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Precision-porous templated scaffolds of varying pore size drive dendritic cell activation

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Abstract

Scaffold based systems have shown significant potential in modulating immune responses in vivo. While there has been much attention on macrophage interactions with tissue engineered scaffolds for tissue regeneration, fewer studies have looked at the effects of scaffold design on the response of immune cells—that is, dendritic cells (DCs). Here, we present the effects of varying pore size of poly (2-hydroxyethyl methacrylate) (pHEMA) and poly(dimethylsiloxane) (PDMS, silicone) scaffolds on the maturation and in vivo enrichment of DCs. We employ a precision templating method to make 3-D porous polymer scaffolds with uniformly defined and adjustable architecture. Hydrophilic pHEMA and hydrophobic PDMS scaffolds were fabricated in three pore sizes (20, 40, 90 μm) to quantify scaffold pore size effects on DCs activation/maturation in vitro and in vivo. In vitro results showed that both pHEMA and PDMS scaffolds could promote maturation in the DC cell line, JAWSII, that resembled lipopolysaccharide (LPS)-activated/matured DCs (mDCs). Scaffolds with smaller pore sizes correlate with higher DC maturation, regardless of the polymer used. In vivo, when implanted subcutaneously in C57BL/6J mice, scaffolds with smaller pore sizes also demonstrated more DCs recruitment and more sustained activation. Without the use of DC chemo-attractants or chemical adjuvants, our results suggested that DC maturation and scaffold infiltration profile can be modulated by simply altering the pore size of the scaffolds.

Keywords

adjuvant; dendritic cells; porous scaffolds

1 | INTRODUCTION

In the development of vaccines and immunotherapies, antigen uptake and activation of dendritic cells (DCs) is key to generating sustained adaptive immune response (Bonifaz et al., 2004). This is usually achieved by programming DCs ex vivo or co-delivering antigens with a selected adjuvant in vivo. One challenge of in vivo DC modulation is to match the DC surface target receptor(s) and the potential adjuvant with the desired immune outcome

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

SUPPORTING INFORMATION

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(Gilboa, 2007). Early studies usually involved bolus delivery of nano- or micro-particles carrying antigen (as protein or its gene equivalent) to DCs (Palucka & Banchereau, 2012). However, the response to such approaches is usually transient due to the lack of proper microenvironment for DC antigen uptake/activation as well as sustained antigen exposure.

Scaffold-based systems were developed to overcome this problem by providing a suitable local environment to induce immune cell recruitment, migration, activation, and subsequent homing to lymph nodes (LNs) (Ali, Huebsch, Cao, Dranoff, & Mooney, 2009; Singh et al., 2011; Stachowiak & Irvine, 2008). Mooney developed a poly-lactide-co-glycolide (PLG) scaffold-based cancer vaccine platform that released granulocyte macrophage colony-stimulating factor (GM-CSF) as a DC chemo-attractant, tumor lysate (B16 melanoma) as antigen, and Cytosine-guanosine oligonucleotide (CpG-ODN) sequences (Ali & Mooney, 2008) as an inflammatory adjuvant. After 7 days implantation, their system was able to augment cytotoxic T-cell immunity and induce regression of established tumors through prolonged presentation of tumor antigen (Ali, Doherty, Mooney, & Emerich, 2011). Compared to conventional systemically injected bolus delivered immunotherapies, porous scaffolds can be used to achieve sustained release of multiple factors, while providing a temporary residence and appropriate microenvironment for cell:vaccine interaction (Xie, Yang, & Kniss, 2001).

The study of DCs response to biomaterials began by attempting to define the innate immune (inflammatory) response to biomaterials (Babensee, 2008). Biomaterial implants, by promoting an inflammatory response would recruit antigen presenting cells (APCs) and induce their activation, thus, acting as a potential adjuvant for co-delivered antigens (Matzelle & Babensee, 2004)—that is, vaccine delivery. Babensee's group identified the key factors contributing to DC interaction with biomaterials. Macromolecules adsorbed on the surface of biomaterials can be recognized through innate immune cell receptors (e.g., Toll-like receptors, TLRs), whereas, the physical/chemical structure of an implant can also induce conformational change and DC activation. Most of these earlier studies were performed on solid biomaterial films or nanoparticles. Recently, implanted scaffold-based gene delivery platforms have demonstrated success in promoting immune response in vivo (Ali & Mooney, 2008; Ali et al., 2011; Kim, Li, Sands, & Mooney, 2014). Consequently, there emerged studies exploring the relationship between DC activation and 3D porous scaffolds, particularly within the context of DC recruitment and vaccine immunization (Kim et al., 2014; Leifer, 2017; Li et al., 2016). While most of these studies have focused on enhancing DC infiltration into the porous scaffolds and migration to LN, the impact of pore size and scaffold construct material on DC activation has not been reported.

In previous studies, we fabricated precision porous scaffolds with controlled, monodisperse pore sizes, and explored their impact on cell differentiation and tissue integration. Cardiac implants with 30–40 μm pores showed maximum vascularization and minimal fibrotic response (Madden et al., 2010). It was later found that such angiogenesis induced by 38 μm pore size scaffolds coincided with a shift in macrophage phenotype toward the anti-inflammatory M2 state (Madden et al., 2010). We hypothesize that pore size also plays a significant role in dendritic cell activation. Here, we investigate how DC activation and recruitment are affected by pore sizes in vitro and in vivo using two chemically different

biomaterials. pHEMA and PDMS were selected because of their distinctive chemical features and extensive use in tissue engineering applications. Hydrophilic pHEMA tends to prevent cellular adhesion and spreading and provides a substratum for cells that do not require anchorage to proliferate, thus, promoting cell differentiation (Son et al., 2013). PDMS has multiple hydrophobic domains, which mimic PAMPs structures and has the potential to induce DCs activation (Babensee & Paranjpe, 2005). Scaffolds of three pore sizes, 20, 40, and 90 μm were fabricated from either pHEMA or PDMS then the response of DCs were characterized in vitro and in vivo.

2 | MATERIALS AND METHODS

2.1 | Scaffold fabrication

All the scaffolds in this study were fabricated (Figure 1) using a patented sphere-templating method (Marshall & Ratner, 2005) that generates 3-D porous polymer scaffolds where pore size is tightly controlled, and pore interconnects are also uniform in size, with both parameters being adjustable. Poly (methyl methacrylate) (PMMA) beads were obtained from either Microbeads AS (Norway) or Polysciences, Inc. (Warrington, PA) and sorted using an ATM Sonic model L3P Sifter (Milwaukee, WI) to obtain a particle size distribution reflecting the sieve mesh size required for each range. A mold was created by placing 1 or 0.5 mm thick Teflon tape strips (2 mm wide) on the top of a 75 mm $\times$ 25 mm $\times$ 1 mm glass slide to serve as a retaining gasket. Sifted beads of the desired size were poured into the cavity formed by the Teflon gasket. A second glass slide was fixed on top of the bead-filled cavity; thus completing the mold. Molds filled with beads were sonicated for 30 min in a water bath sonicator to compact the beads. Beads were then sintered to each other at their contacts points by heating at 110 $^{\circ}\text{C}$ (20 μm), 175 $^{\circ}\text{C}$ (40 μm), or 140 $^{\circ}\text{C}$ (90 μm) for 24 hr. pHEMA precursor consisted of 5 ml 2-hydroxyethyl methacrylate (HEMA) (Polysciences), 0.23 ml tetraethyleneglycol dimethacrylate (TEGDMA) (Polysciences), 2.0 ml deionized water, 3.6 ml ethylene glycol, and 20 mg 2,2-dimethoxy-2-henylacetophenone (Irgacure 651) (BASF, Freeport, LA). The reaction mixture was infiltrated into the glass mold surrounding the template and degassed under vacuum for 30 min. HEMA monomer was cross-linked into pHEMA under broad-spectrum UV for 10 min by free-radical polymerization.

Silicone gel components were obtained from Applied Silicone (Santa Paula, CA). Silicone base component vinyl terminated polydimethylsiloxane and crosslinker silica were thoroughly mixed in 10:1 ratio in advance. The mixture was then poured on top of the template inside of a PTFE beaker (Sigma–Aldrich, Ashtabula, OH). Because of high viscosity ($\sim 100,000$ cps) of the polymer, the beaker was centrifuged at 4,000 rpm for 10 min to ensure thorough infiltration of the PDMS into the template. Afterwards, the beaker was placed inside a vacuum chamber and degassed at 28" Hg for 1 hr. The polymer was then cured at 130 $^{\circ}\text{C}$ for 30 min.

After polymerization, the PMMA bead template surrounded by the polymerized material was removed from the mold and placed in acetone to dissolve the PMMA microspheres. The resulting porous scaffolds, composed of pHEMA gel or PDMS, were cut into 3 mm discs using a biopsy punch. The discs were sterilized in 70% ethanol and then rehydrated in

distilled water. Scaffolds were evaluated for endotoxin using a standard limulus amoebocyte lysate (LAL) gel clot protocol (Lonza; Hayward, CA). All scaffold batches used for in vitro and in vivo study contained endotoxin concentration 0.06 EU/ml. All scaffolds were pre-soaked in complete culture medium overnight prior to experiments.

2.2 | Scanning electron microscopy (SEM) imaging

Scanning electron microscopy (SEM) was used to confirm the pore dimensions of the various scaffolds. Lyophilized samples were sputter coated for 120 s with gold/palladium (~20 nm layer). Scaffolds were then imaged on a scanning electron microscope (FEI SEM XL Siron) at 5 kV with a 5 mm working distance. Both a cross-section and top view were imaged. ImageJ software was used to measure the size of pores and the interconnecting throats based on the SEM images. For each scaffold and template, three top-view images from different batches were analyzed and 10 sample points were measured from each image. At least one sample of template and scaffold from each scaffold batch made were imaged.

2.3 | In vitro assays

2.3.1 | Cell culture and seeding—A JAWSII murine dendritic cell line (ATCC; Manassas, VA) was used for all in vitro studies. All cell culture medium and reagents were obtained from Gibco unless otherwise specified. JAWSII cells were maintained in alpha minimum essential medium (alpha-MEM) supplemented with 2 mM L-glutamine, 20% fetal bovine serum (FBS), 1% penicillin-streptomycin, and 10 ng/ml GM-CSF. All cells were cultured at 37 °C and 5% CO₂. JAWSII cells were seeded onto non-tissue culture treated polystyrene (NTPS) or the various scaffolds in 24-well plates at 500,000 cells/well density in 1 ml complete medium. Cells seeded on NTPS and stimulated with 100 ng/ml lipopolysaccharide (LPS) were used as positive control for matured DCs.

2.3.2 | Viability of cells seeded in scaffolds—A metabolic XTT assay (Life Technologies) and LIVE/DEAD™ stain (BD Biosciences) were used in combination to determine the viability of cells seeded onto 3-D scaffolds and 2-D NTPS plates.

2,3-Bis-(2-Methoxy-4-Nitro-5-Sulfophenyl)-2H-Tetrazolium-5-carboxanilide (XTT) assay: Cell viability measured with XTT assay is based on the cleavage of yellow tetrazolium salt XTT by metabolically active cells to form an orange formazan dye that is quantified using a plate reader. 50 µL of 1 mg/ml XTT (Life Technologies) and 4 µL 1 mM menadione (Sigma-Aldrich) were added to each well. Extra blank wells were prepared containing the same amounts of reagents and culture medium. After 4 hr incubation at 37 °C, supernatant was collected and absorbance was measured at a wavelength of 492 nm using a Tecan Safire-2™ microplate reader. A standard curve was obtained by measuring metabolic activity of a known number of cells. All absorbance results were converted into viable cell numbers then normalized to cells cultured on NTPS based on DNA content quantified using CyQUANT™ assay.

LIVE/DEAD™ stain: Scaffolds and cells were fixed with 2% BD Cyto-fix (BD Biosciences) for 15 min at room temperature. The LIVE/DEAD® assay (Life technologies) was used to visualize viable cells. A total of 2 µM calcein AM and 4 µM EthD-1 solution

was mixed at 1:1 ratio. A total of 100–150 μL of mixed stain reagents were added to the surface of the scaffold, incubated for 30–45 min at room temperature, then wash 3X with PBS, prior to imaging the cells within scaffolds with Olympus FV1000 MPE BX61 multi-photon microscope.

2.3.3 | Maturation cytokines and chemokines concentration—Select cytokine and chemokines related to DC activation were quantified. After 24 hr co-culture with biomaterial scaffolds, the cell culture supernatant was collected and centrifuged for 10 min to remove any cells from suspension. Supernatants samples were then stored at $-20\text{ }^{\circ}\text{C}$ until analysis. The amount of select cytokines (TNF- α , IL-6, IL-12, and IL-10) along with two chemokines, MIP-1 α and RANTES, released into the supernatant was analyzed by ELISA according to the manufacturer's direction (Peprotech, NJ). Cytokine/chemokine levels “per well” were converted to “per cell” concentrations using cell numbers per well using viable cell numbers based DNA content quantified using CyQUANT™ assay. Cytokine/chemokine “per cell” concentrations were then normalized to the corresponding cytokine/chemokine “per cell” concentration for cells cultured on NTPS.

2.3.4 | Cell surface maturation markers; flow cytometry—After 24 or 48 hr culture, scaffolds were removed from the culture medium and JAWSII cells grown inside or on the surface of the scaffolds were retrieved by PBS-based cell dissociation buffer (Invitrogen). After centrifugation, cells were suspended in 200 μL FACS buffer (DPBS containing 2% FBS) at concentration 1×10^6 cells/ml. Nonspecific binding was blocked by incubating with anti Fc γ III/II receptor antibody (1:500) (BD Biosciences) at $4\text{ }^{\circ}\text{C}$ for 5 min. Cells were then incubated with CD86 (BioLegend, San Diego, CA, APC-conjugated, 1:1000), CD80 (BioLegend, PerCP-Cy5.5-conjugated, 1:1000), or MHCII (BioLegend, PE-conjugated, 1:1000) antibodies on ice in the dark for 30–40 min. Cells were washed twice with PBS and re-suspended in 200 μL FACS buffer with 0.2 $\mu\text{g}/\text{ml}$ propidium iodide (Invitrogen). To determine surface antigen expression levels, 10,000 events per sample gated on viable propidium iodide-negative single cells were acquired on BD FACSCantoII flow cytometer (BD Biosciences). Cells were gated according to positive APC, PerCP-Cy5.5, or PE using unstained controls, and the percentage of cells staining positive for each surface antigen was recorded and analyzed using FlowJo software (TreeStar).

2.4 | In vivo APC recruitment and phenotype at implantation site

All animal experiments adhered to federal guidelines and were approved by the University of Washington Animal Care and Use Committee. Groups of female C57BL/6J mice (Jackson Laboratory, 6–8 weeks old; five mice per group) were used for this study. On Day 0, pHEMA and PDMS scaffolds were implanted subcutaneously in the dorsa of selected C57BL/6J mice. Briefly, mice were anesthetized and a 1 cm incision was made on the central dorsal surface. A subcutaneous pocket was made on either side of the incision, biomaterial scaffolds (3 mm diameter disks, 1 mm thick) were inserted, and the incision was closed with clips. To assess cell recruitment and phenotype, scaffolds were excised on Day 1, 3, and 7. The ingrown tissue was digested into single cell suspensions using a collagenase solution (Worthington; Lakewood, NJ, 250 U/ml) that was agitated at $37\text{ }^{\circ}\text{C}$ for 45 min. The cell suspensions were then poured through a 40 μm cell strainer to isolate cells from scaffold

particles. Cells were then stained with primary antibodies conjugated to fluorescent markers for flow cytometry analysis as described in Section 2.3.4.

2.5 | Statistical analysis

For in vitro studies, a two-sided student *t*-test was used to compare LPS stimulated DCs or each sample group to iDCs in pairs. If not indicated, *p*-value less than or equal to 0.05 was considered to be significant. For in vivo studies, a two-way ANOVA was used to determine statistical significance. *p*-value less than or equal to 0.05 was considered to be significant.

3 | RESULTS

3.1 | Scaffold characterization

pHEMA and PDMS scaffolds of three sizes were fabricated. SEM images of (Figure 2a) 40- μ m PMMA bead template, cross-section; (Figure 2b) 40- μ m PMMA bead template, top view; (Figure 2c) 20- μ m pHEMA scaffold, cross-section; (Figure 2d) 90- μ m pHEMA scaffold, cross-section; (Figure 2e) 40- μ m pHEMA scaffold, cross-section; and (Figure 2f) 40- μ m PDMS scaffold, cross-section (higher magnification). The 20- μ m scaffold pore surfaces were rougher (Figure 2c) than the 40- or 90 μ m surfaces due to the fact that the 20 μ m beads themselves had an uneven surface. Both pHEMA and PDMS scaffolds maintained their 3D morphology well after dissolving the PMMA template. For each size, the pores were uniform with very narrow size distribution (0.36–3.21 μ m). The size of the throats between pores ranged from 5.45 to 30 μ m. The throat size increases proportionally to the pore size while the ratio of pore interconnect diameter/pore diameter remained between 30 to 40%.

3.2 | In vitro characterization of DCs response to scaffolds

3.2.1 | In vitro cell viability—Cell viability was primarily determined by cell metabolic activities measured by the XTT assay and normalized by total DNA in each well. In LPS-treated controls, DCs viability decreased by half after 48 hr culture (Figure 3, top) due to apoptosis of terminally differentiated DCs. All cells seeded into porous scaffolds exhibited near 100% viability that was equal to or higher than those cultured on NTPS surface. LIVE/DEAD® staining was used to confirm the results obtained by XTT assay. A significant amount of cell loss was noted in LPS treated samples due to loss of adhesion and apoptosis in terminally differentiated DCs (image not shown). Cells cultured on (Figure 3a) 40- μ m pHEMA scaffold and (Figure 3b) 40- μ m PDMS scaffold have similar viability compared to the cells cultured in (Figure 3c) NTPS for 48 hr. NTPS plates. A majority of the cells in the field stained green indicating viable cells.

3.2.2 | Maturation cytokines and chemokines—After the JAWSII cells were stimulated with LPS or seeded in scaffolds for 24 hr, the pro-inflammatory cytokines and chemokines secreted into the cell culture supernatant were analyzed as indications of the DCs' maturation levels. As a positive control, LPS-treated DCs had strong up-regulation of all the pro-inflammatory cytokines tested in this study (Figure 4). Scaffolds of all sizes also induced pro-inflammatory responses in JAWSII cells. TNF- α is a pro-inflammatory cytokine and a key mediator in innate immunity. After exposure to all biomaterials, DCs secreted 15–

57 times more TNF- α than DCs on NTPS (Figure 4a). IL-6 is known for inducing T_H1 type immune phenotype; IL-6 expression on the various scaffolds ranged from 5 to 31 times the IL-6 expressed from DCs on NTPS (Figure 4b). RANTES levels were only slightly elevated in scaffolds; ranging from 1.3–12 times that seen on NTPS (Figure 4c). MIP-1 α secretion ranged from 21 to 211 times higher than cells seeded on NTPS (Figure 4d). Levels of IL-10 and IL-12 were also quantified and there were no statistical differences observed on scaffolds versus iDC controls. The pro-inflammatory cytokines and chemokines production increases with decreasing pore size, with 20 μ m pore scaffolds inducing the highest response. The difference between the two materials pHEMA and PDMS was not significant. Absolute concentrations of all cytokines expressed are provided in Supplementary Table S1.

3.2.3 | Flow cytometry of cell surface activation markers—Co-stimulatory molecule CD86 is expressed on JAWSII cell surfaces upon stimulation and is one crucial indication of DCs maturation. The percentage of DCs expressing CD86 was measured by flow cytometry and was normalized to the control group (iDCs on NTPS) at a 24 hr time point (<2%). After biomaterials culture or LPS treatment, all cells up-regulated CD86 and expression was increased progressively during maturation. At 24 hr, CD86 expression on LPS-activated DCs (mDCs) was 5x higher than iDC on NTPS. DCs cultivated within the various scaffolds increased CD86 expression 2–4 fold by 24 hr relative to iDCs on NTPS. Expression of CD86 surface markers decreased slightly with increasing scaffold pore size regardless of polymer (Figure 5a). Another activation marker, MHC-II, for scaffold cultures or LPS-activated plate cultures were all ~1.3–2.0x higher than iDC expression. There appears to be little effect of pore size or construction polymer on MHC-II surface markers relative to iDCs (Figure 5b). CD80 surface markers were all elevated for JAWSII cells cultivated on all scaffolds or in plates exposed to LPS; LPS activated cells were 43x higher than iDCs, whereas, scaffold cultivated JAWSII cell CD80 surface markers were 12–38x higher than iDCs, again expression levels decreased with increasing pore size, independent of polymer used (Figure 5c).

3.3 | In vivo cell infiltration and APC phenotype

Cell recruitment was observed as early as 24 hr at the pHEMA scaffold periphery and by 48 hr throughout the scaffolds. By Day 3, different levels of cell accumulation at the scaffold edge were observed (Figure 6). pHEMA scaffolds with 40 μ m pores recruited the highest density host cells. By Day 7, the cell density within the 20- and 90- μ m pore scaffolds increased, but so did the cellular accumulation at the scaffold periphery. In contrast, fluorescent images show cell densities within 40- μ m pHEMA scaffold decreased and there was no apparent exterior cellular accumulation (Figure 6). For a vaccine/therapeutics delivery application, it is important for APCs to have full access to the entire scaffold interior to uptake the therapeutics being released from within the scaffold, as well as an easy exit route to the LN upon antigen uptake. In 20- μ m scaffolds, the rapid external cell accumulation prevented more cells from entering the scaffold. In contrast, the 90- μ m pore size scaffolds allowed for multiple cells in each pore. We can see clearly from SEM images that cell clusters fill the 90- μ m pores on the outer edge at Day 3. Similar trends of cellular recruitment and infiltration with as a function of pore size were observed in the PDMS

scaffolds as well. A fibrous structure was observed on the surface of 40 PDMS but not 40 pHEMA scaffolds on Day 7 (Figure 6, Supplementary Figures S1 and S2).

To further understand the immune cell population at the implantation site, cells were extracted from the scaffolds and were analyzed with flow cytometry. As shown in Figure 7, similar levels of CD11c+ DC subpopulation (~6%) were observed at all scaffolds after 3 days' implantation (Figure 7b). DCs number decreased by Day 7; cell retention increased with decreasing scaffold pore size. (Figure 7e). Majority of the DCs (>60%) showed early signs of maturation by expressing MHCII molecules (Supplementary Figures S3A and S3B) and this did not change over the course of one week. While there was not a significant difference between DCs activation levels at early time point (Figure 7c), both HEMA and PDMS scaffolds with 20 μ m pores showed considerable amounts of activated DCs at Day 7 (Figure 7f). Macrophage population reduced from 11.5–13.2% on Day 3 to 5.5–8.4% on Day 7; no obvious trend in macrophage residency was observed based on pore size or material. (Figures 7A and 7D).

4 | DISCUSSION

With an increasing number of studies reporting enhanced innate and adaptive immune responses when antigen (as protein or the equivalent nucleic acid vector) is delivered from implanted scaffolds (Ali et al., 2009; Stachowiak & Irvine, 2008; Xie et al., 2001), how scaffold 3D architecture may influence immune cells' responses becomes more relevant.

In this report, we investigated how scaffold pore size would impact dendritic cell maturation and enrichment by constructing scaffolds with three distinct precisely-controlled pore sizes. Unlike all other studies of porous scaffold effects on immune cell responses, our precision templating protocol insures our scaffolds display pores of exactly the same dimension uniformly throughout the construct. Thus, pore diameter is truly a unique variable in our study as compared to an averaged pore size garnered from the broad pore size distributions seen with porous scaffolds formed by other methods (e.g., electro-sprayed, gas-foaming, poragen dissolution). We also thought it important to quantify DC interactions with the 3D porous scaffold alone, without the added influence of a released antigen or extraneous adjuvant, in order to identify parameters that might affect scaffold vaccine delivery.

As previously defined, T_H1 promoting immune response is characterized by the secretion of TNF- α , IL-6, RANTES, and MIP-1 α (Banerjee, Biswas, & Biswas, 2008; Caux et al., 2002; Dieu-Nosjean, Vicari, Lebecque, & Caux, 1999; Sallusto et al., 1998). In the study, compared to cells cultured on NTPS, all scaffolds induced the secretion of above mentioned cytokines and chemokines in JAWSII dendritic cell line as well as the upregulation of DC surface maturation markers, MHCII and CD86 (Figure 4). While the expression of all cytokines was significantly lower in DCs cultured in the porous scaffolds relative to LPS treated cells, the absolute values (Supplementary Table S1) of all cytokines are expressed at levels sufficient to recruit and activate T cells. Antigen-independent activation of T cells by cytokines is important for recruiting effector T cells at the site of an immune response and in maintaining the clonal size of memory T cells in the absence of antigenic stimulation (Unutmaz, Pileri, & Abrignani, 1994). There is evidence that RANTES, MIP-1 α , and IL-6

can co-stimulate T cell activation at nanomolar doses (Taub, Turcovski-Corrales, Key, Longo, & Murphy, 1996; Wong & Fish, 1998), which is similar to the levels reported here.

Scaffolds with smaller pore sizes (20, 40 μm) induced more secretion of $\text{T}_{\text{H}}1$ promoting cytokines and chemokines. A previous study with macrophages and pHEMA scaffolds revealed that macrophages inside the pores are significantly different from the ones away from it and displayed pro-inflammatory M1-like phenotype (Sussman, Halpin, Muster, Moon, & Ratner, 2014). Moreover, increasing their average pore size correlated with the onset of an anti-inflammatory M2-like macrophages (Garg, Pullen, Oskertizian, Ryan, & Bowlin, 2013). Since macrophages and DCs share the same precursor cells and both are APCs, it was not surprising to see here that smaller pore size scaffolds also promoted pro-inflammatory response and activation in DCs.

To study whether the pore structure and such pro-inflammatory responses induced by the scaffold could enhance immune cell recruitment and activation in vivo, scaffolds were implanted subcutaneously in mice. After the initial inflammatory response, both macrophages and DC population numbers decreased continuously to Day 7. While scaffold pore size did not affect macrophage decrease, increasing scaffold pore size did decrease DC retention. Flow cytometry analysis revealed that independent of the polymer used, 20 μm pore scaffolds retained the most DCs at Day 7. More importantly, activated DCs were only observed in 20 μm pore scaffolds on Day 7. As the immune response is shaped collectively by the various cell types at the implantation site (Andorko & Jewell, 2017), it is possible that the interplay between DCs and other host cell types at the implant site intensified the adjuvant effect of the porous scaffold in vivo.

While DC maturation is important, DC infiltration is also an important factor to consider when designing an immunizing delivery platform. When implanted in C57BL/6J mice, immune cells (both CD11b and CD11c positive APCs) were found around scaffolds two days after implantation. As more cells migrated to the implantation site, we observed different infiltration patterns based on the material and pore size. As expected, we observed most DCs recruitment occurred during the first week. During this time frame, scaffold pore size has a positive correlation with cell infiltration, which is consistent with previous studies (Stachowiak & Irvine, 2008; Underwood et al., 2011). Cells quickly accumulated on the outer surfaces of 20 μm pore scaffolds but not many cells migrated into the scaffold interior after a week. It was previously reported that keratinocytes distribution within pHEMA scaffolds with 20 μm pores was improved by increasing the throat size from 5 to 8 μm (Underwood et al., 2011). So, it is possible that the junctions between 20 μm pores here are too small for DC migration. Adjusting the pore/throat ratio should be investigated in future studies. It was interesting to see while 90 μm pore allowed for the most cell infiltration in the first week, it also had a considerable amount of cell accumulation and fibrous tissue formation on the scaffold periphery on Day 7. To determine if this cellular accumulation would impact long term tissue integration, we explanted scaffolds after 3 weeks and examined the tissue integration with Masson's trichrome staining. We found that the external cell accumulation persisted leading to the formation of more collagenous, avascular, fibrous tissues around the 90 μm scaffolds; a classic foreign body response brought on by chronic

inflammation. At the same time, tissue reconstruction and vessel formation is observed in the 40 μm pore pHEMA scaffolds.

The surface chemical properties of the scaffolds will affect the presentation, orientation, or conformation of adsorbed proteins, which can lead to different rates of DC activation. Low surface carbon and high surface oxygen chemistry of pHEMA as well as pHEMA's low protein adsorption rate are thought to be associated with low induction of DC maturation (Kou et al., 2012). In contrast, high protein adsorption on PDMS surfaces are thought to be the cause of macrophage inflammatory response, prolonged recruitment of immune cells, and enhanced APC activity (Iribarren, Correa, Sodero, & Riera, 2002; Roch, Kratz, Ma, & Lendlein, 2016). One study murine DCs cultured on PDMS in vitro showed a marginal increase of cytokine and maturation markers (Shokouhi et al., 2010); thus, PDMS is classed as low-activating material. In our study, we did not notice a significant difference between the two materials when co-cultured with cells in vitro or implanted in mice for up to a week. Tissue samples were collected after 3 weeks implantation for (Figure 8a) 40- μm PDMS scaffold; (Figure 8b) 40- μm pHEMA scaffold; (Figure 8c) 90- μm PDMS scaffold. PDMS scaffolds lead to a more prominent fibrous structure (Figures 8a and 8c) around the scaffold while pHEMA scaffolds consistently promoted better tissue integration (Figure 8b). Biomaterial scaffolds of certain chemistry and structures can promote APC activation and evoke an adjuvant effect. However, random protein accumulation at the implantation site can lead to a chronic inflammatory situation, ending in the foreign body reaction (Boehler, Graham, & Shea, 2011). This poses the questions of (a) whether immune cells will have full access to any vaccine vector being released from within the scaffold; and (b) whether differentiated APCs can successfully navigate the fibrous structure, leave the scaffold, and migrate to lymph nodes (LNs).

Early pro-inflammatory signals can recruit and activate immune cells. At the same time, exposure to pathogen or specific antigens are also necessary for the expansion of functional T cells (Joffre, Nolte, Spörri, & Reis e Sousa, 2009). Having demonstrated our scaffolds' ability to recruit immune cells and maintain them at the implantation site, this adjuvant effect could be exploited by the controlled release of an antigen from the scaffolds.

5 | CONCLUSION

Without the need for exogenously-supplied chemo-attractants or adjuvants, utilizing scaffolds with precisely controlled pore sizes throughout the construct, this study demonstrated that varying pore sizes of pHEMA and PDMS scaffolds could differentially affect DCs in vitro maturation and in vivo enrichment. Results suggest smaller pore size enhanced a T_H1 promoting skewed response, which is favorable to DC activation. In vivo, 20 μm pore scaffolds were found to be more favorable for CD11c+ DCs enrichment and activation. Overall, these findings indicate that pore and throat size of a scaffold can modulate DC maturation. These findings contribute to the understanding of the interaction between biomaterial scaffold architecture and immune responses, and will have broader implications in the area of immunotherapy and vaccine delivery.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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FIGURE 1.
Porous templated scaffold construction protocol. Description in text

FIGURE 2.

Top: SEM images of (a) 40-μm PMMA bead template, cross-section; (b) 40-μm PMMA bead template, top view; (c) 20-μm pHEMA scaffold, cross-section; (d) 90-μm pHEMA scaffold, cross-section; (e) 40-μm pHEMA scaffold, cross-section; and (f) 40-μm PDMS scaffold, cross-section (higher magnification). Bottom: Pore size and throat size calculated from SEM image using ImageJ. Results are shown as means ± standard deviation

FIGURE 3.

Top: Viable cells after 48 hr culture within biomaterial scaffolds as measured by XTT assay. Data was normalized relative to cells cultured on NTPS. Results are from single representative experiment conducted in triplicate $\pm$ standard deviation. Cells seeded on biomaterials had viability significantly different from LPS activated DCs Bottom: Cells cultured on (a) 40- μ m pHEMA scaffold; (b) 40- μ m PDMS scaffold; and (c) NTPS for 48 hr. Live cells were stained with calcein AM (green) and dead cells with membrane damage were stained with EthD 1 (red). Similar results were seen for other pore sizes in pHEMA and PDMS scaffolds

FIGURE 4.

Supernatant analysis of maturation cytokines and chemokines “per cell” expression levels relative to that of iDC on NTPS (a) TNF- α ; (b) IL-6; (c) RANTES; (d) MIP1 α . All results are from single representative experiment performed in triplicate $\pm$ standard deviation (* $p < 0.05$, ** $p < 0.01$). Absolute expression levels are provided in Supplementary Table S1

FIGURE 5.

JAWSII cell surface marker expression levels for 24 hr cultures recovered from indicated polymer (pHEMA or PDMS) scaffolds as a function of scaffold pore size, relative to levels seen for iDCs on TCPS. JAWSII cells recovered from scaffolds were stained with antibodies to the indicated cell surface marker and detected by flow cytometry. (a) CD86, (b) MHC-II, and (c) CD80 expression relative to iDCs on NTPS. (* $p < 0.05$, ** $p < 0.01$)

FIGURE 6.

Scaffold cell infiltration in vivo analysis. Fluorescence imaging of DAPI stained cells within pHEMA scaffolds after 3 or 7 days implantation. SEM images of samples after 3 or 7 days implantation. Top row = Day 3; Bottom row = Day 7

FIGURE 7.

Cell surface marker expression on cells extracted from scaffolds. (a,d) CD11b⁺CD11c⁻ macrophages; (b,e) CD11c⁺ DCs; (c,f) MHCII⁺CD86⁺ mature DCs

FIGURE 8.

Masson's trichrome stain of tissue sample after 3 weeks implantation. (a) 40-μm PDMS scaffold; (b) 40-μm pHEMA scaffold; (c) 90-μm PDMS scaffold. Scale Bar = 100 μm