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DIFFERENTIAL FUNCTIONAL EFFECTS OF BIOMATERIALS ON DENDRITIC CELL MATURATION

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Abstract

The immunological outcome of dendritic cell (DC) treatment with different biomaterials was assessed to demonstrate the range of DC phenotypes induced by biomaterials commonly used in combination products. Immature DCs (iDCs) were derived from human peripheral blood monocytes, and treated with different biomaterial films of alginate, agarose, chitosan, hyaluronic acid (HA), or 75:25 poly(lactic-*co*-glycolic acid) (PLGA) and a comprehensive cadre of phenotypic functional outcomes were assessed. Differential levels of functional changes of DC phenotype were observed depending on the type of biomaterial films used to treat DCs. Treatment of DCs with PLGA or chitosan films supported DC maturation with higher levels of DC allostimulatory capacity, pro-inflammatory cytokine release, expression of CD80, CD86, CD83, HLA-DQ and CD44 expression as compared to iDCs, and endocytic ability at a level lower compared to iDCs. Alginate film induced pro-inflammatory cytokine release from DCs at levels higher than iDCs,. Dendritic cells treated with HA film expressed lower levels of CD40, CD80, CD86 and HLA-DR as compared to iDCs. They also exhibited endocytic ability and CD44 expression at levels lower than iDCs, possibly due to an insolublized (cross-linked) form with high molecular weight HA. Interestingly, treatment of DCs with agarose film maintained a DC functional phenotype at levels similar to iDCs except for CD44 expression which was lower than expression levels for iDCs. Taken together, these results can provide selection criteria for biomaterials to be used in immunomodulating applications and can inform potential outcomes of biomaterials within combination products on associated immune responses as desired by the application.

Keywords

biocompatibility; immunomodulation; dendritic cells

1. INTRODUCTION

In tissue engineering applications, immune responses should be minimized, whereas vaccine strategies aim to enhance the protective immune response. Adjuvants function in enhancing an immune response by interacting with antigen presenting cells (APCs) such as dendritic

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cells (DCs) during the innate immune response which, upon their maturation, bridge the innate and adaptive immune response by stimulating T lymphocytes. Hence, the adjuvant effect of biomaterials can be implied by assessing their effect on maturation of DCs.

Biomaterial properties such as hydrophobicity/hydrophilicity direct protein adsorption to and complement activation on the biomaterial surface with these ligands recognized by pattern recognition receptors (PRRs) of DCs to function in a synergistic manner in controlling an immune response to associated antigens [1]. These PRRs include toll-like receptors (TLRs) [2] and C-type lectin receptors [3, 4] and function to recognize conserved structures characteristic of pathogens called “pathogen associated molecular patterns” (PAMP) [5, 6] to induce an immune response to the pathogens. Toll-like receptors and their pathogen-derived ligands are being elucidated and it has been found that exogenous ligands of TLRs include many of the evolutionarily conserved molecules such as lipopolysaccharide (LPS), lipoproteins, lipopeptides, flagellin, double-stranded RNA, unmethylated CpG islands of DNA [2]. For these reasons, TLRs have been considered to essentially act as adjuvant receptors and sustain the molecular basis of adjuvant activity. On the other hand, given the internalization activity of DCs, interactions of DCs with particulate forms of PLGA, polystyrene, and latex have been investigated [7, 8]. Phagocytosis of PLGA or poly(β-amino ester) microspheres showed an adjuvant effects or induction of DC phenotypic changes such as upregulation of co-stimulatory molecules of CD80, CD86, and CD40 [9–12]. However, in the consideration of critical roles of other functional or adjuvant receptors in phenotype changes of DCs, the mechanism involved in biomaterial-induced maturation or other functional changes of DCs are not yet fully elucidated.

In this study, the immunobiological functional DC response to a variety of biomaterials commonly used in combination products such as vaccine delivery vehicles or tissue engineered scaffolds was assessed. The biomaterials used in this study included PLGA as a synthetic polymer and chitosan, alginate, hyaluronic acid (HA), and agarose as natural polysaccharide polymers [13–15]. These biomaterials have been used as scaffolds or cell-carriers for tissue engineering in application areas such as articular cartilage tissue engineering with support of chondrocyte proliferation in the cartilage repair process [13–19]. However, particulate forms of PLGA, chitosan, or alginate are also used to support vaccine immunogenicity resulting in activating T cells or B cells, associated with its adjuvant effect [20–22]. The use of the same biomaterial within combination products, where the desired immunological outcomes are opposite, highlighted the importance of studying how the biomaterial component influences the immune response to associated biological antigens (e.g. derived from transplanted cells). For example, the mixed lymphocyte reaction (MLR) used here indicates the ability of biomaterial-treated DCs to present their alloantigen to stimulate proliferation of allogeneic T cells. This is the most representative assay for measurement of histocompatibility difference in the transplantation, and has been used to assess biomaterial effects on allogeneic splenocyte [23] or T cell [24] proliferation. We have previously shown that PLGA acts as an adjuvant in enhancing humoral response against a co-delivered model antigen [11, 25], and that maturation of human peripheral blood-derived DCs [10] and murine bone marrow-derived DCs [26] was induced upon treatment with this biomaterial (to support this biomaterial adjuvant effect). We have also shown that different biomaterials (for example, PLGA or agarose) differentially affect DC phenotype, suggesting differential immunomodulating capacities by inherently different features of biomaterials [24, 27]. Herein, we extend our previous studies to further characterize effects of different biomaterials on the functional immunological changes of DCs using a comprehensive cadre of immunobiological assays. For some of the biomaterials tested, where available, effect of biomaterial grade (clinical grade vs. research grade) on induced DC phenotype was also examined.

2. MATERIALS AND METHODS

2.1. Preparation of biomaterial films

All biomaterial films were prepared freshly for each experimental procedure. Preparation methods of all biomaterial films were adapted or modified from previously described methods as noted for each biomaterial. Based on commercial availability of the bulk polymer, films of research grade or clinical grade biomaterials were prepared. Specifically, for this study, biomaterial films of research grade polymers were prepared for chitosan, alginate, HA and agarose. Biomaterial films of clinical polymers were prepared for PLGA, chitosan, alginate, and HA (sources noted in film preparation methods described forthwith). Due to our previous research involving DC responses to PLGA and its *in vivo* adjuvant effect, it was included in both the clinical and research grade sets of polymers for assessments of DC responses with independent determination of DC responses to PLGA for each set of polymers. Data of DC responses for each grade set of polymers were obtained. However, for the most part data presented is for research grade materials unless specified as clinical grade. All biomaterial films were tested as discs of 34.8 mm diameter to fit in the 6-well plate.

For the research grade sources of bulk materials the following film preparation procedures were used. Chitosan (research grade; high molecular weight: 400,000 MW, degree of deacetylation: ≥75%, Fluka, Milwaukee, WI) was dissolved in 1% (w/v) chitosan in glacial acetic acid (2% v/v in ddH_2O) (Fisher Scientific, Pittsburgh, PA) for 24 hours at room temperature (r.t.) and then, poured into the Teflon dish of 50 mm diameter (Cole-Parmer, Vernon Hills, IL) in the chemical fume hood. Upon evaporation of the solvent and drying (36–48 hours), chitosan films were then cross-linked by immersion in 20% (v/v) sodium sulfate (Sigma, St. Louis, MO) in ddH_2O (2 hours) and washed by ddH_2O (20 min), followed by immersion in 1 M NaOH (Sigma, 30 min) to neutralize the surface and washed with ddH_2O (20 min) [28]. Chitosan films were punched for a well of the 6-well cell culture plate, and finally washed for 20 min in ddH_2O. Alginate (research grade; 80,000 MW; mannuronic acid content: ≥ 50%; primarily anhydro-β-D-mannuronic acid residues with 1–4 linkage; Sigma) was dissolved to a concentration of 3% (w/v) alginate in ddH_2O for 24 hours at 4°C and then, poured into the Teflon dish of 50 mm diameter in the tissue culture laminar flow hood. Upon drying (36–48 hours), alginate films were cross-linked by immersion in 5% (w/v) calcium chloride (Sigma) in 40% aqueous ethanol for 48 hours and washed with ddH_2O for 10 min [29]. Alginate films were punched for a well of the 6-well cell culture plate, and washed for 30 min in ddH_2O changing water every 10 min. Hyaluronic acid (research grade; 800,000 MW; sodium salt from Streptococcus equi, BioChemika, Fluka) was dissolved to a concentration of 4% (w/v) HA in ddH_2O for 24 hours at 4°C and then, poured into the Teflon dish of 50 mm diameter in the tissue culture laminar flow hood. Upon drying (36–48 hours), HA films were cross-linked by immersion in 50 mM water soluble carbodiimide (Sigma) in 72% aqueous ethanol for 24 hours and washed by ddH_2O for 10 min [30]. Hyaluronic acid films were punched for a well of the 6-well cell culture plate, and washed for 30 min in ddH_2O changing water every 10 min. Agarose (research grade; type V; high gelling; gel strength of ≥ 800 g/cm^2 at 1.0 %; Sigma; molecular weight is not known) was dissolved in ddH_2O to a concentration of 3% (w/v) by heating using a microwave until boiling and visible homogeneity was reached [31]. Agarose films were prepared by dispensing 1 ml of this agarose solution into a well of a 6-well tissue culture plate (Corning, Corning, NY), and allowed to solidify at a temperature of 4°C for at least 30 min, and brought back to r.t. for another 30 min prior to use in treating immature DCs (iDCs). All biomaterial films were UV-sterilized for 30 min per surface in the tissue culture hood prior to use in treating iDCs.

For the research grade biomaterial films, endotoxin contents were measured (Supplemental Materials and Methods) and the effective endotoxin content (EU/ml) of 4.5 mm-diameter films were determined as 0.0007±0.0001 for chitosan, 0.035±0.006 for alginate, 0.004±0.003 HA, and 0.037±0.006 for agarose. A previous study has shown that minimum *E. Coli* endotoxin (LPS) concentration of 1 ng/ml (approximately 10 EU/ml) was required to stimulate human monocyte-derived DCs [32].

For the clinical grade sources of bulk materials the following film preparation procedures were used. Chitosan (clinical grade; Protasan UPB 80/500, 500,000 MW, degree of acetylation: 80–89%, NovaMatrix, FMC Biopolymer, Sandvika, Norway) and HA [clinical grade; 770,000 MW; sodium hyaluronate in European Pharmacopoeia (EP) grade, sodium salt from Streptococcus equi, Genzyme Biosurgery, Cambridge, MA] materials were processed using the identical methods described above for the research grade, whereas alginate (clinical grade; 100,000 MW; mannuronic acid content: ≥50%, sodium alginate, Pronova UP LVM, NovaMatrix, FMC Biopolymer) material were processed using the method described above except that the starting alginate concentration was 3.5% w/v in ddH_2O. For preparation of poly(DL-lactic-*co*-glycolic acid) (PLGA) films, PLGA (clinical grade with ester terminated; molar ratio: 75:25, inherent viscosity: 0.70 dL/g in trichloromethane, 100,000 MW; Birmingham Polymers, Birmingham, AL) was dissolved in 20% (w/v) in dichloromethane (DCM) overnight at r.t. and poured into the Teflon dish of 50 mm diameter in the chemical fume hood [33]. Upon evaporation of the solvent and drying (36–48 hours), PLGA films were punched for a well of the 6-well cell culture plate, and washed for 1 hour in ddH_2O changing ddH_2O every 15 min.

For these clinical grade biomaterial films the effective endotoxin content (EU/ml) of 4.5 mm-diameter films were determined as 0.011±0.007 for PLGA, 0.0025±0.0024 for chitosan, 0.0012±0.0002 for HA, and 0.0997±0.0198 for alginate.

2.2. X-ray photoelectron spectroscopy (XPS)

To examine the chemistry changes of biomaterial surfaces associated with film processing, low resolution XPS survey scans were obtained for raw polymeric materials, films before cross-linking and films after cross-linking and atomic percentages determined and compared. Theoretical values were determined based on known chemical structures. A surface Science Laboratories X-100 spectrometer (Surface Science Laboratories, Mountain View, CA) with monochromatized A1 Kα X rays using 1486.6 eV was used at a fixed take-off angle of 55°. Raw polymeric material samples were prepared by placing the material directly on a normal aluminum foils without any adhesive and then, these foils containing samples were placed on the sample stage. Biomaterial films (10 mm × 10 mm) selected from a region of uniform thickness which had been washed using the endotoxin-free water (LAL reagent water, Cambrex) and dried in the tissue culture hood were directly placed on the sample stage for analysis. All samples for XPS were kept in the vacuum desiccator, at least for 24 hours, before analysis. Biomaterial samples were placed under a nickel mesh, and 5 eV flood gun was used to assist with the compensation for differential charging. Atomic percentages of elements were derived from low resolution spectra. High resolution C1s spectra were obtained and resolved using curve fitting routines provided by the manufacturer, and the binding energy scale was adjusted to place the hydrocarbon peak at 284.6 eV.

2.3. Dendritic cell culture

Peripheral human blood was collected from donors with informed consent using heparin (333 U/ml blood) (Baxter Healthcare Corporation, Deerfield, IL) as the anticoagulant. This procedure was performed at the Student Health Center Phlebotomy laboratory, in

accordance with the protocol (#H05012) of Institutional Review Board (IRB) of Georgia Institute of Technology. Monocytes isolated from peripheral blood mononuclear cells (PBMCs) can be differentiated into iDCs by exposure to granulocyte macrophage colony-stimulating factor (GM-CSF) and interleukin-4 (IL-4) in the DC culture media {abbreviated as DC media, which was prepared by filter-sterilizing RPMI-1640 containing 25m*M* HEPES [4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid)] and L-glutamine (Invitrogen), supplemented with 10% (v/v) heat inactivated fetal bovine serum (FBS, Cellgro MediaTech, Herndon, VA) and 100U/ml Penicillin/Streptomycin (Cellgro MediaTech, Herndon, VA).} [34–36]. Thus derived iDCs were positive for CD40, CD80, CD83, CD86, HLA-DQ, and HLA-DR, expression of which were upregulated upon DC maturation by lipopolysaccharide (LPS) treatment [10, 37]. See Supplemental Materials and Methods for details on derivation of iDCs from human PBMCs using the previously described method with some modifications [35].

On day 5 of culture, non- and loosely-adherent cells containing iDCs were harvested by centrifugation. At the same time, biomaterial films were pre-inserted into well of a 6-well plate secured in place using a segment of sterilized silicone tubing (Cole-Parmer) [10, 24, 27]. Immature DCs were then plated at 0.5×10^6 cells/ml into 6-well plates containing biomaterial films.

In addition to wells of biomaterial films, wells for the negative control of iDC remained untreated while wells for the positive control of mature DCs (mDC) involved addition of 1 μg/ml of LPS (E. coli 055:B5; Sigma) (see Supplemental Materials and Methods for justification of concentration). All biomaterial treatments and control groups were incubated for next 24 hours (or 5 hrs for NF-κB assay) using DC media supplemented with GM-CSF and IL-4 (95% relative humidity, 5% CO2, 37°C) with subsequent harvesting of cells and collection of media.

For each experiment, all biomaterials were included as treatments to allow for comparisons between treatments and controls at the end of the treatment period. Non-/loosely-adherent DCs were harvested by gently rocking and swirling the cell culture plate and collecting the cells by pipetting. The cell suspension was centrifuged (1100 rpm, 10 mins) to collect cells and supernatant media, both for further analysis as described below. Harvested non-/loosely adherent DCs from a single well (of the 6-well plate) for treatment or control group were used for all phenotypic assessments.

In the present study, iDCs or mDCs indicates a specific DC phenotype of immature DCs or mature DCs (treated with LPS), respectively. In addition, use of DC as an abbreviation is to indicate generally the cell type, dendritic cell.

2.4. Dendritic cell counting for non-/loosely-adherent or adherent fraction

DCs in culture consist of a non-/loosely-adherent fraction and an adherent fraction. To characterize adhesion status of DCs in culture or upon biomaterial or control treatment, the non-/loosely-adherent and adherent fractions of cells were harvested and counted. To count adherent cell fraction from each well of controls or treatments with biomaterial films, a cell dissociation solution (CDS) (Sigma) was used. After 24 hours of DC culture on biomaterial films or controls, non-/loosely-adherent DCs were gently collected as same as above and then, adherent DC fraction was removed from the culture dishes of controls or biomaterial films using CDS in accordance with the manufacturer's protocol (see Supplemental Materials and Methods). Dendritic cell numbers in the non-/loosely-adherent or the adherent fraction were counted using the Coulter counter (Coulter Multisizer III, Beckman Coulter, Fullerton, CA) by considering particle sizes in 10~20 μm as DCs. Total numbers of DCs were obtained by adding the numbers of non-/loosely-adherent and adherent DCs and

percentages of non-/loosely-adherent DCs per well of controls or treatments were determined.

To justify that the non-/loosely-adherent DCs were representative of the entire culture for analyses of DC phenotype changes upon DC treatments in this study, DC maturation marker expressions (CD40, CD80, CD86, CD83, HLA-DQ, & HLA-DR) for each of the non-/loosely-adherent and adherent DC fractions were tested with 5,000 events per sample using the flow cytometer (BDLSR, Becton Dickinson, San Jose, CA) for DCs obtained from 3 donors. For all these maturation markers, expression levels on the adherent DC fraction were not significantly different from those seen for non-/loosely-adherent DC fraction for all of the biomaterial treatments or controls, except for the HA film treatment. For DCs treated with HA film, HLA-DQ expression of adherent DCs was significantly higher than that for the non-/loosely-adherent DCs (data not shown). Others have reported that non-adherent iDCs can be differentiated to macrophages upon adhesion on the bottom of culture dish wells [38]. Moreover, non-/loosely-adherent DCs are generally employed for the immunotherapeutic researches on the migratory blood-resident DCs [39, 40]. For all DC treatments, except the treatment with PLGA, non-/loosely-adherent cells make up more than 65% mean value in total DCs present in each well of treatment. Thus, based on these results and reasoning, the non-/loosely-adherent DC fractions were collected for assessment of resultant DC phenotypes.

2.5. Cell morphology

Dendritic cell morphology was examined throughout the culture duration by phase contrast microscopy. On day 6, DCs were processed for Cytospin preparations as described previously [10] (Cytospin Cytocentrifuge, Thermo Shandon, Pittsburgh, PA) and stained with Hematology Stain (Astral Diagnostics, West Deptford, NJ) for light microscopy examination using an Axiovert 135 microscope (Zeiss, Jena, Germany) and imaged using Image-Pro Plus (v.5) software (Media Cybernetics, Inc., Bethesda, MD). A representative image was selected from 6 different Cytospin preparations from 6 separate experiments.

2.6. Cell surface marker expression

The levels of DC surface marker expression were monitored by flow cytometry (FC) as previously described [10] after 24 hours of treatment with biomaterial films and compared to controls. Non-/loosely-adherent DCs treated with different biomaterial films or controls were collected by centrifugation at 1100 rpm for 10 min and suspended in Hank's HEPES buffer (120 m*M* NaCl, 10 m*M* KCl, 10 m*M* $MgCl_2$, 10 m*M* glucose, 30 m*M* HEPES) (all from Sigma) containing 1% (v/v) human serum albumin (HSA) (Calbiochem, Darmstadt, Germany) and 1.5 m*M* $CaCl_2$ (Sigma). Cells were stained with saturating concentrations of fluorescently conjugated mouse anti-human monoclonal antibodies against CD40 (clone B-B20; IgG1κ), CD80 (clone BB1; IgMκ), CD86 (clone BU63; IgG1κ) (all from Southern Biotechnology Associates, Birmingham, AL), CD83 (clone HB15a; IgG2b) (IO Test Immunotech, Marseille, France), HLA-DQ (clone TU169; IgG2aκ), HLA-DR (clone TU36; IgG2aκ), CD32 (clone 3D3; IgG1κ), CD206 (clone 19.2; IgG1κ), CD44 (clone 515; IgG1κ), or Annexin V (recombinant purified protein) (all from BD Pharmingen, San Jose, CA) for 1 hour at 4°C in the dark, filtered using 40 μm cell strainer (Becton Dickinson, Franklin Lake, NJ) and then, analyzed immediately with 5,000 events per sample using a BDLSR flow cytometer (Becton Dickinson, San Jose, CA). Propidium iodide (PI) (fluorescent vital dye) (BD Pharmingen) was applied into the sample tube less than a minute before analysis to exclude dead cells from determination of marker expression on viable cells. Data was obtained together with the negative control of autofluorescence per sample and then analyzed using WinMDI 2.8 (Scripps Research Institute, La Jolla, CA).

2.7. Mixed lymphocyte reaction (MLR)

Allostimulatory capacity of DCs to induce T cell proliferation upon DC treatment with different biomaterials was assessed as previously described[10], using an allogeneic MLR and compared to levels induced by control DCs. After 24 hours of DC treatment with biomaterial films, allogenic T cells were isolated from PBMCs by negative selection using Pan T-cell magnetic isolation (Miltenyi Biotech, Auburn, CA) according to the manufacturer's protocol. These cells were used as responder cells. The T cells were resuspended in RPMI-1640 with 25 m*M* HEPES and L-glutamine (Invitrogen) with 100 U/ml penicillin/streptomycin (Cellgro, Herndon, VA) and heat-inactivated filter-sterilized (0.22 μm) 10% (v/v) human AB serum (Biowhittaker, Walkersville, MD) (complete RPMI-10 media) and plated at a concentration of 10^5 cells/well in a 96-well flat-bottomed plate (Corning) in triplicate per treatment groups or controls. Non-/loosely-adherent DCs treated with biomaterial films or controls were resuspended at 1.6×10^5 cells/ml, and treated with 25μg/ml mitomycin C (Sigma) for 30 min to prevent their proliferation. Upon extensive washing with complete RPMI-10 media, DCs were resuspended in complete RPMI-10 media and added by 1.6×10^5, 0.8×10^5, and 0.4×10^5 cells/ml to responder cells (1×10^6 cells/ml) in triplicate so that DC:T cell ratios were graded as 1:6.25, 1:12.5, and 1:25, respectively. Cells were co-cultured for 4 days at 37 °C, with the addition of 10 μ*M* 5-bromo-2-deoxyuridine (BrdU) for the last 24 hours of culture. Dendritic cell-induced T-cell proliferation was measured using BrdU colorimetric cell proliferation ELISA (Roche Applied Science, Indianapolis, IN) according to the manufacturer's directions.

2.8. Pro-inflammatory cytokine release

The amount of pro-inflammatory cytokines, tumor necrosis factor-α or interleukin-6 (TNF-α or IL-6), released by DCs into the cell culture supernatant after the treatments with biomaterials were analyzed by ELISAs (R&D systems, Minneapolis, MN) according to manufacturer's directions. After 24 hours of DC treatment with biomaterial films or controls, non-/loosely-adherent DC fraction and cell culture supernatants were collected together and then, cleared by centrifugation for 10 minutes at 1,100 rpm. These cleared supernatants were stored at −20°C until analysis. For each well, total cells were collected, adherent cells using CDS as described above, and processed for DNA quantification using picoGreen dsDNA quantification kit (Invitrogen) per manufacturer's directions (see Supplemental Materials and Methods) for normalization of cytokine amounts.

2.9. Preparation of DC nuclear extract and measurement of activity of nuclear factor 3B (NF3B) family of transcription factors

Nuclear extracts from DCs treated with biomaterial films for 5 or 24 hours were prepared using TransFactor Extraction kit (Becton Dickinson Clontech, Palo Alto, CA) according to manufacturer's directions. Briefly, non-/loosely-adherent cells DCs treated with biomaterial films were collected and washed twice with ice cold PBS (pH 7.5) by centrifugation at 450×g for 5 min at 4°C. The cell pellet was resuspended in lysis buffer containing protease inhibitors and allowed to incubate for 15 min on ice. After the incubation, the suspension was centrifuged, and the particulate fraction was resuspended in lysis buffer. Cells were disrupted by forcing the suspension through a 27-gauge needle. The resulting suspension was centrifuged at 11,000×g for 20 min at 4°C. The supernatant and the pellet from this centrifugation were considered as cytosolic and nuclear fractions, respectively. The pellet containing the cell nuclei was resuspended in an extraction buffer, and nuclear membrane disrupted by forcing the suspension through a 27-gauge needle. The suspension was centrifuged at 21,000×g for 5 min at 4°C, and the supernatant from this centrifugation was considered to be the nuclear extract, transferred to a new chilled tube, and stored at −80°C until analysis. Activities of the p50 subunit of NF3B family of nuclear transcription factor

were assessed using TransFactor NFκB Family kit (Becton Dickinson Clontech), an ELISA-based method of detecting transcription factor activities, per manufacturer's protocol. In brief, nuclear extracts were incubated for 60 min in a well pre-coated with consensus binding sequence. Upon washing, the primary antibody corresponding to the p50 subunit of NF-κB family was added, and allowed to incubate for 60 min. The plate was washed and incubated for additional 30 min with the secondary antibody. Binding was detected by the addition of tetramethylbenzidine (TMB) substrate and measured by colorimetric development at 655 nm.

2.10. Endocytic ability

Endocytic ability of DCs upon DC treatment with different biomaterials or controls was assessed as previously described.[41] Briefly, on day 6, non-/loosely-adherent DCs treated with different biomaterials or the iDC or mDC controls were collected by centrifugation at 1100 rpm for 10 min and then, resuspended 500 μl of fresh DC media by 2×10^5 cells/ml. Pre-warmed FITC-dextran (dextran labeled with fluorescein isothiocyanate; 40,000 MW, Sigma) solution (0.01 mg/ml in DC media) was added with 50 μl into each 500 μl of DC suspension prepared. After mixed gently by pipetting, each FITC-dextran/DC tube was incubated in the dark at 37°C for 45 minutes and then, cells were extensively washed using PBS (pH 7.2) twice by centrifuging at 300×g for 10 minutes. After washing steps, cells were resuspended in the identical buffer previously used in the flow cytometer for the surface marker expression and then, scanned with 5,000 events per sample in the flow cytometer. Data was obtained together with the negative control of autofluorescence per sample and then, analyzed using WinMDI 2.8 (Scripps Research Institute).

2.11. Statistical analysis

For statistical analysis, a one-sided Student t-test was used to compare mDCs or each treatment group to iDCs (negative control) in pairs. To observe significant differences between mDCs and all treatment groups in pairs, a general linear model of two-way ANOVA (Tukey test) in pairwise was used for a mixed model with repeated measure. For all statistical methods, the Minitab software (Version 14, State College, PA) was used. If not indicated, *p*-value less than or equal to 0.05 was considered to be significant. See Supplemental Materials and Methods for more information.

3. RESULTS

3.1. Characterization of surfaces of biomaterial films

X-ray photoelectron spectroscopy was used to analyze the surface chemistry (30 nm depth from the surface) of the biomaterial films used here. Atomic percentages of C, N, O, Na, Ca, and Cl associated with the different biomaterial films were obtained from survey scans at low resolution and results shown in Table 1. Alginate films showed residual calcium and chloride with 1.9 and 1.4 in atomic percentage (%), respectively. The other biomaterial films did not show an elemental composition which deviates from that expected based on raw materials and films before cross-linking procedure. Upon cross-linking or film forming procedure, all biomaterial films showed increased compositions of carbon, showing decreased values of O/C or N/C as shown in Table 1, compared to theoretical or raw material values.

3.2. Agarose or HA films induces the highest fraction of non-/loosely-adherent DCs, whereas PLGA films does the lowest fraction

Of the total DCs treated with PLGA, chitosan, alginate, HA, or agarose films, 37 ± 15%, 65 ± 13%, 71 ± 1%, 86 ± 3%, or 86 ± 9% were in the non-/loosely-adherent fraction,

respectively, and the remainder were adherent. For the controls of iDC or mDC, 84 ± 15% or 71 ± 15% of the DCs were in the non-/loosely adherent DC fraction, respectively. All cells were counted in determining this non-/loosely-adherent fraction for controls or treatment groups of which cellular viability was consistently greater than 95% regardless of group (based on the trypan blue exclusion method) (data not shown).

3.3. Dendritic cells treated with PLGA or chitosan films show morphologies similar to mature DCs

Dendritic cells were treated with different research grade biomaterial films, cytospins prepared and their morphologies compared with iDCs and mDCs (Figure 1). As shown, DCs treated with PLGA or chitosan films exhibited dendritic processes similar to mDCs, whereas DCs treated with agarose, alginate or HA films exhibited a morphology similar to iDCs without such processes. Dendritic cells treated with the clinical grade biomaterial films (Supplemental Figure S1) also showed morphologies very similar to DCs treated with the respective research grade films.

3.4. Dendritic cells treated with PLGA or chitosan films express higher levels of co-stimulatory and MHC class II molecules than iDCs, whereas DCs treated with HA film express lower levels

To assess DC phenotype upon 24 hour-treatment with different biomaterial films or controls, surface expression of co-stimulatory and MHC class II molecules were determined using fluorescently labeled antibodies and FC detection. As seen in Figure 2, DCs treated with research grade biomaterials, expressed differential levels of surface markers depending on the biomaterial used for treatment. Treatment of DCs with LPS induced maturation with an increase in expression levels of all surface markers as compared to iDCs. Treatment of DCs with PLGA or chitosan films resulted in significantly higher expression levels of CD80, CD86, CD83, and HLA-DQ, as compared to iDCs. Treatment of DCs with alginate films resulted in significantly higher expression levels of CD83, CD86, and HLA-DQ, as compared to iDCs. However, DCs treated with agarose films did not show a significant difference in their expression of all surface molecules (except for CD83 which was slightly higher), as compared to iDCs. Interestingly, DCs treated with HA films resulted in significantly lower levels of CD40, CD80, CD86, and HLA-DR expression as compared to iDCs. As shown with brackets, significantly different gMFIs for DC expression of CD80, CD86 and HLA-DR were observed among biomaterial treatments. Treatment of DCs with the clinical grade chitosan, alginate or HA films induced some slight differences in DC specific marker expression as compared to that induced by the research grade counterparts (Supplemental Figure S2); but generally, results were consistent.

3.5. Dendritic cells treated with PLGA or chitosan films support allostimulatory T cell proliferation while treatment with HA films does not

Allostimulatory capacities of DCs treated with the different biomaterial films were assessed using an MLR as compared to the controls (Figure 3). Mature DCs supported a high level of T cell proliferation as compared to iDCs. Dendritic cells treated with PLGA or chitosan films supported T cell proliferation to a higher extent than iDCs, whereas DC treated with HA films, supported lower levels of T cell proliferation than iDCs, actually lowering T cell proliferation. In the case of the 1:6.25 DCs: T cells ratio, DCs treated with PLGA or chitosan films resulted in significantly higher levels of allostimulatory T cell proliferation than DCs treated with alginate, HA, or agarose films. The allostimulatory capacity of DCs treated with the clinical grade films also resulted in patterns very similar to those of DCs treated with the clinical grade films (Supplemental Figure S3).

3.6. Dendritic cells treated with PLGA, chitosan, or alginate films secreted significantly higher levels of pro-inflammatory cytokines as compared to iDCs, whereas DCs treated with HA films secreted significantly lower levels

Pro-inflammatory cytokine (TNF-α and IL-6) release into the supernatants of DC treated with biomaterial films was measured using ELISA to assess phenotypic changes in DC maturation as compared to controls. Mature DCs secreted high levels of the autocrine maturation stimulus, TNF-α, as compared to iDCs (Table 2). Similarly, DCs treated with PLGA, chitosan or alginate films secreted significantly higher levels of TNF-α as compared to iDCs. Dendritic cells treated with chitosan films also showed significantly higher levels of TNF-α as compared to all other biomaterial treatments. However, DCs treated with agarose films did not secrete levels of TNF-α that were significantly different from iDCs, whereas DCs treated with HA films released significantly lower levels of TNF-α as compared to iDCs. Dendritic cells treated with biomaterial films secreted IL-6 at levels, as compared to iDCs, in patterns identical to TNF-α. However, no significant difference was observed among all biomaterial treatments for IL-6 release (Table 2).

3.7. Activation of NF-κB transcription factor, subunit of p50, does not show significantly different levels among DCs treated with PLGA, chitosan, alginate, or agarose films

To measure activation of NF-κB transcription factor subunit p50, upon DC treatment with different biomaterial films, nuclear extracts were prepared from DCs treated with different biomaterial films for 5 or 24 hours and levels of p50 were determined, as compared to the controls (Figure 4). The p50 subunit of NF-κB was selected for analysis of DC responses to biomaterials since it was expressed at a high level and was previously shown to be most responsive to biomaterial treatments [24]. Mature DCs exhibited significantly higher activation levels of p50 subunit of NF-κB at both 5 and 24 hours of time points, compared to iDCs. However, no significant difference in levels of activated p50 was observed for DCs treated with biomaterial films as compared to iDCs except Alginate for 5 hours (lower) and Agarose for 24 hours (higher). Activation of subunit p50 also was not induced at significantly differential levels among DCs treated with PLGA, chitosan, alginate, or agarose films.

3.8. DCs treated with PLGA, chitosan, alginate, or HA films underwent apoptosis at a level significantly higher than iDCs, whereas DCs treated with agarose films were similar to iDCs

Apoptosis or necrosis of DCs upon 24 hour treatment with different biomaterial films was measured using flow cytometry analysis of DCs stained with Annexin V (apoptosis) and propidium iodide (PI) (necrosis). Dendritic cells treated with PLGA, chitosan, alginate, or HA films showed significantly higher percentages of cell population positive for Annexin V as compared to iDCs or DCs treated with Agarose films, where the latter were not significantly different as compared to iDCs (Table 3). No significant differences were observed among all controls and treatments for detection of necrosis using PI staining. The percentages of cell populations positive for Annexin V and PI of DCs treated with clinical grade biomaterial films also showed very similar results as for DCs treated with research grade films (Supplemental Table S1).

3.9. Dendritic cells treated with agarose films showed endocytic ability at a level similar to that of iDCs while all other biomaterial films induced levels significantly less than iDCs

As the endocytosis is defined to include phagocytosis, pinocytosis, clathrin-dependent receptor-mediated endocytosis, and clathrin-independent endocytosis [42], FITC-dextran uptake, CD32, and CD206 expressions were measured without further specification of

endocytic mechanism (e.g., phagocytosis) to understand the overall endocytic behaviors of DCs upon treatment with different biomaterial films.

To measure the endocytic ability of DCs upon treatment with different biomaterial films, DCs treated with biomaterial films were co-incubated with FITC-conjugated dextran and then, the intensity of FITC uptaken by DCs was measured using FC. In accordance with the documented result that lower levels of endocytic activity are associated with DC maturation [43], the studies here showed that the endocytic ability of mDCs is downregulated at level significantly less than iDCs (Figure 5). Dendritic cells treated with PLGA, chitosan, alginate, or HA also exhibited the levels less than iDCs whereas DCs treated with agarose did the level which is not different from to iDCs. However, DC treatment with HA films unexpectedly induced endocytic ability that was at a significantly lower level than for iDCs, or for DCs treated with alginate or agarose films.

3.10. Dendritic cells treated with HA films induced lower levels of expression of endocytic and migration receptors

To provide explanation of the functional changes in DC phenotype upon treatment with different biomaterial films, expression levels of endocytic receptors, CD32 (Fcγ Type II) and CD206 (mannose receptor) and migration receptor, CD44, were examined by FC (Figure 6). Both CD32 and CD206 expression on DCs upon treatment with different biomaterial films showed patterns in accordance with those of the endocytic ability shown in Figure 5. Even though the CD44 is well known for receptor specific to the hyaluronan component in the extracellular matrix (ECM) for migration of DCs, the HA films in this study induced significantly lower level of expression than iDCs or DCs treated with other biomaterial films.

4. DISCUSSION

As summarized in Table 4, differential effects of biomaterials on functional changes of DC phenotypes were observed in the present study. Given the results presented herein, a discussion of how the biomaterial chemistry/properties of these diverse biomaterials might play a role in the DC phenotype induced upon treatment is a logical direction. This is done in the Discussion herein, with the caveat of significant differences in biomaterial family represented by these examples, but with useful insight regardless. It is recognized that biomaterial chemistry can determine protein adsorption profiles and conformation. Since biomaterial surface chemistry can be a strong director of cellular interactions and the expected chemistry can be modulated due to biomaterial processing conditions, the chemical compositions of all biomaterial film surfaces were determined by XPS. The film processing induced a change in surface chemistry of the biomaterials as indicated by increasing amounts of carbon upon film formation and cross-linking procedure while those of oxygen decreased, compared to the theoretical values (Table 1). It is estimated that unknown contaminants were introduced (e.g. hydrocarbons) or gas (e.g., CO_2) was adsorbed from the environment during the film forming process [44]. It is also estimated that the hydrophilic sites of biomaterial molecules were rearranged towards the inside of biomaterial films due to the gradient of evaporation rate of distilled water or solvents during the film formation [45]. After the cross-linking procedure, only alginate films showed introduction of chlorine (1.4%) presumably due to residual $CaCl_2$ cross-linking reagent on the film surface.

The extent of protein adsorption varies with surface hydrophilicity/hydrophobicity and charge with greatest extents on hydrophobic surfaces followed by cationic surfaces under the physiological pH [46–48]. Among all biomaterials examined in this study, PLGA has been recognized for a hydrophobic biomaterial and chitosan is natural polysaccharides having carbohydrate units that are mainly composed of glucosamine with a high cationic

charge density [13, 49, 50]. Macrophages interact with proteins adsorbed on the hydrophobic surface of biomaterials such that many studies on effects of macrophages on immune responses have been performed using macrophages adherent to surfaces of biomaterials [51–56].

Dendritic cells follow a similar linage (derived from the monocyte) as macrophage and both cell types have been known to upregulate similar sets of genes in response to pathogens, sharing similarity in more than 96% of basal gene expression [57]. Dendritic cells have been shown to bind RGD peptides using their α_v integrin with effects on their surface marker expression and cytokine release [58]. Previously, we have demonstrated that β2 integrin-mediated adhesion on PLGA films supports PLGA-induced DC maturation with demonstration of direct binding of β2 integrins to the biomaterial using immunohistochemistry following crosslinking of bound integrins and extraction of cellular component [59]. Furthermore, we demonstrated many years ago that direct contact of these non-/loosely adherent DCs with biomaterials (specifically PLGA) was necessary for DC maturation using a transwell assay [10]. Thus, the biomaterial contact-induced DC phenotypes, including allostimulatory capacity, observed in this study are expected to be related to interactions between DC receptors and proteins adsorbed on biomaterial surfaces (even though DCs are loosely adherent to protein-coated biomaterial surfaces but presumably still interacting).

While PLGA does not have a carbohydrate unit recognizable by PRRs on DCs, chitosan consists of carbohydrate units of *N*-acetyl-D-glucosamine (GlcNAc), which can interact with macrophage mannose receptor for mannose- and GlcNAc-glycoproteins [60, 61]. Alginate also consists of guluronic acids and mannuronic acids, the latter of which has been reported to play a critical role in secretion of cytokines from human or murine macrophages in contact with TLR-2 and TLR-4 [62–64]. In this study, even though alginate films (a high content of mannuronic acid) induced adherent DC fraction less than PLGA or chitosan films, they induced a secretion of TNF-α and IL-6 by DCs at levels significantly higher than iDCs. These indicate that in addition to biomaterial features of hydrophobicity and cationic charges, inherent chemistries that can be recognized by specific receptors such as PRRs expressed on DCs, may control resultant DC phenotype upon biomaterial treatment.

In contrast to chitosan and alginate, agarose does not have a specific carbohydrate composition which is recognized by DC PRRs. Due to its non-inflammatory feature, agarose has been employed in the agarose gel immunodiffusion (AGID) assay for the serological infection diagnosis [65] or used as a plate in assessment of chemokinetic behavior of inflammatory lung macrophages [66]. It has also been used as a cell immobilization material for cell transplantation [67, 68] typically with minimal inflammatory responses to the carrier material. Thus the hydrophilicity and presumably low protein adsorption and/or the absence of the carbohydrate component recognizable by DC PRRs play a role in the lack of DC stimulation observed with this biomaterial in this study.

Antigen presentation by DCs to antigen-specific T cells is a successive step to antigen uptake and migration of DCs. CD32 (Fcγ Type II) or CD206 (mannose receptor) are well known for their important roles of mediating endocytosis by DCs [41, 69, 70]. It has also been well known that if DCs undergo maturation, overall ability of antigen uptake of DCs is downregulated as seen in the uptake results (Figure 5). CD44 has been extensively studied *in vitro* or *in vivo* with interactions of the hyaluronan components in the ECM or soluble fragments of low molecular weight HA (≤ 200,000 Da) that enhanced DC clustering, migration and/or maturation [71–74]. In addition, an interaction of CD44 with intermediate-sized HA (MW ~ 200,000 Da) induced apoptosis of DCs through nitric oxide (NO) production by DCs [75]. Interestingly, patterns of CD44 expression results were very similar

to those of co-stimulatory molecule expression (Figure 2) or MLR functional outcome (Figure 3) results as far as differential DC maturation upon DC treatment with different biomaterial films.

Hyaluronic acid is a negatively charged high molecular weight glycosaminoglycan, which is ubiquitously distributed throughout our body. For instance, high molecular weight HA, in a hydrogel form, plays a critical role as a lubricant in the joints. As such, HA is very hydrophilic taking up water to 1000-times its own weight. Interestingly, even though CD44 specifically recognizes HA components, DCs treated with HA films in this study expressed lower levels of CD44. In addition, similarly to agarose films (Table 4), these HA films did not support DC maturation (actually suppressed) in co-stimulatory molecule expressions, MLR, or cytokine release. However, the apoptosis and endocytic ability levels were induced on the contrary to agarose films; these two results are actually close to those from PLGA films. It is conceivable that negatively charged and insolublized (cross-linked) film forms processed with high molecular weight (≥ 800,000 MW) as used HA in this study may be the reason for these effects on DCs as seen in CD44 expression, endocytic ability, and apoptosis. Thus, it is expected that HA is of unique therapeutic interest in its potential immunosuppressive effects which may be associated with apoptosis of immune cells, in disease situations [76, 77].

The results of the positive percentages of the Annexin V/PI staining (Table 3) indicate that biomaterials differentially regulate DC maturation and apoptosis/necrosis. For instance, HA (cross-linked) films exhibited effects on DC apoptosis at level significantly different from agarose films even though both of these two biomaterial films induced DC maturation at levels similar or less than iDCs. While all biomaterials examined here can be employed when DC necrosis should be minimized, each biomaterial can selectively be used for inducing DC apoptosis to further reduce immune response. For example, HA is possibly the best for reducing immune responses in association with its effects on DC maturation and DC apoptosis.

Even though LPS has been known for its role in DC activation and survival [78, 79], at the same time, the LPS induced a certain level of apoptosis of human monocyte-derived DCs [80], and macrophage apoptosis by LPS was shown as time- and dose-dependent [81]. As mentioned in the Supplemental Materials and Methods, to induce fully matured DCs, LPS was added by 1 μg/ml in the cell suspension in the present study. This LPS concentration may have affected the Annexin V positive percentages to levels different from iDCs (Table 3). However, it should also be addressed that the Annexin V and PI positive percentages from the entire cell population of mDCs are not statistically different from iDCs. Moreover, the Annexin V positive percentage of mDCs is much less than that of some biomaterials in the present study or that of LPS-treated DCs shown in the literature [80]. Thus, the LPS (1 μg/ml) in the present study cannot be considered a strong inducer of DC apoptosis or necrosis. In addition, biomaterial films examined in the present study induced Annexin V and PI positive percentages that ranged from 20 to 30% and 2 to 4%, respectively (Table 3). Interestingly, HA films, similarly to other polysaccharide films except agarose, induced Annexin V and PI positive percentages similar or higher than PLGA films or controls.

The extents of marker expression were different between the research grade and clinical grade biomaterials as shown in Figure 2 and Supplemental Figure S2, respectively. It is conceivable that difference between the research and clinical grades (e.g., purification in the biomaterial processing) might affect differential expression of specific markers on DCs with differential maturation extents in the context of biomaterial treatment. However, in consideration of overall results of the examination of cytospin, MLR, or annexin V/PI staining upon DC treatment with the clinical grade biomaterials as well as surface marker

expressions, trends of DC responses to the clinical grade biomaterials were consistent with the responses to the research grade biomaterials.

While NF-κB activation can peak after 15 minutes of cell treatment with biomaterials but wane by 24 hours depending on cells and biomaterials [82], cytokines released into the supernatant can be accumulated through 24 hours of cell culture [83]. These collectively support that NF-κB activation is time-sensitive and independent from DC maturation mechanisms specifically induced by biomaterial films as suggested previously [84, 85]. The complexity of the immune cell also means that there can be results that do not exactly "fit". The incongruence of NF-κB activation for DCs treated with agarose films, as observed in the previous [24] and present study, emphasizes the need for multiple biological evaluations of various aspects for material biocompatibility and consideration of time courses.

Certainly biological significance of *in vitro* measured outcomes is a ubiquitous issue for all *in vitro* studies. *In vitro* experiments do however allow for measurement of important biological outcomes, particularly for human primary immune cells, and for isolation experimental variables or mechanistic elucidation. Correlation of differential biomaterial effects on DC phenotype *in vitro* with the observed ability to modulate humoral adaptive immune responses to co-delivered exogenous antigen *in vivo* begins to translate the *in vitro* results to *in vivo* immunomodulation. Specifically, biomaterial-specific *in vitro* induced DC phenotype could be translated into *in vivo* host responses by the demonstration that PLGA, but not agarose scaffolds, enhanced the humoral immunity against a co-delivered model antigen *in vivo* [86]. Demonstration of *in vivo* significance of these findings is expected through current efforts to assess DC dependence for the biomaterial adjuvant by conditionally ablating DC in mice with transgenic expression of the diphtheria toxin (DT) receptor under control of the CD11c receptor in B6.FVB-Tg(Itgax-DTR/EGFP)$_{57}$Lan/J during the time period of OVA delivery from PLGA scaffolds with analysis of resultant anti-OVA humoral response [87].

Further, elucidating the physicochemical properties of biomaterials that are important in determining DC phenotype will required more controlled systems of biomaterials with graded variations in material properties. Correlation of these biomaterial *in vitro* effects on DC phenotype with their *in vivo* immunomodulatory ability are expected to provide guidelines for design and selection criteria for biomaterials in the combination products where immunological responses are of consequence. For example, *ex vivo* culture and adoptive transfer regimes in which DCs would be treated with PLGA films or HA films would be useful potentially inducing cancer immunity or tolerance, respectively.

5. CONCLUSION

We were able to observe differential effects of biomaterials on functional changes of DC phenotypes upon DC treatment with different biomaterial films. Results showed that PLGA or chitosan films supported DC maturation whereas HA inhibited DC maturation. Agarose was also noteworthy in its maintenance of an iDC phenotype. Through the comprehensive cadre of functional immunological assays used herein to assess DC phenotype, this study highlights the complexity of immune cell phenotypic outcomes but from which statements of biomaterial-induced DC phenotype can be made. These are made as supported by statistical analysis, a large human donor set and several complementary assays. In summary, consideration of the immunomodulatory aspect of the biomaterial component of a combination device is an important biocompatibility aspect for which biomaterials should be appropriately selected or design for host responses as dictated by the intended application. These studies imply that biomaterials can be used to drive DCs with different immunological functions, including non-immunostimlatory, which have the potential to

direct immune responses in the context of combination productssuch as can be of use in immunological adoptive therapies. Not only are adjuvant effects of biomaterials possible, but also non-immunostimulatory which may be particularly useful for tolerance induction in tissue engineering/transplantation scenarios.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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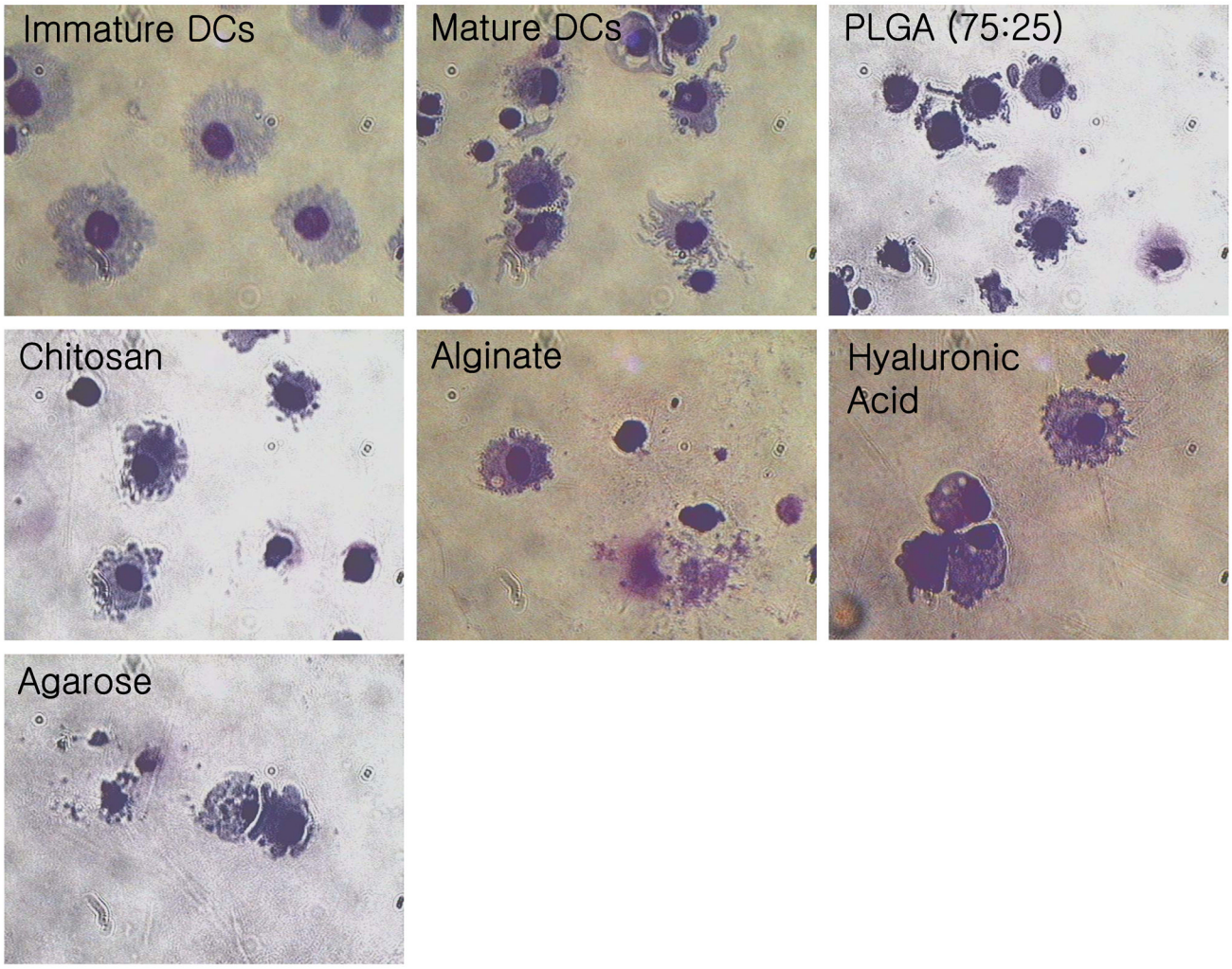

Figure 1.
Dendritic cell treated with PLGA or chitosan films possess cell morphologies similar to mDC induced with LPS treatment. DCs derived from peripheral blood monocytes in the presence of GM-CSF and IL-4, treated with PLGA or chitosan films showed similar morphology to that of mDCs, with the presence of dendritic processes. The morphology of DCs treated with agarose, alginate, or hyaluronic acid films was similar to untreated iDCs. Original magnification: 40×.

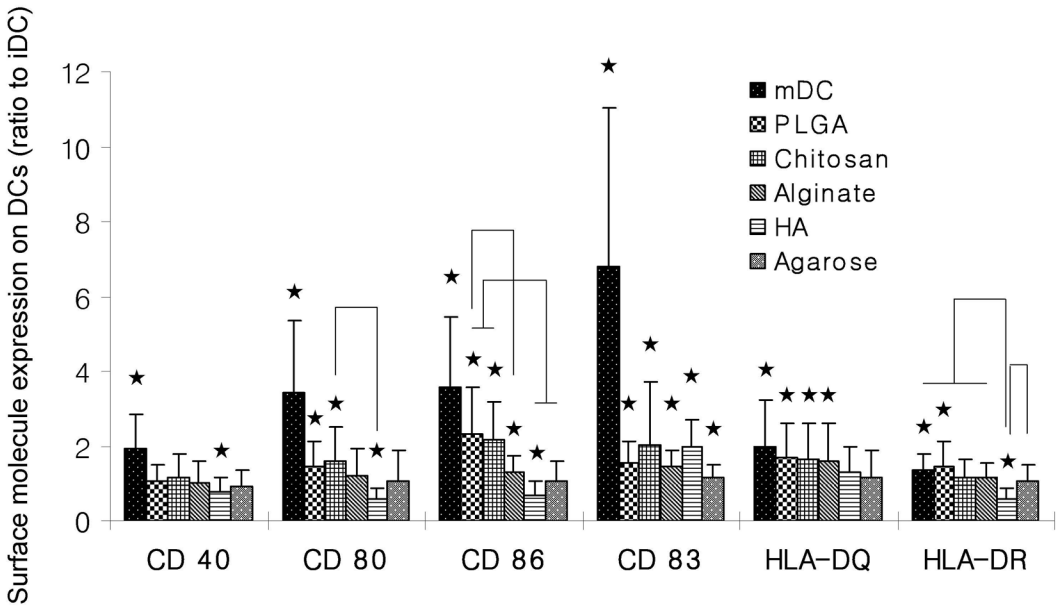

Figure 2.

Geometric mean fluorescence intensity (gMFI) of flow cytometry analysis on co-stimulatory and MHC class II molecules for DCs treated with research grade biomaterial films. For each treatment, for each donor and for each surface marker, gMFIs were normalized to the respective value for iDCs and ratios to the iDCs are shown with mean±SD, n=6 donors [3 replicates from 6 independent experiments with different donors (18 runs in total)].★: $p \leq 0.05$, different from iDCs; Brackets: $p \leq 0.05$, statistically different between two biomaterial treatments; '⊥' indicates 'or'.

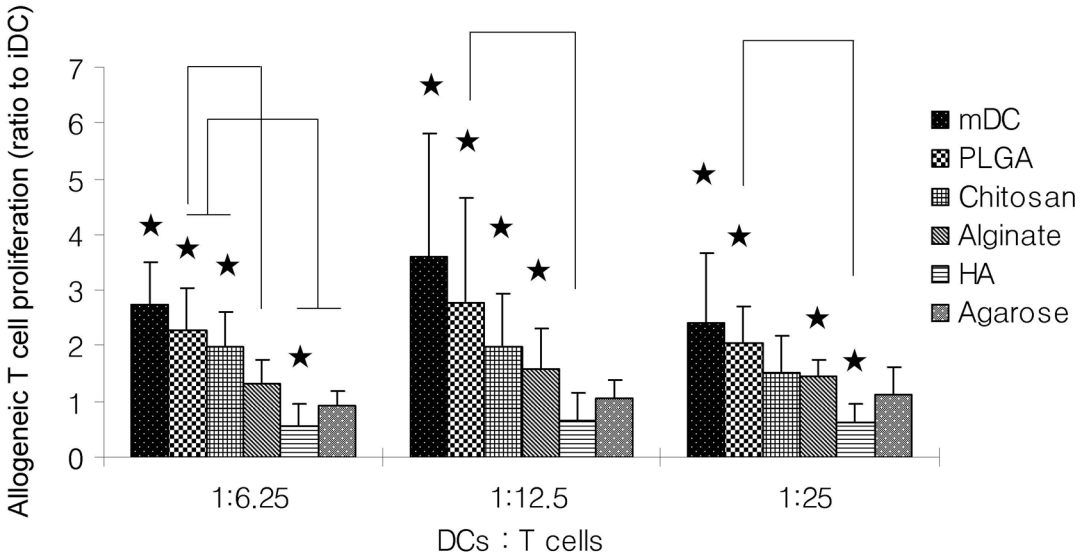

Figure 3.

Allostimulatory capacities in Mixed Lymphocyte Reaction (MLR) for DCs treated with different biomaterial films. Ratios to the iDCs are shown with mean±SD, n=6 donors (6 independent experiments with different donors). ★: $p \leq 0.05$, different from iDCs; Brackets: $p \leq 0.05$, statistically different between two biomaterial treatments; '⊥' indicates 'or'.

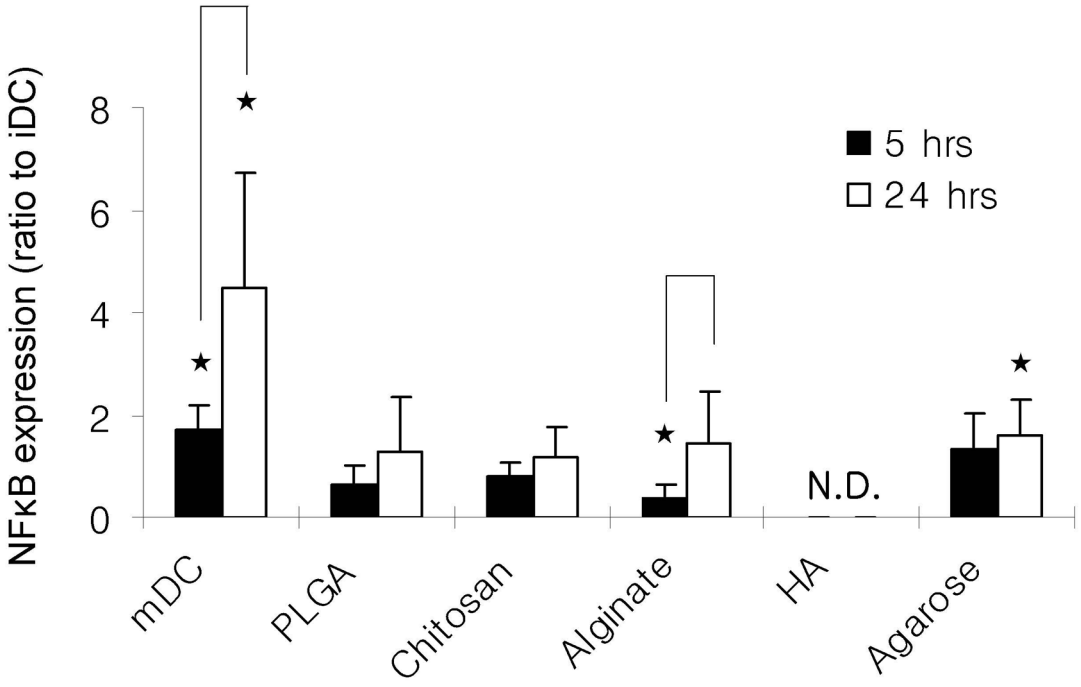

Figure 4.

Activation of NF-κB (subunit of 50) upon DC treatment with biomaterials as a function of time (5 and 24 hours). Ratios to the iDCs are shown with mean±SD, n=6 donors (6 independent experiments with different donors). ★: $p \leq 0.05$, different from iDCs. For statistical comparisons between 5 and 24 hours within a same treatment, one-sided Student t-test was also used. Brackets: $p \leq 0.05$, statistically different between 5 and 24 hours of biomaterial treatments for a same treatment; N.D.: not detectable.

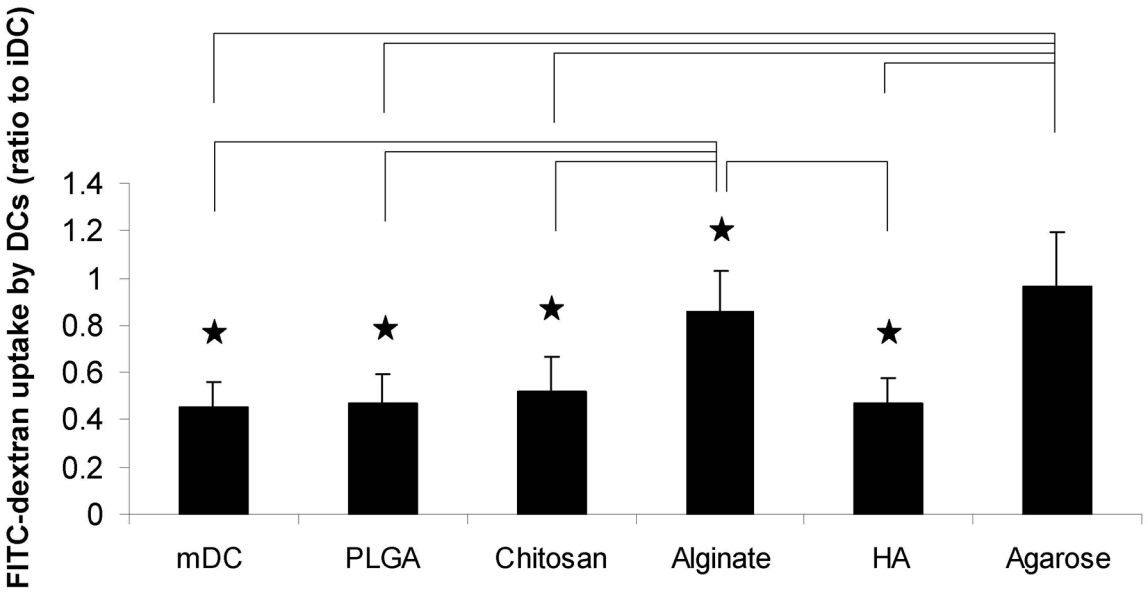

Figure 5.
Geometric mean fluorescence intensity (gMFI) of flow cytometry analysis of FITC-dextran uptake by DCs treated with biomaterial films. Ratios to the iDCs are shown with mean±SD, n=6 donors (6 independent experiments with different donors). ★: $p \leq 0.05$, different from iDCs; Brackets: $p \leq 0.05$, statistically different between two biomaterial treatments.

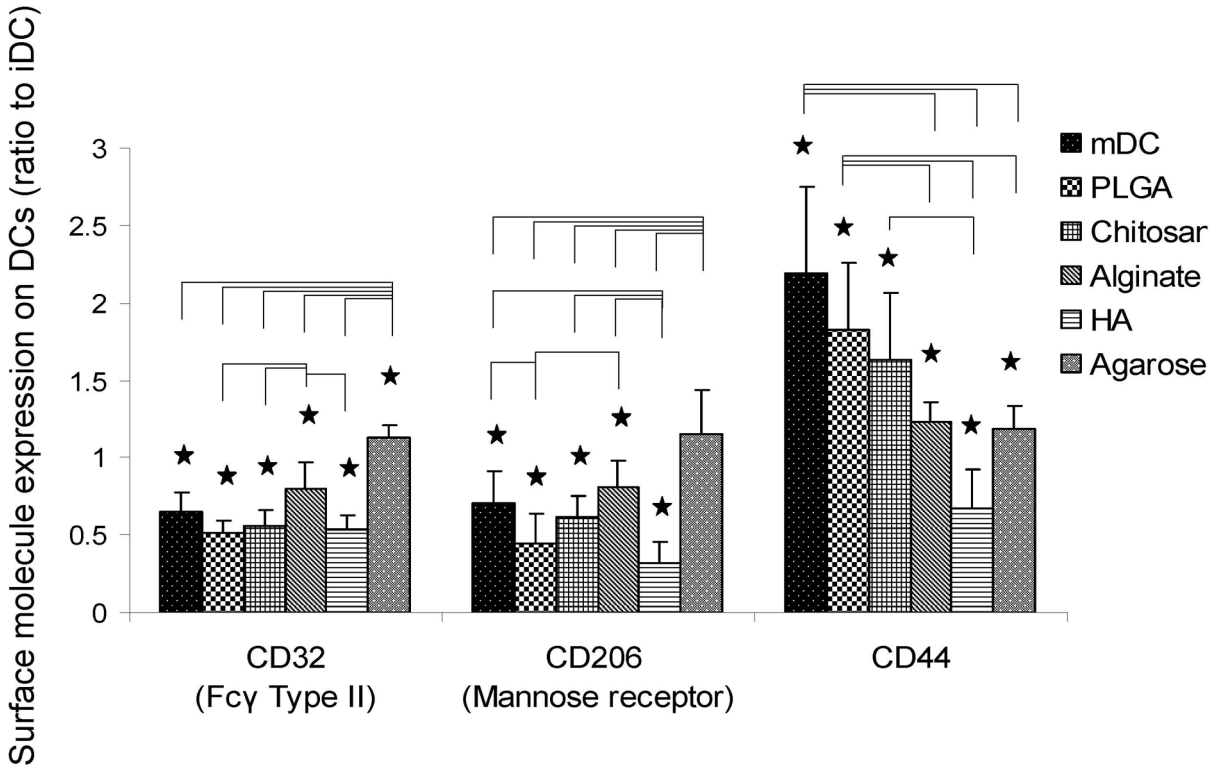

Figure 6.

Geometric mean fluorescence intensity (gMFI) of flow cytometry analysis of CD32, CD206, and CD44 expression on DCs treated with biomaterial films. Ratios to the iDCs are shown with mean±SD, n=6 donors (6 independent experiments with different donors). ★: $p \leq 0.05$, different from iDCs; Brackets: $p \leq 0.05$, statistically different between two biomaterial treatments.

Table 1

Low resolution XPS survey scans of biomaterial film surfaces used for DC treatment. More than five measurements for each sample were averaged (mean ±SD). Ratios of O/C or N/C were obtained only using mean value of each atomic percentage. All biomaterials examined here are a research grade except PLGA which is a clinical grade.

Samples	Atomic percent (%)						Ratios	
	C	N	O	Na	Ca	Cl	O/C	N/C
PLGA (75:25) (theoretical)	55.6		44.4				0.80	
PLGA (raw material)	68.4±1.9		31.6±1.2				0.46	
PLGA (film)	64.3±2.1		35.7±1.1				0.56	
Chitosan (theoretical)	54.5	9.1	36.4				0.67	0.17
Chitosan (raw material)	59.1±1.9	10.4±0.2	30.5±1.1				0.52	0.18
Chitosan (film before X-link)	70.1±2.3	4.1±0.1	25.8±0.3				0.37	0.06
Chitosan (film after X-link)	69.0±1.7	3.9±0.1	27.1±0.2				0.39	0.06
Alginate (theoretical)	44.5		48.1	7.4			1.08	
Alginate (raw material)	47.9±1.5		36.3±0.3	15.8±0.3			0.76	
Alginate (film before X-link)	63.8±1.1		28.9±0.1	7.3±0.1			0.45	
Alginate (film after X-link)	67.5±0.7		29.2±0.1		1.9±0.1	1.4±0.1	0.43	
HA (theoretical)	51.9	3.7	40.7	3.7			0.78	0.07
HA (raw material)	52.8±0.9	1.9±0.1	37.1±1.3	8.2±0.2			0.70	0.04
HA (film before X-link)	59.2±2.1	3.5±0.2	32.3±0.7	5.0±0.1			0.55	0.06
HA (film after X-link)	57.8±0.4	9.8±0.2	29.6±0.5	2.8±0.0			0.51	0.17
Agarose (theoretical)	55.8		44.2				0.79	
Agarose (raw material)	61.3±2.0		38.7±0.9				0.63	
Agarose (film)	64.2±0.7		35.8±0.1				0.56	

Table 2

Differential levels of tumor necrosis factor (TNF) –α and Interleukin -6 (IL-6) upon DC treatment with biomaterial films. TNF-α or IL-6 released from each treatment group for each donor was normalized to total DNA amount and then, ratios to the iDCs are shown with mean±SD, n=6 donors (6 independent experiments with different donors).

Controls & treatments	TNF-α (ratio to iDCs)	TNF-alpha (pg/ml)	IL-6 (ratio to iDCs)	IL-6 (pg/ml)
		Range		Range
iDCs	1	7.6 – 192.7	1	15.4 – 61.9
mDCs	76.9±62.9 *	240.0 – 11726.7	106.7±55.4 *	2123.7 – 5740.2
PLGA	5.6±3.5 */**	40.7 – 771.7	6.9±3.4 *	42.0 – 415.0
Chitosan	80.0±80.1 *	126.1 – 6829.0	11.6±7.8 *	42.0 – 628.4
Alginate	14.9±11.5 */**	117.8 – 2341.0	6.2±4.7 *	26.9 – 360.0
HA	0.2±0.2 */**	4.0 – 14.8	0.1±0.0 *	1.5 – 0.3
Agarose	1.6±1.5 **	21.2 – 37.0	1.6±0.9	12.6 – 75.0

* $p \leq 0.05$, statistically different from iDCs (=1);

** $p \leq 0.05$, statistically different from Chitosan (between two biomaterial treatments).

The concentrations (pg/ml) indicate the original concentrations measured from the cell culture supernatants without normalizations per control or treatment with the cytokine concentration range defined by the lowest and highest values observed from two different donors out of six donors per group.

Table 3

Cell populations positive for Annexin V or PI upon DC treatment with biomaterial films. Each value indicates the positive percentages compared to the negative control (unstained) per control or treatment. mean±SD. n=9 donors (9 independent experiments with different donors).

Controls & treatments	Annexin V (%)	PI (%)
iDCs	10.4±2.0	2.6±0.8
mDCs	12.6±3.8	2.7±0.7
PLGA	22.6±9.3 */***	2.7±0.5
Chitosan	26.2±9.6 */**/***	2.9±0.9
Alginate	25.8±8.0 */***	3.7±0.5
HA	26.8±5.1 */***	3.4±0.7
Agarose	9.8±3.9	2.8±1.1

* $p \leq 0.05$, statistically different from iDCs;

** $p \leq 0.05$, statistically different from mDCs;

*** $p \leq 0.05$, statistically different from Agarose (between two biomaterial treatments).

Table 4

Summary of the results. Selected DC phenotypes are shown by statistical significance only compared to iDCs.

Controls & treatments	CD40	CD80	CD86	CD83	MLR*	TNF-α or IL-6	Endocytic ability	Annexin V	PI
mDCs	↑	↑	↑	↑	↑	↑	↓	↑	–
PLGA	–	↑	↑	↑	↑	↑	↓	↑	–
Chitosan	–	↑	↑	↑	↑	↑	↓	↑	–
Alginate	–	–	↑	↑	–	↓	↓	↑	↑
HA	↓	↓	↓	↑	↓	↑	↓	↑	–
Agarose	–	–	–	↑	–	–	–	–	–

*The result only from 1:6.25 of DC:T ratio is shown here.

'↑': phenotype changes at levels significantly higher than iDCs; ' – ': phenotype changes at levels similar to iDCs; '↓': phenotype changes at levels significantly lower than iDCs.