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Treatment of Experimental Autoimmune Encephalomyelitis by Codelivery of Disease Associated Peptide and Dexamethasone in Acetalated Dextran Microparticles

Kevin J. Peine,[†,∥] Mireia Guerau-de-Arellano,[‡] Priscilla Lee,[†,‡] Naveen Kanthamneni,[∥] Mary Severin,[‡,§] G. Duane Probst,[∥] Haiyan Peng,[‡] Yuhong Yang,[‡] Zachary Vangundy,[⊥] Tracey L. Papenfuss,[⊥] Amy E. Lovett-Racke,[#] Eric M. Bachelder,[∥] and Kristy M. Ainslie*[,∥,¶]

[†]Molecular, Cellular and Developmental Biology Graduate Program, The Ohio State University, Columbus, Ohio, 43210, United States

[‡]Department of Neuroscience, The Wexner Medical Center, The Ohio State University, Columbus, Ohio, 43210, United States

[§]Integrated Biomedical Science Graduate Program, The Ohio State University, Columbus, Ohio, 43210, United States

[∥]Department of Pharmaceutical Sciences, College of Pharmacy, The Ohio State University, Columbus, Ohio, 43210, United States

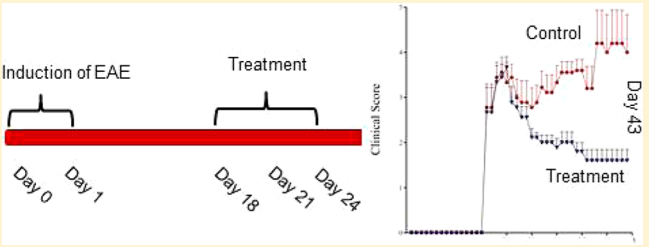
[⊥]The College of Veterinary Medicine, The Ohio State University, Columbus, Ohio, 43210, United States

[#]Microbial Infection and Immunity, The Wexner Medical Center, The Ohio State University, Columbus, Ohio, 43210, United States

[¶]Division of Molecular Pharmaceutics, Eshelman School of Pharmacy, University of North Carolina, Chapel Hill, North Carolina 27599, United States

Supporting Information

ABSTRACT: Multiple sclerosis (MS) is an autoimmune, demyelinating disease of the central nervous system that can cause loss of motor function and is thought to result, in part, from chronic inflammation due to an antigen-specific T cell immune response. Current treatments suppress the immune system without antigen specificity, increasing the risks of cancer, chronic infection, and other long-term side effects. In this study, we show treatment of experimental autoimmune encephalomyelitis (EAE), a model of MS, by coencapsulating the immunodominant peptide of myelin oligodendrocyte glycoprotein (MOG) with dexamethasone (DXM) into acetalated dextran (Ac-DEX) microparticles (DXM/MOG/MPs) and administering the microparticles subcutaneously. The clinical score of the mice was reduced from 3.4 to 1.6 after 3 injections 3 days apart with the coencapsulated microparticulate formulation (MOG 17.6 μg and DXM 8 μg). This change in clinical score was significantly greater than observed with phosphate-buffered saline (PBS), empty MPs, free DXM and MOG, DXM/MPs, and MOG/MPs. Additionally, treatment with DXM/MOG/MPs significantly inhibited disease-associated cytokine (e.g., IL-17, GM-CSF) expression in splenocytes isolated in treated mice. Here we show a promising approach for the therapeutic treatment of MS using a polymer-based microparticle delivery platform.



KEYWORDS: *multiple sclerosis, microparticle, immunotherapy, acetalated dextran*

INTRODUCTION

Multiple sclerosis (MS) is a chronic inflammatory disease in the central nervous system (CNS) affecting approximately 2.5 million people worldwide.[1] MS is thought to be induced by macrophage (Mφ), and the T cell infiltrates to localized areas, causing demyelination of axonal regions,[2] possibly in a myelin-specific manner.[3] T cells secreting interferon γ (IFN-γ), interleukin 17 (IL-17), or TH17 cells have been determined to exacerbate the disease.[4,5] New and emerging treatments for MS have successfully targeted this subset of cells responsible for inflammation in the CNS; however most treatments have serious side effects.

Current common treatments for MS are generally effective at decreasing relapses; however there are serious concerns due to their nonspecific suppression of the immune system. Natalizumab (Tysabri) is a monoclonal antibody treatment that blocks leukocyte migration into the CNS[6] but can lead to immunosuppressive related diseases such as progressive multifocal leukoencephalopathy (PML).[7] Another treatment, beta IFN (Betaseron; Extavia, Avonex, and Rebif), creates neutralizing antibodies toward endogenous IFN[8] and has been shown to decrease relapse rates, but it does not stop overall disease progression.[9] Fingolimod (GILENYA) is an oral

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treatment that inhibits migration of naïve T cells out of the peripheral lymph nodes,[10] but side effects related to immunosuppression have been seen clinically.[11] Long-term treatment with immunosuppressive drugs can lead to an increased risk of cancer[12] and infection,[13] illustrating the need to develop new therapies that limit not only relapse rates and disease progression, but also provide antigen specific immunosuppression.

Therapies have been developed to treat in an antigen specific manner, thereby limiting suppression of the entire immune system. Antigen specific tolerance has been accomplished through interaction with mucosal surfaces by oral,[14,15] nasal,[16] and sublingual[17] delivery to treat animal models. Additionally, tolerance has been achieved through *ex vivo* antigen pulsing of dendritic cells.[18] The mechanism of how antigen specific tolerance forms varies but usually involves the generation of either regulatory CD4 T cells that can inhibit cellular inflammatory responses[19] or inflammatory cell anergy.[20] Even though these methods have been successful preclinically, they have failed once they have reached the clinics, likely due to an inaccurate choice of antigen or issues with dosing quantities or timing.[21] Based on this lack of success, new methods for treatment that can be applied to deliver a broad array of antigens or allow for sustained release of antigens are desired.

Recently, Kang et al. have shown that, injecting both an immunodominant peptide of insulin with dexamethasone (DXM), they were able to prevent the onset of Type 1 Diabetes in a regulatory T cell manner.[22] By immunizing with an antigen and an "immune tolerizing" adjuvant, Kang et al. were able to generate immune tolerance toward a self-antigen. Others have built on this success, by using biomaterial-based antigen-specific immunomodulatory formulations that protect mice from experimental autoimmune encephalomyelitis (EAE),[23−27] a model of MS, and inflammatory arthritis via antigen specific t-regulatory cell activation.[28] Studies using potential treatments for EAE have rarely examined the outcome after administration at a clinically relevant time point. A recent review by Vesterinen et al. showed approximately 4% of EAE papers, out of the 126 studied, examine treatment efficacy beyond 2 weeks post induction of EAE.[29] Unfortunately, this implies the vast majority of studies begin treatment prior to symptom onset. Two recent studies use particle-based postinduction treatments to ameliorate a relapsing and remitting form of EAE. Yeste et al. utilized nonbiodegradable gold nanoparticles injected intraperitoneally to treat EAE, and Getts et al. induced tolerance through intravenous injection of microparticles with surface conjugation of an encephalogenic peptide.[24,26] Although both studies are promising, alternate methods optimizing particle formulation and function should be explored. The goal of this work was to build on this existing research to formulate a clinically relevant antigen specific therapy utilizing a biodegradable polymer, FDA-approved immunosuppressive drug, and a disease-associated antigen. Traditionally, only phagocytic cells such as antigen presenting cells (APCs) can internalize microparticles.[30] By encapsulating an immunosuppressive drug and an antigen in a microparticle, only APCs can internalize the particles, which then could induce an adaptive immune response that results in tolerance against the autoimmune antigen.

Expanding on the previous work we formulated microparticles with the novel acid sensitive polymer, acetalated dextran (Ac-DEX).[31−33] Ac-DEX is derived from dextran by modifying the hydroxyl groups with pendant acetals. Since acetals are sensitive to acidic conditions, in low pH conditions present in the lysosome of APCs, Ac-DEX microparticles degrade releasing their cargo inside the lysosome. Previous nanoparticulate formulations[24,26] used surface bound proteins which may be exposed to degradation *in vivo*, such as low pH or enzymatic degradation. Our proposed acetalated dextran (Ac-DEX) formulation can be an improvement upon this because not only is the protein or peptide encapsulated, but we have also shown enhanced protein stability across a broad range of temperatures when a protein is encapsulated in Ac-DEX.[34] We use Ac-DEX to formulate a first of its kind polymer-based particulate delivery vehicle coencapsulating a major MS target antigen, myelin oligodendrocyte glycoprotein (MOG), and an immunosuppressive drug, DXM, in an effort to treat MS. The efficiency of encapsulated DXM delivery to APCs was determined *in vitro* using nitric oxide (NO) and cytokine measurements. To determine the protective properties of our particles *in vivo*, they were administered as a treatment to C57Bl/6 mice with EAE, and their effect on IL-17 and GM-CSF production by these mice was examined through an antigen recall assay.

MATERIALS AND METHODS

Materials. All reagents were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise noted. Water (dd-H_2O) for buffers was purified using a Millipore (Billerica, MS) Milli-Q Integral water purification system, which was made basic by addition of triethylamine (TEA) (0.01% v/v). DXM (98%) was purchased from Alfa Aesar (Ward Hill, MS), and MOG_{35-55} peptide was purchased from CS Bio Co. (Menlo Park, CA). Antibodies used for ELISA and FLOW cytometry were acquired from BD Biosciences (San Jose, CA), unless otherwise specified. Fluorescence measurements were detected using a Molecular Devices (Sunnyvale, CA) FlexStation 3, courtesy of the Department of Chemistry and Biochemistry at the Ohio State University and Molecular Devices SoftMax Pro Software (Sunnyvale, CA).

Animals. Mice used for experiments were 10 week old C57Bl/6 females purchased from Taconic Farms (Hudson, NY). All animals were kept in a sterile facility according to The Ohio State University Institutional Guidelines. All animal procedures were in accordance with and approved by the Institutional Animal Care and Use Committee (IACUC) of The Ohio State University.

Cell Lines. RAW 264.7 macrophages were purchased from ATCC (Manassas, VA) and cultured according to the manufacturer specifications. Bone marrow derived dendritic cells (BMDCs) were prepared as previously described.[14]

Synthesis and Analysis of Acetal Coverage of Ac-DEX. Ac-DEX was synthesized using 71 kDa dextran as previously described.[33] Cyclic acetal coverage was determined to be 51.9% by nuclear magnetic resonance as previously described.[31]

Preparation of Empty or DXM-Loaded Ac-DEX Microparticles. To formulate microparticles (MPs) containing DXM, Ac-DEX (100 mg) and DXM were dissolved in chloroform and ethanol (95:5 v/v, respectively) and mixed with 3% polyvinyl alcohol (PVA) (MW ∼ 13−23 kg/mol, 87−89% hydrolyzed) in phosphate-buffered saline (PBS). This solution was probe sonicated (Branson Sonifier 450, Branson, Los Angeles, CA) in an ice bath with a flat tip for 30 s with max energy 30W. MPs were stirred for 2 h in 0.3% PVA in PBS and were washed and collected by centrifugation at 18 000 rpm for 16 min on a Beckman Coulter Avanti J-E centrifuge (Brea,

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CA). Empty MPs (/MPs) were formulated in the same manner, excluding the addition of DXM.

Preparation of MOG or MOG/DXM Co-Encapsulated Ac-DEX MPs. DXM (1 mg) and Ac-DEX (100 mg) were dissolved in chloroform and ethanol (95:5 v/v, respectively), and MOG peptide (1 mg) in PBS was added. The mixture was probe sonicated in an ice bath with a flat tip at with max energy at 30 W for 30 s; 3% PVA in PBS (2 mL) was added, and the mixture was probe sonicated for a second time. MPs were stirred for 2 h in 0.3% PVA in PBS and were washed and collected by tangential flow filtration using a mPES MidiKros Filter Module (500 kD pore size, 235 cm^2 surface area) (Spectrum Laboratories, Rancho Dominguez, CA). MOG MPs (MOG/MPs) were formulated by the same mechanism without the addition of DXM.

Scanning Electron Microscopy (SEM). The microparticle size and morphology were characterized by SEM using an FEI NOVA nanoSEM (Hillsboro, OR). SEM sample preparation and analysis was done as previously described.[30]

Quantification of DXM. Quantification of DXM for *in vitro* assays was performed by liquid chromatography−mass spectrometry (LCMS) with a Thermo Scientific Accela Pump and Finnigan TSQ Quantum Discovery Max and analyzed using LCquan software. MPs were dissolved in acetonitrile (1 mg/mL), purified by centrifugation for 5 min at 15 000*g*, and passed through a 0.2 μm filter. Samples were run in a mobile phase of acetonitrile−ddH_2O−formic acid (50:49.9:0.1 v/v) through a Thermo Scientific 2.5 μm × 100 × 100 C18 column with a C8 guard column in isocratic mode with a flow rate of 0.2 mL/min. Desoxymethasone, the internal standard, and dexamethasone were quantified using ion reaction monitoring at 25% collision energy with ion transitions m/z 377 → 339 and m/z 393 → 237, respectively.

Quantification of DXM in DXM/MPs and DXM/MOG/MPs was determined by high-performance liquid chromatography (HPLC) with a method adapted from Zhang et al.[35] MPs were dissolved in a methanol−ddH_2O solution (80:20 v/v) (1 mg/mL), and DXM concentration was determined using an Agilent 1100 series HPLC (Santa Clara, California) with a Thermo Scientific 150 mm × 4.6 mm, pore size 5 μm, Aquasil C18 column (Waltham, MS). Samples were passed through the column at a flow rate of 1 mL min^{-1} in a mobile phase of methanol−ddH_2O (80:20 v/v). DXM was detected at a wavelength of 240 nm. Sample peaks were compared to a standard curve and analyzed using Aglient Chemstation software. DXM/MP encapsulation was 3.5%, and DXM/MOG/MP was 1.5%.

Quantification of Encapsulated MOG. Samples of either MOG MPs (MOG/MP), MOG, or /MPs (1 mg) were suspended in 990 μL of PBS, and the pH was lowered with 5 μL of 50% formic acid (v/v). Samples were incubated on a 37 °C shaker plate overnight, and the solution was returned to neutral pH using 13.25 M sodium hydroxide. The encapsulation efficiency was determined using a fluorescamine assay per the manufacturer's instructions.

***In Vitro* Release of Dexamethasone.** DXM/MPs were suspended in either sodium acetate buffer (pH 5.0) or PBS (pH 7.4) at a concentration of 1 mg/mL. Samples were incubated on a 37 °C shaker plate, and aliquots were withdrawn at each time point, centrifuged (15 000*g* for 5 min), and supernatant collected and freeze-dried. DXM quantification was performed using LCMS (above).

Nitrite Analysis. Nitrite concentrations in supernatants from RAW 264.7 macrophages cultured with LPS and DXM were determined using Griess reagent from Promega (Madison, WI). Macrophages were seeded in a 96-well plate at 5 × 10^4 cells/well with Thermo Scientific HyClone DMEM/high glucose (Logan, UT) with 5% fetal bovine serum and 1% penicillin/streptomycin (complete media) and left overnight to adhere. Cells were cultured with LPS (10 μ/mL) for 24 h, then treated with empty MPs or varying concentrations of DXM (0−0.1 μM), in the form of DXM/MPs or free DXM, for 24 h. Supernatants were collected after 24 h and centrifuged at 15 000 rpm for 10 min to remove residual particles and cells, then analyzed with Griess reagent in accordance with the manufacturer's protocol.

Measurement of Il-6 Secreted by Bone Marrow Derived Dendritic Cells (BMDCs). BMDCs were seeded at a concentration of 5 × 10^4 cells/well in a 96-well plate, stimulated with LPS (1 μg/mL) for 24 h, and then treated with various concentrations of DXM or DXM/MPs (0−0.5 μM). Supernatants were collected after 24 h and centrifuged at 15 000 rpm for 10 min to pellet residual cells and particles. The level of IL-6 was measured by ELISA per the manufacturer's specifications.

Immunization and Treatment of EAE. Mice were immunized with a complete Freund's adjuvant (CFA) and MOG peptide emulsion along with pertussis toxin, purchased from Hooke Laboratories (Lawrence, MS), per the manufacturer's suggestions. After immunization, mice were given clinical scores as previously described[36] and treated 18, 21, and 24 days post immunization with 100 μL injections of either PBS, /MPs, DXM/MPs (8 μg DXM), MOG/MPs (17.6 μg MOG), DXM/MOG/MPs (8 μg DXM and 17.6 μg MOG), or free DXM (8 μg) with free MOG (17.6 μg). On day 32 post immunization mice were euthanized and their spinal cord, spleens, and inguinal lymph nodes removed.

Measurement of Secreted IL-17 and Granulocyte-Macrophage Colony-Stimulating Factor. Splenocytes from treated mice were plated at 5 × 10^6 cells/well in a 12-well plate and stimulated for 24 h with 2 μg/mL MOG. Supernatants were isolated, and ELISA was performed on Immulon 2 plates (Fisher Scientific) to determine the levels of IL-17 and Granulocyte-macrophage colony-stimulating factor (GM-CSF) as previously described.[37]

Fluorescence Activated Cell Sorting Analysis. Cells from the spinal cord, spleen, and inguinal lymph node of the *in vivo* mouse study were seeded at approximately 5 × 10^6 cells/well in a 12-well plate and stimulated with 2 μg/mL MOG for 24 h. Approximately 1 × 10^6 cells were removed and placed in a 96-well plate for staining of intracellular IL-17, IFN-γ, and FoxP3. Cells were treated with FACS buffer (1X PBS, 1% ethylenediaminetetraacetic acid (EDTA), and 0.2% heat shocked sterile FBS) and FC-receptor blocker (BD Biosciences), followed by addition of surface antibodies. Cells were fixed and permeabilized with Cytofix/Cytoperm (BD Biosciences) for 30 min at 4 °C, then stained for IL-17, IFN-γ, and FoxP3. Flow cytometry was performed with a FACSCanto II flow cytometer (BD Biosciences) and analyzed using FlowJo.

RESULTS

Particle Formulation and Analysis. Figure 1 shows electron micrographs of DXM/MOG/MPs (Figure 1A), DXM/MPs (Figure 1B), and MOG/MPs (Figure 1C). Table 1 reports encapsulation efficiencies of DXM, MOG, or both in

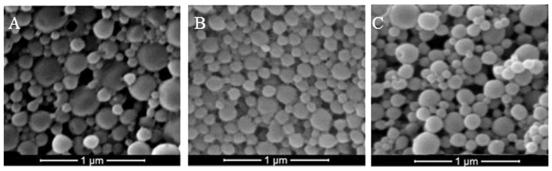

Figure 1. Scanning electron micrographs of acetalated dextran (Ac-DEX) microparticles encapsulating (A) dexamethasone (DXM) + myelin oligodendrocyte glycoprotein$_{35-55}$ (MOG), (B) DXM, or (C) MOG.

Table 1. Encapsulation Efficiencies for Ac-DEX Particles Containing DXM, MOG, or Both

	Ac-DEX			
	encapsulation efficiency		μg drug per mg MPs	
formulation	DXM	MOG	DXM	MOG
DXM/MOG/MP	1.5%	21.8%	1.4	4.8
DXM/MP	3.5%	n/a	3.6	n/a
MOG/MP	n/a	42.0%	n/a	7.5

Ac-DEX MPs as well as quantity of drug per milligram of particles. DXM was loaded with higher efficiency into DXM/MPs compared with DXM/MOG/MPs (3.5% versus 1.5%), and MOG/MPs showed increased encapsulation of MOG compared with DXM/MOG/MPs (42% versus 21.8%). The release of DXM from DXM/MPs incubated in either pH 5.0 or pH 7.4 buffers is shown in Figure S1.

***In Vitro* Immunosuppressive Function of Dexamethasone.** Figure 2 shows the effect of DXM on nitric oxide (NO)

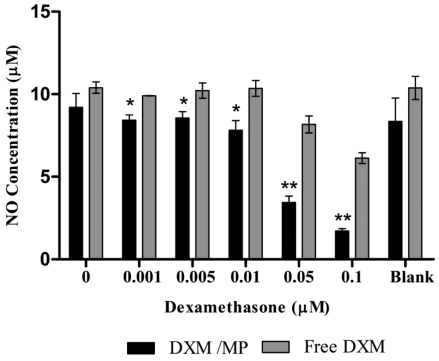

Figure 2. Nitric oxide (NO) release by macrophages when cultured with lipopolysaccharide plus free or encapsulated dexamethasone (DXM) in Ac-DEX. The significance with respect to free DXM is presented as * $p < 0.01$ and ** $p < 0.001$. Data are presented as the average ± standard deviation.

release from Mϕ stimulated with LPS. Mϕ were cultured with ranging doses of DXM (0–0.1 μM) in the form of free DXM or DXM/MPs. There was no significant difference between any of the groups at the 0 μM or /MPs groups. DXM/MPs significantly decreased the amount of NO compared with free DXM ($p < 0.01$ except $p < 0.001$ for 0.05 and 0.1 μM). Inhibition of IL-6 production (Figure 3) in LPS stimulated C57Bl/6 BMDCs was significantly decreased at 0.01 and 0.1 μM with DXM/MPs when compared with free DXM ($p < 0.05$ and $p < 0.005$, respectively).

***In Vivo* Treatment of EAE.** Figure 4 shows average clinical score data for mice treated with either PBS, /MPs, DXM/MPs, MOG/MPs, or DXM/MOG/MPs 18 days post immunization.

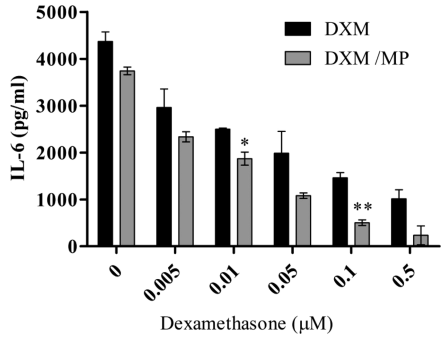

Figure 3. Interleukin (IL)-6 production in culture from C57Bl/6 bone-marrow derived dendritic cells cultured in the presence of lipopolysaccharide and with free dexamethasone (DXM) or DXM encapsulated in Ac-DEX microparticles. The significance with respect to free DXM is presented as * $p < 0.05$ and ** $p < 0.005$. Data are presented as the average ± standard deviation.

Mice receiving injections of DXM/MOG/MPs had significantly lower clinical scores compared to mice receiving any other treatment type. All other treatment groups showed no significant difference in clinical score from each other. As analyzed with an ELISA, splenocytes stimulated with 2 μg/mL MOG peptide after removal from DXM/MOG/MPs treated mice showed significantly lower levels of IL-17 (Figure 5) when compared with mice treated with /MPs ($p < 0.05$), DXM/MOG ($p < 0.05$), DXM/MPs ($p < 0.005$), or MOG/MPs ($p < 0.05$). Also, with the exception of DXM/MOG, splenocytes from mice treated with DXM/MOG/MPs also had significantly lower levels of GM-CSF (Figure 5), when compared with mice treated with PBS ($p < 0.05$), /MPs ($p < 0.05$), DXM/MPs ($p < 0.005$), or MOG/MPs ($p < 0.05$). There was no significant difference in in FoxP3, IL-17 or IFN-γ levels measured by flow cytometry in the spinal cord (Figure S2), inguinal lymph node (Figure S3), or spleen (Figure S4 and S5) cells.

DISCUSSION

Scanning electron micrographs show the particles encapsulating MOG, DXM, or DXM and MOG display relatively spherical morphology and heterogeneity (Figure 1). Encapsulation efficiencies (EE) for DXM/MPs and MOG/MPs were greater than when the therapeutics were coencapsulated (DXM/MOG/MPs; Table 1). Variability with amount of therapeutics encapsulated in polymeric particles has been shown to affect EE. Uchida and Goto showed that the EE of OVA in PLGA microparticles increased proportionally with theoretical drug loading; however after a certain point, large loading attempts resulted in decreased efficiencies.[38] Additionally, Fan et al. were able to encapsulate DXM in poly(D,L-lactic acid) (PLA) microparticles with efficiencies near 70%, but as attempts at increased drug loading were made, the efficiencies also decreased.[39] This data suggests there may potentially be a maximum loading, which could explain our diminished loading of DXM and MOG in DXM/MOG/MPs compared with DXM/MPs and MOG/MPs.

MP size seems to have little effect on DXM EE. Hickey et al. were able to encapsulate DXM (8 μg/mg PLGA) in PLGA microspheres ranging from 1 to 50 μm; however they still only achieved 3–4% EE.[40] Krishnan et al. used nanoprecipitation to formulate copolymer particles using poly(ethylene glycol) (PEG) and poly(ϵ-caprolactone) (PCL). Although DXM encapsulation did slightly increase the particle size upon

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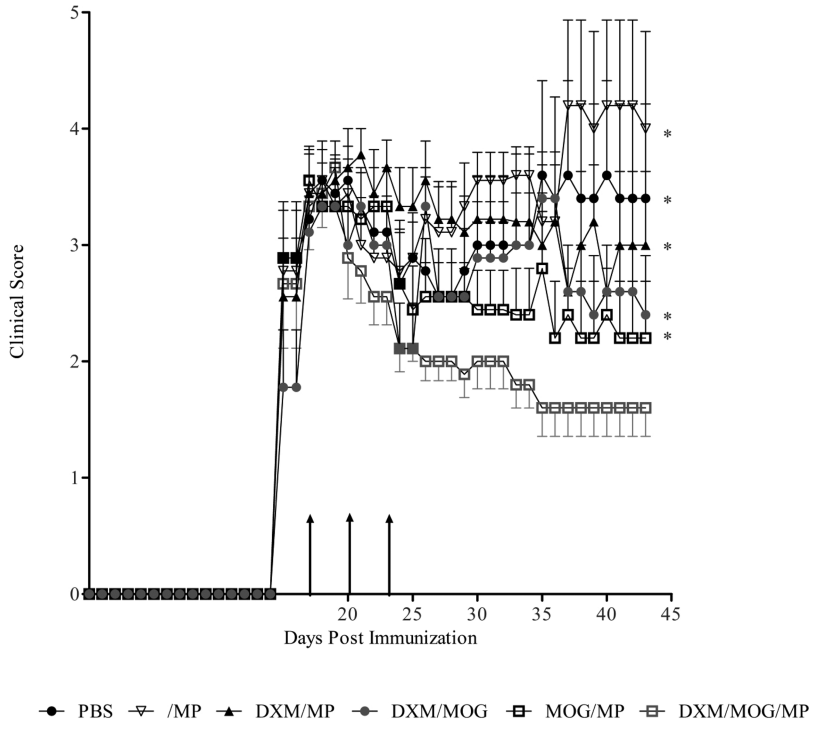

Figure 4. Clinical scores of 9 week old C57Bl/6 female mice immunized with experimental autoimmune encephalomyelitis (EAE) on day 0. Mice were treated after symptoms began by subcutaneous injections of PBS, empty microparticles (/MP), dexamethasone (DXM) MPs (DXM/MP), DXM with myelin oligodendrocyte glycoprotein peptide (MOG), MOG MPs (MOG/MP), or DXM/MOG/MP on days 18, 21, and 24 postimmunization (as indicated with arrows). Treatments with DXM contained 8 μg of DXM and treatments with MOG contained 17.6 μg of MOG. The statistical significance with respect to DXM/MOG/MP is presented as * $p < 0.05$. Data are presented as the average ± standard deviation.

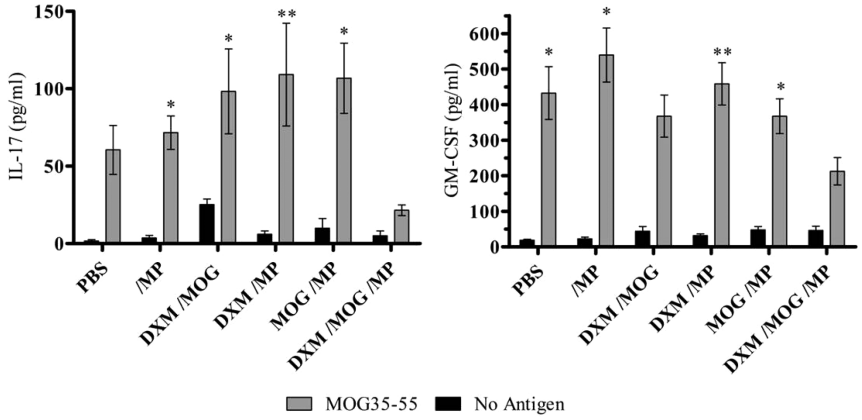

Figure 5. Antigen recall measurements of interleukin (IL)-17 and granulocyte macrophage colony-stimulating factor (GM-CSF) production from splenocytes isolated from C57Bl/6 mice immunized for experimental autoimmune encephalomyelitis (EAE) and treated with PBS, empty microparticles (/MP), dexamethasone MPs (DXM/MP), free DXM, and myelin oligodendrocyte glycoprotein (MOG) MPs (MOG/MP) or DXM/MOG/MPs on days 18, 21, and 24 postimmunization. The significance with respect to DXM/MOG/MP is presented as * $p < 0.05$ and ** $p < 0.005$. Data are presented as average ± standard deviation.

loading (from 111 to 127 nm), the particles displayed a high EE of DXM (52.6%), while still maintaining small size and dispersion.[41] It is clear that DXM loading efficiencies and particle sizing vary greatly based on polymer usage, formulation technique, and drug loading quantity; therefore further work should be performed to optimize formulation methods used in this paper.

To evaluate the bioactivity of DXM/MPs we monitored the release of pro-inflammatory nitric oxide (NO) in Mϕ stimulated with LPS, *in vitro*. Macrophages cultured with DXM/MPs had significantly lower NO production compared to those cultured with free DXM (Figure 2). DXM/MP treatment also diminished the response of LPS-stimulated BMDC's *in vitro*, which was illustrated by decreasing levels IL-6 (Figure 3). Inhibition of IL-6 has been shown to have a protective effect in mice immunized with EAE, possibly through the inhibition of TH_{17}-mediated CNS infiltration of autoreactive cells.[42] Increased DXM levels were able to decrease the amount of IL-6 produced by BMDCs; however, only at 0.01 and 0.1 μM were DXM/MPs able to significantly inhibit IL-6 production compared to free DXM. This trend suggests MPs

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containing DXM may inhibit IL-6 more efficiently at high levels.

Our study found mice therapeutically treated with DXM/MOG/MPs at 18, 21, and 24 days post immunization displayed significantly decreased mean clinical scores compared to mice receiving PBS, /MPs, DXM/MPs, MOG/MPs, or free DXM with free MOG (Figure 4). Since diagnostics recognizing MS prior to symptom onset have yet to be developed, the MP treatment was administered after the mean clinical score for each group was approximately 3.5. A clinical score of 4 represents mice that no longer have hind limb function, while clinical scores of 3 indicate severely deficient motor function of the hind limbs. Mice receiving DXM/MOG/MPs improved to a mean clinical score of 1.8, 16 days after the final injection, and maintained this clinical score throughout the remainder of the trial (Figure 4). A clinical score between 1 and 2 indicates the mice have limpness in their tails, but their hind limb function ranges from normal to slightly inhibited. This improvement in clinical score was significantly lower than all experimental groups, showing MPs loaded with MOG and DXM are superior at ameliorating disease compared with the other groups. In particular, DXM/MOG/MPs were superior to DXM/MPs, suggesting that antigen is required in amelioration of the disease. This possibly occurs through inhibition of disease-associated cytokine production. Our data indicates that MOG stimulated splenocytes from EAE immunized mice treated with DXM/MOG/MPs had significantly lower production of both IL-17 and GM-CSF (Figure 5); however, these mice did not have a significant difference in intracellular splenocytes IFN-γ production (Figure S5). Additionally, there was no significant difference in FoxP3$^+$ T-regulatory cells or intracellular IFN-γ and IL-17 in the spinal cord or inguinal lymph nodes (Figures S2 and S3). Other previous studies have indicated that FoxP3$^+$ T-regulatory cells have a primary role in autoimmune treatment, when the tolerogenic adjuvant and antigen are given prophylactically or at the time of disease induction. Kang et al. previously showed that administration of unencapsulated DXM and protein antigen allowed for protection against delayed-type hypersensitivity and diabetes onset.[43] With prophylactic administration of the compounds, induction of tolerogenic DCs and FoxP3$^+$ T-regulatory cells occurred.[43] Fissolo et al. reported that a MOG-based DNA vaccine administered prophylactically to EAE immunized mice had a significant protective effect, decreasing the overall clinical score through FoxP3$^+$ T-regulatory cell expansion and concomitant decreases in IL-17 and IFN-γ expression.[44] Although we saw no changes in regulatory T cell populations with treatment, interestingly, our DXM/MOG/MPs induced regulatory T cell formation and limited the progression of EAE, when given prophylactically (data not shown). Previous work with biomaterials and an immunomodulating peptide has shown both prophylactic EAE protection[23] and protection after immunization, but prior to EAE symptom onset.[27] Administration of gold-based nanoparticles containing a tolerogenic small molecule and an epitope of MOG resulted in decreased IFN-γ, IL-6, and IL-17, as well as increased levels of FoxP3$^+$ T-regulatory cells.[26] As previously stated, we saw no significant change in FoxP3$^+$ populations with our treatment study, and the mechanism of treatment has not been previously reported with a similar study. Therapeutic treatment with the aforementioned MOG-based DNA vaccine at 10 and 24 days after disease immunization displayed significant inhibition of EAE progression and diminished symptoms; however Fissolo et al. make no mention of mechanisms with regard to therapeutic action.[44] Although both prophylactic and therapeutic treatments have been shown to work, very little has been elucidated on a therapeutic mechanism. Wegmann et al. showed dendrimers containing a synthetic peptide therapeutically protected mice against experimental allergic encephalomyelitis; however, unlike our study they showed an increase in IL-17 generated by splenocytes.[25] In a recent promising study, Getts et al. have suggested that encephalogenic peptide-based EAE therapies work through an increase in T cell anergy.[24] T cell anergy is a process where T cells are inactivated in the periphery, possibly through co-stimulatory inhibition between APCs and T cells.[45] Co-stimulatory inhibition has been associated with DXM.[45] Classically, anergeric cells do not respond to antigen; however, Figure 5 shows that with antigen stimulation significant levels of cytokines are produced. This antigen specific response indicates that perhaps anergy might not be the mechanism of action, but further work would need to be performed to fully rule-out anergy as a mechanism of action for therapeutic-based EAE treatments.

Here we report that one possible contribution to the protective effects of DXM/MOG/MPs is the significant decrease in production of IL-17 and GM-CSF from antigen stimulated splenocytes (Figure 5). IL-17 is an important cytokine in autoimmune disorders, and Yan et al. have recently shown CNS-specific inhibition of IL-17 protects against EAE without diminishing the entire immune system.[46] A study by Komiyama et al. also suggests IL-17 may play a more significant role in EAE than that of IFN-γ.[47] Additionally, GM-CSF has functioned to increase activation of CNS-associated APCs,[48] to enhance the survival of Th17 cells,[49] and may possibly function in IL-17 and IFN-γ independent roles to exacerbate disease.[50] Although this study suggests IL-17 and GM-CSF playing a possible role in the protective functions of DXM/MOG/MPs in EAE treatment, there are other possible mechanisms of tolerance that need to be explored.

A further mechanism could include expression of transforming growth factor β (TGF-β), a cytokine that has previously been shown to inhibit the trafficking of disease-causing effector cells.[51] Also, it is possible that a less well-studied regulatory T cell subset could be involved, such as CD8$^+$FoxP3$^-$ cells, which suppress EAE through TGF-β mediated mechanisms.[52] Other ways that T-effector cell function may be inhibited is by T cell anergy (*vide supra*) or T cell deletion. Glucocorticoids, such as DXM, have been shown to induce thymocyte death.[53] Repetitive exposure to antigen has also been shown to induce T cell deletion.[54] Due to the significantly lowered mean clinical score of the DXM/MOG/MPs group and the lowered mean clinical score of the MOG/MPs group, it is possible that repetitive MOG or DXM exposure may result in some level of T cell deletion. Future work should be performed to try and elucidate through what mechanisms therapeutic treatments act to provide protection from autoimmune disorders such as MS.

■ CONCLUSIONS

Here we report encapsulation of MOG peptide and DXM into Ac-DEX MPs to therapeutically treat EAE, a model of MS. We showed encapsulated DXM was more efficient at decreasing *in vitro* immune responses to LPS using both Mϕ and primary BMDCs. Furthermore, mice immunized for EAE were treated with Ac-DEX MPs coencapsulating DXM and MOG peptide. This therapy significantly reduced the disease clinical score and

expression of IL-17 and GM-CSF, two inflammatory and disease-associated cytokines. Additionally, we show DXM/MOG/MPs were superior to free DXM and MOG at ameliorating disease which indicated encapsulation of these compounds provides for more efficient delivery to the desired cellular populations, *in vivo*. Treatment of EAE after symptom onset by subcutaneous injection of DXM and disease-associated peptide in a polymeric delivery vehicle provides promising new possibilities for the treatment of MS.

■ ASSOCIATED CONTENT

Supporting Information

Dexamethasone release profile, flow cytometry looking at *in vivo* T-regulatory cells and intracellular staining for IL-17 and IFN-γ in the spleen, spinal cord, and inguinal lymph node. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*Kristy M. Ainslie, 242 Parks Hall, 500 W 12th Ave, Columbus, OH 43210. E-mail: ainslie@pharmacy.ohio-state.edu. Phone: 614-688-3797. Fax: 614-292-7766.

Notes

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■ ABBREVIATIONS

MS, multiple sclerosis; EAE, experimental autoimmune encephalomyelitis; MOG, myelin oligodendrocyte glycoprotein; DXM, dexamethasone; Ac-DEX, acetalated dextran; MPs, microparticles; Mϕ, macrophages; CNS, central nervous system; PML, progressive multifocal leukoencephalopathy; NO, nitric oxide; BMDCs, bone marrow derived dendritic cells; PVA, poly(vinyl alcohol); PBS, phosphate-buffered saline; SEM, scanning electron microscope; LCMS, liquid chromatography−mass spectrometry; HPLC, high-performance liquid chromatography; GM-CSF, granulocyte-macrophage colony-stimulating factor; LN, lymph nodes; CFA, complete Freund's adjuvant; EDTA, ethylenediaminetetraacetic acid; EE, encapsulation efficiency; PLA, poly(D,L-lactic acid); TGF-β, transforming growth factor β; PEG, poly(ethylene glycol); PCL, poly(ϵ-caprolactone)

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