD4+Rorγt+T-bet+ T cells (X10³)
dMP MOG35-55
S+U MPs
16
14
12
10
8
6
4
2
0
35
30
25
20
15
10
5
7
3
1

FIG. 6B

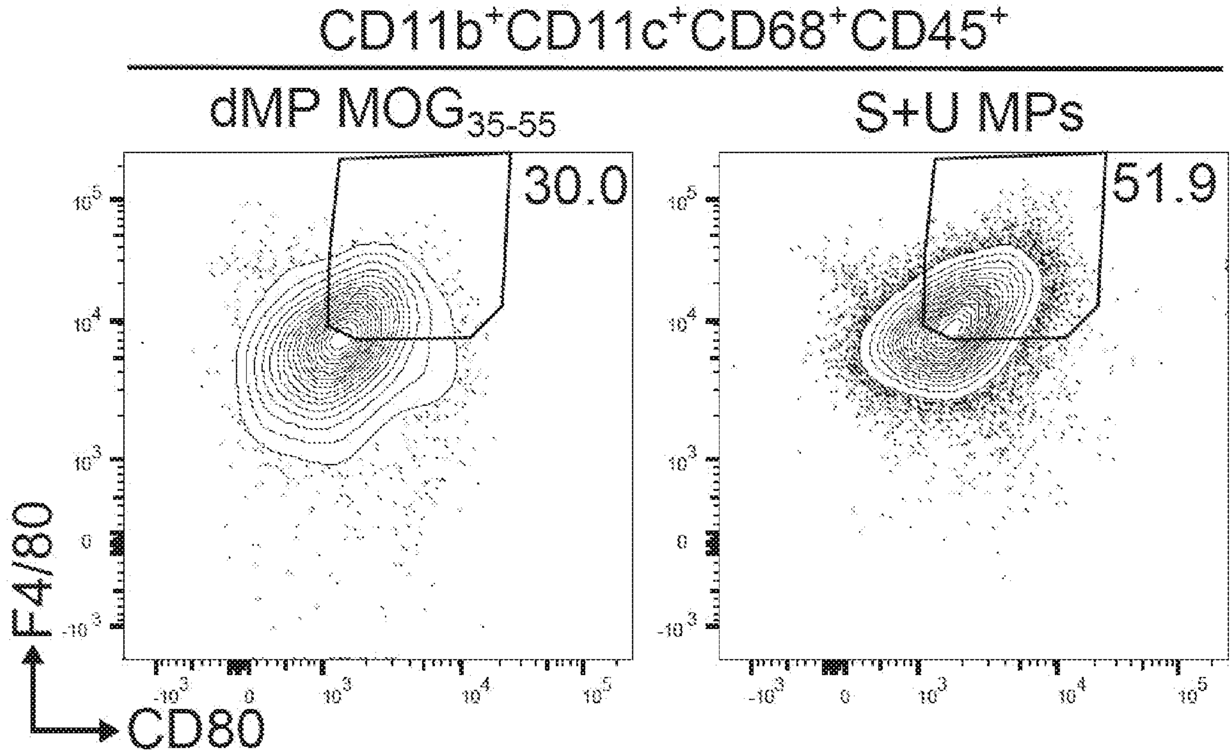
CD11b+CD11c+CD68+CD45+
dMP MOG35-55
S+U MPs
30.0
51.9
F4/80
CD80
10^5
10^4
10^3
0
-10^3

FIG. 7A

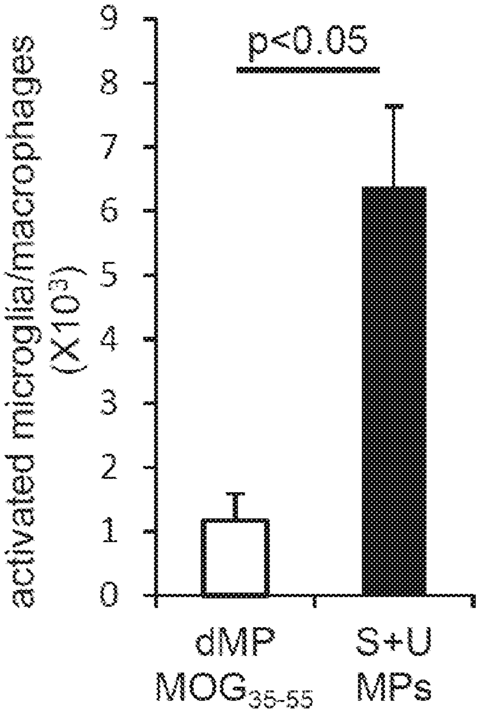
activated microglia/macrophages (X10^3)
p<0.05
0
1
2
3
4
5
6
7
8
9
dMP MOG35-55
S+U MPs

FIG. 7B

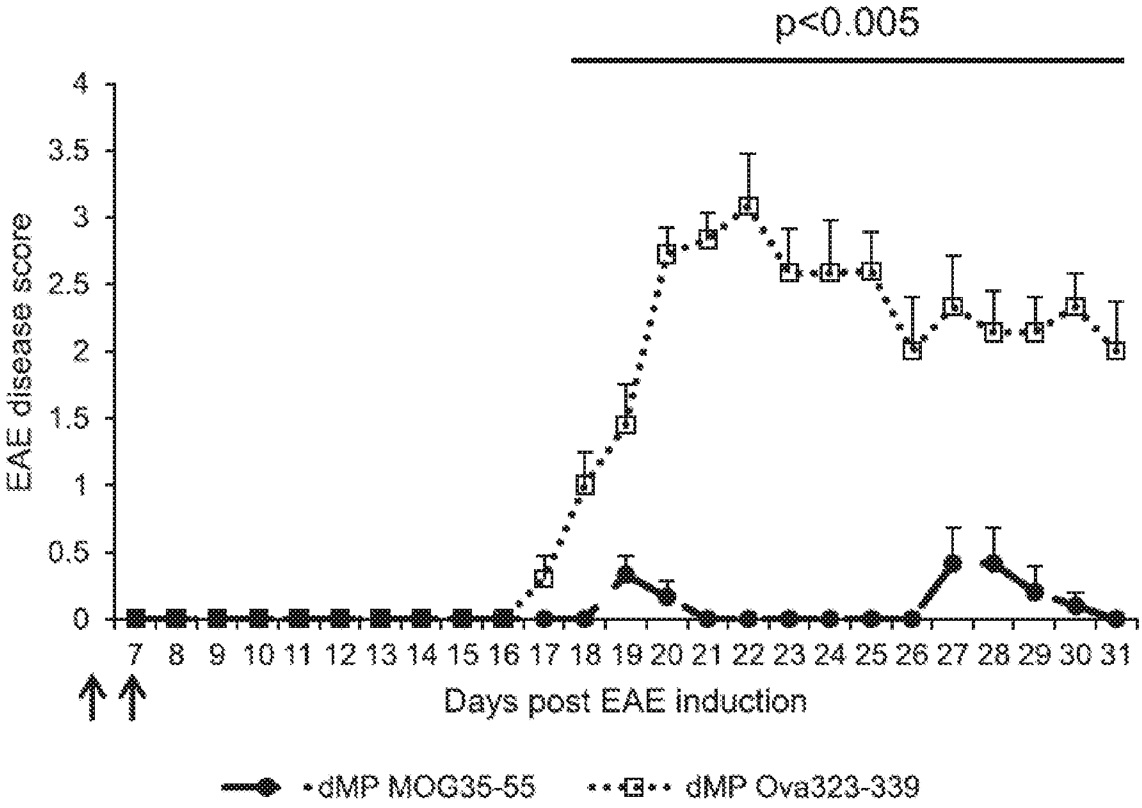
p<0.005
EAE disease score
4
3.5
3
2.5
2
1.5
1
0.5
0
7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31
Days post EAE induction
dMP MOG35-55
dMP Ova323-339

FIG. 8A

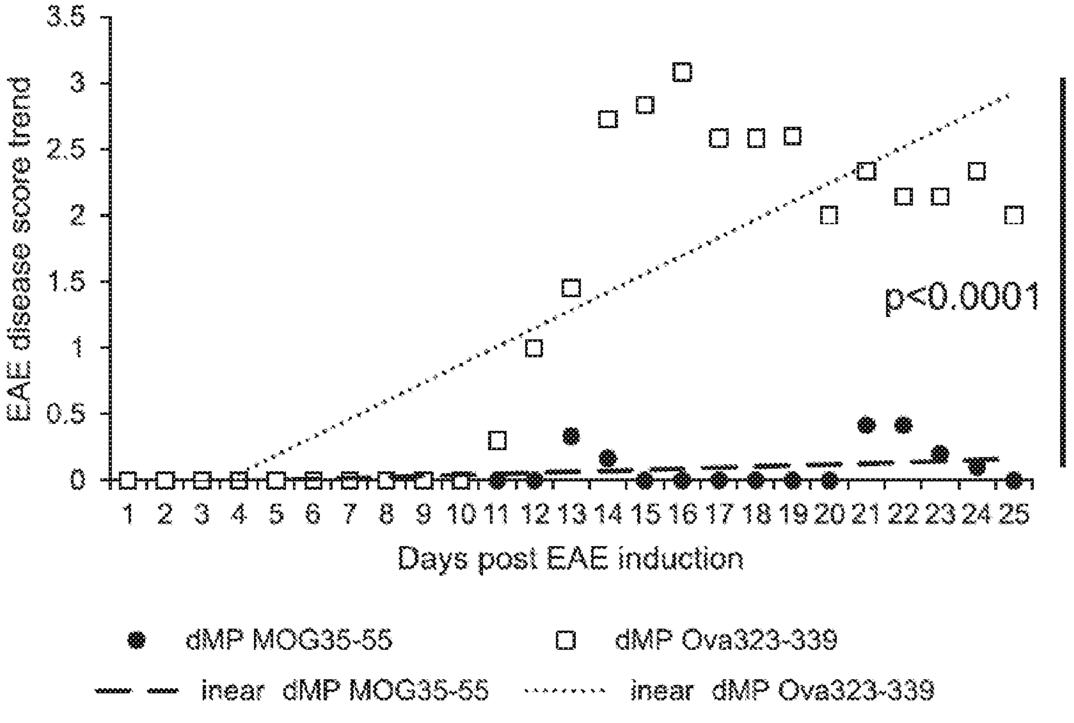
EAE disease score trend
3.5
3
2.5
2
1.5
1
0.5
0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25
Days post EAE induction
p<0.0001
dMP MOG35-55
dMP Ova323-339
inear dMP MOG35-55
inear dMP Ova323-339

FIG. 8B

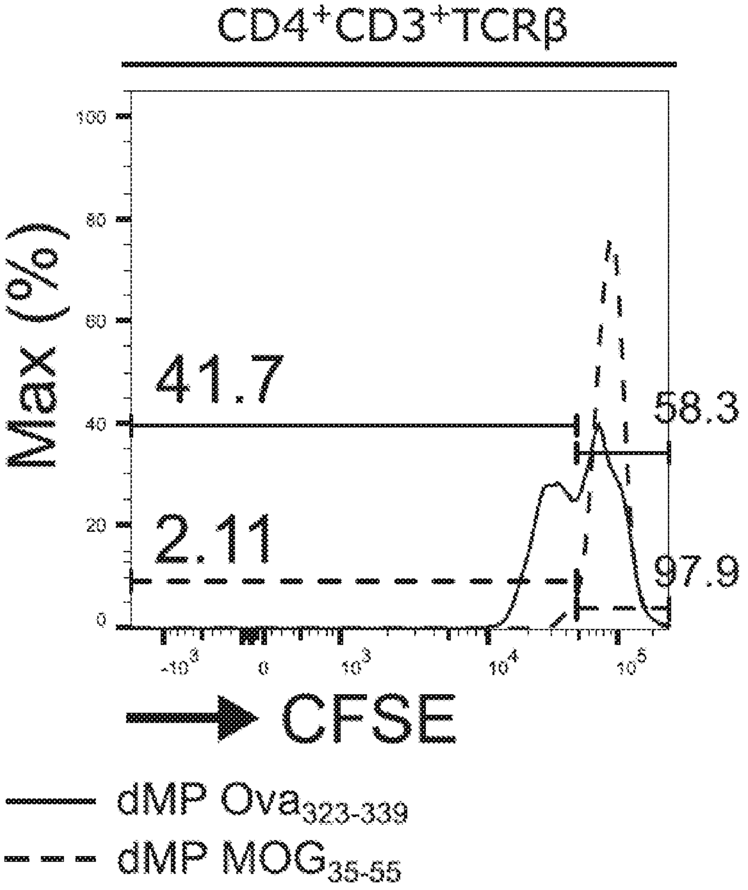
CD4$^+$CD3$^+$TCRβ
Max (%)
100
80
60
40
20
0
41.7
58.3
2.11
97.9
-10^3
0
10^3
10^4
10^5
CFSE
dMP Ova$_{323-339}$
dMP MOG$_{35-55}$

FIG. 9A

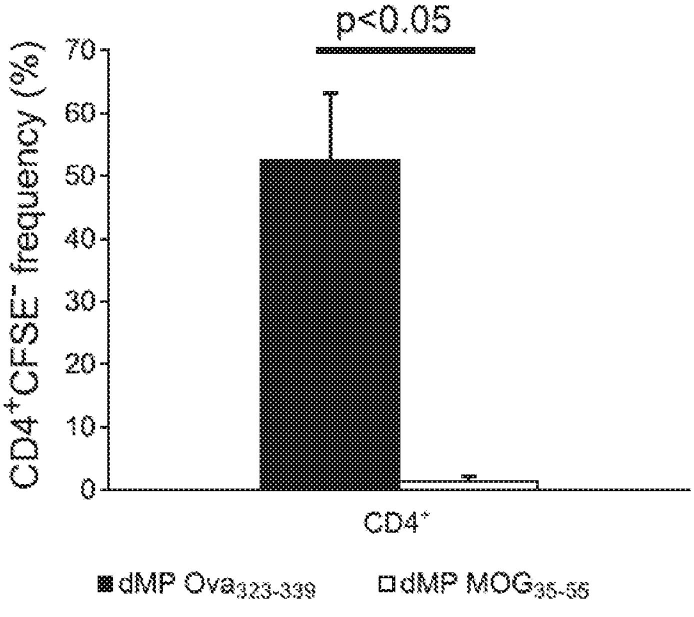
p<0.05
CD4$^+$CFSE$^-$ frequency (%)
70
60
50
40
30
20
10
0
CD4$^+$
■ dMP Ova$_{323-339}$
□ dMP MOG$_{35-55}$

FIG. 9B

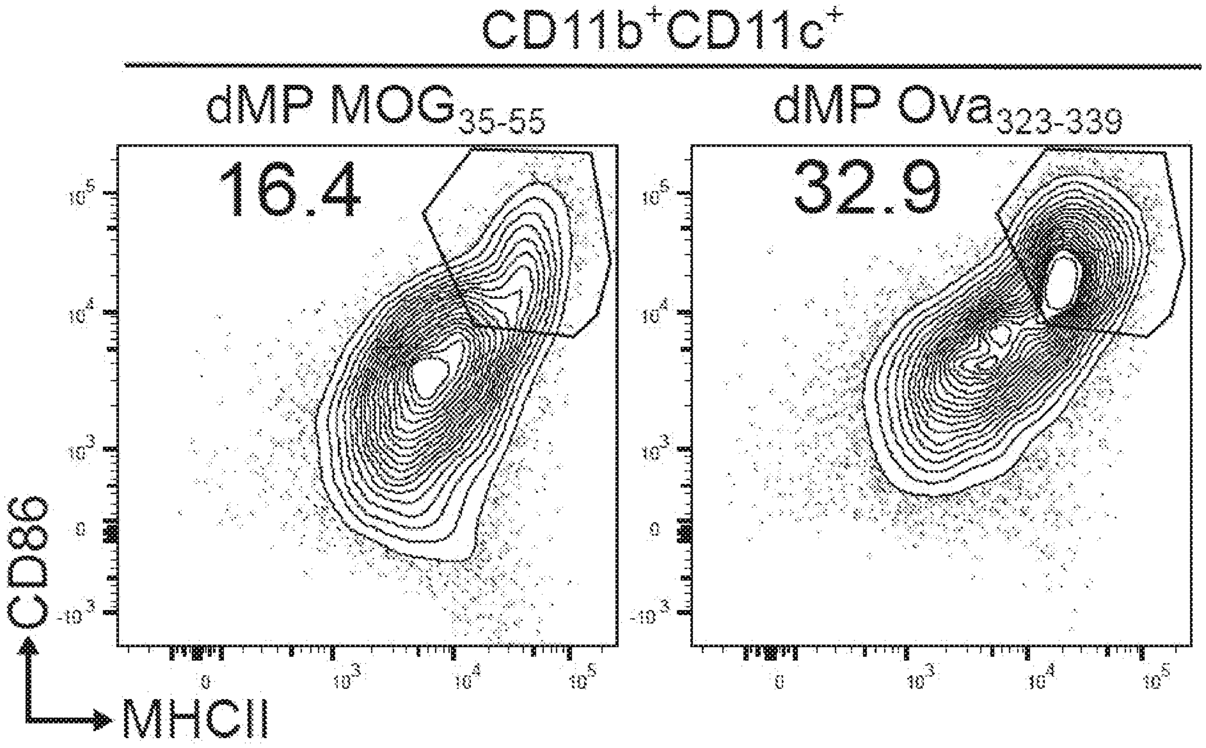

FIG. 10A

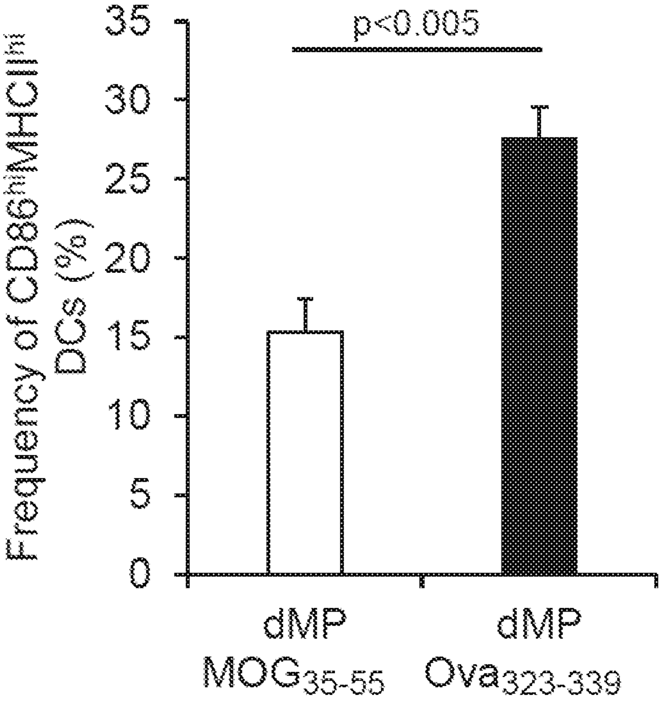

FIG. 10B

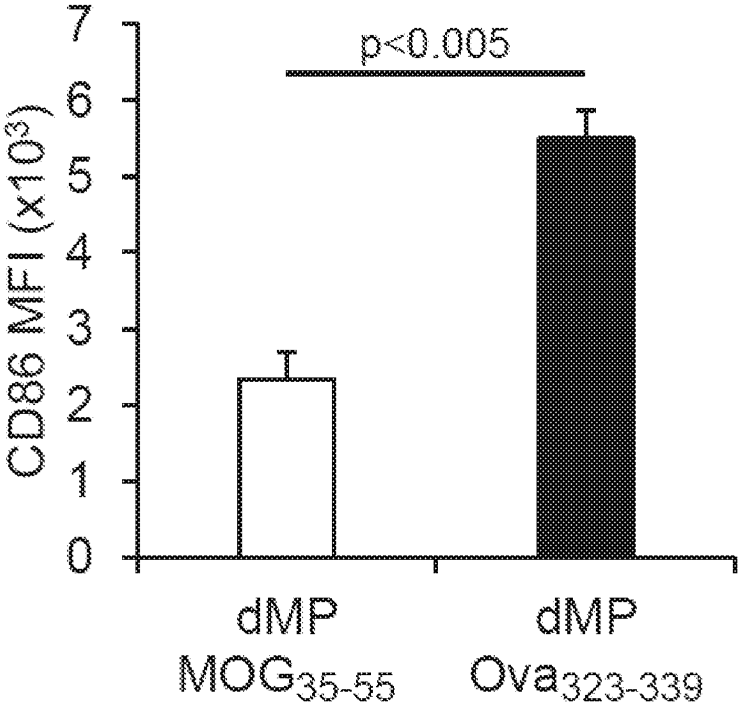

FIG. 10C

Description	Sequence	aa	M_r
MBP$_{13-32}$	KYLATASTMDHARHGFLPRH	20	2351.1
MBP$_{83-99}$	ENPVVHFFKNIVTPRTP	17	2036.3
MBP$_{111-129}$	LSRFSWGAEGQRPGFGYGG	19	2070.6
MBP$_{131-155}$	ASDYKSAHKGLKGVDAQGTLSKIFK	25	2691.1
MBP$_{146-170}$	AQGTLSKIFKLGGRDSRSGSPMARR	25	2718.2
PLP$_{40-60}$	TGTEKLIETYFSKNYQDYEYL	21	2647.0
PLP$_{89-106}$	GFYTTGAVRQIFGDYKTT	18	2066.5
PLP$_{139-154}$	HCLGKWLGHPDKFVGI	16	1848.6
PLP$_{178-197}$	NTWTTCQSIAFPSKTSASIG	20	2141.6
PLP$_{190-208}$	SKTSASIGSLCADARMYGVLP	20	2071.8
MOG$_{1-20}$	GQFRVIGPRHPIRALVGDEV	20	2215.8
MOG$_{11-30}$	PIRALVGDEVELPCRISPGK	20	2190.8
MOG$_{35-55}$	MEVGWYRPPFSRVVHLYRNGK	21	2633.2
CNP$_{343-373}$	EVGELSRGKLYSLGNGRWMLTLAKNMEVRAI	31	3534.2
CNP$_{356-388}$	GNGRWMLTLAKNMEVRAIFTGYYGKGKPVPTQG	33	3683.4

FIG. 11

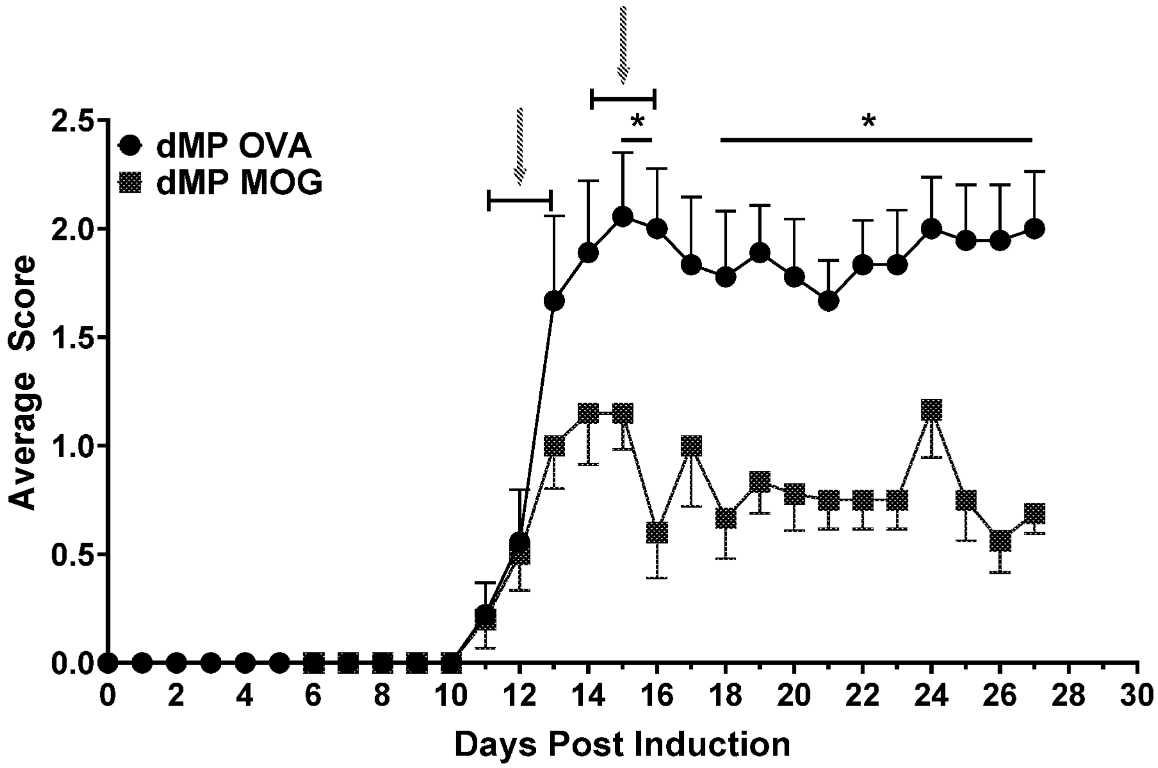
2.5
2.0
1.5
1.0
0.5
0.0
Average Score
● dMP OVA
▨ dMP MOG
*
*
0
2
4
6
8
10
12
14
16
18
20
22
24
26
28
30
Days Post Induction

FIG. 12

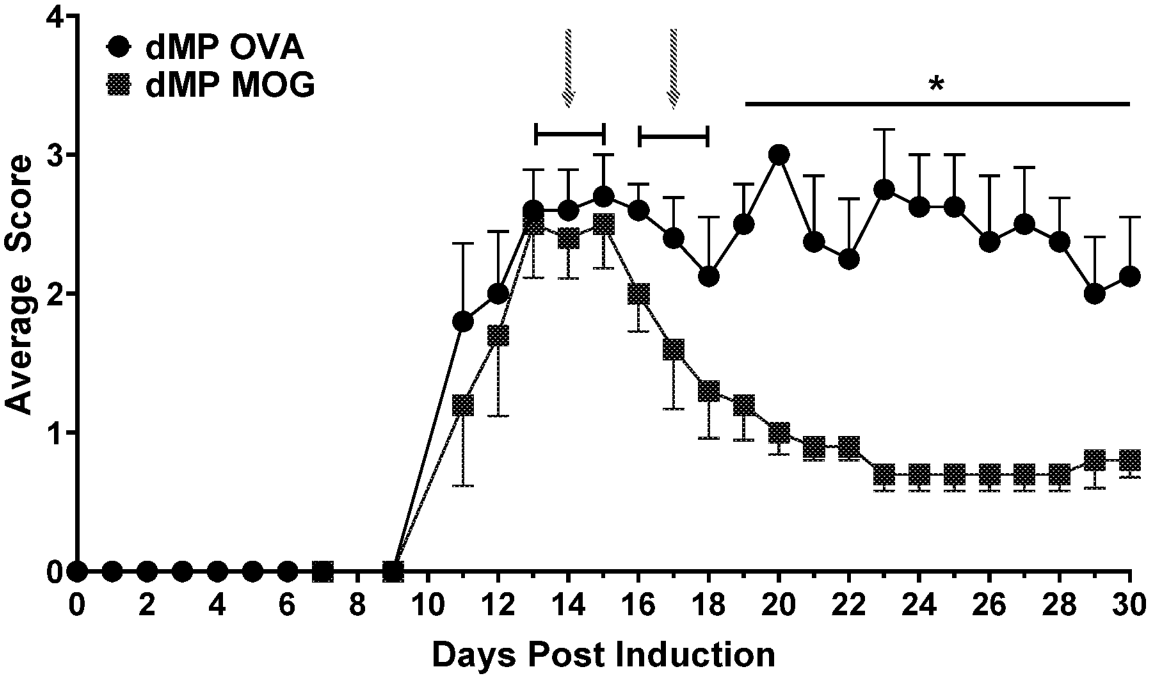
dMP OVA
dMP MOG
*
Average Score
0
1
2
3
4
Days Post Induction
0
2
4
6
8
10
12
14
16
18
20
22
24
26
28
30

FIG. 13

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US19/40534

A. CLASSIFICATION OF SUBJECT MATTER

IPC - A61K 9/16, 9/51, 39/39, 47/34; A61P 25/28 (2019.01)

CPC - A61K 9/167, 9/51, 39/39, 39/0008, 47/34; A61P 25/28

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
See Search History document

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
See Search History document

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
See Search History document

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X -- Y	(CHO, JJ et al.) An antigen-specific semi-therapeutic treatment with local delivery of tolerogenic factors through a dual-sized microparticle system blocks experimental autoimmune encephalomyelitis. Biomaterials. October 2017, Epub 24 July 2017, No. 143; pages 79-92; abstract; page 3, 1st-2nd paragraphs; page 5, 3rd paragraph; page 7, 3rd paragraph; page 13, 2nd paragraph; DOI: 10.1016/j.biomaterials	1, 14 ------- 2, 3/1-2, 15-17
Y	WO 2018/013625 A1 (CONCERT PHARMACEUTICALS, INC.) 18 January 2018; paragraphs [92], [109], [113], [115]	2, 3/1-2
Y	WO 2010/147484 A1 (INNATE THERAPEUTICS LIMITED, et al.) 23 December 2010; page 4, lines 25-28; page 5, lines 1-4; page 9, lines 20-21; claims 1, 3, 11	15-17

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"D" document cited by the applicant in the international application
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search 23 September 2019 (23.09.2019)	Date of mailing of the international search report 17 OCT 2019
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-8300	Authorized officer Shane Thomas Telephone No. PCT Helpdesk: 571-272-4300

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US19/40534

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☒ Claims Nos.: 4-13
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.