

RESEARCH ARTICLE

Optimization of Interleukin-10 incorporation for dendritic cells embedded in Poly(ethylene glycol) hydrogels

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Abstract

Translational research in biomaterials and immunoengineering is leading to the development of novel advanced therapeutics to treat diseases such as cancer, autoimmunity, and viral infections. Dendritic cells (DCs) are at the center of these therapeutics given that they bridge innate and adaptive immunity. The biomaterial system developed herein uses a hydrogel carrier to deliver immunomodulatory DCs for amelioration of autoimmunity. This biomaterial vehicle is comprised of a poly (ethylene glycol)-4 arm maleimide (PEG-4MAL) hydrogels, conjugated with the immunosuppressive cytokine, interleukin-10, IL-10, and cross-linked with a collagenase-degradable peptide sequence for the injectable delivery of immunosuppressive DCs to an anatomical disease-relevant site of the cervical lymph nodes, for intended application to treat multiple sclerosis. The amount of IL-10 incorporated in the hydrogel was optimized to be 500 ng in vitro, based on immunological endpoints. At this concentration, DCs exhibited the best viability, most immunosuppressive phenotype, and protection against proinflammatory insult as compared with hydrogel-incorporated DCs with lower IL-10 loading amounts. Additionally, the effect of the degradability of the PEG-4MAL hydrogel on the release rate of incorporated IL-10 was assessed by varying the ratio of degradable peptides: VPM (degradable) and DTT (nondegradable) and measuring the IL-10 release rates. This IL-10-conjugated hydrogel delivery system for immunosuppressive DCs is set to be assessed for in vivo functionality as the immunosuppressive cytokine provides a tolerogenic environment that keeps DCs in their immature phenotype, which consequently enhances cell viability and optimizes the system's immunomodulatory functionality.

KEYWORDS

dendritic cells, interleukin-10 (IL-10), poly (ethylene glycol)-4 arm maleimide (PEG-4MAL) hydrogels

In honor of Dr. James M. Anderson: It is my (JEB) pleasure and honor to provide a manuscript for this special edition of the *Journal of Biomedical Materials Science Part A* honoring Dr. James M. Anderson. Dr. Anderson's research was an inspiration to me. He has been a colleague, mentor, and friend. I also had the honor of inheriting writing his Biocompatibility Chapter in the textbook *Biomaterials Science: An Introduction to Materials in Medicine*, in its 4th edition. Thank you, Dr. Anderson for your leadership, excellence, and mentorship of the Biomaterials community.

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1 | INTRODUCTION

Multiple sclerosis (MS) is an autoimmune disease that damages the myelin sheath through neurodegeneration, which exposes the brain's axons, hence making them susceptible to damage.¹ In the United States, approximately 12,000 people are diagnosed with

neurological diseases each year, with MS reported to show the highest prevalence.^{2,3} The economic burden of this condition on the healthcare sector is a mean annual cost per patient of over 60,000 USD for severe disease cases.⁴ Additionally, approximately 70% of MS patients remain unemployed after 15 years due to the physical impairment challenges, which further exacerbate their livelihood.⁵ Current interventions commonly employ disease-modifying therapies that slow disease progression and reduce the frequency of relapses.^{6–10} Whereas these interventions have alleviated MS disease condition, they remain flawed with inefficiencies, and most pharmaceutical agents act through systemic immunosuppression, which is associated with an increased risk of developing side effects such as susceptibility to other autoimmune diseases, opportunistic infection, and other complications.^{11–13} Moreover, drugs that are used to alleviate the onset or prevent progression of MS have been reported to have variable patient responsiveness and require long-term monitoring.¹⁴ Therefore, alternative approaches are needed to enhance MS disease control and therapeutic outcomes.

Using immunotherapeutics, researchers are investigating strategies that treat autoimmune disease without necessarily shutting down systemic immunity, by focusing on modulating immune cells in an antigen-specific and localized manner.¹⁵ Delivery of immunomodulatory cells using biomaterials is a promising strategy that has shown improved effectiveness of these cellular therapeutics, which promotes cell viability and functionality through localizing cells to a targeted site.¹⁶ Dendritic cells (DCs) are pivotal in mediating human immune responses, as they attenuate many autoimmune diseases such as MS and Type 1 diabetes.¹⁷ DCs induce adaptive immunity through the activation of primary T cells response and polarization of naïve T cells.¹⁸ Therefore, they play a fundamental role in the perpetuation and prevention of autoimmune disorders, as they regulate immunity and tolerance, respectively.^{19–21} The maturation state and location of DCs dictate their specific functions in the immune system.²² Upon the presence of inflammatory stimuli or antigen uptake, DCs switch to T-cell stimulation mode known as DC maturation.^{23,24} Mature DC (mDC) phenotype expresses chemokine receptors and moves to the lymph nodes where they interact with T cells, provoking a T-cell immunity.²⁵ In MS cases, mDCs migrate to CNS expressing major histocompatibility complex (MHC) class II and co-stimulatory molecules such as CD86,²⁶ they acquire myelin antigens, and subsequently activate myelin-specific T cells in the lymph nodes, which exacerbates the immune response, hence advancing the MS condition.^{27,28} Studies with experimental autoimmune encephalomyelitis (EAE), which serves as the murine model of MS, have shown that delivery of tolerogenic DCs and maintenance of an anti-inflammatory environment alleviates the severity of MS disorder.^{29,30} Prevention of DC maturation and activation leads to a tolerogenic DC phenotype which plays a key role in the immunomodulatory function of T-cell responsiveness.²⁶ Immature DCs (iDCs) may uptake antigen but only show lower expression of the MHC class II and co-stimulatory molecules, thereby downregulating T-cell immune responses.³⁰ Previously, the Babensee group has demonstrated that local delivery of DCs treated in culture with interleukin-10 (IL-10), which is a cytokine with robust anti-

inflammatory properties,³¹ to cervical lymph nodes (cLNs), embedded in poly (ethylene glycol)-4 arm maleimide (PEG-4MAL) hydrogels maintained an iDC phenotype via surface marker expression and immunosuppression effect in EAE model.¹⁶ Interleukin-10 has been shown to play a role in the inhibition of antigen-induced T-cell proliferation and inhibition of the production of proinflammatory cytokines and chemokines by activated human monocytes and neutrophils.³² Because of these anti-inflammatory effects, IL-10 is widely used for immunosuppression in biomaterial drug delivery systems.^{33–35} For instance, the Botchwey group incorporated IL-10 into other biomaterials, including PEG-4MAL, which was used for the recruitment of proregenerative cell phenotypes in wound healing complications.³⁶ However, in their studies, IL-10 was entrapped in the polymer and not covalently bound as seen in the Babensee group for controlled release as the polymer was degraded by proteases.

The IL-10 conjugation supports the localized delivered DCs by making the cell microenvironment more immunosuppressive at least for early time points as the DCs are delivered and acclimatized to their in vivo environment. The studies demonstrated here optimized the IL-10 concentrations for DCs embedded in PEG-4MAL hydrogels for appropriate immunomodulatory applications in related previous in vivo studies.^{16,37,38} The PEG-4MAL-based hydrogels offer broad multifunctionality for the incorporation of biomolecules such as adhesion peptides or immunosuppressive cues such as IL-10 for an induced cellular response embedded in the hydrogel or to affect an in vivo host response. Furthermore, the tunability of mechanical properties of PEG-4MAL hydrogels makes it a common drug or cell delivery vehicle in clinical applications.³⁹ For instance, PEG-4MAL hydrogel viscoelasticity can be tuned by varying polymer weight percent used for synthesis or molecular weight of the PEG-4MAL macromolecule.⁴⁰ Similarly, the degradability of the hydrogel is achievable by varying the proportions of protease degradable versus nondegradable peptide sequences as crosslinkers.⁴¹ Thus, with such flexibility, embedded cells can remodel the biomaterial surrounding this microenvironment to achieve a desired clinical application. The Michael-type addition chemistry associated with the maleimide reaction with thiol groups is orthogonal chemistry to biological chemistry, thus it is not deleterious to cellular incorporation during the hydrogel formation.

The authors, therefore, hypothesize that this biomaterial delivery system functionalized with immunosuppressive cytokine IL-10 at physiologically relevant concentrations³¹ will promote DC-mediated amelioration of autoimmunity against the MS condition.

It is important to note that cellular therapies skew T-cell compartments toward tolerance in an antigen-specific manner for autoimmune treatment. Henceforth, the design of biomaterials requires favorable parameters such as providing a proinflammatory microenvironment that supports DC hydrogel delivery in a nonimmunogenic surrounding while ensuring an iDC phenotype that maintains cell viability, and condition T cells toward regulatory T cells for effective treatments by the biomaterial system. An immunosuppressive cytokine: IL-10 has been shown to induce a tolerogenic phenotype of DCs.^{42,43} Whereas IL-10 has shown promising results in previous

studies, its potential is yet to be fully studied in animal models for autoimmune diseases such as MS.⁴⁴ The biomaterial delivery system used here is composed of IL-10 at varying concentrations, which is covalently tethered to PEG-4MAL polymers in a bulk hydrogel carrying therapeutic DCs. This unique biomaterial cell delivery system optimizes cell viability, function, and localization of delivered DCs, building on structure–function relationships between PEG-4MAL scaffolds and embedded DCs. The purpose of incorporating IL-10 at different concentrations in PEG-4MAL polymers is to assess the extent of tolerogenicity and immature phenotype of embedded DCs and identify an optimal *in vitro* IL-10 dose for future studies. Previous studies have shown that anti-inflammatory immunological cytokines such as IL-10 induce tolerance in DCs.⁴⁵ Albeit, a major challenge to current DC therapies is the possibility of compromising the viability and tolerogenic state of DCs *in vivo* due to systemic inflammation during autoimmunity.⁴⁶ Thus, the methods described herein aimed to conduct *in vitro* studies to characterize PEG-4MAL hydrogels and to assess the viability and immunosuppressive phenotype of DCs embedded with IL-10 conjugated at varying concentrations to PEG-4MAL hydrogels, as treated with simulating proinflammatory insults.

2 | MATERIALS AND METHODS

2.1 | Animals

All live animal procedures were conducted under the approval of the Institutional Animal Care and Use Committee (IACUC) of the Georgia Institute of Technology, with protocol number: BABENSEE-A100488-1/27/2025. Mice were housed in the Physiological Research Laboratory (PRL) Animal Facility of the Georgia Institute of Technology. The animals were 8–12-week-old male C57BL/6J mice (Jackson Laboratories, Inc.).

2.2 | Differentiation of DCs

Bone marrow was harvested from 8-week-old male C57BL/6J mice, red blood cells were lysed, and the remaining cells were washed twice in phosphate-buffered saline (PBS) (Thermo Fisher) with 10% fetal bovine serum (FBS) (Corning). Bone marrow-derived dendritic cells were then cultured (1×10^6 cells/mL) in Dulbecco's modified Eagle medium (DMEM) (Thermo Fisher) with 10% FBS, 1% penicillin streptomycin (Corning), supplemented with 20 ng/mL granulocyte-macrophage colony-stimulating factor (PeproTech), and 20 ng/mL interleukin-4 (IL-4) (PeproTech), media refreshed every 3 days, for 6 days. On day 6, nonadherent cells were removed, and fully differentiated adherent iDCs were released from plates using CellStripper (Corning) for 15 min at 37°C, 5% CO₂ in a humidified incubator. DCs were then treated with 50 ng/mL IL-10 (DC-IL10⁵⁰), 200 ng/mL IL-10 (DC-IL10²⁰⁰), 500 ng/mL IL-10 (DC-IL10⁵⁰⁰), 50 ng/mL TNF- α and 10 ng/mL IFN- γ (all from R&D systems) (stimulated DCs, STIM-DC), 1 μ g/mL lipopolysaccharide (LPS) (Sigma-Aldrich) (mDC), 1 μ L/mL Vitamin D3 (stimulated DCs with VD3; Thermo Fisher), or no treatment (iDC) and cultured (10^6 cells/mL) in 24-well plates for 24 h at 37°C, 5% CO₂ in a humidified incubator (Figure 1). See the various DC types and/or their treatments summarized in Table 1.

2.3 | IL-10 thiolation and quantification

Recombinant murine IL-10 (PeproTech) was thiolated with 30 molar excess of Traut's reagent (Sigma Aldrich) in PBS with 5 mM ethylenediaminetetraacetic acid (EDTA) (Gibco) at pH 8.0 for 1 h, according to the manufacturer's protocol.⁴⁷ Protein samples were then washed twice by diluting with PBS (pH 8.0) and passing through a 10 kDa cellulose filter (Amicon Ultra; Millipore) at 14,000 g for 10 min. To quantify the extent of thiolation, samples were stained for free thiols using the Measure-iT Thiol Quantification Kit (Thermo Fisher). The

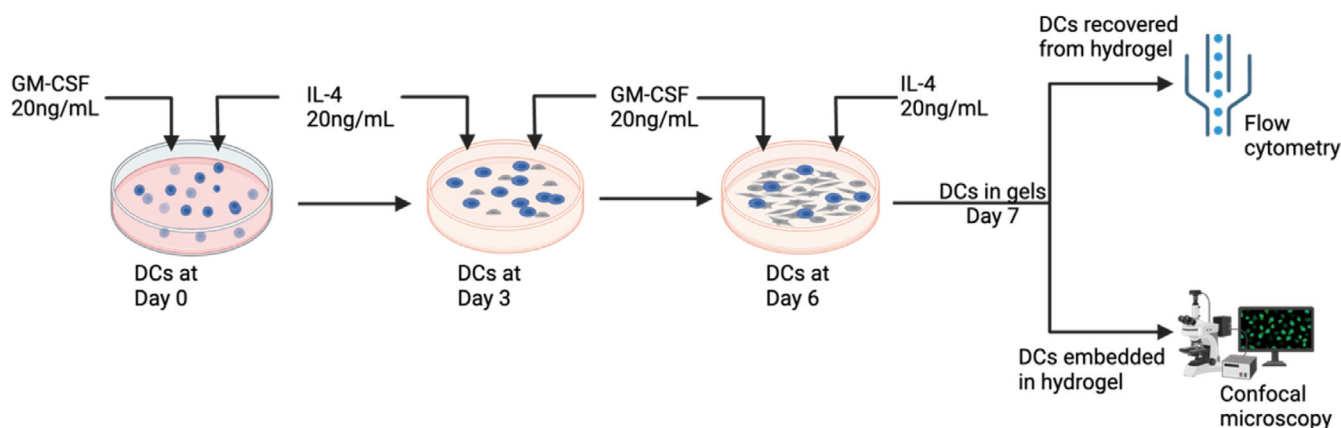


FIGURE 1 Schematic of bone marrow-derived dendritic cells (BMDCs) in culture, treatment for DC differentiation, and analysis techniques. About 50% of culture media was replaced with fresh media containing granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-4 every 3 days, for 6 days. On day 6, fully adherent and differentiated DCs were pretreated with specific stimuli/treatments and embedded in hydrogels. DCs were analyzed with flow cytometry or confocal microscopy. IL, interleukin.

TABLE 1 Types of dendritic cells and the associated description.

Type	Description
BMDC	Bone marrow-derived DCs
iDCs	Immature dendritic cell. This is nonactivated DC phenotype
mDCs	Mature dendritic cells. This is the activated DC phenotype
VD3-DCs	Dendritic cells treated with Vitamin-D3 stimuli. This is a control group for tolerogenic DC phenotype
T&I STIM-DCs	Dendritic cells treated with TNF- α and IFN- γ stimuli. This is a control group for moderately activated DC groups
LPS STIM-DCs	Dendritic cells treated with lipopolysaccharide stimuli. This is a control group for severe activation of DC groups
Embedded DCs	Dendritic cells embedded in PEG-4MAL hydrogels
Unembedded DCs	Dendritic cells that were NOT embedded in PEG-4MAL hydrogels

Abbreviations: DC, dendritic cell; PEG-4MAL, poly (ethylene glycol)-4 arm maleimide.

fluorescence intensity (Ex/Em: 494/517 nm) of thiolated IL-10 (HS-IL-10), native IL-10, blank wells, and reduced glutathione standards were quantified on a spectrophotometer (Synergy H4; BioTek), according to manufacturer's protocol.

2.4 | PEG-4MAL hydrogel preparation

PEG-4MAL macromer (20 kDa; Laysan Bio) at 4.5% w/v polymer density was dissolved in PBS (Thermo Fisher) containing 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES; Thermo Fisher), and mixture was maintained at pH 5–5.5. GRGDSPC (RGD) cell adhesive peptide (>95% purity; GenScript) was dissolved in 1× PBS containing 10 mM HEPES at pH 5–5.5 and added to the PEG-4MAL to produce a solution of RGD-functionalized PEG-4MAL, which was incubated at 37°C, 5% CO₂ in a humidified incubator for 10 min. Following incubation, the protease-degradable crosslinking peptide GCRDVPMSMRGGDRCG (VPM; GenScript) was dissolved in 1× PBS containing 10 mM HEPES at pH 5–5.5 and added. Hydrogels were then polymerized at 37°C, 5% CO₂ in a humidified incubator for 5 min. Hydrogels were synthesized with or without thiolated IL-10 incorporated (PEG-IL10) in selected study experiments unless mentioned otherwise. For hydrogel embedding DCs, cells were added to the mixture of PEG-4MAL and RGD before the addition of the crosslinker. The mixture ratio of PEG-4MAL, RGD, VPM, and cells/buffer was 2:1:1:1, respectively, to obtain a 50 μ L gel (Figure 2). In degradability studies (explained later), hydrogels were synthesized with and/or without dithiothreitol (DTT; Thermo Fisher) as a crosslinker instead of VPM, or gels synthesized with both crosslinkers at varying ratios.

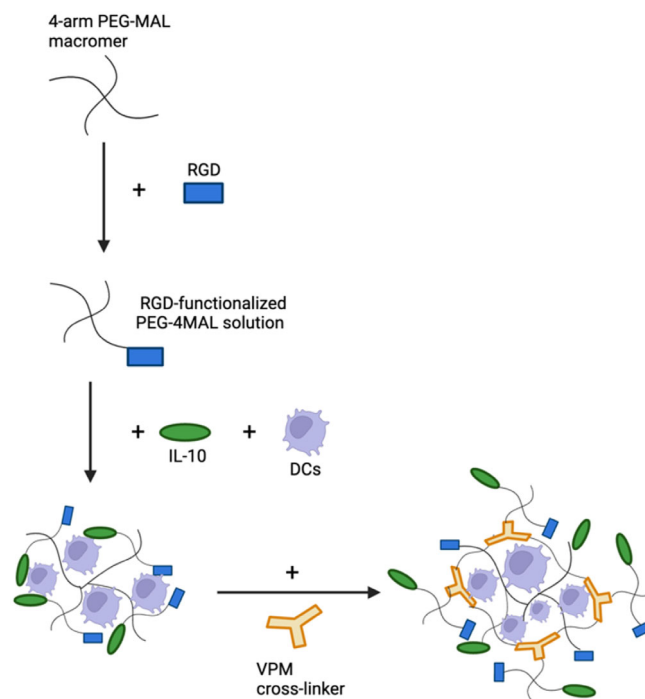


FIGURE 2 Schematic detailing synthesis of poly (ethylene glycol)-4 arm maleimide (PEG-4MAL) hydrogels. The 4-arm PEG macromers were functionalized with RGD and mixed with or without IL-10 pretreated dendritic cell (DC) suspension at varying loading amounts (in control samples, phosphate-buffered saline was used instead of cells, or DCs were pretreated with specified stimuli), and finally crosslinked using VPM. Substitutions of VPM with dithiothreitol were made in some experiments, or using both crosslinkers at varying ratios, and are described where that was the case. IL, interleukin.

2.5 | Characterization of hydrogels with rheology

PEG-4MAL hydrogels were prepared with varying IL-10 concentrations: 50, 200, or 500 ng. Once fully crosslinked, hydrogels were removed and swelled overnight in 1× PBS (pH 7.4) at 4°C. Studies on the viscoelasticity properties were examined for the hydrogels using a rheometer (MCR-302, Anton Paar; CP10-2). Samples were maintained at 37°C while a frequency sweep (100–0.1 rad/s) was performed at a constant strain of 1%. Storage modulus (G') and loss modulus (G'') were determined by averaging values acquired within the linear range. PEG-4MAL hydrogels are viscoelastic materials that are primarily treated as linearly elastic based on higher G' values and lower G'' values (loss factor ~ 0.05 for most samples).

2.6 | Embedding DCs in hydrogels

Specified modifications to PEG-4MAL hydrogel preparation were considered for incorporating cells. Following the addition of prepared RGD solution to PEG-4MAL solution to form an RGD-functionalized

PEG-4MAL mixture maintained at pH 5–5.5, and incubated at 37°C, 5% CO₂ in a humidified incubator for 10 min, 10 µL of a solution containing DCs in 1× PBS and 10 mM HEPES was added to the hydrogel solution, maintaining a cell number of 10³ cells per 50 µL hydrogel. Immediately, following the addition of DCs, the protease-degradable crosslinking peptide GCRDVPMRGGDRCG (VPM; GenScript) was added. The mixture ratio of PEG-4MAL, RGD, VPM, and cells was 2:1:1:1, respectively, to obtain a 50 µL gel (Figure 2). Hydrogels were polymerized at 37°C, 5% CO₂ in a humidified incubator for 5 min. DC-laden hydrogels were incubated at 37°C, 5% CO₂ in a humidified incubator for specified culture periods in the same DMEM-based media with cytokines, as specified previously above, with varying IL-10 concentrations: 50, 200, or 500 ng. Control groups for unembedded DCs were cultured the same way as fully differentiated adherent iDCs as described in previous subsections, then treated accordingly in specified conditions.

2.8 | Percentage incorporation of IL-10

Recombinant murine IL-10 was fluorescently tagged with AlexaFluor-488 protein labeling kit (Invitrogen; Thermo Fisher) with reference to the manufacturer's protocol. The labeled IL-10 was thiolated using Traut's reagent (as described above), and 50 µL hydrogels were prepared with 50, 200, or 500 ng of IL-10 incorporated accordingly. Gels were washed thoroughly in 1× PBS and allowed 2 h to fully form before they were completely digested with 1 mL collagenase I at 2 mg/mL (Gibco). The amount of incorporated IL-10 was measured in the supernatant using AF488 fluorescence (Ex495/Em519) on a spectrophotometer (Synergy H4; BioTek) according to the manufacturer's protocol. Percent incorporation was calculated by dividing the amount measured in the digested polymer over the amount initially incorporated in the hydrogel, multiplied by 100.

That is,

$$\%IL-10\text{ recovered} = \frac{\text{Total IL-10 detected upon release} + \text{Total IL-10 obtained upon complete digestion}}{\text{Amount of IL-10 initially incorporated}} \times 100,$$

$$\%IL-10\text{ incorporation} = \frac{\text{Amount measured in the digested polymer}}{\text{Amount of IL-10 initially incorporated in the hydrogel}} \times 100.$$

2.7 | BCA protein assay

A bicinchoninic acid (BCA) protein assay was conducted to quantify the total protein (IL-10) amount in the samples. The experiment was carried out with a bovine serum albumin (BSA) (Thermo Scientific) standard reference, for its ability to increase signal in calorimetric assays. Diluted BSA standards were prepared for a working range of 20–2000 µg/mL. Similarly, dilution series from test samples were prepared for a working range of 5–250 µg/mL. A BCA protein assay kit (Thermo Scientific) was used to prepare BCA working reagents according to the manufacturer's protocol. The standard and test samples were mixed with BCA working reagent at respective dilutions, and incubated for 30 min at 37°C, 5% CO₂ in a humidified incubator. The absorbance at 562 nm was detected on a spectrophotometer (Synergy H4; BioTek). The total protein concentration was determined using a standard curve as per the manufacturer's protocol. The quantification is validated by the reduction of Cu²⁺ to Cu⁺ by the protein in an alkaline medium with the highly sensitive and selective colorimetric detection of the cuprous cation (Cu⁺) by BCA.⁴⁸

2.9 | IL-10 release kinetics

Recombinant murine IL-10 (PeproTech) was first tagged with AlexaFluor-488 (AF488) according to the manufacturer's protocol. It was then thiolated and PEGylated, as described above. Hydrogels consisting of PEG-IL10 at varying concentrations were synthesized and swelled in PBS (pH 7.4). Hydrogels with IL-10 tethered or IL-10 entrapped were plated in PBS or collagenase I (2 mg/mL). Supernatants were collected intermittently and stored at –80°C until use. Once all hydrogels had fully degraded, supernatants were collected, and AF488 fluorescence (Ex495/Em519) was measured on a spectrophotometer (Synergy H4; BioTek), the fluorescence signal was used to determine the IL-10 release concentrations in the supernatants using a standard curve according to the manufacturer's protocol.

2.10 | Bioactivity of PEG-IL-10 using MC/9 cell assay

A murine liver mast cell line responsive to soluble IL-10, MC/9 cells (ATCC), was expanded in DMEM with 10% FBS, refreshing media

TABLE 2 Types of IL-10 groups and the associated description.

Type	Description
Native IL-10	Unmodified IL-10
HS-IL-10	Thiolated IL-10
IL-10 ⁵⁰	IL-10 concentration at 50 ng/mL
IL-10 ²⁰⁰	IL-10 concentration at 200 ng/mL
IL-10 ⁵⁰⁰	IL-10 concentration at 500 ng/mL
IL-10 ¹⁰⁰⁰	IL-10 concentration at 1000 ng/mL
DC-IL-10	Dendritic cells treated with IL-10
PEG-IL-10	IL-10 in the PEG-4MAL hydrogel
Tethered IL-10	Covalently bound IL-10 to the backbone of PEG-4MAL hydrogels
Untethered IL-10/ entrapped IL-10	Unbound IL-10 in hydrogels

Abbreviations: IL, interleukin; PEG-4MAL, poly (ethylene glycol)-4 arm maleimide.

every 2–3 days as previously described.⁴⁷ To assess the bioactivity of PEG-IL10 compared with native IL-10, MC/9 cells were collected and plated in DMEM with 3% FBS and supplemented with 5 pg/mL co-stimulatory murine IL-4 (Thermo Fisher) at 25,000 cells/well in a 96-well plate. Cells were either treated with soluble IL-10 (PeproTech), embedded in PEG-IL10 at 0, 50, 200, 500, and 1000 ng, or cultured on top of PEG-IL-10 hydrogel, or treated with supernatant from release kinetics experiment, or treated with LPS. After 48 h of incubation at 37°C, 5% CO₂ in a humidified incubator, a cell proliferation assay was carried out by adding 10 µL of the cell counting kit-8 (CCK-8) solution (absorbance 240 nm; Sigma Aldrich) to MC/9 cell suspension of 100 µL/well for respective treatment groups in a 96-well plate and incubated at 37°C, 5% CO₂ in a humidified incubator for 4 h. The proliferation of MC/9 cells in response to treatment was measured on a spectrophotometer (Synergy H4; BioTek) based on cellular metabolic byproducts using a calibration curve from the data obtained in reference to the manufacturer's protocol.

See Table 2 for all IL-10 groups used in this manuscript and their associated description.

2.11 | Flow cytometry

Cells were harvested by removing media and adding 1 mL collagenase I (2 mg/mL) to each well to digest the PEG-4MAL hydrogels embedding DCs. Hydrogels were incubated at 37°C, 5% CO₂ in a humidified incubator for 15 min to enhance their digestion. Contents of wells were then pipetted directly into media with 25% FBS to quench collagenase I activity. Cells were collected by centrifugation at 300× g and prepared for flow cytometry (FC).

DCs were processed for FC using a method previously described with minor modifications.^{49,50} DCs were washed with 1× PBS and then reconstituted in 100 µL of fluorescence-activated cell sorting (FACS) buffer (Hank's Buffered Salt Solution; 1% BSA; and 1 mM EDTA). TruStain FcX (anti-mouse CD16/32; BioLegend) was added,

and cells were incubated on ice for 10 min. The DCs were then stained with APC-CD86 (GL-1; BioLegend), BV785-CD11c (N418; BioLegend), and PE-PD-L1 (MIH7; BioLegend) for 30 min on ice, protected from light. Then, DCs were washed twice with FACS buffer and analyzed on a flow cytometer (FACSARIA III; BD). Flow cytometer gating strategies are illustrated in Figure A1. To assess viability, propidium iodide (PI) was added 5 min before analysis on a flow cytometer to determine the percentage of live and dead cells. Additionally, supplemental experiments for CCK-8 cell viability assay were considered by adding 10 µL of the CCK-8 solution (absorbance 240 nm; Sigma-Aldrich) to all treatment groups, and incubated at 37°C, 5% CO₂ in a humidified incubator for 24 h, and then analyzed on a spectrophotometer (Synergy H4; BioTek).

2.12 | PEG-IL-10-embedded DCs resistance to inflammatory stimulation

iDCs were embedded in native PEG-4MAL or PEG-IL-10 hydrogels with conjugated IL-10 at varying concentrations. Some of the embedded iDCs in native PEG-4MAL hydrogel groups were cultured in media consisting of varying treatment conditions of either 1 µL/mL VD3, 150 ng/mL TNF-α and 30 ng/mL IFN-γ, or 2 µg/mL LPS. A separate iDC embedding hydrogel group was cultured in media with IL-10⁵⁰⁰ added directly to the culture media. Control groups consisted of unembedded DCs, these were cultured the same way as fully differentiated adherent iDCs (as described in the differentiation protocol under Materials and Methods Section 2 above), then treated accordingly with either IL-10⁵⁰, IL-10²⁰⁰, IL-10⁵⁰⁰, 1 µL/mL VD3, 150 ng/mL TNF-α and 30 ng/mL IFN-γ or 2 µg/mL LPS, or IL-10⁵⁰⁰ added to culture media. All groups were incubated at 37°C, 5% CO₂ in a humidified incubator for 48 h. However, following the first 24 h of incubation, some groups were selected for additional treatment with inflammatory stimuli; with either 150 ng/mL TNF-α and 30 ng/mL IFN-γ (T&I STIM-DCs) or 2 µg/mL LPS (LPS STIM-DCs) and resumed incubation for the other 24 h. After 48 h of the cumulative incubation period, all hydrogels were digested with collagenase I, and the recovered cells were analyzed via FC to assess their resistance to the maturation stimuli by quantifying key surface marker expression levels.

2.13 | Confocal microscopy

Live/dead cells were visualized by staining with calcein acetoxymethyl (AM) and ethidium homodimer-1 (EthD-1) and imaging conducted on a confocal microscope (Nikon Eclipse Ti-E C2+), respectively. Live/dead cell visualization and phenotype of DCs were assessed after 24 h of treating embedded cells in PEG-4MAL or PEG-IL10 hydrogels at varying treatment conditions. Media were removed, and PEG-4MAL hydrogels embedding DCs were washed twice with 1× PBS by gentle pipetting. Samples were then stained with 1 µM calcein AM and 1 µM EthD-1 in media without FBS for 20 min protected from light. Intact hydrogels were then washed two more times, and media without FBS was added for imaging.

2.14 | Degradability of PEG-4MAL hydrogel using varying ratios of crosslinkers

The PEG-4MAL hydrogel was modified with cell-adhesive peptide GRGDSPC (RGD) (>95% purity; GenScript) and degradable peptide crosslinker GCRDVPM SMRGDRCG (VPM; GenScript) to facilitate DC interaction with the hydrogel as needed and host remodeling for immune cell trafficking into and out of the gel, respectively. A nondegradable crosslinker, DTT (Thermo Fisher), was substituted for VPM at various ratios to control the rate of hydrogel degradation and presumably the release profile of conjugated IL-10. The varying ratios of VPM (more degradable) and DTT (nondegradable crosslinker) for different gel formulation groups included hydrogel + IL-10 (VPM: DTT; 1:0), hydrogel + IL-10 (VPM:DTT; 0:1), and hydrogel + IL-10 (VPM:DTT; 1:1) at different IL-10 conjugation concentrations. The release rate of IL-10 was analyzed for each of these hydrogel formulations, and consequently, hydrogels were digested to run FC analysis for CD86 biomarkers (with PI exclusion for cell viability identification) to determine the extent of hydrogel degradability kinetics, and its effect on DC viability and phenotype. CD86 is a surface marker expressed by activated DCs that have attained a mature-like DC phenotype.⁵¹

2.15 | Statistical analysis

Experimental values are reported as mean and standard deviation for all samples, with statistical analysis conducted using one or two-way analysis of variance coupled with Sidak's pairwise tests using GraphPad (Prism Inc.). All datasets were normally distributed. Three independent experiments were conducted in triplicates for each study, except for the confocal microscopy imaging study, which was done in duplicate. *p*-values <0.05 were considered statistically significant.

3 | RESULTS

3.1 | IL-10 is successfully thiolated

The chemical process of thiolation is illustrated in Figure A2. The mean concentration of free thiols was determined using the glutathione standard curve (Figure A5). The mean concentration of free thiols on HS-IL-10 samples was 2.2 μ M versus negligible values for unthiolated IL-10 and filtrate (Figure 3A). This magnitude of thiolation suggests that, on average, ~10 lysine residues were thiolated on each IL-10 protein, representing numerous free thiols present to be linked to a maleimide group on PEG-4MAL. The mean concentration of the IL-10 in the samples tested was 50 ng. Therefore, the mean concentration of free thiols represents the lysine residues, which are the expected sites for thiolation on IL-10. Additionally, the BCA assay indicated that thiolated IL-10 (HS-IL-10) was sufficiently quantified with absorbance at 562 nm in comparison to the BSA protein standard (Figure 3B).

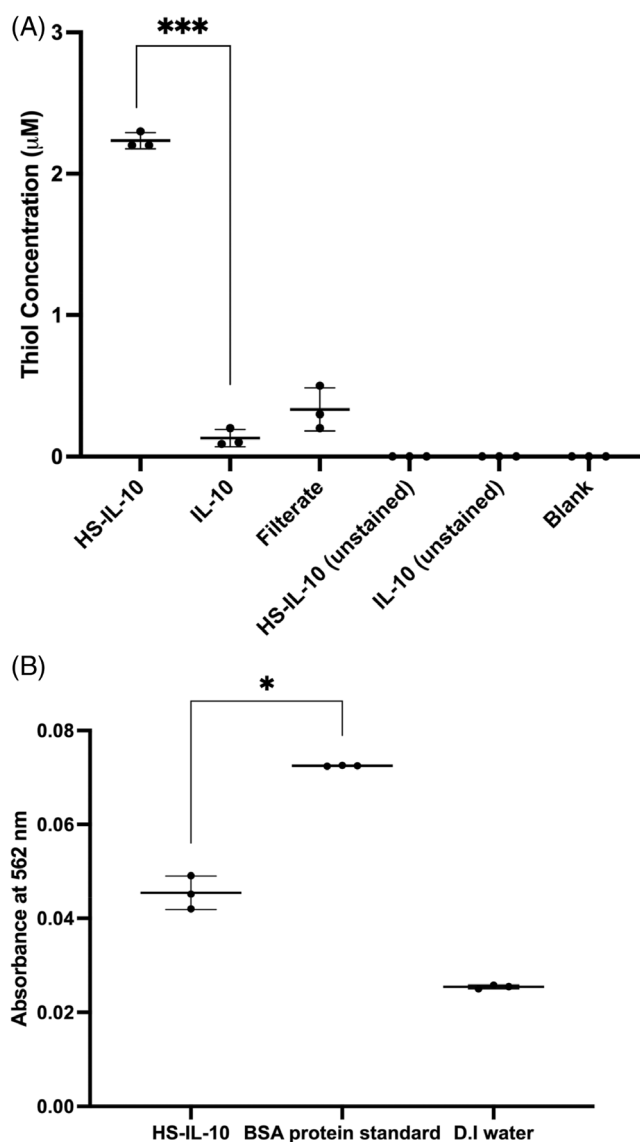


FIGURE 3 Quantification of thiolation of IL-10 using Traut's reagent. (A) Concentration of free thiols on modified IL-10 proteins, determined at 2.2 μ M. Filtrate condition consisted of the flow-through liquid collected through the filter during the thiolation process. Unstained samples were processed without the Measure-iT Thiol Quantification Kit dye. (B) Bicinchoninic acid (BCA) assay used to determine total protein in thiolated IL-10 sample. Data are presented as the mean with SD of three independent experiments conducted in triplicates. ****p* < .001; **p* < .05 via one-way analysis of variance. IL, interleukin.

3.2 | A high percentage of incorporated IL-10 recovered after gel digestion

After 2 h of hydrogel synthesis, about 95% of the incorporated IL-10 was recovered upon hydrogel digestion across all incorporated concentrations (Figure 4A), with approximately 47, 189, and 480 ng of IL-10 amounts recovered from hydrogels initially incorporated with IL-10 loading amounts of 50, 200, or 500 ng, respectively. Additionally, intermittent IL-10 release measurements indicated

increased amounts of IL-10 detected in the supernatant within 24 h (Figure 4B). PEG-IL-10 in PBS with added collagenase I released higher IL-10 compared with gels in PBS (without collagenase I), see Figure A3.

3.3 | The incorporation of IL-10 at varying concentrations did not significantly affect PEG-4MAL hydrogel mechanical properties

Rheological studies using a cone and plate rheometer indicated that incorporation of IL-10 at varying concentrations: 50, 200, or 500 ng did not significantly alter the storage and loss moduli of hydrogels as compared with gels without IL-10, hence, maintaining the viscoelastic properties of the gels across all PEG-4MAL IL-10 gel groups and the native PEG-4MAL hydrogels (Figure 5).

3.4 | Controlled release of IL-10 from PEG-IL10 hydrogels with varying IL-10 concentrations

Hydrogels with IL-10 or HS-IL-10 at varying IL-10 concentrations: 50, 200, and 500 ng with or without collagenase I treatment had release profile trends over 96 h, with nearly 90% of protein released after just 6 h. Hydrogels with untethered IL-10 in PBS also exhibited rapid release of protein within the first 6 h with about 60% protein release, whereas HS-IL-10 with tethered IL-10 released only ~40% after 6 h in PBS. For HS-IL-10 hydrogels with tethered IL-10, another ~30% of IL-10 was gradually released between 6 and 96 h into PBS. Collagenase I (2 mg/mL) was added to hydrogels at 96 h to release IL-10 still bound to hydrogels, after which the remaining IL-10 was released into the PBS. The initial burst release of HS-IL-10 from hydrogels in PBS is likely due to only a fraction of the HS-IL-10 being biochemically incorporated in PEG-4MAL hydrogels, not covalently bound (Figure 6).

3.5 | PEGylated IL-10 retains its bioactivity

While IL-10 is classified as a class II anti-inflammatory cytokine (cytokines that can either be host-protective or have pathogenic activity), liver mast cells exhibit pleiotropic behavior and have been shown to proliferate in response to IL-10.⁵² The bioactivity of PEG-IL10 was measured by the proliferation of MC/9 cells, a murine liver mast cell line commonly used in IL-10 bioactivity assays.⁵³ Absorbance was generally closely similar for native, unmodified IL-10 and PEGylated IL-10 at both 4 and 24 h after the addition to the MC/9 cells to assess

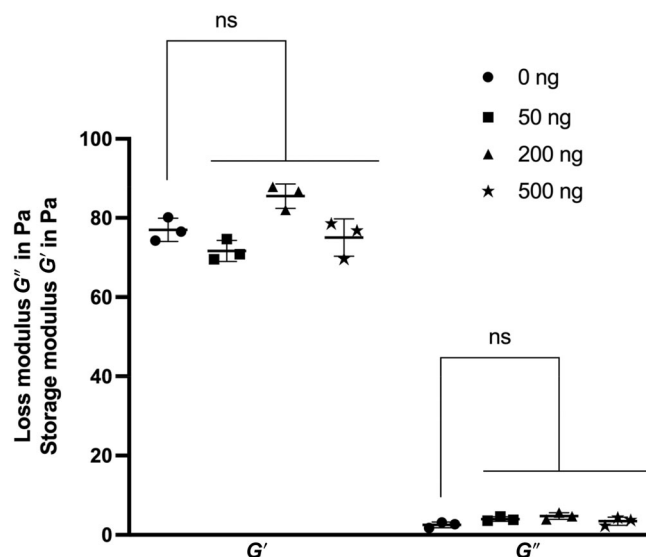


FIGURE 5 Rheology properties for hydrogels incorporated with varying IL-10 amounts. Both storage modulus (hydrogel stiffness) and loss modulus (viscous behavior of the gel) were indistinguishable across all hydrogels with or without IL-10. Data are presented as the mean with SD of three independent experiments conducted in duplicates. ns means no significant differences, via one-way analysis of variance. IL, interleukin.

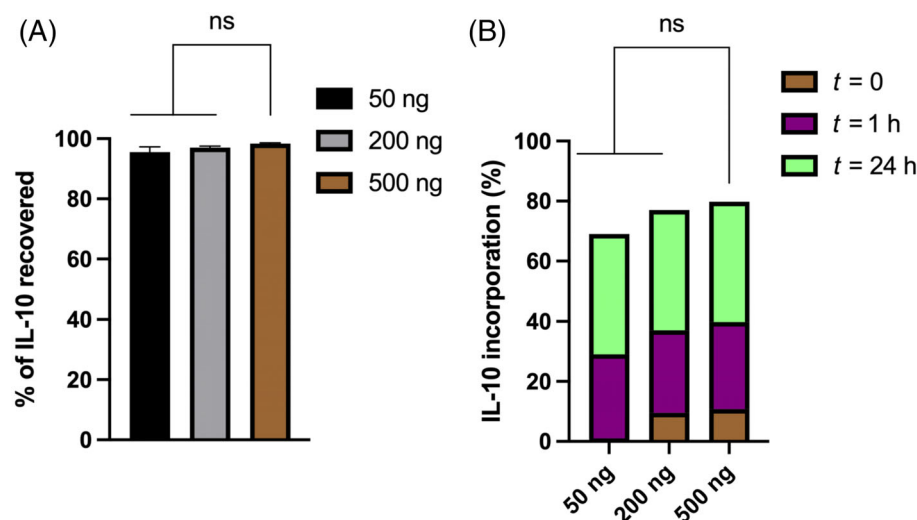


FIGURE 4 Data from percentage incorporation. (A) Percentage incorporation for recovered IL-10 following gel digestion 2 h after the hydrogel was synthesized. (B) Percentage incorporation for released IL-10 measured at varying time intervals. Data are presented as the average of three independent experiments conducted in duplicates. ns means no significant differences, via one-way analysis of variance. IL, interleukin.

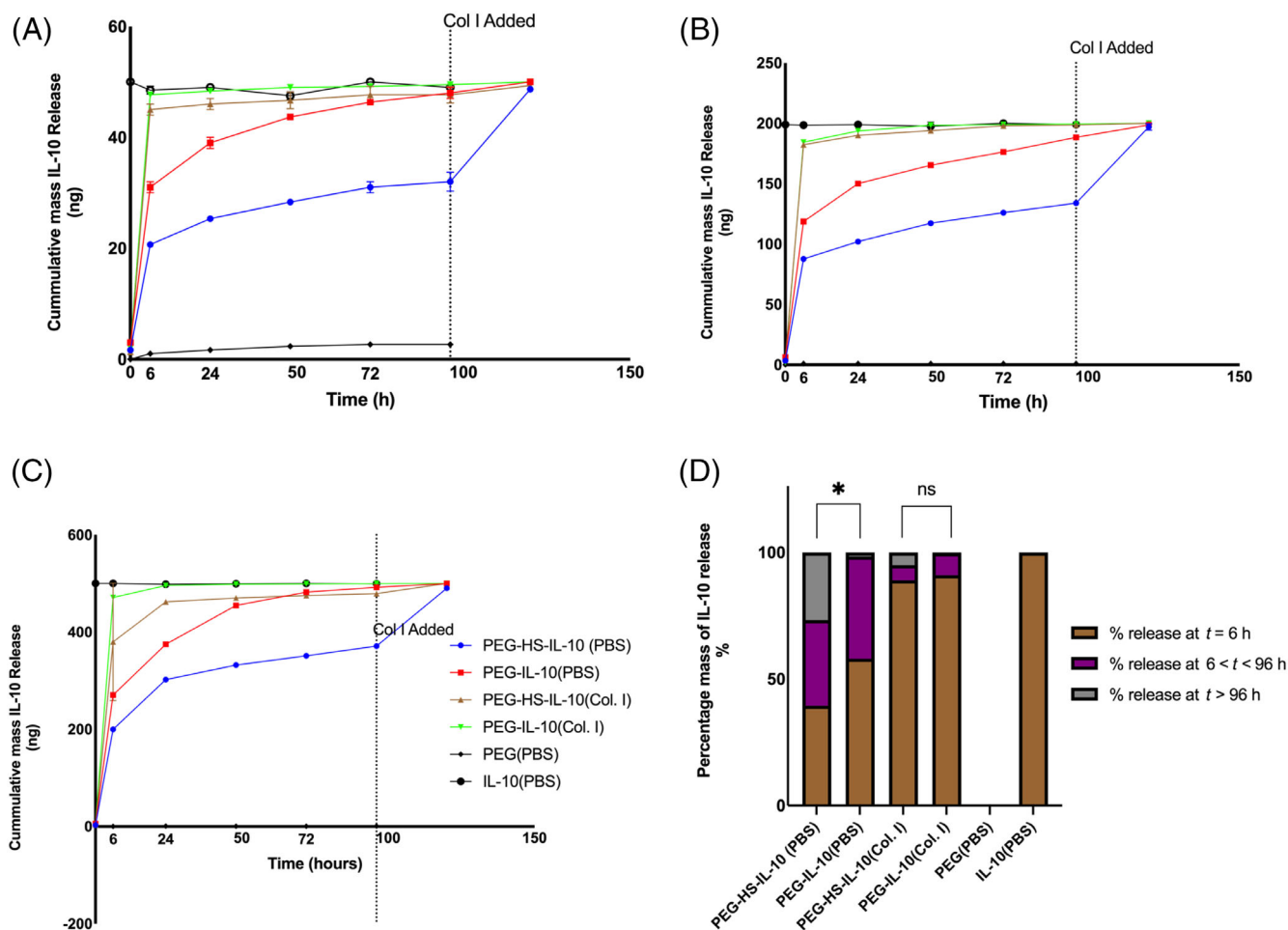


FIGURE 6 Controlled release of IL-10 from PEG-IL10 hydrogels at varying IL-10 incorporation concentrations. Generally, all hydrogels exhibited a burst release of IL-10 in the first 6 h, however, hydrogels with thiolated IL-10 (PEG-HS-IL-10) began to release IL-10 in a more controlled manner compared with other hydrogels with other conditions after the first 6 h. All hydrogels were digested at $t = 96$ h to release all remaining IL-10. There were not many differences in the IL-10 release profiles among hydrogels with varying IL-10 incorporation. (A) 50 ng IL-10 incorporated, (B) 200 ng IL-10 incorporated, (C) 500 ng IL-10 incorporated, and (D) percentage mass release for 500 ng IL-10 incorporated for varying time intervals. Data are presented as the average of three independent experiments. $*p < .05$, ns means no significant differences, via one-way analysis of variance. IL, interleukin; PEG, poly (ethylene glycol).

the resultant proliferation. To further validate the bioactivity of PEG-IL10, the anti-inflammatory cytokine, IL-10 was incorporated at varying amounts: 0, 50, 200, 500, or 1000 ng in PEG hydrogels. A significant increase in the proliferation rate of MC/9 cells was observed from 0 ng through the 500 ng concentration for MC/9 cells embedded in the PEG-IL-10 condition (Figure 7), with just a gradual increase observed from 500 to 1000 ng (Figure 7). These results were fundamental in determining optimal IL-10 incorporation for hydrogel embedding in subsequent experiments. Importantly, the supernatant that was stored after 96 h from release kinetics studies as described above showed a significant effect on the bioactivity of PEG-IL-10 compared with cells cultured on PEG-IL-10 for 24 h. LPS-treated cells consistently affected the proliferation of mast liver cells across all IL-10 concentrations. LPS has been reported in previous research studies to guide tolerance in mast cells.^{54,55} Therefore, LPS stimulation on PEG-1 L-10 cell embedding gels was included as a control group to determine the extent to which PEG-4MAL hydrogels across all IL-10

concentrations can impact the LPS effect on mast cells. It was observed that LPS had a mild downregulated effect on the proliferation of mast cells 24 h following treatment on hydrogel embedding cells (Figure 7).

3.6 | FC and expression of DCs biomarker: increased immunosuppressive phenotype of DCs observed at higher hydrogel-incorporated IL-10 concentrations. The viability of DCs is significantly lower with unembedded DCs compared with those in gels

Some DC groups expressed the cluster of differentiation 86 (CD86) cell surface markers upon treatment with maturation stimuli for 24 h determined by FC. The CD86 surface marker is a co-stimulatory molecule whose expression on DCs increases upon treatment with

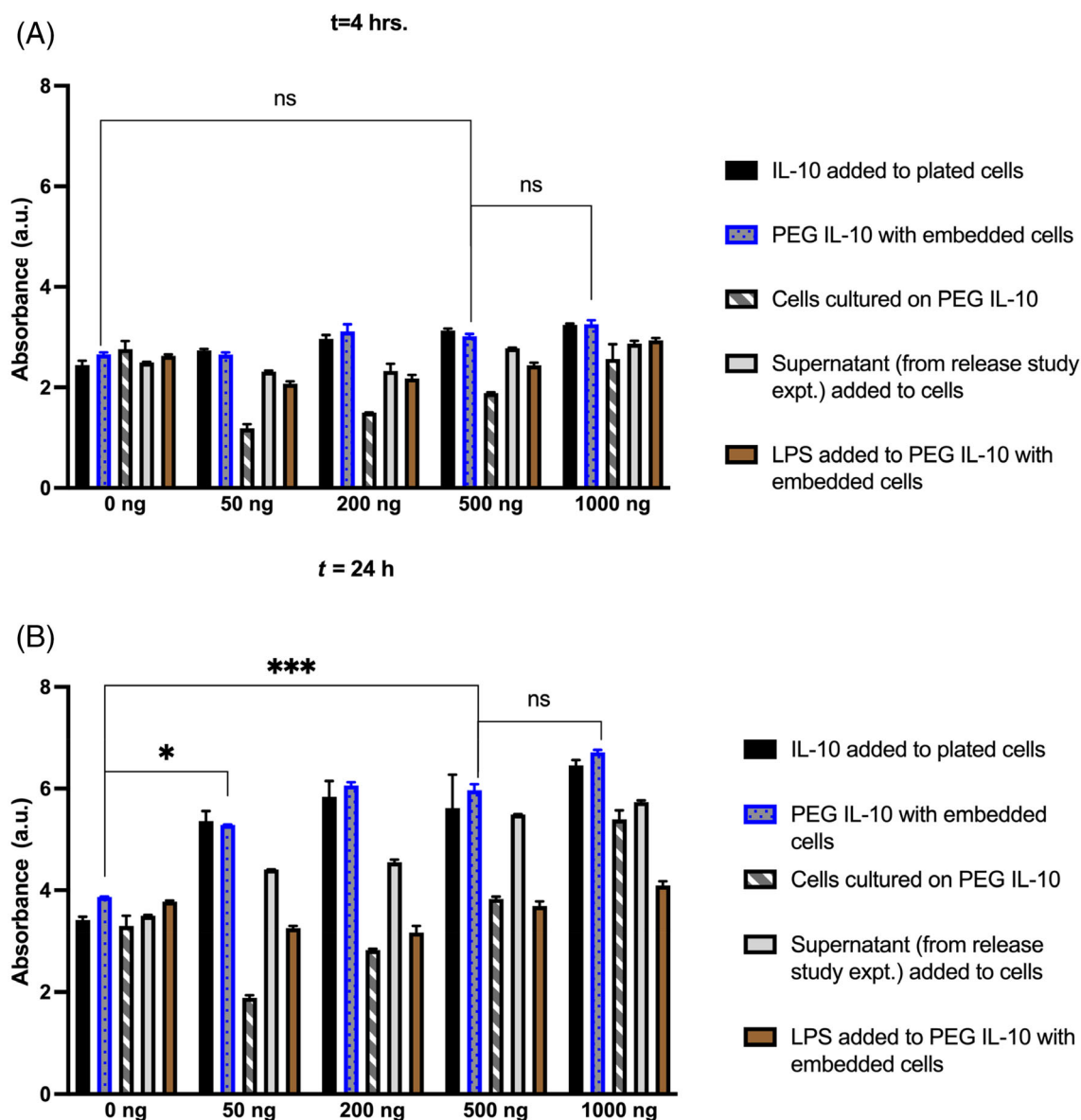


FIGURE 7 Bioactivity of PEG-IL10 at varying IL-10 concentrations based on the proliferation of MC/9 cells at 4 and 24 h of incubation with treatments measured by CCK-8 metabolic assay. The proliferation of MC/9 cells increased with increasing IL-10 loading amounts for PEG-IL-10 hydrogels with embedded cells at $t = 24$ h. However, an indistinguishable increase was observed from 500 to 1000 ng, this result was key in determining optimal IL-10 incorporation in hydrogel at 500 ng loading amount. Data are presented as the mean with SD of three independent experiments conducted in triplicates. *** $p < .001$; * $p < .05$, ns represents no significant difference, via one-way analysis of variance. IL, interleukin; LPS, lipopolysaccharide; PEG, poly (ethylene glycol).

maturation stimulus such as LPS.⁵⁶ Similarly, cell-embedded hydrogels were also analyzed for the expression CD11c which is a marker expressed by all DCs and can also be used to determine subsets of DCs.⁵⁷ Both TNF- α /IFN- γ -treated DCs and LPS-treated DCs (mDCs) had significantly increased expression of CD86, with mDCs having the highest expression (Figure 8B), as compared to iDCs. Embedding these DCs in hydrogel significantly lowered their expression upon stimulation with maturation stimuli. Additionally, DCs treated with IL-10⁵⁰⁰ concentration or VD3-DCs exhibited a more immature phenotype, with lower levels of CD86 expression, similar to that of iDCs, as

compared to all other treatment groups. Overall, hydrogel-embedded DCs showed a relatively lower CD86 expression across all treatment groups compared with the unembedded DCs. To further validate the viability, PI was added to test samples 5 min before running FC to assess viability. While observed viability was similar across treatments, better cell viability was observed with hydrogel-embedded DCs than in unembedded DCs (Figure 8A). Similarly, additional supplemental experiments with CCK-8 cell viability assay showed that embedded cells maintained higher cell viability compared with the unembedded DCs (Figure A4).

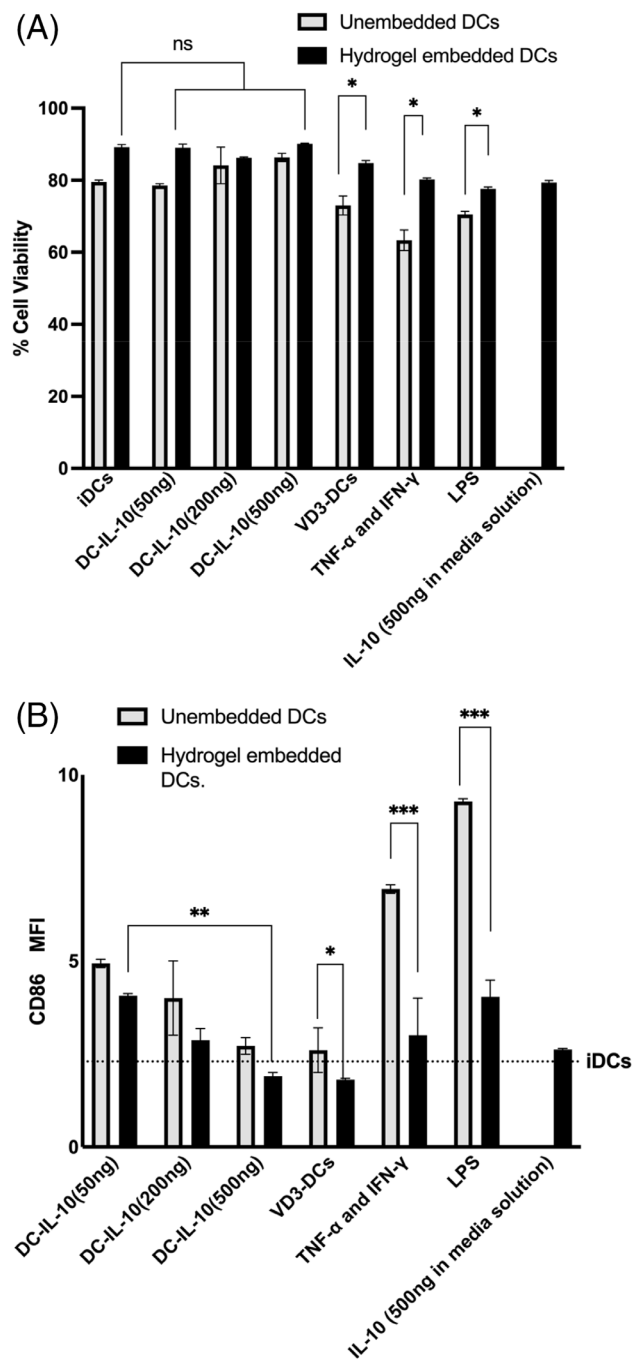


FIGURE 8 Cell viability and dendritic cell (DC) phenotype expressed as CD86 MFI after stimulation for 24 h with varying DC treatment conditions. (A) Percentage of cell viability. In general, all conditions had comparable cell viability. However, DCs embedded in poly (ethylene glycol) (PEG) hydrogels showed better cell viability compared with unembedded cells, especially with harsh conditions/stimuli. Live cells are defined by propidium iodide negative cells. (B) CD86 biomarker expression. DCs embedded in PEG hydrogels at 500 ng IL-10 loading amount expressed lower CD86 biomarkers compared with other conditions. Similarly, PEG hydrogel-embedded DCs expressed lower CD86 markers compared with unembedded DCs. MFI stands for mean fluorescence intensity. Data are presented as the mean with SD of three independent experiments conducted in triplicates. *** $p < .001$; ** $p < .01$; * $p < .05$, ns represents no significant difference, via two-way analysis of variance. IL, interleukin; LPS, lipopolysaccharide.

3.7 | Confocal microscopy: higher DC viability for hydrogel-embedded DCs observed at higher IL-10 concentrations

The viability of DCs was assessed after 12 h of embedding in PEG-4MAL or PEG-IL10 hydrogels treated at varying conditions. PEG-IL10 hydrogels incorporated at higher IL-10 concentrations maintained slightly better DC viability compared with PEG-IL10 hydrogels with lower IL-10 concentrations. DCs recovered from PEG-IL10 hydrogels had similar viability with more fluorescence (green) of live cells in comparison with the red channel (dead). Conversely, DCs recovered from PEG-4MAL hydrogels visually showed similar levels of live and dead cell fluorescence on confocal images. Moreover, PEG-IL10 hydrogels with IL-10⁵⁰⁰ and VD3-DCs exhibited greater cell viability compared with DCs embedded in hydrogels with IL-10²⁰⁰ and IL-10⁵⁰ (Figure 9). VD3 has been reported to promote a tolerogenic DC phenotype by activating glucose metabolism in these cells⁵⁸ while IL-10⁵⁰⁰ is likely to maintain a more anti-inflammatory environment than IL-10²⁰⁰ and IL-10⁵⁰, this could explain why we observed better cell viability in these treatment groups compared with other groups with different conditions. DCs in PEG-4MAL hydrogels treated with LPS showed higher cell death compared with TNF-α and IFN-γ-treated cells. TNF-α and IFN-γ have a moderate stimulation and activation effect on iDCs compared with LPS which has been previously reported to expose iDCs to severe and harsh conditions, which affect the cells' responsiveness, thereby leading to an “exhausted” state.^{59,60} A treatment group with cells incubated at 37°C, 5% CO₂ in a humidified incubator in IL-10⁵⁰⁰ solution for 12 h was observed to have more dead cells compared with cells that were embedded in PEG-IL-10⁵⁰⁰. This condition was fundamental in showing the role played by PEG-4MAL hydrogel in protecting DCs from a potentially harsh environment that could affect DC viability.

3.8 | Embedding DCs in hydrogel with incorporated IL-10 contributes to DC resistance against inflammatory cytokines

Following the 48 h of incubation (with or without proinflammatory stimulation), hydrogels were digested, and the recovered DCs were analyzed via FC to assess their response to the inflammation stimuli. It was generally observed that unembedded DCs expressed higher levels of CD86 marker compared with the embedded DCs. Additionally, DC-IL-10⁵⁰⁰ hydrogel groups that were treated with T&I STIM and LPS STIM inflammatory stimuli exhibited significantly lower CD86 expression compared with iDCs in native PEG-4MAL hydrogels treated with the same inflammatory stimuli (Figure 10). There was a moderate extent of resistance observed with VD3-DCs hydrogels due to a relatively lower CD86 marker compared with the unembedded VD3-DCs group. VD3 maintains a tolerogenic DC phenotype, and the results here suggest that hydrogel encapsulation augments its effect. Positive control groups for TNF-α/IFN-γ and LPS show significant increases in the expression of CD86 markers in unembedded DC groups

Calcein AM (live cells) and Ethidium homodimer-1 (dead cells)

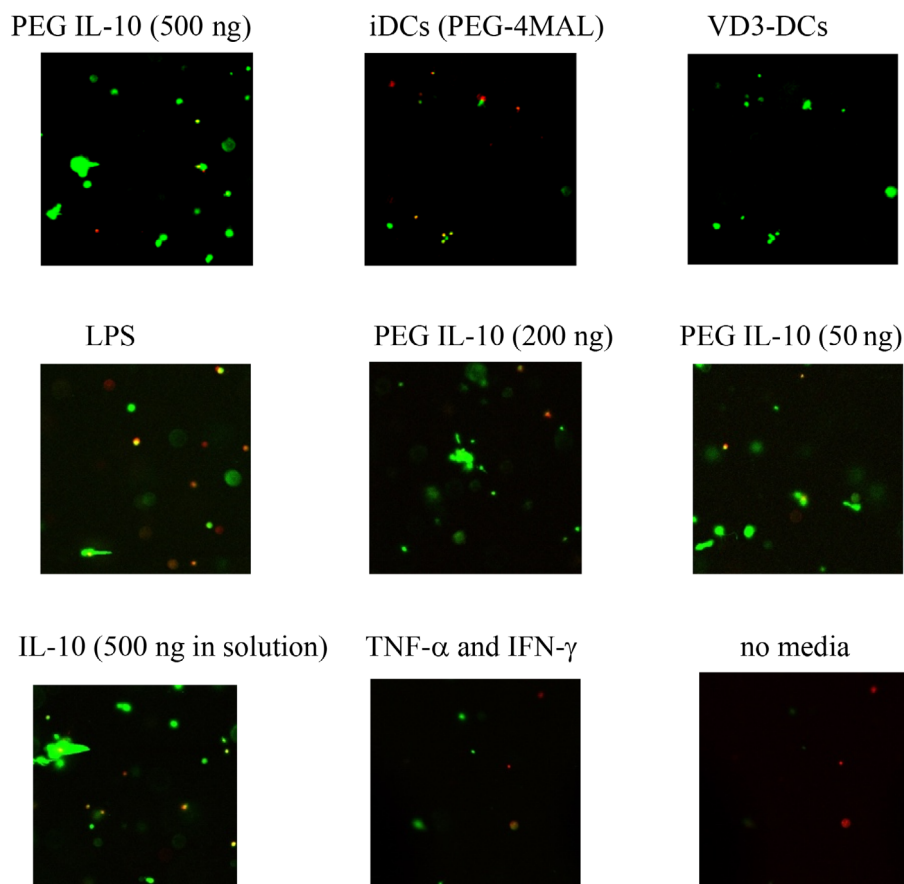


FIGURE 9 Live/dead cell visualization of dendritic cells (DCs) on confocal microscopy after 24 h of treating embedded DCs in poly (ethylene glycol)-4 arm maleimide (PEG-4MAL) or PEG-IL10 hydrogels with varying conditions. DC embedded in PEG hydrogels with varying IL-10 loading amounts appeared to have more live cells (green) than DCs embedded in PEG hydrogels without IL-10. Lipopolysaccharide (LPS) and no media conditions appeared to have more dead cells (red) than other treatment groups. The live/dead stain dyes consisted of calcein AM (green) and ethidium homodimer-1 (red). iDC, immature dendritic cell.

compared with those observed in embedded hydrogels. DC hydrogels in the IL-10⁵⁰⁰ solution showed lower CD86 markers compared with the unembedded DCs in the IL-10⁵⁰⁰ solution. Generally, results obtained in this study suggest that both IL-10 treatment and PEG-4MAL embedding contribute to the DCs' ability to resist a negative proinflammatory secondary stimulation of LPS and/or TNF- α /IFN- γ , at IL-10⁵⁰⁰ incorporation. DCs embedded in PEG-4MAL-IL-10 hydrogels are resistant to maturation due to an inflammatory insult. They are thus maintained as immunosuppressive, and their phenotype would not be adversely affected by being subjected to a proinflammatory environment, for example upon implantation in an autoimmune environment.

3.9 | Hydrogel degradability kinetics: effects on cell viability, phenotype, and IL-10 release rates

PEG-4MAL hydrogels were synthesized using a percentage of the crosslinker DTT: 0%, 50%, and 100% with VPM constituting the remaining crosslinker proportion accordingly with embedded DCs. The viability of cells collected from 0% DTT, 50% DTT, and 100% DTT-crosslinked PEG-4MAL hydrogels after 24 h of embedding was similar to iDC controls with approximately 80% of live cells across the

different IL-10 concentrations (Figure 11A). Expression of CD86 among embedded DCs was lowest and similar to iDC controls for IL-10⁵⁰⁰ incorporated hydrogels, with no significant variation associated with crosslinker ratios (Figure 11B). This data suggest that hydrogel degradability kinetics do not significantly affect DC viability and phenotype, allowing for flexibility in crosslinker choices when designing PEG-4MAL hydrogels for in vivo applications. Additionally, release kinetics studies were conducted by synthesizing hydrogels with the incorporation of varying IL-10 amounts at different crosslinker ratios. Generally, hydrogels with 100% DTT released IL-10 much slower compared with other ratio groups (Figure 11C-E). This is attributed to the nondegradability nature of DTT compared with VPM, which is a degradable crosslinker.

4 | DISCUSSION

In developing adoptive cell therapies for treating autoimmune diseases such as MS, promoting viability and phenotypic stability of tolerogenic DCs is a significant challenge in retaining their effectiveness. This study illustrates a multifunctional biomaterial system based on PEG-4MAL hydrogel with the incorporation of IL-10 to support DC viability and maintain an iDC phenotype, which is necessary for

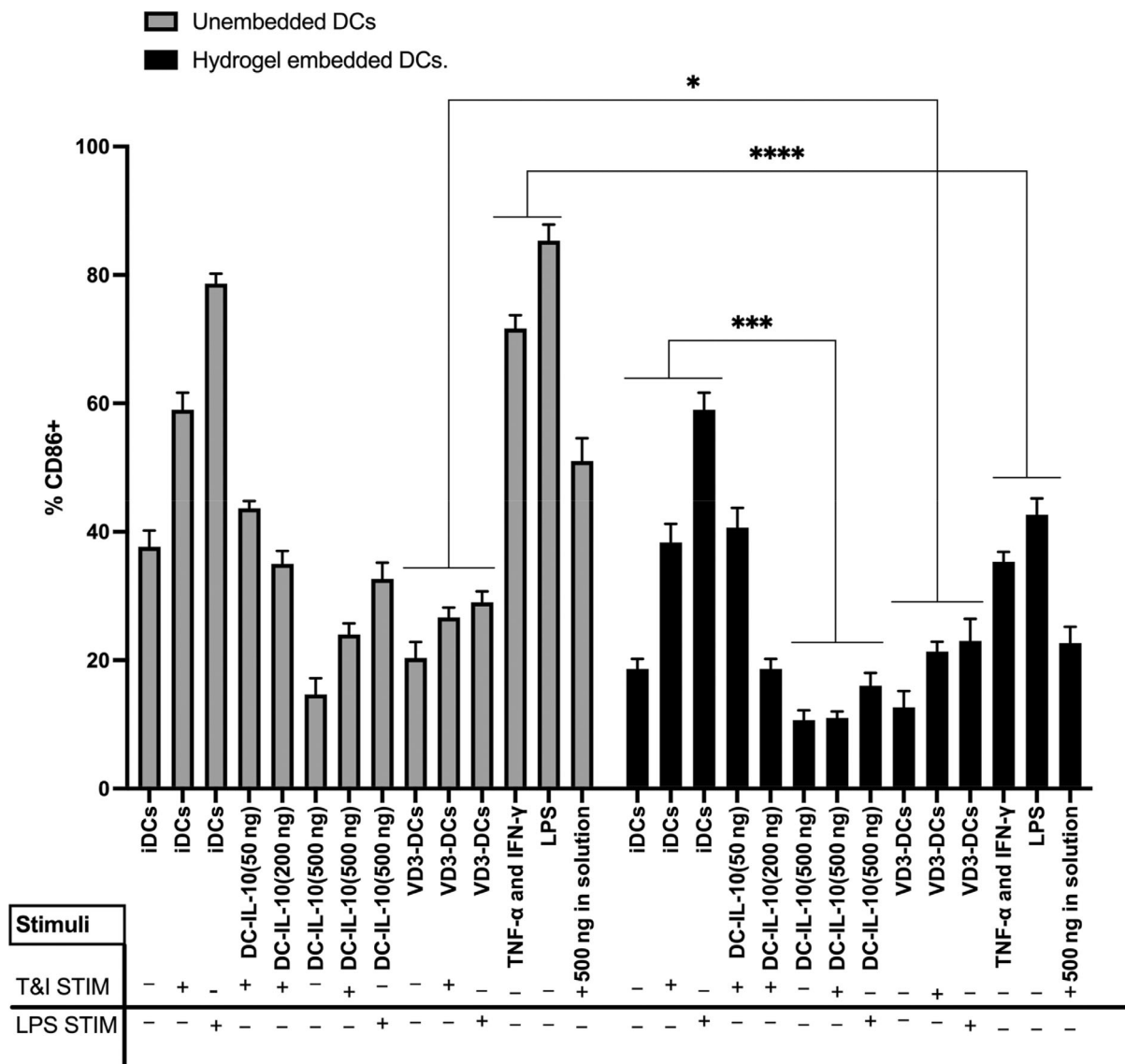


FIGURE 10 Resistance to inflammatory stimulation of dendritic cells (DCs) receiving various treatments with or without embedding in poly (ethylene glycol)-4 arm maleimide (PEG-4MAL) or PEG-IL10 hydrogels at varying IL-10 concentrations. DCs were treated with different conditions as shown in the figure and then incubated for 48 h, however, selected groups were stimulated with either TNF- α and IFN- γ (T&I STIM) or LPS (LPS STIM) 24 h after initial incubation time before completing the overall 48 h of incubation. Stimulated groups are denoted by + or - for unstimulated groups. CD86 expression by DCs was assessed by flow cytometry 48 h after complete incubation. Gray bars denote unembedded DCs in 24-well plates, and black bars denote hydrogel-embedded DCs. Data are presented as the mean with SD of three independent experiments conducted in triplicates. **** $p < .0001$; *** $p < .001$; * $p < .05$ via two-way analysis of variance. iDC, immature dendritic cell; IL, interleukin; LPS, lipopolysaccharide.

sustaining regulatory immunomodulation upon their implantation in an autoimmune/proinflammatory environment.^{61,62} Delivery of DCs with IL-10 has been shown to promote tolerogenic DC phenotype thereby attenuating autoimmune responses.^{63,64} The results described herein build on previous research studies by Babensee et al⁶⁵⁻⁶⁷ and have shown the parameters most relevant to the support of DCs in a tolerogenic context by tuning biophysical and biochemical biomaterial properties to enhance the viability and prevent phenotypic maturation of DCs.

PEG-4MAL hydrogel characterization, IL-10 thiolation, IL-10 release profiles, degradability, DC viability, and phenotype were modulated and assessed through manipulation of varying IL-10 incorporation concentrations, crosslinker type, and treatment with DC controls. It was crucial to demonstrate reproducible mechanical properties for hydrogels with the incorporation of different concentrations of IL-10. Moreover, hydrogels incorporated at varying IL-10 amounts did not affect gel mechanical properties upon conducting rheology studies (Figure 5). Beskid et al showed that tuning polymer weight

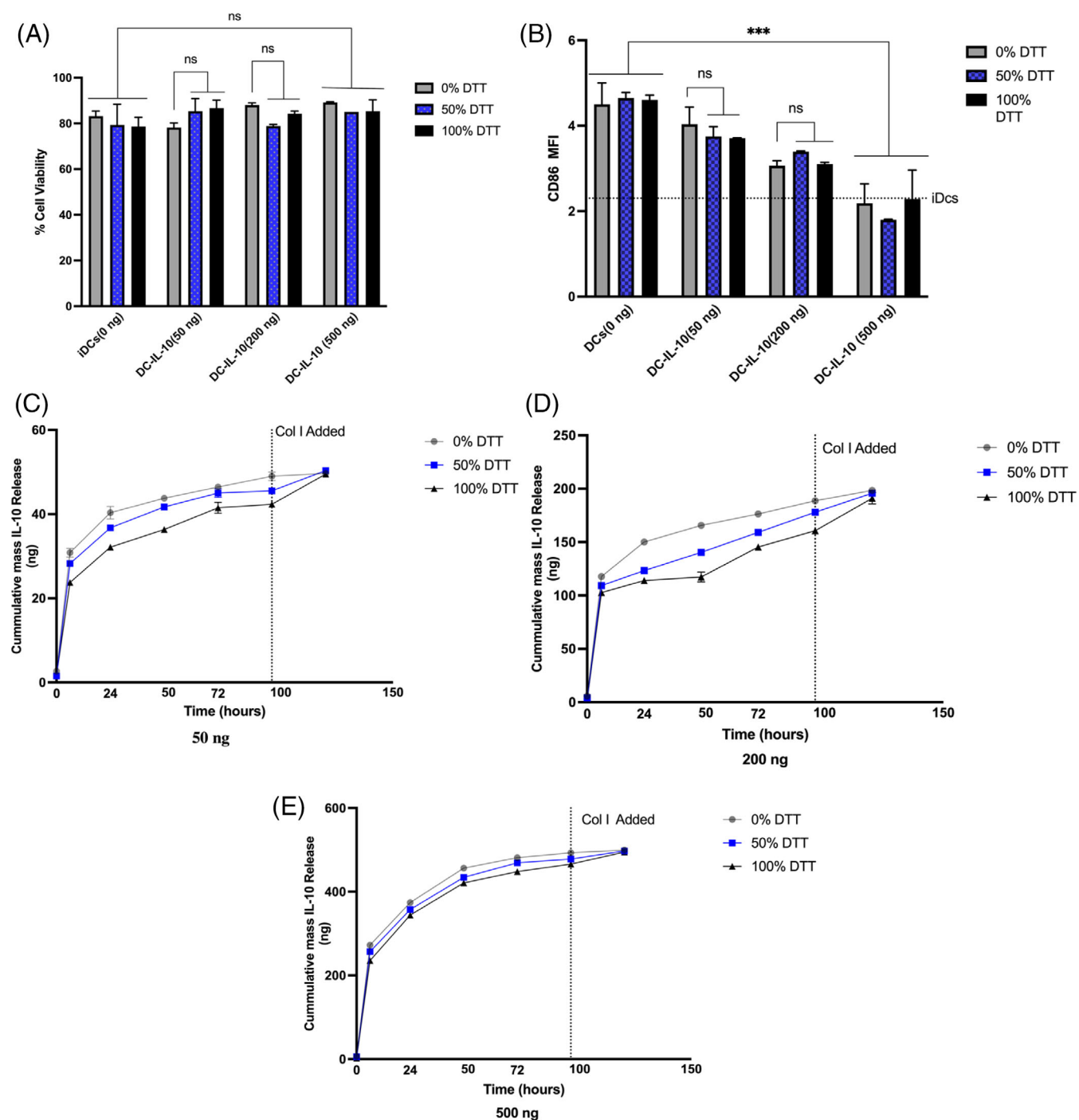


FIGURE 11 Viability and phenotype of dendritic cells (DCs) after 24 h embedding in poly (ethylene glycol)-4 arm maleimide (PEG-4MAL) hydrogels with ratios, and IL-10 controlled release profiles for PEG-4MAL hydrogels synthesized at varying ratios of dithiothreitol (DTT) (nondegradable):VPM (degradable) crosslinkers. (A) Percentage cell viability of hydrogels synthesized at varying ratios of DTT:VPM. Generally, there were no observable significant differences in the extent of live cells across all hydrogel groups with varying ratios of the two crosslinker types. Live cells were defined by propidium iodide negative cells. (B) CD86 expression was assessed by flow cytometry in DC PEG-4MAL hydrogels, which were synthesized at varying ratios of DTT:VPM. Lower expression of CD86 was observed with increasing IL-10 loading amount. However, crosslinker type did not affect CD86 expression in DCs. MFI stands for mean fluorescence intensity. (C–E) IL-10 release kinetics from hydrogels synthesized at varying ratios of DTT:VPM and incorporated with 50, 200, and 500 ng IL-10, respectively. All hydrogels exhibited a burst release of IL-10 at 6 h, however, hydrogels synthesized with 100% DTT released IL-10 much slower compared with what is seen in other conditions. Data are presented as the mean with SD of three independent experiments conducted in triplicates. *** $p < .001$, ns represents no significant differences via two-way analysis of variance. IL, interleukin.

percentages affects the gel viscoelastic properties, which ultimately impacts DCs viability and phenotypic needs of the biomaterial system.^{47,68} Therefore, it is crucial to optimize system parameters for reproducible DC outcomes. For instance, other factors of hydrogels that may contribute to irregularities include reagent purity, molarity, pH, hydrolyzation of maleimide functional groups, and handling technique.⁶⁹ Both hydrogel stiffness (represented by storage modulus) and loss modulus were indistinguishable across all hydrogels, regardless of the amount of IL-10 incorporated, as compared with hydrogels without the incorporated IL-10 (Figure 5). Furthermore, we sought to assess the potential benefit of functionalization of PEG-4MAL hydrogels with IL-10 at varying concentrations for DC phenotypic outcomes upon their embedding in the gels. Again, hydrogels were synthesized without or with 50, 200, or 500 ng of IL-10 (herein referred to as IL-10⁵⁰, IL-10²⁰⁰, and IL-10⁵⁰⁰, respectively) to match the IL-10 dose of DC controls, VD3-DCs, TNF- α and IFN- γ , LPS-treated DCs for direct comparison. DCs have been studied and utilized in many different applications and research purposes, with varying treatment protocols and controls.^{70,71} Here, DC phenotypes were established for both unembedded and hydrogel-embedded DCs based on IL-10 treatment at IL-10⁵⁰, IL-10²⁰⁰, and IL-10⁵⁰⁰, VD3 treatment, stimulation with TNF- α and IFN- γ (TNF- α /IFN- γ), LPS, or no treatment DCs. The DCs in this study were differentiated using a well-established culture protocol adapted from Pulendran et al.^{72,73} VD3-treated DCs were included as a tolerogenic control that functions primarily through PD-L1 presentation,^{74,75} while DCs treated with IL-10 have been shown to induce tolerance through both PD-L1 presentation and secretion of IL-10.^{42,76} At 24 h postembedding, it was observed that DCs embedded in PEG-4MAL hydrogels without IL-10 appeared to show increased cell death and expressed higher levels of CD86 maturation marker⁷⁷ compared with DCs embedded in PEG-4MAL hydrogels with IL-10, which maintained an immature state and remained viable with increasing IL-10 concentrations (Figure 8). DCs treated with 500 ng IL-10 concentration showed a similar mean fluorescence intensity (MFI) of CD86 marker as the iDCs, whereas DCs treated with 50 and 200 ng of IL-10 had a higher CD86 MFI (were less immature) (Figure 8). This result demonstrates that higher IL-10 concentrations can maintain an immature-like DC phenotype when compared with lower incorporation IL-10 concentrations. DCs stimulated with TNF- α /IFN- γ expressed relatively lower MFI for CD86 surface marker compared with DCs stimulated with LPS. Studies conducted in the Babensee laboratory and other research groups have shown that LPS has an augmented effect on iDCs activation compared with TNF- α /IFN- γ ,^{78–80} which is corroborated by higher expression of CD86 with LPS-treated DCs.

Overall, embedding of DCs in hydrogels appeared to protect DCs from a severe proinflammatory environment. Additionally, increased IL-10 concentration provided a more anti-inflammatory microenvironment for the DCs thereby maintaining an immature phenotype compared with DCs embedded without IL-10.^{81,82} Moreover, the viability of DCs embedded in PEG-4MAL hydrogels with IL-10 was evaluated against unembedded DC groups and other hydrogel DCs controls treated with VD3, TNF- α and IFN- γ , and LPS. Based on calcein AM

and EthD-1live/dead staining of DCs within hydrogels and the CCK-8 viability assay, DCs embedded in PEG-4MAL hydrogels with IL-10 were observed to have more live cells than unembedded DC groups and other DCs controls, 24 h postembedding (Figures 8 and A4). Thus, the benefit of IL-10 functionalization on DC viability was further validated. The percentage of live cells was similar across different IL-10 concentrations. This benefit of IL-10 incorporated in the hydrogel translates into improved potency for the proposed cell therapy in MS conditions.^{83–85}

MS patients also have increased levels of inflammatory cytokines and hyper-activated immune systems that can lead to dysregulated cytokine networks and the development of unrelated autoimmune diseases.⁸⁶ It has been established that PEG-4MAL hydrogels with IL-10 support viability and an immature phenotype in DCs,⁴⁷ so we sought to assess the extent to which these hydrogels could protect embedded DCs from inflammation. To assess the resistance of these DCs to inflammatory stimulation, DCs were embedded in PEG-4MAL hydrogels with varying IL-10 amounts or DCs embedded in PEG-4MAL hydrogels without IL-10 for 24 h before inflammatory stimulation with either a moderate stimulus (150 ng/mL of TNF- α and 30 ng/mL IFN- γ “T&I STIM”) or severe stimulus (2 μ g/mL of LPS “LPS STIM”) on day 7. Forty-eight hours after inflammatory stimulation, DCs in hydrogels without IL-10 and unembedded DC groups had significantly high proportions of CD86 marker expression in response to T&I STIM treatment. Conversely, DCs in hydrogels with IL-10 exhibited lower proportions of CD86+ cell surface markers when treated with moderate inflammatory stimuli. Moreover, increased IL-10 concentration had an augmented effect on inhibition of DC maturation, thereby, expressing lower CD86 MFI in comparison with DCs in hydrogels with lower IL-10 amounts. In response to the severe stimulus, however, DCs in hydrogels with IL-10 were also significantly higher in the frequency of CD86 cell marker, albeit to an amount less than DCs in hydrogels without IL-10, with only a moderate stimulus. While IL-10 treatment and hydrogel embedding do not entirely protect DCs from severe stimulation (2 μ g/mL of LPS), these DCs are much less activated than unembedded iDCs controls and DCs in hydrogels without IL-10 or at low IL-10 concentrations, suggesting substantial resistance to inflammatory stimulation.^{47,87} Additionally, LPS is regarded as a very harsh stimulus that is not generally experienced in vivo, especially at the tested concentrations,^{88,89} therefore, such an augmented effect will likely be minimized in the in vivo microenvironment.⁹⁰

Maleimide functional groups on PEG-4MAL polymers allow a wide array of proteins and peptides to be covalently linked to the polymer backbone through free thiols found in free cysteine residues.⁴⁰ However, native IL-10 does not have any free cysteines and must be thiolated before being linked to the PEG-4MAL.^{47,91} Traut's reagent (2-iminothiolane) was used to add free thiols to IL-10 by reacting with primary amines on lysine residues on IL-10. The number of free thiols per IL-10 was found to be $\sim$ 10, which is more than sufficient to enable covalent tethering to the PEG-4MAL.^{40,41,92} One concern with determining the extent of thiolation is that IL-10 might be able to bind multiple maleimide groups and thus act as a crosslinker.

Recent studies from the Babensee laboratory conducted a series of experiments to determine the optimal conditions for covalently tethering IL-10 to PEG-4MAL, including the ratio of PEG-4MAL to IL-10, thiolation chemistry, incubation time, and IL-10 species.⁴⁷ Thiolated IL-10 was reacted with PEG-4MAL in a molar ratio of 1:1000 (HS-IL-10:PEG-4MAL) and successful PEGylation of IL-10 was visualized and imaged on a molecular imager gel XR system (BioRad).⁴⁷ Therefore, current studies leveraged the results obtained from previous studies for conjugating IL-10 onto the PEG-4MAL backbone for the system's optimization in this project.

Cell-based therapies offer the benefit of responsiveness to the disease state regarding the release of immunomodulatory molecules and the ability to interact with immune cells to exert their effect.^{93,94} A key design parameter of these immunosuppressive hydrogels is the ability to have continued IL-10 release for a significant duration (at least 3 days).^{35,95} Thus, it is essential to design a biomaterial that has a controlled release of incorporated IL-10 as seen in related studies.⁹⁶ To assess the release of IL-10 from hydrogels, fluorophore-tagged IL-10 was thiolated, PEGylated, and hydrogels were synthesized as described in the Materials and Methods Section 2. Hydrogels were loaded with either IL-10⁵⁰, IL-10²⁰⁰, or IL-10⁵⁰⁰ so the effect of these hydrogels could be directly compared with DC controls. Hydrogels were degraded with collagenase I, and the fluorescence intensity of supernatants was analyzed over time. Hydrogel groups in collagenase I released almost all incorporated IL-10 after just 6 h, whereas hydrogels in PBS released more than half of the incorporated IL-10 within the first 6 h across all concentration groups. On the other hand, hydrogels with tethered IL-10 in PBS had an initial burst release of ~40% of the incorporated IL-10, which likely consists of the unreacted IL-10. Following this burst release, these hydrogels then exhibited a much more controlled release of ~30% of the incorporated IL-10 over the next 4 days. After 96 h, collagenase I was added to these hydrogels to release any remaining IL-10, which totaled the amount of IL-10 released by hydrogels with untethered protein. The concentration of IL-10 incorporated in hydrogels did not affect the rate of IL-10 release studies. The results obtained in this kinetics release study suggest that hydrogels incorporated with thiolated IL-10 can provide a controlled release of immunosuppressive molecules for at least 96 h, which play a vital role in maintaining a tolerogenic immunosuppressive microenvironment, which is important for inhibiting DC maturation, thereby maintaining an iDC phenotype.⁹⁷ Additionally, the duration for controlled IL-10 release is comparable to the dosage frequency of other drug-delivery biomaterial systems used in MS conditions.^{98–100}

To test the bioactivity of PEG-4MAL hydrogels consisting of IL-10, we utilized a murine mast cell line (MC/9 cells) that proliferates in response to IL-10. For this study, DCs were not used because they are nonproliferate cells unless stimulated by specific conditions¹⁰¹; therefore, MC/9 cells were instead used to test the bioactivity of PEG-4MAL hydrogels consisting of IL-10. The MC/9 cells were treated in various conditions at different IL-10 concentrations. Treatment groups consisted of either cells treated with native IL-10, embedded in PEG-4MAL hydrogels with IL-10, cultured on PEG-4MAL hydrogels

consisting of IL-10, treated with supernatant that was obtained from release kinetics studies, or LPS-treated PEG-4MAL hydrogels with embedded cells, and incubated at 37°C, 5% CO₂ in a humidified incubator for 2 days before conducting CCK-8 metabolic assay. Proliferation was measured at both 4 and 24 h following the addition of CCK-8 metabolic assay reagents. MC/9 cells treated with native IL-10 and those embedded in hydrogels with IL-10 exhibited almost similar levels of proliferation, which suggests that the bioactivity of PEG-4MAL hydrogels with IL-10 had not been affected by the PEGylation reaction. Additional treatment groups with supernatant from release studies showed significant proliferation of MC/9 cells. This result supported the validation for IL-10 release from hydrogels. Similarly, cells cultured on PEG-4MAL hydrogels consisting of IL-10 also exhibited proliferation, which further asserts that IL-10 is released from hydrogels for the cells to respond to. Moreover, increasing IL-10 amounts augmented the proliferation effect on the MC/9 cells. Albeit, a significantly lower increase in the proliferation of MC/9 cells was observed with hydrogels at IL-10¹⁰⁰⁰ concentration compared with hydrogels with IL-10⁵⁰⁰ amounts. PEG-4MAL hydrogels with excessively high IL-10 loading of 1000 ng most likely contributed to toxicity within the hydrogel, which ultimately attenuated the proliferation effect of MC/9 cells. Alternatively, the saturation of IL-10 receptors could lead to no further cell proliferation at very high IL-10 dosages. Related studies show an estimated physiologically relevant IL-10 concentration of up to >300 pg/mL in cases of autoimmunity,^{102–104} with studies here supporting the hypothesis that IL-10 concentration at 500 ng per 50 μ L gel is an optimal incorporation amount for the biomaterial delivery system designed herein. Also, the effect of LPS-treated PEG-4MAL hydrogels on the proliferation of embedded MC/9 mast cells was comparable across varying IL-10 concentrations. MC/9 cells respond to LPS stimulation by inducing secretion of Th2-associated cytokines such as IL-5, 10, and 13, which affect mRNA regulation in mast cells.⁵¹ Additionally, IL-13-mediated signaling pathways are reported to have a synergic inhibition effect on cell proliferation in the presence of other signaling mediators.¹⁰⁵ In current studies, LPS was added to PEG-1L-10 hydrogels across varying IL-10 concentrations, and there was limited protection from the hydrogel and IL-10 on MC/9 cells against LPS, with a relatively lower proliferation rate of mast cells observed in LPS-treated gels compared with PEG-IL-10 embedding cells without LPS treatment after 24 h. This effect is attributed to the severe harsh environment that LPS presents on the hydrogel, thereby likely to affect its mesh size and degradability, which increases the risk of exposure to the embedded mast cells, hence affecting its proliferation.

The structure of the PEG-4MAL polymer allows manipulation through several parameters, such as polymer density and macromer size, adhesive peptide, and crosslinker type.^{16,47} We next aimed to evaluate the effect of crosslinker ratio and IL-10 concentration variations on embedded DC viability and phenotype. The effect of hydrogel degradability on embedded DCs was evaluated by synthesizing hydrogels with various ratios of VPM to DTT crosslinkers at different IL-10 concentrations. Cells recovered from hydrogels with various crosslinker concentrations exhibited similar cell viability and CD86

expression to iDC controls. Crosslinker composition did not significantly affect DC viability or phenotype based on analysis by FC. Furthermore, release kinetics studies were conducted with VPM:DTT at ratios 1:0, 1:1, and 0:1 to assess the rate of IL-10 release from hydrogels incorporated with varying amounts of IL-10. Overall, hydrogel groups incorporated with 100% DTT exhibited lower IL-10 release over 96 h compared with 50% and 0% DTT incorporated hydrogels. This is because VPM is a more degradable crosslinker compared with the nondegradable DTT,¹⁰⁶ hence the difference in IL-10 release profiles. Whereas no significant differences were observed with cell viability and CD86 expression with varying VPM and DTT crosslinkers at different IL-10 concentrations, observable variations were reported on the IL-10 release kinetics study at varying VPM:DTT ratios. This study demonstrates the tunability of PEG-4MAL hydrogels and the system's ability to achieve desirable delivery of immunosuppressive molecules at varying dosages by varying crosslinker types.

4.1 | Limitations and future directions

The results presented in this study are promising in the development of novel immunological cell-biomaterial delivery systems for tolerance-induction and immunomodulation therapies; however, some limitations were encountered during conducting this study. For instance, the design parameters optimized here are typically in vitro without the complexity of an autoimmune situation, and yet, the biomaterial approach is modular, and modifications are made easily to the system to engineer the system for enhanced DC functionality in MS conditions. This establishes a baseline in understanding the critical fundamental components of the system fully which can be observed in in vivo readouts such as the delivery of hydrogel-embedded DCs with optimized IL-10 subcutaneously by cLNs in the EAE model. Potentially, there may be a need to change the dose of IL-10 or optimize the release kinetics for in vivo applications, although this is not expected to deviate a lot from the results obtained in this study. Furthermore, the studies conducted here did not assess the level of toxicity that could result from increasing IL-10 dosages or loading amounts. Whereas the project aims at optimizing the cell-biomaterial system functionality, this should not be achieved at the expense of other physiological performance.¹⁰⁷ Finally, studies conducted here did not determine the nature of released IL-10 from the hydrogel, whether tethered or unthiolated and evaluate how this could impact DC outcomes like cell viability and phenotype. These study limitations shall be addressed in future studies building on the results obtained herein.

There are many promising next steps to this project, including studies focused on replicating select parameters that were optimized from the research outcomes herein for future in vivo functionality of the system. The biomaterial approach is modular, and modifications are made easily to the system to further engineer the system for enhanced DC functionality in normal mice to examine these design

parameters, first without the complexity of an autoimmune situation. This establishes a basic standard for response to understand critical fundamental components of the system more fully. Lastly, the effectiveness of optimized IL-10 concentrations in PEG-4MAL hydrogel-delivered DCs in ameliorating symptoms in mice with EAE model shall be evaluated by immunoprofiling EAE mice receiving treatment with the optimized cell-biomaterial system. These in vivo studies shall contribute to existing research for DCs engineered systems in developing better treatment regimens at optimized IL-10 loading amounts, with comparison to other related studies.¹⁰⁸

5 | CONCLUSION

The biomaterial approach used herein has a high potential to provide an immunological breakthrough in tolerance-induction and immunomodulation therapies. The unique aspect used here includes designing a biomaterial system at varying IL-10 amounts for optimal IL-10-secreting DCs released in a controlled manner to induce tolerance by the tolerogenic DCs, through conditioning T cells and other DCs recruited to the gel, and to the cervical LNs for future in vivo studies. Moreover, FDA-approved PEG products have been reported to have no local or systemic toxicity following degradation, with just minimal inflammation, and about 96% urine clearance within 24 h.¹⁰⁹ Generally, this tolerogenic biomaterial system is promising to serve as a model for developing cell-delivery scaffolds in immunosuppressive applications, such as the treatment of MS condition, islet transplantation for the treatment of Type I diabetes (T1D), and also in other related complex immunological diseases.³⁶

AUTHOR CONTRIBUTIONS

This manuscript was written mainly by Fredrick Bulondo with revisions by Julia E. Babensee. Fredrick was the lead person in conducting this research study with the help of other people. Julia E. Babensee is the principal investigator for the laboratory in which this research was conducted and the overall project supervisor. Both authors have approved the final version of the manuscript.

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CONFLICT OF INTEREST STATEMENT

The authors declare that there are no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are openly available in [Figshare] at [[10.6084/m9.figshare.25506823](https://doi.org/10.6084/m9.figshare.25506823)].

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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