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Title
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Permalink
https://escholarship.org/uc/item/6153c9tz

Journal
Lab on a Chip, 21(18)

ISSN
1473-0197

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Publication Date
2021-09-14

DOI
10.1039/d1lc00096a

Peer reviewed

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Author manuscript

Lab Chip. Author manuscript; available in PMC 2022 September 14.

Published in final edited form as:

Lab Chip. ; 21(18): 3598–3613. doi:10.1039/d1lc00096a.

GRAS-microparticle microarrays identify dendritic cell tolerogenic marker-inducing formulations

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Abstract

Microarrays, miniaturized platforms used for high-content studies, provide potential advantages over traditional *in vitro* investigation in terms of time, cost, and parallel analyses. Recently, microarrays have been leveraged to investigate immune cell biology by providing a platform with which to systematically investigate the effects of various agents on a wide variety of cellular processes, including those giving rise to immune regulation for application toward curtailing autoimmunity. A specific embodiment incorporates dendritic cells cultured on microarrays containing biodegradable microparticles. Such an approach allows immune cell and microparticle co-localization and release of compounds on small, isolated populations of cells, enabling a quick, convenient method to quantify a variety of cellular responses in parallel. In this study, the microparticle microarray platform was utilized to investigate a small library of sixteen generally regarded as safe (GRAS) compounds (ascorbic acid, aspirin, capsaicin, celastrol, curcumin, epigallocatechin-3-gallate, ergosterol, hemin, hydrocortisone, indomethacin, menadione, naproxen, resveratrol, retinoic acid, α-tocopherol, vitamin D3) for their ability to induce suppressive phenotypes in murine dendritic cells. Two complementary tolerogenic index ranking systems were proposed to summarize dendritic cell responses and suggested several lead compounds (celastrol, ergosterol, vitamin D3) and two secondary compounds (hemin, capsaicin), which warrant further investigation for applications toward suppression and tolerance.

Introduction

The immune system safeguards the body against foreign pathogens while retaining the ability to distinguish self from non-self.[1] In healthy patients, self-tolerance is maintained by

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Conflicts of interest

There are no conflicts to declare.

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inactivating or culling self-reactive lymphocytes.[2,3] However, in organ-specific autoimmune diseases, such as type 1 diabetes (T1D)[4–6] or multiple sclerosis,[7] a breakdown of immune tolerance to self-antigens induces chronic, tissue-specific inflammation. Such inflammation is mediated by the activation of naïve T cells into auto-reactive effector T cells.[8] Since removal of self-antigen is impossible and systemic immunosuppression increases infection risk, there persists a need for an antigen-specific therapy, commonly termed a tolerogenic vaccine. This technique aims to reeducate the immune system to induce peripheral tolerance of the presented antigen when co-presented with immunomodulating factors.[9]

One strategy to develop tolerogenic vaccines is to suppress auto-reactive T cells by targeting the most potent T cell activators, dendritic cells (DCs).[10–12] Dendritic cells are the most effective antigen-presenting cell (APC) that are efficiently able to acquire, process, and present antigen to T cells.[13–15] Traditionally, DCs were thought to only activate T cells as part of a pro-inflammatory response.[16] However, their anti-inflammatory capacity to suppress T cell activation, and therefore T-cell mediated immunity is also critical.[14] This immune suppression requires the induction of specific subsets of DC phenotypes, termed tolerogenic DCs (tolDCs), which are capable of inducing T-cell anergy, apoptosis, and the upregulation of regulatory T-cells (Tregs).[6,17–20] Maturation resistance, high production of anti-inflammatory cytokines, low production of pro-inflammatory cytokines, and production of ‘pro-tolerogenic’ proteins characterize tolDCs.[21–24] Previous work has shown that exposing DCs to various biologics,[21,25] pharmacological agents,[26,27] or genetic modifications[28,29] can effectively polarize DCs toward such tolerogenic phenotypes.

The success of tolDC-generating compounds has stimulated interest into investigating traditional anti-inflammatory and generally recognized as safe (GRAS) compounds for their potential to induce suppressive tolDC phenotypes. Compounds such as aspirin, naproxen, and curcumin have been shown to elicit various immune responses, yet haven’t been fully investigated for their ability to promote tolDCs.[30,31] High-content screening on primary lymphocytes using traditional culture methods can be challenging due to cell source limitations. To address this, single-cell microwell arrays have been developed to analyze human antigen-specific B-cells producing human monoclonal antibodies[32] while microfluidic systems have demonstrated their utility in quantifying secretion rates in APCs following immunological challenge.[33] Further, cell-based microarrays incorporating loaded polymeric microparticles (MPs) were developed.[34] By incorporating cell patterning microarray techniques[35,36] with a carrier biomaterial, both targeting of agents to DCs intracellularly *via* MP uptake as well as local extracellular controlled release can be achieved.[10,11,37–39] Utilizing this cell-based microarray approach could provide a comprehensive assessment of DC tolerogenicity by enabling a better understanding of how DC maturation, cytokine production, and tolerogenic markers interact. Here, MP–DC microarrays were used to identify several compounds with high tolerogenic potential in MP formulation as evidenced by inducing maturation resistance, high anti-inflammatory and low pro-inflammatory cytokine production, and high pro-tolerogenic marker expression in DCs. Further, two complementary models are proposed to rank the tolerogenic potential of compounds using these criteria in an effort to evaluate tolDC generation in a systematic manner.

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Methods

Dendritic cell isolation

Female mice were purchased from University of Florida Animal Care Services (ACS) (Gainesville, FL). All animals were housed in a specific pathogen free-environment in University of Florida ACS facilities, and experiments were performed in accordance with detailed protocols approved by University of Florida Institutional Animal Care and Use Committee (IACUC). Dendritic cells were isolated from the bone marrow of 8 week old C57Bl6/j mice using a 10 day protocol.[40] Briefly, bone marrow was isolated from the femur and tibia. Red blood cells were lysed by ACK lysing buffer (Whittaker) and the isolated precursor cells were incubated with DC-media consisting of 20 ng mL^{-1} of GM-CSF (R&D Systems), DMEM/F12 (1 : 1) with L-glutamine (Cellgro, Herndon, VA) and 10% fetal bovine serum (Bio-Whittaker), 1% sodium pyruvate (Lonza, Walkersville, MD) and 1% non-essential amino acid (Lonza, Walkersville, MD) for 2 days in a T-75 flask. The floating cells were collected after 48 h and re-seeded with fresh DC-media in a 6-well low-attachment plate (Corning Inc., NY) for 4 days with fresh media supplied after 2 days. After 4 days of culture on the low attachment plate the cells were re-suspended in fresh media and seeded onto tissue-culture treated 6-well plates (Corning, Inc., NY) for 4 days with fresh media supplied after 2 days. After 10 total days of culture DCs were ready for investigation and used within 10 days for a maximum total culture of 20 days. When ready, DCs were incubated with 10 mM solution of Na_2EDTA (Fisher Scientific) in PBS for 30 minutes prior to lifting. Dendritic cells thus isolated were then tested *via* flow cytometry for purity (CD11c+ >90%) and viability (7-AAD >99%). All culture media, buffers, and other components were purchased sterile.

Microparticle fabrication

A 50 : 50 polymer composition of poly(D,L-lactide-*co*-glycolide) (PLGA) (MW ~ 44 000 g mol^{-1}; Purac Biomaterials, Netherlands) was used to generate microparticles. Poly-vinyl alcohol (PVA) (MW 15 000 g mol^{-1}) (MP Biomedicals, Santa Ana, CA, USA) and was used as an emulsion stabilizer. NANOpure H_2O was used as the aqueous phase to form emulsions while methylene chloride (Fisher Scientific, NJ, USA) was the organic solvent to dissolve PLGA. Microparticles were formed using either a standard water–oil–water (double emulsion) or oil–water (single emulsion) solvent evaporation technique.

Briefly, for the single emulsion technique, PLGA was dissolved in methylene chloride at 10% concentration. Known amounts of a fluorescent dye or hydrophobic drug of interest (aspirin, capsaicin, celastrol, curcumin, ergosterol, hemin, hydrocortisone, indomethacin, menadione, α-tocopherol, resveratrol, retinoic acid, and vitamin D3) were added to produce dispersion in the solution. This solution was then added to 50 ml of 5% PVA solution in NANOpure H_2O and was homogenized using tissue-miser homogenizer (Dremel, Wisconsin, USA) at 25 000 rpm for 2 minutes to form the emulsion. Following, the emulsion was added to 500 ml of 1% PVA solution. The particles thus formed were agitated using a magnetic stirrer (Fisher Scientific, NJ, USA) for 24 h to evaporate residual methylene chloride. The remaining solution was centrifuged at 10 000*g* for 10 min to collect MPs which were subsequently washed three times with NANOpure H_2O. The water was

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aspirated from the centrifuged MPs, which were then flash-frozen in liquid nitrogen and kept under vacuum overnight. The MPs were stored at −20 °C until used.

To produce MPs using a double emulsion technique, PLGA was dissolved in methylene chloride at 10% concentration. Meanwhile, hydrophilic drugs of interest (ascorbic acid, epigallocatechin-3-gallate, and naproxen) were dissolved in NANOpure H_2O. The drug solution was then added to the dissolved PLGA in methylene chloride at a 1 : 10 ratio while homogenizing at 25 000 rpm for 1 minute to form a primary emulsion. The primary emulsion was added to 10 mL of 5% PVA solution in NANOpure H_2O and the homogenizing was continued at 25 000 rpm for 2 minutes to form the secondary emulsion. Then, the secondary emulsion was added to 100 ml of 1% PVA solution. The particles thus formed were agitated using a magnetic stirrer for 24 h to evaporate residual methylene chloride. The remaining solution was centrifuged at 10 000*g* for 10 min to collect MPs which were subsequently washed three times with NANOpure H_2O. The water was aspirated from the centrifuged MPs, which were then flash-frozen in liquid nitrogen and kept under vacuum overnight. The MPs were stored at −20 °C until used.

Preparation of microarrays with specific non-fouling regions

Arrays with PEG-based non-fouling backgrounds and amine-terminated silane adhesion islands were manufactured as previously reported.[34,41,42] Briefly, glass coverslips were cleaned in an oxygen plasma etcher (Terra Universal, Fullerton, CA). Arrays of (3-aminopropyl)trimethoxysilane (NH_2-terminated silane) (Sigma-Aldrich, St. Louis, MO) were printed on clean coverslips using a Calligrapher Miniarrayer printer (Bio-Rad, Hercules, CA) with 1200 μm center to center distances and a pin diameter of 400 μm. The silane printed coverslips were then coated with 175 Å of titanium (Ti; 99.995% pure) and 225 Å of gold (Au; 99.999% pure) (Williams Advanced Materials, Buffalo, NY). Following coating, gold-coated arrays were sonicated to remove gold from the amine spots, exposing NH_2-terminated silane islands. The coverslips were incubated with 0.1 M, methyl-terminated alkanethiol ($CH_3(CH_2)_{11}SH$) (Sigma) for 30 min. Substrates were then incubated in 10% Pluronic® F-127 (BASF Corporation, USA) for 3 h. After, microarrays were dried and MP formulations were printed in randomized configurations. The microarrays were placed in 35 mm Petri dishes containing PBS with 2% (vol/vol) penicillin and 2% (vol/vol) streptomycin for 30 min to rehydrate the nonfouling PEG background and as a noncaustic sterilization step. Randomization was performed *via* an Excel routine. Each microarray island was given a number that was then randomly sorted in Excel and assigned to the various compounds at different concentrations.

Microparticle characterization

Particle size was characterized *via* dynamic laser diffraction (Beckman Coulter, Brea, CA). A total of 10 mg of particles were re-suspended in 10 mL of de-ionized (DI) water *via* sonication for 2 min (Branson 2510, Paragon Electronics, FL). The sample solution was then run through the instrument and the size was verified through measuring the angular variation in intensity of light scattered as a laser beam passes through a dispersed particulate sample.

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To quantify loading efficiency, a known weight of MPs loaded with drug was first dissolved in DMSO. The total compound load was then quantified by measuring the absorbance at a wavelength specific to the molecule of interest *via* spectrophotometry (Nanodrop Technologies Inc., DE, USA) and comparing absorbance to a standard curve made from known concentration of drug in DMSO. The standard curve was made by dissolving known weight of drug in DMSO and serially diluting 1 : 1 with DMSO 12 times ($n = 3$). Blank PLGA particles were then added to each dilution to account for PLGA absorbance. Loaded MPs were tested for endotoxin levels using the ChromoLAL Assay kit (Associates of Cape Cod, Inc., Falmouth, MA.) according to manufacturer's instructions.

Microparticle printing optimization

To determine the correlation between the source particle density and the number of particle printed onto the microarrays, rhodamine-loaded MPs were first suspended at 10 mg ml^{-1} in NANOpure H_2O with 0.05% methyl cellulose. The methyl cellulose was incorporated to produce a uniform density of printed MPs on the microarray. The MP concentration was then calculated as MP per ml *via* quantification on a hemocytometer using fluorescent microscopy and Axiovision software. Serial 1 : 2 dilutions of MPs in NANOpure H_2O were generated and suspensions were added into separate wells in a 384-well plate. MPs were then printed onto the arrayed NH_2-terminated substrate. Following printing, microarrays were washed three times in PBS and then allowed to dry overnight. Fluorescence micrographs of the entire arrays were obtained and the number of MPs on each island was quantified using Axiovision software.

Dendritic cell seeding and immunocytochemistry

Dendritic cells were cultured with varying ratios of particles to cells for each compound. This was achieved by printing either 1500, 450, or 150 particles to specific islands on the microarray (hi, med, low # of MPs per spot). The number of DCs were observed to be 150 ± 20 per island, thus resulting in MP : DC ratios of 10, 3, and 1. Once printed, the dried microarrays were rinsed 3X with PBS and re-hydrated for 30 min with PBS without calcium/magnesium under UV to sterilize. Meanwhile, the DCs were incubated with EDTA for 30 minutes, lifted, and re-suspended in PBS containing 0.025 g L^{-1} of magnesium chloride at 10^6 cells per ml. The microarrays were washed once more in PBS and placed in 35 mm Petri dishes with 2 ml PBS. The DCs were then gently seeded onto the microarrays and placed on a rocker plate for 10 minutes to allow adhesion onto the particle-coated, amine-terminated islands. Afterward, the microarrays were gently rinsed in complete PBS to remove cells from the background regions, and placed in new Petri dishes with cell media. The microarrays were then placed in the incubator for 48 h. Microarrays that were used to quantify MHCII, CD80, CD86, and IL-12p40 production were pulsed with lipopolysaccharide (LPS) (200 ng ml^{-1}) for the final 24 h.

Following, DCs were fixed with 4% paraformaldehyde (USB Corporation). Cells were then fluorescently stained either for surface markers CD80 (BD Biosciences), CD86 (BD Biosciences), MHC-II (BD Biosciences), PD-L1 (BD Biosciences); intracellular cytokines IL-10 (BD Biosciences), IL-12p40 (BD Biosciences); and the intracellular enzyme indoleamine 2,3-dioxygenase (IDO, Millipore). For surface staining, the microarrays were

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first blocked with 1% heat-denatured BSA. Biotinylated primary antibodies were then incubated in permeabilization buffer (includes saponin) (eBioscience) (1 : 100) with CD16/32 (FC Block) (1 : 100, BD Bioscience). Intracellular staining for IL-10, and IL-12p40 was performed by first incubating seeded immunoarrays with GolgiPlug (1 μL ml^{-1}) for the final 4 h of culture. For all intracellular staining, microarrays were incubated with 4% paraformaldehyde followed by FOXP3 fixation/permeabilization buffer for 2 h (eBioscience). The microarrays were then washed and incubated with several blocking solutions including avidin blocking solution (Pierce), biotin blocking solution (Pierce), 2% mouse serum (Invitrogen), and 2% goat serum (Invitrogen) all in permeabilization buffer. Afterward, the primary antibodies were incubated in permeabilization buffer (1 : 100) with CD 16/32 (FC Block, 1 : 100, BD Bioscience) followed by biotinylated secondary antibodies (BD Bioscience).

For all microarrays, a streptavidin-cross-linked alkaline phosphatase was used after incubation with the biotinylated antibody, followed lastly by the precipitating fluorescent substrate, ELF97 (Invitrogen). Nuclei were counterstained with propidium iodide. Immunoarrays were then mounted and imaged on a Carl Zeiss Axiovert 200m fluorescent microscope using a 10× objective and compiled using Axiovision software.

Statistical analysis

Statistical analyses were performed using a one-way ANOVA (Version 12, Systat Software, Inc., San Jose, CA) and pairwise comparisons were made using Tukey's honestly-significant-difference ($\alpha = 0.05$). Samples were nested within each microarray and then within each experiment. Each MP formulation was printed on six islands per microarray. For each marker, three microarrays were evaluated.

Results

Dendritic cell characterization on the immunoarray

Expression of cell surface markers, cytokine production, and IDO enzyme production upon interaction with the different on-chip particle formulations is quantified using standard immunocytochemistry and fluorescent microscopy (Fig. 1a). Cell attachment is tightly confined to the particle-coated silane islands as confirmed *via* nuclei (shown in blue) staining of adhered DCs. Dendritic cells are readily stimulated on-chip as evidenced by the dose-dependent IL-12p40 response to LPS-loaded MPs (Fig. 1b). Intracellular (Fig. 1c) and extracellular (Fig. 1d) marker staining was optimized for each assay. DC viability was assessed after culturing on the microarrays using the LIVE/DEAD assay and found to be 99% viable (data not shown).

Microparticle characterization

A ~1 μm microparticle size was selected as being readily printed and surface adsorbed on-chip in a reproducible manner.[34] This size also allows delivery of agents both intracellularly through DC phagocytosis[11,43,44] as well as on-chip local controlled release by cell-adjacent MPs.[34] An average particle diameter of 1.6 μm (±0.6 μm, polydispersity index PDI = 0.13) (Fig. 2a) was fabricated. Loading efficiency, the percentage of drug that is encapsulated into

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the MPs *versus* the amount that is attempted to be loaded, was quantified. This parameter is of particular interest with expensive reagents and biomolecules, where a significant loss of the payload is economically unfeasible. Accordingly, encapsulation efficiency and loading amount per mass of polymer for each compound was quantified although not necessarily maximized (Table 1). Endotoxin levels were quantified and determined to be below guideline levels for preclinical vaccine formulations (Table 1).[45]

Microparticle printing optimization

To achieve controllable deposition of MPs on the microarray, the relationship between the density of MPs in suspension loaded into the source plate and the amount of MPs delivered per spot was quantified. Rhodamine was encapsulated in PLGA MPs, providing a fluorescent readout. A stock solution of 10 mg MPs per ml was made in diH_2O with 0.05% w/v methylcellulose. Methyl cellulose was incorporated to increase viscosity and facilitate uniform distribution of printed MPs on the microarray. It was determined that 0.05% w/v was the optimum concentration to achieve uniform particle surface distribution while also readily washing away. Thus, MPs remained adsorbed, while also providing the amine functionalized silane islands promoting cell attachment. Serial 1 : 2 dilutions were made with the particles in suspension and were subsequently robotically printed (Calligrapher, BioRad). Automated fluorescent microscopy was utilized to quantify the number of particles per spot. The number of surface-bound particles was correlated to MP suspension density using the following experimentally determined relationship:

$$\text{MPs per spot} = 1.96x + 11.98 \quad \left(R^2 = 0.998\right)$$

where *x* represents the source plate MP concentration (mg ml^{-1}). This relationship was used deliver 1500, 450, and 150 particles to specific islands on the microarray (hi, med, low # of MPs per spot). The number of DCs were observed to be 150 ± 20 per island, thus resulting in MP : DC ratios of 10, 3, and 1.

Screening GRAS compounds on DC microparticle microarrays

A library of compounds (Table 2) was successfully formulated into PLGA MPs and screened on the microarray platform. We sought to evaluate the efficacy of these compounds to promote markers of tolDC phenotype. Specifically, we investigated the ability of these compounds to inhibit DC maturation and pro-inflammatory cytokine production when exposed to the TLR4 ligand, lipopolysaccharide (LPS). Further, we evaluated the production of anti-inflammatory cytokine IL-10 and immunosuppressive enzyme IDO, as well as expression of the inhibitory molecule, PD-L1, on DCs when exposed to the compound library.

Maturation resistance

Either 1500, 450, or 150 # of MPs per spot (hi, med, low) were printed to each island on the microarray. The number of DCs were observed to be 150 ± 20 per island, resulting in MP : DC ratios of 10, 3, and 1. To evaluate the ability of the various compounds to induce DC maturation resistance, MP-printed microarrays with adherent DCs were incubated for 48

h in normal DC medium. At that time, LPS (200 ng ml^{-1}) was added to the medium and the MP–DC microarrays were incubated for an additional 24 h. The cells adherent to the microarrays were then fixed in paraformaldehyde and stained for MHCII, CD80, and CD86.

Surface expression of MHC class II molecules, a critical component of DC maturation was quantified first. MHCII complexes enable presentation of foreign peptides to antigen-specific T-cells, initiating the adaptive immune response.[71] Exposure to LPS activates TLR4 in DCs resulting in upregulation of MHCII. As expected, DCs exposed to LPS and treated with unloaded PLGA MPs resulted in relatively high MHCII expression, whereas several compounds significantly inhibited MHCII presentation (Fig. 3a). Of note, celastrol, ECGC, hemin, α-tocopherol, and vitamin D3 all inhibited LPS-induced MHCII expression, an expected finding.[56,59,64,70,72–74] Interestingly, ascorbic acid, ergosterol, and naproxen were also effective at inhibiting MHCII expression, a previously unreported finding.

In addition to MHCII expression, DC activation is characterized by expression of co-stimulatory proteins, notably CD80 and CD86.[75] Taken together, the presence of these two co-stimulatory proteins comprise 'signal 2' of DC-mediated T cell activation.[76] CD80 is expressed earlier in DCs upon exposure to TLR signaling. Dendritic cells screened on the microarray with unloaded MPs again displayed high expression of CD80 (Fig. 3b). Exposure to ascorbic acid, celastrol, epigallocatechin gallate,[56,77] ergosterol, indomethacin, menadione, naproxen, resveratrol,[66,78] retinoic acid, and vitamin D3 (ref. 79 and 80) all significantly inhibited CD80 expression. In contrast, DCs cultured in the presence of curcumin-loaded MPs upregulated CD80 expression compared to DCs cultured with unloaded MPs.

Microparticle microarray analysis of CD86 expression in DCs revealed that most of the compounds tested inhibited LPS-induced CD86 expression for at least one concentration tested. Only DCs cultured with α-tocopherol- and resveratrol-loaded MPs were unable to significantly decrease LPS-induced CD86 expression (Fig. 3c).

Cytokine production

Cytokines are small proteins secreted by cells to mediate intercellular communication.[81] Both pro- and anti-inflammatory cytokines are crucial to regulating immune responses. Production of the pro-inflammatory cytokine IL-12p40 (Fig. 3d) and the anti-inflammatory cytokine IL-10 (Fig. 3e) were evaluated *via* the MP-microarray.

It was found that ascorbic acid, curcumin, ergosterol, indomethacin, and α-tocopherol all significantly mitigated IL-12p40 production while hydrocortisone and naproxen both increased DC production of IL-12p40. Ascorbic acid,[46,82] curcumin,[83–85] and α-tocopherol[46] has been previously reported to modulate IL-12p40 production whereas this is the first instance of IL-12p40 production mediated by ergosterol, indomethacin, hydrocortisone, or naproxen in murine bone marrow DCs.

The anti-inflammatory cytokine interleukin-10 (IL-10) is produced by DCs and inhibits the activity of Th1 cells, NK cells, and macrophages[86] in a critical process to maintain peripheral tolerance.[81] This study revealed aspirin, capsaicin, epigallocatechin

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gallate, hemin, menadione, naproxen, and vitamin D3 to upregulate production of the immunosuppressive protein IL-10. Whereas aspirin,[87] epigallocatechin gallate,[77] and vitamin D3 (ref. 21 and 88) have been shown to increase IL-10 production in DCs previously, and capsaicin-induced macrophage production of IL-10 has been reported,[89] this is the first report of menadione, naproxen, and hemin upregulating IL-10 in murine bone marrow derived DCs.

Indoleamine 2,3-dioxygenase (IDO) production

Indoleamine 2,3-dioxygenase (IDO) is an intracellular heme-containing enzyme that catalyzes the rate-limiting step in tryptophan degradation.[90] Increasing evidence has linked IDO production with tolDCs through the generation of regulatory T cells[91–99] and suppression of activated T cells.[100] We aimed to quantify the relationship between these compounds and their potential in promoting IDO production in DCs (Fig. 3f). Celastrol, hemin, retinoic acid, and vitamin D3 were found to significantly upregulate IDO. By contrast, ascorbic acid, ECGC, and ergosterol significantly inhibited IDO production. Among all the compounds that modulated IDO production, only hemin,[101] ECGC,[102] and vitamin D3 (ref. 103) has been previously reported.

PD-L1 expression

Program death-ligand 1 (PD-L1) is found constitutively in low levels on DCs where it binds with the receptor PD-1 on T cells.[24,104] This interaction inhibits T cell activation and proliferation and serves as a critical mechanism in maintaining immune tolerance. However, in several diseases this interaction is modulated, in either a positive or negative way, to disrupt normal immune function. For instance, upregulation of PD-L1 has been associated with increased cancer risk, whereby tumor cells effectively evade the immune response allowing for unfettered malignant proliferation and metastasis.[105,106] By contrast, blocking the PD-1/PD-L1 interaction has been shown to precipitate T1D onset[107,108] in addition to other autoimmune diseases.[109,110] Upregulating PD-L1 expression in DCs has been shown to promote a tolerogenic phenotype and was therefore of interest. Our results confirmed previous findings that retinoic acid[111] and vitamin D3 (ref. 112) significantly increased PDL1 expression while also reporting, for the first time, PD-L1 expression is inducible by celastrol, ergosterol, hemin, and resveratrol (Fig. 3g).

Tolerogenic index

Tolerogenic dendritic cells are characterized by maturation resistance, low pro- and high anti-inflammatory cytokine production, and high expression of immunomodulating molecules. We sought to create a model that summarized our findings and encapsulates these criteria in a comprehensive, concise manner. To do so, protein expression values were first grouped by the following categories: 1) maturation marker expression following LPS challenge, 2) cytokine production, and 3) tolerogenic protein expression. Values were averaged within these groups and then equally weighted and combined to form a tolerogenic index. Two relatively simple models are proposed, the first uses absolute values to represent the magnitude of each response (Fig. 4), and the second model bins values based on significance (Fig. 5). That is, a value of 0 is given for compounds that do not differ significantly from control, +1 is given to compounds that differ significantly in a

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desirable (tolerogenic) manner, and −1 is given to compounds that differ significantly in an undesirable (immunogenic) manner.

Utilizing these models, it is thus possible to suggest the tolerogenic-producing potential of the compounds and rank them accordingly. The first model ranks ergosterol, vitamin D3, capsaicin, and celastrol as the compounds with the highest tolerogenic capacity (Fig. 4c). The second model ranks vitamin D3, hemin, ergosterol, and celastrol the most tolerogenic (Fig. 5c). Thus, these complementary models both identified MP-loaded ergosterol, vitamin D3, and celastrol as the compounds most capable of inducing tolerogenic markers in DCs on-chip.

Discussion

The development of miniaturized platforms has shown great promise to facilitate biological inquiry in both industrial and academic research. Multiple approaches have been developed that enable high-content phenotyping, isolation, and clonal expansion of antigen-specific antibody-presenting cells.[33,113,114] While such approaches offer a robust platform to facilitate lymphocyte analysis, they do not allow for the selective combinatorial analysis of small molecule libraries on antigen presenting cells such as DCs. Others have developed platforms to investigate the pharmacological effects of compounds on hepatic spheroids[115] or in tumor models.[116] These approaches typically quantify viability, apoptosis, and mitochondrial activity to identify promising drug candidates. By contrast, DC–MP microarrays reported here bridge these two approaches by screening compound libraries to identify candidates that polarize DCs into tolerogenic subsets.

Determining the tolerogenic potential of compounds remains difficult given the numerous signaling pathways involved. Traditional *in vitro* methods rely on co-culture systems such as mixed lymphocyte reactions (MLRs) to evaluate the phenotypic changes DCs initiate in T cells[117] before moving into relevant *in vivo* models. We previously identified a promising tolerogenic MP formulation by using this approach.[11,12,118–120] After careful selection of several promising immunomodulatory factors and robust analyses, a formulation that elicited the most pronounced immunosuppressive effects was found[11] and then evaluated in a prediabetic mouse model.[12] Having demonstrated prevention of T1D,[12] the dual microparticle system was then further optimized and demonstrated reversal of T1D.[118] This reengineered formulation also demonstrated efficacy in a multiple sclerosis mouse model.[119] More recent work again optimized the formulation and again showed encouraging results at longer endpoints and in more clinically relevant animal models.[120] Despite success, the nature of such long-term *in vivo* work is cumbersome to identify and optimize candidate formulations for tolerogenic vaccines. Hence, the present work offers a platform on which to identify candidate molecules at relevant concentrations using a cell subpopulation that has demonstrated a causal link toward inducing tolerance. Following lead generation in tolDCs alone, traditional co-culture techniques are warranted to move forward in verifying promising compounds in animal and human models.[121]

For the purpose of this study, we defined DC activation as high expression of MHCII, CD80, and CD86. Lymphocytes require two distinct signals for full activation to occur.[8] The

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first signal is provided by the interaction of the T cell receptor (TCR) on the lymphocyte with MHC antigens on antigen presenting cells.[122] The second, co-stimulatory, signal is required to avoid inducing an apoptotic or anergic response by the lymphocyte. The interaction of CD28 on the lymphocyte with B7 (CD80/86 in DCs) proteins on the APC provides this necessary co-stimulatory second signal.[123] Thus, compounds that exhibited significant down-regulation of CD80 and CD86 yet maintained normal MHCII expression could potentially induce anergic T cell responses.[124] Such complexity underscores the current limitations of identifying tolDCs by protein expression alone and point to a caveat of our approach. Indeed, the compounds identified *via* microarray screening will necessitate validation *via* traditional *in vitro* methods. For example, incubating dendritic cells with compound-loaded MPs followed by co-culture with T cells would be informative. Further, specific T cell phenotypes can then be quantified to determine the suppressive effects of MP-treated DCs on effector T cell responses and Treg induction.

Previous work has demonstrated the ability of the microparticle microarray to deliver controlled doses of adjuvants that elicit corresponding DC phenotypic changes.[34] Extending this established platform, we sought to investigate the tolerogenic effects of a small compound library on primary bone marrow derived DCs. Significant differences were observed for the expression of every protein investigated when compared to the control of unloaded MPs. This confirms that compound-loaded particles have a measurable effect on DC activation using this platform approach.

A comprehensive, quantitative output of tolerogenic potential in DCs is useful when interpreting the results of compound screening. Therefore, in this work we proposed two models: the first incorporates the magnitude of protein expression for each marker, while the second model provides a score of −1, 0, or +1 based on the *post hoc* significance ($\alpha = 0.05$) of each condition compared to the control of unloaded particles. In both models, values were equally weighted by normalizing to the "No Particle" control group and combined to form a tolerogenic index, where activation markers were assigned negative value and suppressive markers were assigned positive value (*i.e.*, IL10 + IDO + PDL1 − MHCII − CD80 − CD86 − IL12). Thus, higher values correlate to an increased tolerogenic phenotype. Both approaches offer benefits and drawbacks.

The first model is more sensitive to the magnitude of individual protein expression for each response investigated. This model is permissive to favorably weighting compounds that induce large changes in a single marker as opposed to moderate changes across all markers. Thus, it may disproportionately promote compounds that elicit large changes in one or two markers while not necessarily influencing others. The second model remedies this bias by binning results based on statistical significance. Thus, compounds that upregulate multiple markers are promoted over compounds only affecting one or two markers. The disadvantage to the second model lies in its inability to distinguish between compounds eliciting large or small effects so long as both are statistically significant. Distinguishing between these two approaches in future work can aid in discerning biologic mechanisms of DC-directed tolerance. Refinement of the tolerogenic indices proposed here will no doubt be required as the results from validation experiments, described above, are obtained. Adjusting the tolerogenic models in this way can be used to elucidate relative importance of

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specific markers on downstream suppressive actions (*e.g.*, on directing naïve T cells toward effector and regulatory phenotypes) which could advance understanding of fundamental immunology principles.

GRAS compound loading was not optimized for this study. The intent of testing three GRAS-MP doses was to traverse such dose-finding efforts. However, future investigation could attempt to optimize fabrication parameters in order to improve or standardize loading amounts. Parameters such as the solvent, viscosity, temperature, and the use of stabilizers contribute to the encapsulation efficiency.[125] For simplicity, this study utilized either one of two loading protocols for all the compounds dependent upon the water solubility of the loaded molecule.

It is worth noting that compounds that did not induce significant responses may potentially still be of interest. Screening using this platform mimics lead compound identification where dosing and release kinetics are typically not fully optimized.[126] For instance, the dose–response of capsaicin has been shown to be quite narrow,[89] suggesting that the absence of a pronounced effect during these screens could be due to dosing considerations. In the present investigation, one aspect that was not controlled for pertains to the uptake of surface-adsorbed MPs for intracellular release of the compounds *versus* local release of factors from nearby MPs. We previously characterized that cultured DCs on arrays readily phagocytosed surface-adsorbed MPs, while also being modulated by agents released locally from nearby MPs.[34,41] Targeting MP localization relative to cells may provide more directed effects, for instance by promoting extra- *vs.* intracellular delivery by modifying particle size. That is, manufacturing larger MPs for local release and reserving smaller, phagocytosable MPs for compounds targeting intracellular receptors.[11,12] Targeting specific sites of action in this way may be considered once promising compounds are identified. Additionally, future iterations could consider the role of cell numbers in such cellular microarray studies. Presumably, higher DC : MP ratios would result in increased phagocytosis and therefore increase the ratio of compound that is delivered intra- *vs.* extracellularly.

Another limitation of the present study is that it does not differentiate subpopulations that differ from the mean intensity per cell. Thus, subtle effects or changes that are only present in a subset of cells would be missed. However, high-content screening often looks at the global response for lead identification.[126] Cell to cell variability can confound results due to heterogeneity of phenotype or compound targeting.[127] Instead, an iterative screening approach should be used where initial lead formulations can be optimized for targeting and dosing. This coupled with more granular analyses to confirm candidate molecules and identify mechanisms of action offers a translational, cell-based screening approach.

Despite such limitations, approaches such as these are important to empower smaller laboratories to investigate and develop effective compounds of interest. The current microarray architecture was designed for imaging *via* fluorescent microscopy to allow for widespread adoption. However, future iterations could also be designed for compatibility with commercially available microarray scanners for high content analyses. Lastly, the cell-based microarray is not limited to investigation of tolDCs for autoimmune disorders but rather could be modified to explore any number of compounds for myriad diseases.

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Conclusion

Results demonstrate that the microarray platform is suited to investigate libraries of compounds for their immunomodulatory effects on DCs. Small molecules can be readily encapsulated into PLGA MPs and printed onto the microarray in controllable densities. Dendritic cell responses are quantified with high sensitivity. Several compounds elicited DC maturation and cytokine responses that have been previously reported, validating the approach. More importantly, several compounds displayed the ability to induce tolerogenic phenotypes in DCs, which have not previously been reported and supporting further investigation. Of note, MP-loaded celastrol, ergosterol and vitamin D3, in particular warrant more in-depth study. Such discoveries underscore the potential of the cell-based microarray as a novel technique to identify lead compounds in academic laboratory settings.

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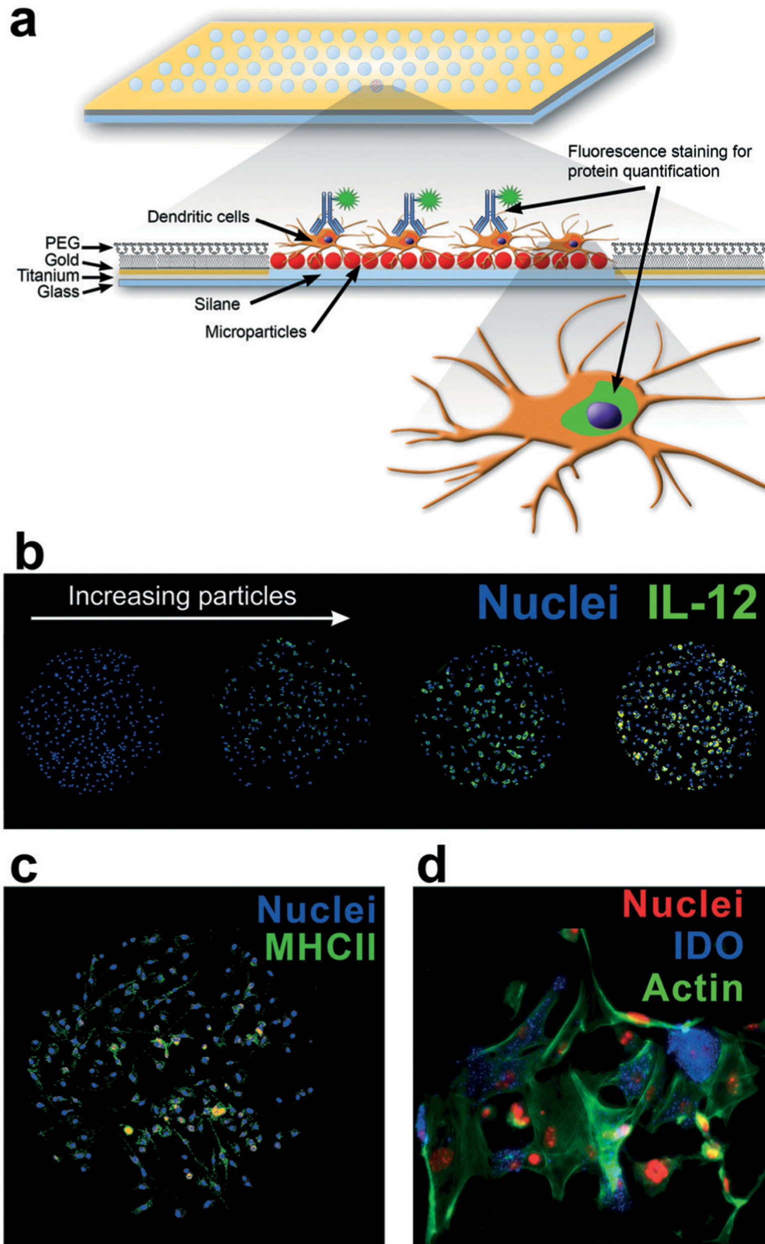

Fig. 1.

Schematic and fluorescence micrographs of the dendritic cell (DC) microarray. (a) The microarray platform consists of 400 μm silane islands surrounded by an anti-fouling background. Microparticles are printed on the discrete islands where DCs can then adhere enabling co-localization of cells with microparticles. Following incubation, immunocytochemistry of the entire microarray allows for quantification of protein expression. (b) Representative islands displaying increasing IL-12 production (FITC, green) of DCs cultured with increasing amounts of LPS-loaded microparticles. LPS is known to upregulate IL-12 production in DCs, and nuclei were stained with Hoechst (DAPI, blue) demonstrating homogeneity of cell seeding across islands. Representative images demonstrating (c) dendritic cell staining of MHCII on an island of a microarray and

(d) intracellular staining of indoleamine 2,3-dioxygenase (IDO) at higher magnification, illustrating cell morphology and marker localization.

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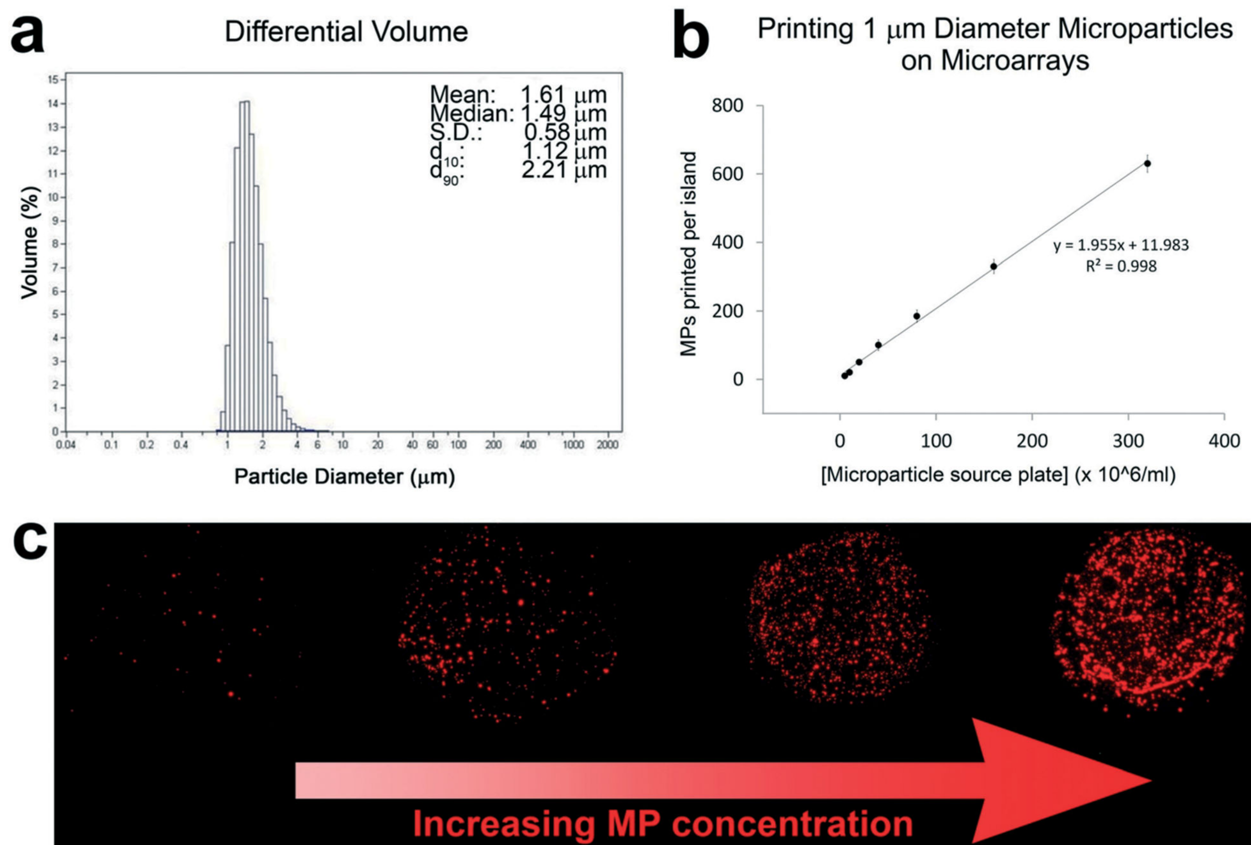

Fig. 2.

Evaluation of size distribution and delivery of PLGA microparticles. (a) Size distribution curve of microparticles quantified *via* laser diffraction (based on volume estimation) found an average diameter of 1.612 µm ± 0.579 µm. (b) Average delivered microparticles with standard deviations were plotted as a function of source plate concentration for 1 µm diameter microparticles. (c) Fluorescence images demonstrating controllable delivery of rhodamine-loaded microparticles. Increasing the source plate concentration shows an increasing deposition of microparticles on the microarray until saturation.

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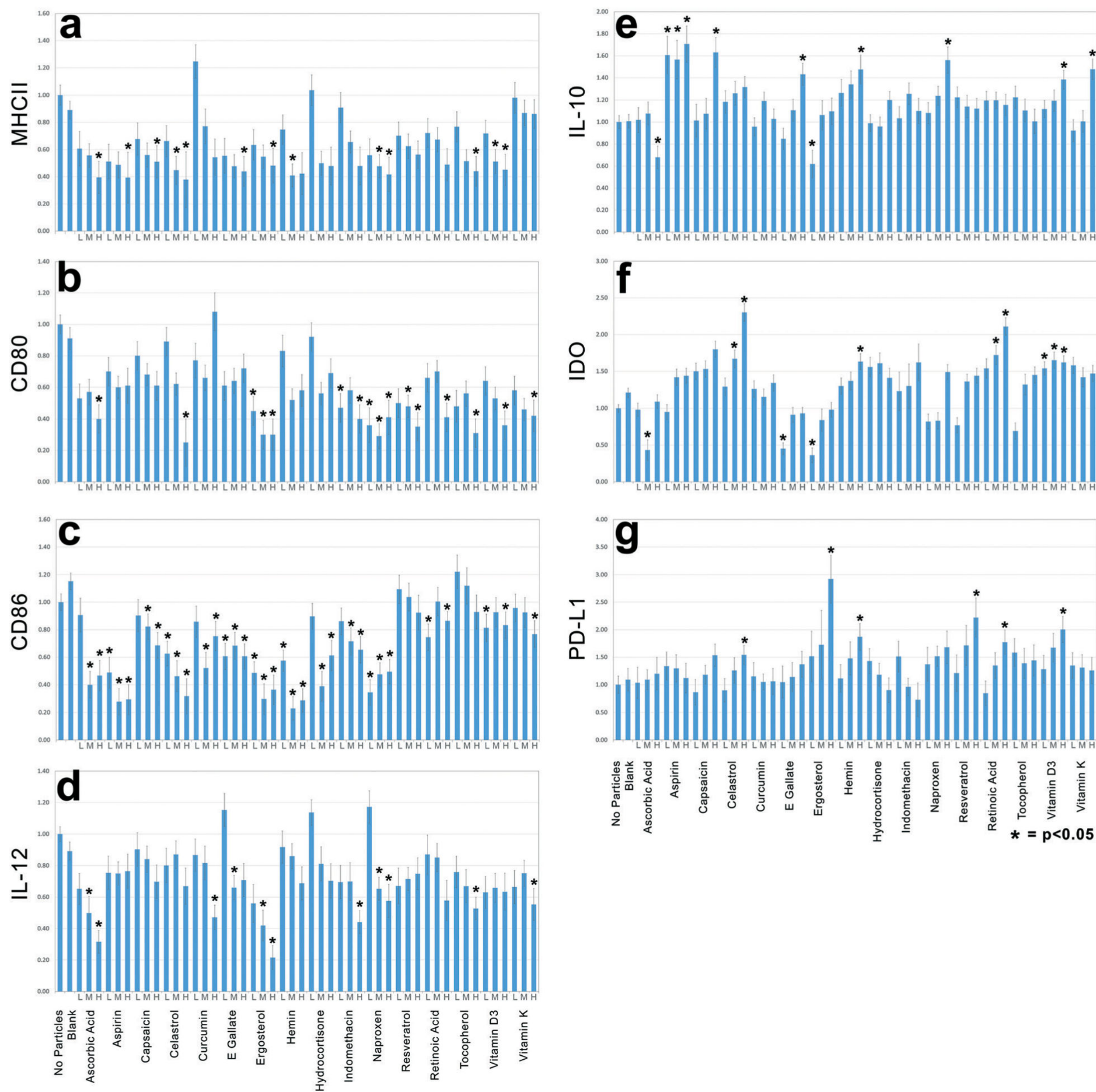

Fig. 3.

Protein expression of DCs cultured on microarrays. Arrays were printed with a small library of compound-loaded microparticles. Dendritic cells were then cultured on the microarrays for 48 h. Expression of (a) MHCII, (b) CD80, and (c) CD86 was quantified following exposure of LPS during the final 24 h of culture. Production of (d) IL-12, (e) IL-10, (f) IDO, and (g) PD-L1 were also quantified *via* immunocytochemistry and fluorescent microscopy (n = 6 for each MP formulation per array, n = 3 arrays). Error bars represent standard error of the mean (SEM).

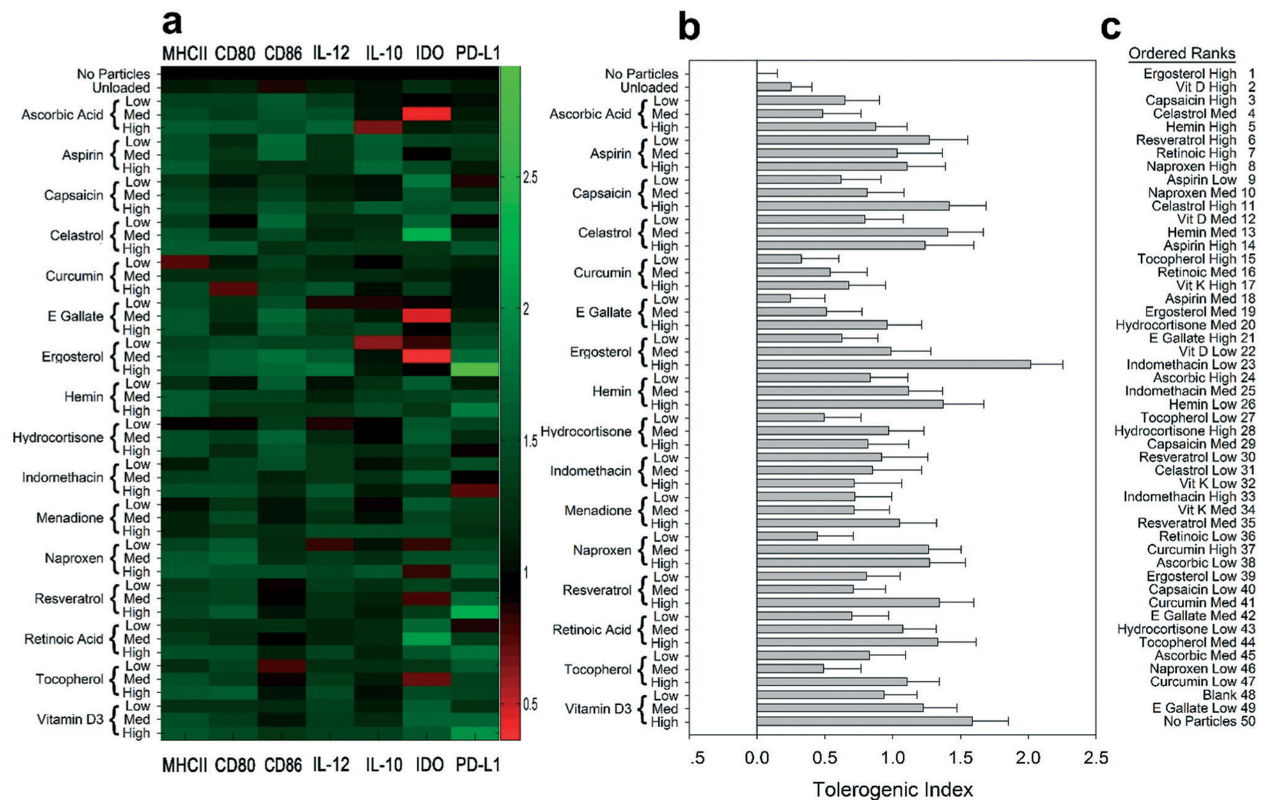

Fig. 4.

Magnitude-composite tolerogenic potential. (a) Heat map displaying relative expression of MHCII, CD80, CD86, IL-12, IL-10, IDO, and PDL1. Dendritic cell expression of these markers and cytokines was equally weighted and combined by the following categories: 1) activation marker expression following LPS challenge (MHCII, CD80, and CD86), 2) cytokine production (IL-12 and IL-10), and 3) tolerogenic protein expression (IDO and PD-L1). (b) Values were normalized to the "No Particle" control group and summed to form a tolerogenic index. Activation markers (MHCII, CD80, CD86, IL-12) were assigned negative value and suppressive markers expression (IL-10, IDO, PD-L1) were assigned positive value (*i.e.*, IL10 + IDO + PDL1 − MHCII − CD80 − CD86 − IL12). Thus, higher values correlate to an increased tolerogenic phenotype. (c) Compounds and their relative concentration were ranked by order of most to least tolerogenic based on the tolerogenic indices.

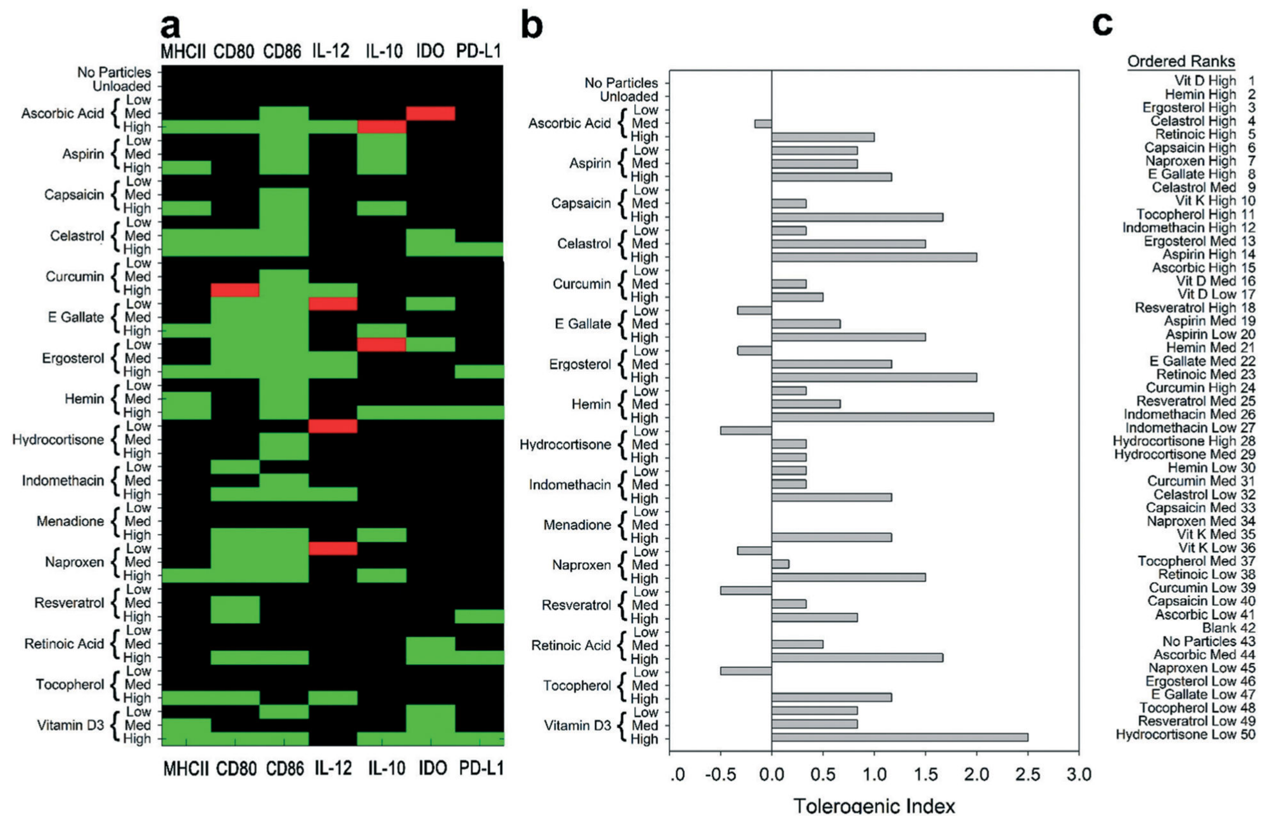

Fig. 5.

Binned-composite tolerogenic potential. (a) Heat map displaying relative expression of MHCII, CD80, CD86, IL-12, IL-10, IDO, and PD-L1. Dendritic cell expression of these markers and cytokines was equally weighted and combined by the following categories: 1) activation marker expression following LPS challenge (MHCII, CD80, and CD86), 2) cytokine production (IL-12 and IL-10), and 3) tolerogenic protein expression (IDO and PD-L1). (b) Values for each marker were averaged and then binned based on significance from control. A value of 0 is given for compounds that do not differ from control, +1 is given to compounds that significantly promote a tolerogenic phenotype (*i.e.*, increased: IL-10, IDO, PD-L1; decreased: MHCII, CD80, CD86, IL-12), and −1 is given to compounds that differ significantly promote an immunogenic phenotype (*i.e.*, increased: MHCII, CD80, CD86, IL-12; decreased: IL-10, IDO, PD-L1), and then values for all markers summed ($\alpha = 0.05$). (c) Compounds and their relative concentration were ranked by order of most to least tolerogenic based on the tolerogenic indices.

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Table 1

Loading efficiency and endotoxin levels of PLGA microparticles

Compound	Loading efficiency (% *vs*. amount loaded)	Loading amount (ng mg^{-1} PLGA)	MP endotoxin level (EU per mg)
Ascorbic acid	39.7	397	0.139
Aspirin	60.4	302	0.093
Capsaicin	54.2	271	0.107
Celastrol	46.7	234	0.086
Curcumin	84.4	422	0.719
ECGC	76.9	769	0.116
Ergosterol	90.3	452	0.086
Hemin	53.9	270	0.345
Hydrocortisone	72.4	362	0.093
Indomethacin	70.8	354	0.107
Menadione	76.4	382	0.102
Naproxen	73.3	733	0.195
Resveratrol	35.0	175	0.077
Retinoic acid	32.1	161	0.130
α-Tocopherol	66.3	332	0.230
Vitamin D3	35.7	179	0.251

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Table 2

Small molecule library evaluated using a Tol-DC microarray

Compound	Description	Ref.
Ascorbic acid	Treatment of ascorbic acid has demonstrated maturation resistance in DCs while reducing the production of pro-inflammatory cytokines IL-12 and IFN-γ	46
Aspirin	Aspirin is a non-steroidal anti-inflammatory drug (NSAID). Studies have shown that aspirin can upregulate tolDCs as well as Tregs	47–49
Capsaicin	Capsaicin is a ligand for vanilloid receptor 1 (VR1). Dendritic cells have demonstrated expression of VR1, which upregulate activation and initiate their migration to draining lymph nodes	50
Celastrol	Celastrol inhibits NF-κB-regulated gene expression and has been shown to induce expression of heme oxygenase-1	51–54
Curcumin	Curcumin is a polyphenolic compound that promotes retinoic acid and IL-10 production in DCs, and induces differentiation of naïve CD4+ T cells into intestine protective Tregs	55
Epigallocatechin-3-gallate	Epigallocatechin-3-gallate (EGCG) has been shown to down-regulate expression of MHCII, CD86, and CD80 in DCs exposed to LPS	56, 57
Ergosterol	Ergosterol is a biological precursor to vitamin D2 that inhibits activation and proliferation in primary human T cells	58
Hemin	Hemin induces heme oxygenase 1 (HO-1) which inhibits DC maturation and production of pro-inflammatory cytokines while retaining IL-10 production	59
Hydrocortisone	Hydrocortisone reduces production of pro-inflammatory cytokines IL-12, TNF-α, and IL-6 in DCs exposed to LPS. Further, it can inhibit LPS-induced DC maturation and induce a tolerogenic capacity in DCs	60, 61
Indomethacin	Indomethacin is a commonly used NSAID that inhibits both COX-1 and COX-2, as well as prostaglandin production	62
Menadione	Menadione is a potent inhibitor of IDO that can induce apoptosis in activated T cells	63
Naproxen	Naproxen inhibits COX-1 and COX-2 and displays anti-inflammatory properties	31
α-Tocopherol	α-Tocopherol is an antioxidant compound that has been shown to induce maturation resistance in DCs when exposed to soluble TLR agonists. Further, α-tocopherol treated DCs have displayed an ability to induce anergy in T cells which then secrete elevated levels of anti-inflammatory cytokines such as IL-10	64
Resveratrol	Resveratrol has been shown to inhibit progression of T1-D in NOD mice *via* prevention of CCR6-mediated migration of inflammatory cells. Further, resveratrol-treated DCs have demonstrated resistance to LPS-induced maturation and pro-inflammatory cytokine production	65, 66
Retinoic acid	Specific subsets of DCs secrete retinoic acid to modulate the differentiation of naïve T cells. When delivered in combination with TGF-β, retinoic acid promotes the differentiation of naive T cells to Foxp3+ Tregs	67–69
Vitamin D3	The active metabolite of vitamin D3, 1a,2,5$(OH)_2$D3, has been recognized as an immunosuppressive agent on DCs that inhibit maturation of LPS- and TNF-α-conditioned immature DCs	70