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PHENOTYPE AND POLARIZATION OF AUTOLOGOUS T CELLS BY BIOMATERIAL-TREATED DENDRITIC CELLS

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Abstract

Given the central role of dendritic cells (DCs) in directing T cell phenotypes, the ability of biomaterial-treated DCs to dictate autologous T cell phenotype was investigated. Here, we demonstrate that differentially biomaterial-treated DCs differentially directed autologous T cell phenotype and polarization, depending on the biomaterial used to pre-treat the DCs. Immature DCs (iDCs) were derived from human peripheral blood monocytes, and treated with biomaterial films of alginate, agarose, chitosan, hyaluronic acid, or 75:25 poly(lactic-co-glycolic acid) (PLGA), followed by co-culture of these biomaterial-treated DCs and autologous T cells. When autologous T cells were co-cultured with DCs treated with biomaterial film/antigen (ovalbumin, OVA) combinations, different biomaterial films induced differential levels of T cell marker (CD4, CD8, CD25, CD69) expression, as well as differential cytokine profiles [interferon (IFN)- γ , interleukin (IL)-12p70, IL-10, IL-4] in the polarization of T helper types. Dendritic cells treated with agarose films/OVA induced CD4+CD25+FoxP3+ (T regulatory cells) expression, comparable to untreated iDCs, on autologous T cells in the DC-T co-culture system. Furthermore, in this co-culture, agarose treatment induced release of IL-12p70 and IL-10 at higher levels, as compared to DC treatment with other biomaterial films/OVA, suggesting Th1 and Th2 polarization, respectively. Dendritic cells treated with PLGA film/OVA treatment induced release of IFN- γ at higher levels compared to that observed for co-cultures with iDCs or DCs treated with all other biomaterial films. These results indicate that DC treatment with different biomaterial films has potential as a tool for immunomodulation by directing autologous T cell responses.

Keywords

biomaterial; dendritic cells; T cells; polarization; immunotherapy

1. INTRODUCTION

In the context of tissue engineering, adaptive immune responses should be minimized, whereas the strategy for DNA- or protein-based vaccines aims to enhance the protective immune responses. Adjuvants enhance a resultant adaptive immunity by interacting with antigen-presenting cells (APCs) such as DCs that stimulate T lymphocytes. Biomaterials

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commonly used in combination products, such as vaccine delivery vehicles or tissue engineering scaffolds, exhibited their potential as an adjuvant; particulate forms of biomaterials support vaccine immunogenicity resulting in T cell or B cell activation, associated with an adjuvant effect.¹⁻³ At the same time, other biomaterials minimize immune responses in articular cartilage tissue engineering, supporting chondrocyte proliferation and cartilage healing.⁴⁻¹⁰

In vitro effects of biomaterials on induced DC phenotype have been extensively studied using inherently different biomaterials, and differential levels of DC maturation were observed with phenotype changes of DCs depending on the type of biomaterials used to treat the immature DCs (iDCs). For example, *in vitro* DC maturation is induced by positive charges¹¹ or hydrophobicity¹² of biomaterial surfaces, associated with DC adhesion (integrin-mediated) on biomaterial surface,¹³ while more hydrophilic surfaces of biomaterials such as agarose did not support DC maturation.¹⁴ In addition, carbohydrate profiles associated with the adsorbed protein layer on surfaces of defined chemistries¹⁵ or surface roughness/energy of biomaterials¹⁶ affect DC maturation. Therefore, biomaterials in combination products can modulate DC phenotypes as these cells are the most effective APCs that initiate T-cell mediated immunity efficiently as they bridge innate and adaptive immunity.¹⁷

Dendritic cells are the only antigen-presenting cells (APCs) that stimulate naïve T cells.¹⁷⁻¹⁹ Upon maturation, DCs migrate to the secondary lymph organs to present the antigenic peptides to T cells so that the adaptive immune response is initiated.¹⁷⁻²¹ Depending on DC phenotype changes, T cell-mediated immune responses are differentially modulated. For example, the reduction of antigen endocytosis by DCs inhibits DC capacity to stimulate T cells,²² while the up-regulation of major histocompatibility complex (MHC) and co-stimulatory molecules on DCs induces effective T cell stimulation.¹⁷ Dendritic cells can control the adaptive immune response by presenting the exogenously introduced antigens in the context of MHC molecules for activation of naïve T cells; MHC class II (the antigenic peptide-binding groove) elicits CD4+ T cell responses while a cross-priming with MHC class I results in CD8+ T cell responses.^{23,24} In addition, upon interaction between DCs and T cells, the resultant immunity can be polarized toward either T helper (Th) type 1 (cellular response), Th type 2 (humoral response), or Th type 17 (anti-microbial immunity) depending on the release of cytokines such as interferon (IFN)- γ /interleukin (IL)-12, IL-10/IL-4, or IL-17, respectively.²⁵⁻²⁷ Immunosuppressive CD4+CD25+ T cells can also be induced in combination with forkhead box P3+ (FoxP3+) expression, which is a transcriptional regulator and specific marker of natural T regulatory cells.^{24,28} Thus DC phenotypic attributes such as antigen uptake/presentation, co-stimulatory molecule expression, or cytokine release are essential in determining T cell phenotype.²⁴

In our previous *in vivo* studies, biomaterial effects on T cell immunity have been demonstrated. Scaffolds or microparticles prepared from poly(lactic-co-glycolic acid) (PLGA) with an incorporated model antigen (ovalbumin, OVA) act as an adjuvant in enhancing a predominately Th2-dependent humoral immune response to an extent depending on the form of the carrier vehicle.²⁹⁻³¹ However, OVA delivery from an agarose scaffold did not enhance the OVA-specific humoral immune response, consistent with the

lack of DC maturation upon treatment with this biomaterial *in vitro*. Thus, these *in vivo* studies suggest an effect of DCs, influenced by the biomaterial contact, on resultant T cell response, to associated exogenous antigen. These *in vivo* studies only examined humoral immune responses but likely require DC interaction with the implanted biomaterial with resultant phenotypic outcomes wherein the immune response to the associated antigen is influenced. This is the subject of the *in vitro* study undertaken here.

As such, DCs respond to biomaterials only when they directly contact with biomaterials as shown in our previous study.³² When biomaterials are introduced into the host, DCs are affected by the biomaterial stimulus (much like a danger signal during the innate immune response³³), and exhibit phenotype changes so that they can then present the antigens, that they uptake during the innate response, to T cells that are effectively stimulated for further adaptive immune responses. Since an adjuvant effect of PLGA was observed in our previous *in vivo* studies, one of the key consequences of DC interaction with biomaterials would be that DCs modulate phenotypes and functions of T cells in association with the antigens internalized by DCs during the innate response to the biomaterials.

Use of an *in vitro* system, allows for the controlled study of the consequence of these specific DC/biomaterial interactions on T cells and validates what we have previously observed *in vivo* as far as differential adjuvant effects of PLGA and agarose.³¹ As such, in the study presented herein, a systematic *in vitro* study was performed to assess effects of DC treatment with different biomaterials on human T cell activation and polarization, using a direct contact co-culture between biomaterial-treated DCs and T cells. Furthermore, additional *in vivo* effects of these selected biomaterials are suggested by this study. For instance, agarose-treated DCs induced immunosuppressive T regulatory cells and such an effect would be particularly useful for acceptance of cell transplants in the context of agarose, whereas PLGA may be useful for immunogenic therapy for boosting immune responses. In this way, these immunomodulating capacities of biomaterials would provide key information for selection of biomaterials for desired extents of immune responses in the combination products for tissue engineering, vaccine delivery, or immunotherapeutic tools.

For this study, non-adherent mononuclear cells (nMNCs) obtained from human peripheral blood mononuclear cells (PBMCs) were used to observe the differential effect of co-culture with DCs pretreated with different biomaterials. Such nMNCs are comprised of multiple fractions such as naïve T cells, memory T cells, CD4+ or CD8+ T cells, other CD3+ T cells, and other non-T cells.³⁴ It is important to note that the secretion patterns of cytokines that can direct T cell polarization of Th1 or Th2 are different between homogeneous and heterogeneous immune cell fractions derived from PBMCs.³⁵ In addition, to understand total T lymphocyte activation prior to moving forward to understand responses of specifically pre-isolated T cell subsets, the experimental model used here adopted that of previous studies wherein unpurified nMNCs obtained from PBMCs were employed to understand total lymphocyte activation and polarization in a specific human disease or cancer.^{36–40} In the present study, resultant T cell phenotype and polarization were determined by assessing T cell surface marker expression and profile of cytokines released. The biomaterials used in this study included PLGA, chitosan, alginate, hyaluronic acid (HA), and agarose that had previously shown differential effects on DC phenotypes.¹⁴

2. MATERIALS AND METHODS

2.1. Preparation of biomaterial films

All biomaterial films were freshly prepared for each experimental procedure. And all biomaterial films were punched and tested as discs of 34.8 mm diameter so as to fit in a 6-well plate. Preparation methods of all biomaterial films were adapted or modified from the previously described methods. Briefly, poly(DL-lactic-co-glycolic acid) (PLGA) (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 room temperature (r.t.) and poured into the Teflon dish of 50 mm diameter (Cole-Parmer, Vernon Hills, IL) in the chemical fume hood.⁴¹ Upon evaporation of the solvent and drying (36–48 hours), PLGA films were punched of an appropriate size, and washed for 1 hour in ddH₂O changing ddH₂O every 15 min. Chitosan (high molecular weight: 400,000 MW, degree of deacetylation: 75%, Fluka, Milwaukee, WI) was dissolved with 1% (w/v) chitosan in glacial acetic acid (2% v/v in ddH₂O) (Fisher Scientific, Fairlawn, NJ) for 24 hours at r.t. and then, poured into the Teflon dish of 50 mm diameter 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 ddH₂O (2 hours) and washed by ddH₂O (20 min), followed by immersion in 1 M NaOH (Sigma, 30 min) to neutralize the surface and washed with ddH₂O (20 min).⁴² Chitosan films were punched of an appropriate size, and finally washed for 20 min in ddH₂O. Alginate (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 ddH₂O 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 ddH₂O for 10 min.⁴³ Alginate films were punched of an appropriate size, and washed for 30 min in ddH₂O changing water every 10 min. Hyaluronic acid (800,000 MW; sodium salt from *Streptococcus equi*, BioChemika, Fluka) was dissolved to a concentration of 4% (w/v) HA in ddH₂O 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 ddH₂O for 10 min.⁴⁴ Hyaluronic acid films were punched of an appropriate size, and washed for 30 min in ddH₂O changing water every 10 min. Agarose (type V; high gelling; gel strength of 800 g/cm² at 1.0 %; Sigma; molecular weight is not known) was dissolved in ddH₂O to a concentration of 3% (w/v) by heating using a microwave until boiling and visible homogeneity was reached.⁴⁵ 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 culture with iDCs. All biomaterial films were UV-sterilized for 30 min per surface in the tissue culture hood prior to use in DC cultures.

Endotoxin contents of biomaterial films were determined using a chromogenic Limulus Amebocyte Lysate assay (QCL-1000 Chromogenic LAL Endpoint Assay, Cambrex,

Walkersville, MD). Endotoxin assays were performed on a smaller piece of film (4.5 mm in diameter), which had undergone the same washing and sterilization procedures as films used to treat DCs. The smaller film pieces were suspended in endotoxin-free water and endotoxin assay performed. Standards in tissue culture treated polystyrene wells and sample wells of different biomaterials were treated with endotoxin-free water. Limulus amoebocyte lysate was added in the presence of biomaterial and incubated for 10 min at 37°C. Chromogenic substrate (Ac-Ile-Glu-Ala-Arg-pNA) was added to each well and incubated for 6 min. Glacial acetic acid (25% v/v) (J.T. Baker, Philipsburg, NJ) was added as a stop solution and the mixture was transferred into flat-bottom microplate and the absorbance was measured at 405 nm. Endotoxin content in the samples was read off the endotoxin standards generated from the manufacturer's kit. Each sample was run in triplicate for quantification. The effective endotoxin contents (EU/ml) of this 34.8 mm diameter film per each biomaterial were 0.676 ± 0.4 for PLGA, 0.042 ± 0.1 for chitosan, 2.096 ± 0.4 for alginate, 0.216 ± 0.2 for HA, and 2.195 ± 0.3 for agarose. A previous study showed that a minimum E. Coli endotoxin (LPS) concentration of 1 ng/ml (approximately 10 EU/ml) was required to stimulate human monocyte-derived DCs.⁴⁶

2.2. Dendritic cell culture and treatment with biomaterials

Dendritic cells were derived from human peripheral blood monocytes, and treated with biomaterial films in the same way as previous study.¹⁴ Briefly, 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) (1000 U/ml) and interleukin-4 (IL-4) (800 U/ml) in the DC culture media, prepared by filter-sterilizing RPMI-1640 containing 25mM 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).^{47–49} Thus derived iDCs were positive for CD40, CD80, CD83, CD86, HLA-DQ, and HLA-DR, expressions of which were up-regulated upon DC maturation by lipopolysaccharide (LPS) treatment.^{18,32}

On day 5 of culture for this iDC derivation, non- and loosely-adherent cells containing iDCs were collected and washed twice using PBS (pH 7.2) by centrifugation at 1100 rpm for 10 min (see Supplemental Materials for more detailed description of the DC population). Immature DCs were then resuspended in the DC culture media at 0.5×10^6 cells/ml, which also included a model antigen, ovalbumin (OVA) (Grade VII, Sigma) at a concentration of 150 µg/ml as previously described.^{50,51} In our preliminary studies, when DCs were pre-treated with biomaterials in the absence of a model antigen, this did not induce CD4, CD8, CD25, or CD69 expressions in nMNCs at levels significantly different from their treatment with control of iDCs (data not shown). It is likely that this is due at least in part that the biomaterial provides a maturation (or not for agarose) stimulus to the DCs, much like during the innate immune response, during which it takes up an associated antigen, and it is then

the presentation of that antigen to T cells, in the context of the DC phenotype, that then determines the activation and polarization of the T cell (adaptive immune) response. Thus, we employed a model antigen to stimulate T cells in the protocol of the study presented herein. Ovalbumin is the most representative model antigen especially for experiments in murine adaptive immunity because it has extensively been proved to induce specific T cell response such as CD4⁺ or CD8⁺ T cell activation depending on the transgenic condition of mice. Even though pre-existing T cells specific to OVA are present at very low frequency in the healthy human blood, CD4⁺ T cells derived from the healthy human PBMCs have been stimulated by mature DCs (mDCs) loaded with OVA.⁵² In addition, OVA was used in the studies for non-specific human T cell activations.^{51,53} For these reasons, OVA was pulsed with biomaterial-treated APCs only for 24 hours as a model antigen in the study presented herein, to effectively stimulate T cells in the following co-culture of DCs and nMNCs.

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)^{12,14,32,54} and then iDC/OVA suspension was plated by 3 ml into each well of 6-well plate containing biomaterial films.

In addition to wells of biomaterial films, wells for the negative DC control of iDC remained untreated while wells for the positive DC control of mDC involved addition of 1 µg/ml of LPS (*E. coli* 055:B5; Sigma).¹⁴ Immature DCs and mDCs were also suspended in DC media containing OVA as appropriate DC controls. Thus, unless specified, all of iDC, mDC and DC treatment with biomaterial films include a model antigen, OVA, in the culture media hereafter. All biomaterial treatments and DC control groups were incubated for next 24 hours using DC media supplemented with GM-CSF and IL-4 (95% relative humidity, 5% CO₂, 37°C). After 24 hour-treatment, DCs were isolated from the biomaterial films and extracellular OVA by extensive washing for the following co-culture with autologous nMNCs while supernatant was collected for cytokine analysis. In this way, only DCs and OVA peptides presented on the DC surface, which was processed from the OVA previously internalized by DCs for 24 hour-treatment, were in contact with T cells. According to justification previously described,¹⁴ the harvested non-/loosely-adherent DC population was used for the co-culture with nMNCs in this study.

In this study, total three different categories are used to describe DC treatments and controls as shown Table 1(a) – iDC or mDC indicates a specific DC phenotype of immature DC or mature DC (treated with LPS) respectively as controls, whereas DC treated with each biomaterial film (or each biomaterial film-treated DC) represents a specific DC phenotype upon treatment with each biomaterial film for 24 hours. And each of these three categories of DCs was treated with OVA at the same time as biomaterial treatment for 24 hours.

2.3. Autologous T cell preparation

A preparation method of autologous T cells was adapted from the previously described methods.^{37–39} On the day of blood collection from the donors, PBMCs were incubated for 2 hours for selecting the adherent mononuclear cells as described above. After this 2 hour-incubation for the plastic adherence, the adherent monocytes on the culture dish were used for generation of DCs in next 5 days as described above. At the same time, nMNCs were

collected and used for the autologous T cell population after they were strained using a cell strainer with 40 μm pore size (Becton Dickinson, San Jose, CA). Purity of these strained nMNCs was confirmed by using a fluorescently conjugated mouse anti-human monoclonal antibodies against CD3 (clone HIT3a; IgG2a κ) (BD Pharmingen, San Jose, CA), followed by scanning 10,000 cells (events) per donor on a BDLSR flow cytometer (Becton Dickinson). For the nMNCs, the percentage of CD3+ T cells was $75\pm 5\%$ (n=6 different donors).

2.4. Co-culture of DCs and autologous nMNCs (T cells)

The procedure for the co-culture of DCs and autologous nMNCs was adapted from the previously described methods.^{37–39} Briefly, from an identical donor, blood was collected twice at two different time points for two separate DC preparations and a single collection of autologous nMNCs as shown in Figure 1a & 1b. On day 0, the first blood collection was made from a donor and the adherent mononuclear cells have been cultured for 5 days to generate iDCs as described above, followed by DC treatment with different biomaterial films for another 24 hours (1st set of DCs). On day 6, the second blood collection was made from the identical donor and then, processed for generation of 2nd set of DCs to be treated with biomaterial films (on day 11) as described above. At the same time (on day 6), the autologous nMNCs were also collected, and then co-cultured with the 1st set of DCs after non-/loosely-adherent DCs were isolated from the biomaterial films and extracellular OVA by extensive washing as above.

The ratio of DCs and nMNCs (1:6.25) in the co-culture was adapted from that of the mixed lymphocyte reaction (MLR) in the previous studies because this ratio showed a very explicit result of allogeneic T cell proliferation among DCs treated with the different biomaterial films examined here.^{14,32} Using this ratio, DCs and nMNCs were resuspended at concentration of 5×10^4 cells/ml and 3.125×10^5 cells/ml, respectively, together in the complete RPMI-10 media (the co-culture media), which was prepared with RPMI-1640, 25 mM HEPES, L-glutamine (Gibco BRL, Invitrogen), 100 U/ml penicillin/streptomycin (Cellgro) and heat-inactivated filter-sterilized (0.22 μm) 10% (v/v) human AB serum (Biowhittaker, Walkersville, MD). This suspension of DCs and nMNCs was plated into the 96-well flat-bottomed plate (Corning) (200 μL /well) and cytokines of IL-2 (10 units/ml) and IL-7 (5 ng/ml) (both from Peprotech) were added to each well of the co-culture to promote naïve T cell survival and population size maintenance.^{55,56} For the negative control of the co-culture system, only nMNCs were cultured using the same concentration of the nMNCs in the DC-T cell co-culture wells. At the same time, this negative control (only nMNCs) was partially treated with transforming growth factor (TGF)- β (5 ng/ml) (Peprotech) to induce FoxP3 expression as a positive control for FoxP3 measurement upon DC-T cell co-culture.⁵⁷

After 3 days (on day 9), the complete RPMI-10 media was exchanged with fresh media by 50% (100 μL) per well of the 96-well plate. In this media exchange, the cytokines of IL-2 (25 units/ml), IL-7 (10 ng/ml) and TGF- β (5 ng/ml) (only for the positive control wells of FoxP3 expression) were newly added into each well of the co-culture.

On day 11, the 2nd set of DCs generated from the second blood collection on day 6 was treated with newly prepared biomaterial films and then, after 24 hours (on day 12), non-/

loosely-adherent DCs were collected and washed twice as above. On the same day (day 12), the complete RPMI-10 media was exchanged with fresh media by 50% (100 μ l) one more time, which includes the cytokines of IL-2 (25 units/ml) and IL-7 (10 ng/ml) as well as the 2nd biomaterial-treated DC harvest at a concentration of 5×10^4 cells/ml. This addition of DCs was performed as the 2nd stimulation of nMNCs to achieve clonal expansion^{20,21} of T cells that were previously stimulated by biomaterial/OVA-treated DCs, which enables us to have final T cell numbers enough for various examinations and future *ex vivo* immunotherapeutic tool development^{58,59} using biomaterials. It can be considered analogous to a booster in an immunization. Another addition of TGF- β (5 ng/ml) was also made into the positive control wells (only nMNCs) for induction of FoxP3 expression. After 2 days (on day 14), the whole procedure was finalized by collecting all cells for examination of T cell marker or FoxP3 expression and supernatant for analysis of cytokine profiles.

2.5. Samples and controls

The present study herein has various controls depending on the time point or treatment with biomaterial films as shown in Table 1. From day 6 to day 14, in the 96-well plate for the co-culture system, 2 different groups were used; Group 1 was for the co-culture of nMNCs and DCs treated with biomaterial films, while Group 2 was for the culture of only DCs treated with biomaterial films in the absence of autologous nMNCs (The Group 2 had cell culture conditions identical to the Group 1 but autologous nMNCs were not added).

In addition, only untreated nMNCs were cultured from day 6 through day 14 in the absence of DCs to obtain a background level of phenotype changes of nMNCs as shown in Table 1(a). Then, in measurement of the T cell markers, all observations were normalized to the negative control of the untreated nMNC control without co-culture with DCs whereas nMNCs co-cultured with iDCs or mDCs were still used as internal controls. In this format, we also compared pair-wise between nMNCs co-cultured with iDCs, mDCs, and biomaterial-treated DCs using ANOVA (see 'Statistical Analysis').

For the cytokine release experiments, cytokines secreted from only DCs treated with biomaterials were measured before co-culture with nMNCs, and data was normalized by iDCs in the same way as our previous study.¹⁴ However, once these DCs are co-cultured with nMNCs, to again consider the effect of the untreated nMNC during 8 days of culture duration in our own experimental condition, cytokine release from the co-culture of nMNCs and DCs was normalized to the negative control of the untreated nMNCs, followed by another normalization by cytokine amount present in culture of only DCs treated with respective biomaterial (Group 2 as shown in Table 1b) and we compared pair-wise between nMNCs co-cultured with iDCs, mDCs, and biomaterial-treated DCs using ANOVA.

2.6. T cell surface marker expressions

The level of T cell surface marker expression was determined for T cells following co-culture with differentially treated DCs, by flow cytometry, as previously described³² and compared to controls. Whole cell population from each co-culture well was collected by centrifugation at $300 \times g$ for 10 min and suspended in Hank's HEPES buffer (120 mM NaCl, 10 mM KCl, 10 mM MgCl₂, 10 mM glucose, 30 mM HEPES) (all from Sigma) containing

1% (v/v) human serum albumin (HSA) (Calbiochem, Darmstadt, Germany) and 1.5 mM CaCl₂ (Sigma). Cells were stained with saturating concentrations of fluorescently conjugated mouse anti-human monoclonal antibodies against CD3 (clone HIT3a; IgG2a κ), CD4 (clone L200; IgG1 κ), CD8 (clone RPA-T8; IgG1 κ), CD25 (clone M-A251; IgG1 κ), CD69 (clone FN50; IgG1 κ) (all from BD Pharmingen) for 1 hour at 4°C in the dark, filtered using 40 μ m cell strainer (Becton Dickinson) and then, analyzed immediately with 10,000 events per sample using a BDLSR flow cytometer (Becton Dickinson). Data were obtained together with the negative control of autofluorescence per sample and then, analyzed using FLOWJO version 7.2.5 (Tree Star, Inc. Ashland, OR).

2.7. FoxP3 expressions

On day 14, the level of FoxP3 expression was determined for T cells following co-culture with DCs treated with PLGA or agarose films by flow cytometry, using the FITC anti-human FoxP3 Staining Kit (eBioscience, San Diego, CA) according to the manufacturer's protocol. Rat IgG2a κ FITC (eBioscience) was used as isotype control. Whole cell population from the each co-culture well was collected by centrifugation at 300 $\times$ g for 10 min and then, cells were fixed and permeabilized using a fixation/permeabilization kit (eBioscience). Cells were stained with FoxP3 (clone PCH101; IgG2a κ) or the isotype control together with CD3, CD4, and CD25 (same as above) to gate CD4+CD25+ T cells from CD3+ population. After staining procedure, cells were filtered using 40 μ m cell strainer (Becton Dickinson) and then, analyzed immediately with 10,000 events per sample using a BDLSR flow cytometer (Becton Dickinson). Data was obtained together with the negative control of autofluorescence per sample and then, analyzed using FLOWJO version 7.2.5 (Tree Star).

2.8. Cytokine release

The amount of cytokines, IFN- γ , IL-12p70, IL-10, and IL-4 produced in the biomaterial treatment of DCs or in the co-culture of differentially-treated DCs and T cells, was analyzed by Cytometric Bead Array (CBA) Human Inflammation Kit (BD Pharmingen) according to manufacturer's directions. Cell culture supernatants were cleared by centrifugation for 10 minutes at 400 $\times$ g and stored at -20°C until analysis. Each cytokine amount was normalized to the respective total DNA quantified using a picoGreen dsDNA quantification kit (Invitrogen) per manufacturer's directions. The CBA analysis was performed using the flow cytometry and then, analyzed using FLOWJO version 7.2.5.

2.9. Statistical analysis

For statistical analysis, one sided Student t-test was used to compare sample group to appropriate control group in pairs. To observe significant differences between all sample groups in pairs, the general liner model of two-way ANOVA in pairwise (Tukey) 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.

3. RESULTS

3.1. T cell marker and FoxP3 expression

Upon DC treatment with different biomaterial films, autologous nMNCs co-cultured with these DCs exhibited differential expressions of T cell markers depending on the type of biomaterial films used to treat DCs as shown in Figures 2, 3, 4, and 5.

In Figure 2, iDCs or DCs treated with agarose films induced CD4 expression on CD3+ T cells at levels higher than the negative control of untreated CD3+ T cells. Furthermore, when nMNCs were co-cultured with DCs treated with agarose films, they exhibited significantly higher levels of CD4 expression on CD3+ T cells compared to all other biomaterial films. Interestingly, DCs treated with HA films induced significantly lower levels of CD4 expression on CD3+ T cells compared to iDCs or the negative control of untreated CD3+ T cells. However, CD8 expression levels of CD3+ T cells, when nMNCs were co-cultured with iDCs, mDCs, or DCs treated with all biomaterial films, did not show any significant difference compared to the negative control of untreated CD3+ T cells or between iDCs, mDCs, and treatments (Figure 2).

Dendritic cells treated with PLGA, chitosan, alginate, or agarose films, induced significantly higher levels of CD25 expression compared to the negative control of untreated CD3+ T cells, whereas iDCs, mDCs, or DCs treated with HA films induced levels similar to the negative control (Figure 2). Dendritic cells treated with agarose films induced significantly higher levels of CD25 expression on CD3+ T cells compared to DCs treated with PLGA films or HA films while DCs treated with alginate films did only when compared to DCs treated with HA films. Dendritic cells treated with each of all biomaterial films or mDCs induced significantly higher levels of CD69 expression on CD3+ T cells as compared to the negative control of untreated CD3+ T cells (no significant difference between biomaterial film treatments was observed) (Figure 2).

Frequencies of double positive CD4+CD8+, CD4+CD25+, or CD4+CD69+ T cell subsets in the peripheral blood from human subjects are significantly modulated depending on clinical conditions of the patients.^{60–64} However, the mechanism underlying their frequency changes is still understudied.^{63–66} In the study presented herein, to understand effects of DC-biomaterial contact on frequency changes of CD4+CD8+, CD4+CD25+, or CD4+CD69+ T cell subsets, expansions of these T cell subsets from the peripheral nMNCs depending on biomaterial treatments of DCs were determined as shown in Figure 3, 4, and 5.

Examination of representative quadrant dot plots for autologous T cell (CD3+) markers after co-culture with differentially treated DCs (Figure 3), showed that CD4+ quadrant percentages for CD3+ T cells changed depending on different biomaterial films used to treat the co-cultured DCs while CD8+ quadrant percentages did not change appreciably as shown in the column of CD4 and CD8 dot plots. Immature DCs, mDCs, and DCs treated with alginate or agarose films induced significantly higher levels of the double positive CD4+CD8+ T cells, as compared to the negative control of untreated CD3+ T cells (Figure 4). However, DC treatment with the other biomaterial films (PLGA, chitosan, or HA) induced levels of the double positive CD4+CD8+ expression on co-cultured T cells that

were similar to the negative control (Figure 4). Treatment of DCs with agarose films induced significantly higher levels of CD4+CD25+ expression on co-cultured CD3+ T cells compared to mDCs or DC treatment with other biomaterial films (PLGA, chitosan, or HA) (Figure 4). Interestingly, all DC controls and treatments induced CD4+CD69+ expression levels that were significantly higher than the negative control (untreated CD3+ T cells) (Figure 4). Furthermore, DC treatment with agarose films supported levels of CD4+CD69+ expression that was significantly higher than that induced by DCs treated with alginate or HA films (Figure 4).

To further characterize the induced CD4+CD25+ T cell phenotype, FoxP3 expression was assessed on the autologous T cells co-cultured with agarose- or PLGA-treated DCs. These two materials were chosen for further examination as these materials demonstrated distinct effects on DC phenotypes in a previous study¹⁴ and here. When autologous nMNCs were co-cultured with DCs treated with PLGA or agarose films, they exhibited differential levels of FoxP3 expression (Figure 5). From the CD4+CD25+ population gated in Figure 5a, FoxP3 expression is shown in Figure 5b. As a result, iDCs or DCs treated with agarose films induced FoxP3 expression on CD4+CD25+ T cells at levels similar between each other, whereas both of them did at levels higher than mDCs or DCs treated with PLGA films (Figure 5c). T cells treated with TGF- β , a protein known as specific inducer of FoxP3 expression,⁵⁷ exhibited a level of FoxP3 expression that was similar to CD3+ T cells co-cultured with iDCs or with agarose-treated DCs (Figure 5c).

3.2. Cytokine release in Th1/Th2 polarization

As another measurement of differential effects of DC treatment with different biomaterial films on autologous CD3+ T cell polarization, cytokine release profiles were determined in the supernatants of DCs treated with biomaterial films before or after co-culture with autologous nMNCs.

As shown in Figure 6, before co-culture with nMNCs, DCs treated with different biomaterial films released different levels of specific cytokines depending on the type of biomaterial used for treatment. Levels of IFN- γ secretion from iDCs, mDCs, or all biomaterial treatments were not significantly different each other (Figure 6). Dendritic cells treated with agarose films secreted IL-12p70 at levels significantly higher than iDCs, while mDCs or DCs treated with all other biomaterial films did at levels similar to iDCs. For IL-10, mDCs or DCs treated with chitosan films released significantly higher levels than iDCs while DCs treated with agarose films induced significantly lower levels, as compared to iDCs. In addition, the chitosan-treated DCs released IL-10 at levels significantly higher than DCs treated with HA or agarose films. Dendritic cells treated with agarose films resulted in levels of IL-4 release that were significantly lower than iDCs, mDCs, or all other treatments. Dendritic cells, which were treated with biomaterials and left in the culture wells during the full co-culture period (until day 14) without added autologous T cells, showed cytokine profiles (data not shown) similar to what was seen at day 6 (Figure 6).

Cytokine profiles for co-culture of biomaterial-treated DCs with autologous nMNCs on day 14 (after 8 days of co-culture) were assessed and results shown in Figure 7. These cytokine amounts were normalized in two ways – by cytokine amount present in cultures of only

untreated nMNCs (negative control) and then, by that present in cultures of only DCs treated with respective biomaterial (Group 2 as shown in Table 1b).

Upon autologous nMNC co-culture with differentially biomaterial-treated DCs (Figure 7), the profiles of released cytokines were observed in patterns different from those of only DCs (treated with the respective biomaterial film) (Figure 6). Co-culture of autologous nMNCs with mDCs induced IFN- γ release at levels significantly higher than DCs treated with other biomaterials (chitosan, HA, or agarose films) (Figure 7). Furthermore, co-culture of autologous nMNCs with DCs treated with PLGA films released IFN- γ at significantly higher levels compared to that observed for co-cultures with iDCs or DCs treated with all other biomaterial films (Figure 7). Co-culture of autologous nMNCs with DCs treated with agarose films induced significantly higher levels of IL-12p70 release as compared to co-cultures with iDCs, mDCs, or DCs treated with all other biomaterial films except alginate films. Similarly, in this co-culture, release of IL-10 was at significantly higher levels as compared to co-cultures with mDCs, or DCs treated with all other materials except for HA films. In the co-cultures with T cells, treatment of DCs with any of the biomaterial films did not induce IL-4 release significantly different between treatment groups (Figure 7).

4. DISCUSSION

4.1. CD4+/CD8+ T cell activation

When DCs were treated with biomaterial films in the presence of the antigen (OVA), autologous CD3+ T cells co-cultured with these DCs showed differential expression levels of T cell markers depending on biomaterials as shown in Figures 2 – 5. The CD4+ T cell responses were modulated by DCs treated with different biomaterial films, whereas the CD8+ T cell responses were not affected as shown in Figure 2. It is conceivable that the system of DC treatment with biomaterial films and subsequent co-culture with autologous CD3+ T cells more effectively modulated CD4+ T cell responses than CD8+ T cell responses, the latter of which would depend on cross-presentation of extracellular antigen component.²⁴ It has previously been reported that CD3+CD4+CD8+ T cell subset is found by around 3% (out of total CD3+ lymphocytes) in the peripheral blood from healthy human subjects.⁶⁷ And there is considerable evidence of an increased frequency of CD4+CD8+ T cell subset in the peripheral blood from human subjects with a wide range of unrelated diseases or a variety of clinical conditions.^{60,61,68–70} Above all, double positive CD4+CD8+ T cell subset originates from peripheral expansion of CD4+ T cells for both healthy and infected human subject.^{60,62} However, the expansion and role of extra-thymic CD4+CD8+ T cell subset remain largely unknown and uncharacterized.⁶⁵

Antigen uptake by the biomaterial-treated DCs would affect antigen presentation to T cells. Enhanced endocytic ability of DCs treated with LPS is transient so that, after around 1 hour of LPS treatment, the endocytic ability decreased over time.⁷¹ In our previous study, after 24 hours of DC treatment with biomaterial films, DCs isolated from treatment with agarose films up-regulated receptors for endocytosis and maintained an endocytic capacity similar to iDCs which was reduced with maturation of DCs.¹⁴ In the present study, DCs were treated with biomaterial films in the presence of OVA antigen for 24 hours, and then, DCs were isolated from biomaterial films and extracellular OVA antigen prior to co-culture with T

cells. Therefore, it is conceivable that active endocytic behavior of iDCs or DCs treated with agarose films, during the 24 hours of DC treatment in the presence of OVA, induced higher levels of CD4 as well as CD4+CD8+ expression of autologous T cells presumably due to enhanced peptide-MHC class II complexes on DCs.

Ligation of CD44 surface molecules on DCs using blocking antibodies, has been shown to inhibit proliferation of co-cultured CD4+ T cells.⁷² Furthermore, previously we observed that DCs treated with HA films unexpectedly expressed lower levels of the hyaluronan-specific receptor, CD44, possibly because these HA films were of a high molecular weight HA, cross-linked and thus insoluble.¹⁴ This may lend explanation for the result that DCs treated with HA films induced significantly lower levels of CD4 expression on co-cultured T cells as compared to iDCs or the negative control of untreated CD3+ T cells (Figure 2).

4.2. CD25+/CD69+ T cell activation

The α chain of IL-2 receptor, CD25, has been widely accepted as an activation marker of T cells along with another T cell activation marker, CD69. While CD4+CD25+FoxP3+ T regulatory cells dominantly suppress activation of autoreactive T cells that lead to autoimmunity,^{62,65,69,73,74} CD4+CD69+ T cells expand systemically upon T cell activation induced by disease condition.⁶³ Frequencies of these two T cell subsets fluctuate across individuals but both are mostly less than 10% (out of total CD3+ lymphocytes) in the peripheral blood from healthy human subjects.^{63,64,75} However, these frequencies are also modulated depending on immunological disease conditions.^{63,64} For example, breast cancer patients have more CD4+CD25+ T cells in their blood than do healthy subjects⁶⁴ – this is considered as immunosuppressive mechanism by which cancer cells escape effector T cells and survive since tumors employ strategies to suppress effector T cell activations in the host immune system by creating a tolerogenic microenvironment mediated through CD4+CD25+ regulatory T cells.⁷⁶ However, number of CD4+CD25+ or CD4+CD69+ T cell subset decreased or increased, respectively, after the patients were vaccinated with antigenic peptide and adjuvant.⁶⁴ These indicate that APCs such as DCs, which are preferentially activated by adjuvant and present antigenic peptide to naïve T cells in vaccination of the host, might modulate CD69 upregulation on T cells in addition to their well-known capacity of controlling activation and expansion of CD4+CD25+ T cells.^{66,77} However, the mechanism underlying frequency changes of these two T cell subsets, depending on diseases, vaccination, or DC-T cell contact still remains to be determined.^{63,64,66} The modulation of CD4+CD25+ and CD4+CD69+ levels through CD3+ T cell co-culture with biomaterial-treated DCs appears to mimic that seen *in vivo* in different immune activating or immunosuppressive situations.

Interestingly, it has been reported that upregulation of co-stimulatory molecule (CD80 or CD86) on DCs was not required for activation of CD69+ T cells.⁷⁸ Similarly to this, we also observed that iDCs or DCs treated with agarose induced CD25/CD69 expression on T cells at levels higher or similar to mDCs or other biomaterials treatments, whereas they exhibited co-stimulatory molecule expression lower than mDCs or other biomaterial treatments in our previous studies.^{12,14,54} These all indicate that co-stimulatory molecule expression of DCs

may be not necessary for induced expression of both CD25 and CD69 activation markers on T cells.

4.3. Cytokine release in Th1/Th2 polarization

Treatment of DCs with different biomaterial films modulated cytokine release from either the culture of DC alone or the co-cultures with autologous T cells (Figure 6 or 7, respectively). For DC treatment with biomaterials (without autologous T cell addition), the cytokine profiles were the same at day 14 (data not shown) as they were at day 6 (Figure 6). However, as seen in Figure 7, once autologous T cells were added in co-cultures (from day 6 through day 14) with biomaterial-treated DCs, the cytokine profiles were changed from those of the control cultures of only DCs without T cells. Interleukin-12p70, a heterodimeric and bioactive form of IL-12, is well known as a Th1 cytokine⁷⁹ while IL-10 is well known as a regulatory and immunosuppressive cytokine of the Th2 response.⁸⁰ Interestingly, a biomaterial employed in an acute cutaneous partial-thickness wound model (*in vivo*) concurrently induces both IL-10 and pro-inflammatory cytokines such as IL-12p70 expressions to modulate the balance between pro-and anti-inflammatory reactions during the course of wound healing.⁸¹ Thus, in consideration of observations in CD4+CD25+Foxp3 expression in Figure 5, both Th1 and Th2 cytokine expressions by the co-culture of agarose-treated DCs and nMNCs in Figure 7 indicate that agarose can be involved in a wound healing or immunosuppressive reactions rather than Th1-biased or immunogenic T cell polarizations.

Interferon- γ is also known as a cytokine representative for Th1 response and has been recently accepted for immunotherapeutic tool for targeting tumors.⁸² It has been reported that stimulation of T cells by DCs treated with CD40 ligand, adjuvants (aluminum hydroxide or LPS), or keyhole limpet hemocyanin (KLH) induced significantly high levels of IFN- γ production, in association with DC maturation phenotypes such as increased upregulation of co-stimulatory molecules or decreased endocytic ability.^{38,83–85} These reported effects are consistent with the results in this study and our previously reported result of an up-regulation of co-stimulatory molecules and decrease of endocytic capacity for DC treated with PLGA to levels very similar to the positive control of DCs treated with LPS.¹⁴ Thus the IFN- γ result in Figure 7 may be directly related with these DC phenotype changes upon treatment with PLGA.

4.4. T regulatory cell phenotype upon co-culture with iDCs or DCs treated with agarose

It has been generally accepted that if DCs present the signal 1 (peptide-MHC complexes) to T cells without the signal 2 (co-stimulatory molecule up-regulations), this condition leads to T cell anergy which leads to a tolerogenic response.^{86,87} Thus, given that DC treatment with agarose films maintained similar levels of endocytic ability to iDCs and did not result in upregulation of co-stimulatory molecule expression compared to iDCs,¹⁴ it would seem logical to follow that DCs treated with agarose film would induce T cell CD4+CD25+FoxP3+ expression at levels very similar to iDCs (Figure 5).

However, at the same time, agarose treatment of DCs modulated release of the immunogenic Th1 cytokine, IL-12p70, as well as the immunosuppressive Th2 cytokine, IL-10, at levels

significantly higher than iDCs and/or DCs treated with most other biomaterial films (Figures 6 and 7). Ghosh et al.⁸⁸ reported that Th1 or Th2 cytokine release was modulated depending on toll-like receptors (TLRs) or T cell receptor (TCR) activation on human PBMCs (including DCs, B, and T cells). Therefore, to further understand cytokine release from biomaterial-treated DC and T cell co-culture, future work needs to be performed focused on signaling pathways related to engagements of pattern recognition receptors (PRRs) (such as TLRs) or TCR activation, depending on DC treatment with different biomaterial films.

4.5. Translation of observations

In this study, DCs pre-treated with each biomaterial were statistically compared to untreated iDCs for all observations of effects on phenotypic outcomes of T cell polarization and phenotype. Because DCs were derived from adherent MNCs while T cells were derived from non-adherent MNCs obtained from the same origin (PBMCs) of each donor (of total 6 donors), this protocol suggests insight to T cell phenotypic outcomes to be expected upon biomaterial introduction to a host. For instance, we previously found that biomaterial-specific modulation of *in vitro* 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*.³¹ Therefore, PLGA may be useful for anti-cancer (immunogenic) therapy through the Th1 polarization induced by IFN- γ . Conversely, DCs treated with biomaterials such as agarose, which maintains DCs as immature and shifts T cell responses towards Th1, Th2 and T regulatory cell induction, are expected to be useful in the context of wound healing or avoiding immune responses (or inducing tolerance)^{89–91} to cellular antigens associated with tissue engineering or cell transplantation scenarios. An *ex vivo* approach is also conceivable. For instance, it is envisioned that autologous DCs and/or T cells, would be treated *ex vivo* with appropriately selected biomaterials such as PLGA or agarose, which could be in the presence of antigen for which immune responses would want to be boosted (e.g. tumor cell lysates) or induced for tolerance (e.g. transplanted cells), and then adoptively transferred into the host for desired immunomodulatory outcomes. Interestingly, the agarose film used in our previous and this study exhibited the highest endotoxin levels (2.195 ± 0.3 EU/ml) among all biomaterial films but this or lower levels of the endotoxin from all biomaterial films were not related with DC phenotype changes upon treatment with biomaterial films in our previous study.¹⁴ Moreover, there is no scientific report currently available regarding effects of these low levels of endotoxin on phenotype changes of human monocyte-derived DCs. For these reasons, the observed biomaterial effects on DC phenotypes are expected to be due to the different biomaterials used to treat the cells and not endotoxin (albeit low levels) associated with these materials.

Thus, the *in vitro* study presented herein, we accessed T cell marker expression and release of different cytokines that define T cell polarizations, and in thus explain the immunological T cell consequences of DC contact with these biomaterials with a co-delivered antigen, as exemplified in the differential adjuvant effects of PLGA and agarose observed *in vivo*.³¹ Implications of this study can be further probed using DC depletion protocols in mice, T cell subset transgenic mice, translation-relevant antigen/biomaterial combinations for *in vitro* and *in vivo* studies, and co-culture with defined T populations, such as memory T cells for their expansion.

5. CONCLUSION

Differentially biomaterial-treated DCs were able to direct distinct CD4⁺ helper T-cell polarizations. Upon co-culture with autologous T cells, DCs treated with PLGA film induced release of the Th1 cytokine, IFN- γ . Interestingly, DCs treated with agarose films induced Th1 (IL-12p70) and Th2 (IL-10) polarization as well as CD4⁺CD25⁺FoxP3⁺ T cells in the co-cultures with autologous T cells. Dendritic cells treated with HA films actually suppressed the CD4⁺ T cell expression in co-cultures. The observations in the study presented herein provide an explanation bridge from our previous *in vitro* results¹⁴ of DC maturation upon treatment with PLGA films and our previous *in vivo* results^{29–31} wherein PLGA exhibited specific adjuvant effects associated with an antigen model, OVA.

All observations in the study presented herein may not be directly translated into *in vivo* environment wherein multicellular interactions and a variety of proteins are involved in a biomaterial implantation. However, the two key cellular players in mounting T cell stimulation to antigen are used here. In consideration of our previous findings that a direct contact between DCs and biomaterial surface or proteins adsorbed on the PLGA surface was necessary for DC maturation,^{13,32} the hydrophobic surface of PLGA, as compared to the hydrophilic surface of agarose, is at least partially involved in T cell phenotype and polarization observations that are clearly different between these two biomaterials.

Thus, the observed multifunctional effects of DCs treated with the different biomaterial films on autologous T cell-mediated phenotype and polarization suggest a means of determining T cell-mediated immune responses in the context of biomaterials. An understanding of T cell activation or polarization associated with biomaterial-induced DC phenotype is expected to provide key information for selection of biomaterials for use in the combination products for tissue engineering or vaccine delivery. Furthermore, this study indicates a novel methodology to develop applications in immunotherapeutic tools. For example, pretreating immune cells with desired biomaterial to either boost immunogenicity or induce tolerance *ex vivo*, and then adoptively transferring this immunity back into the host for desired immunomodulatory outcomes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1a

Figure 1b

Figure 1.

Schematic representation of the study procedure (Figure 1a) and time line (Figure 1b).

During 14 days, the study has been performed based on three main procedures as shown by pattern-coded blocks.

□: 1st blood collection and DC culture on biomaterial films (with OVA)

□□□: 2nd blood collection and collection of non-adherent mononuclear cells (nMNC) and DC culture on biomaterial films (with OVA)

■: Co-culture (DC & T) procedure

Figure 2.

Geometric mean fluorescence intensity (gMFI) of CD4, CD8, CD25, & CD69 expression for autologous CD3+ T cells upon co-culture with DCs treated with different biomaterial films and OVA antigen. Treatment control ratios to the negative control of untreated CD3+ T cells (nMNCs) are shown with mean $\pm$ SD, n=6 donors (6 independent experiments with different donors). ★: $p < 0.05$, compared to control and higher than control; ☆: $p < 0.05$, compared to control and lower than control; Brackets: $p < 0.05$, statistically different between two T cells for DC treated with different biomaterial films.

Figure 3.

Representative quadrant dot plots for autologous T (CD3+) cell markers after co-culture with DCs treated with different biomaterial films and OVA antigen. Since PLGA and Agarose have been shown with opposite results on DC phenotype changes from our previous study, results of T cell markers from only these two biomaterial films are shown here. Representative plots from one donor were selected from all 6 donors. Y-axis for all plots indicates CD4 expression while X-axis for all plots in each column indicates CD8, CD25, or CD69 expressions as shown. The quadrant setup were decided based on the unstained CD3+ MNCs (top) and then applied to all controls and treatments for direct

comparison. Numbers shown together with the cross in each plot indicate the percentage of dots for each quadrant for dual label expression.

Figure 4.

Percentage numbers in double positive quadrant dot plots (Figure 3) of CD4 & CD8, CD4 & CD25, CD4 & CD69 expression on autologous CD3+ T cells upon co-culture with DCs treated with different biomaterial films and OVA antigen. Treatment control ratios to the negative control of untreated CD3+ T cells (nMNCs) are shown with mean±SD, n=6 donors (6 independent experiments with different donors). ★: $p < 0.05$, compared to control and higher than control; Brackets: $p < 0.05$, statistically different between two T cells for DCs treated with different biomaterial films.

Figure 5.

FoxP3 expressions on autologous CD3⁺ T cells upon co-culture with DCs treated with different biomaterial films (PLGA or agarose) and OVA antigen. Representative plots for one donor (different from the donor selected for Figure 3) were selected from all 6 donors. Y-axis for all plots indicates CD25 expression while X-axis for all plots indicates CD4 expression as shown (Figure 5a). Numbers shown in each quadrant indicate the percentage of events for each quadrant. Representative histograms (from all 6 donors) for intracellular Foxp3 expression on CD4⁺CD25⁺ T cells gated from the quadrant dot plots above (solid line) and isotype control for Foxp3 antibody (dotted line) (Figure 5b). Geometric mean fluorescence intensity (gMFI) of Foxp3 expressions on CD4⁺CD25⁺ T cells gated above for all donors (Figure 5c). TGF MNC indicates nMNCs treated with TGF- β to induce Foxp3⁺ from CD4⁺ T cells. gMFIs of the isotype for Foxp3 were subtracted from gMFIs of Foxp3 per control or treatment and then, treatment control ratios to the negative control of untreated CD3⁺ T cells (nMNCs) are shown with mean $\pm$ SD, n=6 donors (6 independent experiments with different donors). ★: p = 0.05, statistically higher than control (=1);

Bracket: $p < 0.05$, statistically different between two T cells for DC treatments with different biomaterial films.

Figure 6.

Geometric mean fluorescence intensity (gMFI) of cytometric bead array (CBA) for interferon (IFN)-gamma, IL-12p70, IL-10, IL-4 release for DCs treated with different biomaterial films with OVA antigen. To set the stage before co-culture with T cells, cytokines were measured using the supernatant saved on day 6 (after 24 hour-treatment of DCs with biomaterial films and OVA antigen). Treatment control ratios to the negative control of immature DCs are shown with mean $\pm$ SD, n=6 donors (6 independent experiments with different donors). ★: $p < 0.05$, compared to iDCs and higher than iDC; ☆: $p < 0.05$, compared to iDCs and lower than iDC; Brackets: $p < 0.05$, statistically different between two DCs treated with different biomaterial films.

The original concentration ranges of these cytokines (before normalization to the negative control) are shown in Table S1.

Figure 7.

Geometric mean fluorescence intensity (gMFI) of cytometric bead array (CBA) for interferon (IFN)-gamma, IL-12p70, IL-10, IL-4 release upon co-culture of autologous T cells and DCs treated with different biomaterial films in the presence of OVA antigen. Cytokines were measured using the supernatant saved on day 14 (after 8 days of DC-T co-culture). To compare the final cytokine levels in large variations (donor effects) between blood donors, data were corrected for cytokine levels present in cultures of only DCs with respective treatments (each biomaterial-treated DC control culture collected at day 14) and subtracting cytokine levels present in cultures of T cells alone (MNC negative control). Normalized ratios are shown with mean $\pm$ SD, n=6 donors (6 independent experiments with different donors). Brackets: p < 0.05, statistically different between two T cells co-cultured with DCs treated with different biomaterial films.

The original concentration ranges of these cytokines (before normalization to the negative control) are shown in Table S2.

Table 1

Samples and controls used in the present study herein – DC treatment and co-culture with autologous non-adherent mononuclear cells (nMNCs) (Table 1a) and controls to the co-culture (Table 1b).

Table 1a – iDC or mDC indicates a specific DC phenotypes of immature DC (treated only with OVA) or mature DC (treated with LPS/OVA) respectively as controls, whereas DC treated with each biomaterial film (or each biomaterial film-treated DC) does a specific DC phenotype upon treatment with each biomaterial film/OVA for 24 hours. These all DCs were isolated from each biomaterial film and extracellular OVA after 24 hour-treatment, and then co-cultured with nMNCs from day 6 through day 14 (Group 1). On day 12, another DCs (2nd DC set derived from 2nd blood collection) were added into co-culture wells to re-stimulate autologous nMNCs. In addition, only untreated nMNCs (untreated nMNC control) were cultured from day 6 to day 14 in the absence of DCs. Arrow indicates a mixture (co-culture) of each DC control or treatment and nMNCs.

Table 1b – all DC controls and treatments were made and then all DCs were isolated from each biomaterial film and extracellular OVA after 24 hour-treatment in the same way as above (Table 1a). However, each DC control or treatment was left without co-culture with nMNCs from day 6 through day 14 as controls (Group 2) to respective co-culture above.

(a)		
DC controls or treatment with biomaterial films/OVA for 24 hours (1 st DC set for day 5 to day 6 or 2 nd DC set for day 11 to day 12)	Mixed with nMNCs on day 6 or day 12	Group 1 (from day 6 through day 14)
No DC	→	Only nMNCs (untreated nMNC control)
Untreated DC but only treatment with OVA as a control	→	Co-culture with nMNCs (control)
iDC treated with LPS/OVA as a control	→	Co-culture with nMNCs (control)
iDC treated with PLGA film/OVA	→	Co-culture with nMNCs
iDC treated with chitosan film/OVA	→	Co-culture with nMNCs
iDC treated with alginate film/OVA	→	Co-culture with nMNCs
iDC treated with HA film/OVA	→	Co-culture with nMNCs
iDC treated with agarose film/OVA	→	Co-culture with nMNCs
(b)		
DC controls or treatment with biomaterial films/OVA for 24 hours (1 st DC set for day 5 to day 6 or 2 nd DC set for day 11 to day 12)	Left unmixed with nMNCs	Group 2 (from day 6 through day 14)
Untreated DC but only treatment with OVA as a control	→	Only each DC control or treatment in the absence of nMNCs
iDC treated with LPS/OVA as a control	→	
iDC treated with PLGA film/OVA	→	
iDC treated with chitosan film/OVA	→	
iDC treated with alginate film/OVA	→	
iDC treated with HA film/OVA	→	
iDC treated with agarose film/OVA	→	