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Lymphocyte Adhesion and Interactions with Biomaterial Adherent Macrophages and Foreign Body Giant Cells

David T. Chang¹, Erica Colton², Takehisa Matsuda³, and James M. Anderson^{1,2,4}

¹Department of Biomedical Engineering, Case Western Reserve University, Cleveland, OH, 44106

²Department of Pathology, Case Western Reserve University, Cleveland, OH, 44106

³Genome Biotechnology Laboratory, Kanazawa Institute of Technology, Ishikawa 921-8501, Japan

⁴Department of Macromolecular Science, Case Western Reserve University, Cleveland, OH 44106

Abstract

To characterize the effects of adherent macrophages and biomaterial surface chemistries on lymphocyte adhesion and activation, lymphocytes were co-cultured with monocytes alone and together, directly and separated by a porous membrane transwell on hydrophobic, hydrophilic/neutral, hydrophilic/anionic, and hydrophilic/cationic biomaterial surfaces. Surface adherent cells were quantitatively analyzed after 3 days utilizing immunofluorescence and phase contrast imaging. After periods of 3, 7, and 10 days, secreted interferon- γ (IFN- γ) was quantified by ELISA. Limited direct biomaterial-adherent lymphocytes were identified regardless of the presence of macrophages or foreign body giant cells (FBGC). The majority of adherent lymphocytes, which were T cells (> 95%) rather than natural killer cells, predominantly interacted with adherent macrophages and FBGCs; greater than 90% were interacting on surfaces with higher levels of adherent macrophages and FBGCs and greater than 55% were interacting on surfaces with lower levels of macrophages and FBGCs. The hydrophilic/anionic surface promoted higher levels of macrophage- and FBGC-adherent lymphocytes but was nonselective for lymphocyte subtype interactions. The hydrophilic/neutral surface was selective for CD4+ T lymphocyte interactions while the hydrophobic surface was selective for CD8+ T lymphocyte interactions. IFN- γ was produced in direct and indirect co-cultures but not in lymphocyte- and monocyte-only cultures suggesting that lymphocytes are activated via macrophage-derived cytokines rather than direct biomaterial contact. Direct lymphocyte interactions with adherent macrophages/FBGCs enhanced IFN- γ production relative to indirect co-cultures. These results suggest that lymphocytes prefer interactions with adherent macrophages and FBGCs, resulting in lymphocyte activation, and these interactions can be influenced by biomaterial surface chemistries.

Keywords

biomaterials; lymphocyte activation; adhesion; macrophages and foreign body giant cells; cytokines

Introduction

Implantation of synthetic biomaterials elicits a foreign body reaction consisting of monocyte adhesion, differentiation to macrophages, and subsequent macrophage fusion to form foreign body giant cells (FBGC). Lymphocytes transiently appear at the implant site during the inflammatory response but the lymphocyte response to biomaterials is still unclear.¹ Lymphocytes have been shown to influence macrophage behavior at biomaterial surfaces *in vitro* through enhancement of monocyte/macrophage adhesion, macrophage fusion, and macrophage activation.^{2,3} These effects are mediated by indirect and direct lymphocyte interactions with monocytes, macrophages, and FBGCs, but we still do not have a full mechanistic understanding of these interactions. There is also evidence of lymphocyte activation from exposure to implanted prostheses such as silicone gel breast implants and left ventricular assist devices (LVAD) can impact immune function.⁴⁻⁶ Patients with LVADs have shown an increased risk of infection as well as the presence of auto-reactive antibodies.⁷ The elucidation of lymphocyte behavior and interactions are vital as we continue to develop novel biomaterials, devices, and other therapeutic technologies utilizing synthetic materials.

Lymphocytes are white blood cells that perform a variety of actions in the immune system to protect the body from foreign antigen. Subtypes of lymphocytes include T lymphocytes (T cells), B lymphocytes (B cells), and natural killer cells (NK). Lymphocytes within the same class can be capable of performing several functions and thus have more specific nomenclature based on their differentiating characteristics. T cells can directly kill infected cells through induction of apoptosis. These cells possess CD8 markers on their cell surface and are referred to as CD8⁺ T suppressor/cytotoxic cells. T cells carrying CD4 markers are known for their ability to affect other cell types by producing cytokines and chemokines. CD4⁺ T cells can be type 1 T helper cells (Th1) which secrete IFN- γ and TNF- β or type 2 T helper cells (Th2) which produce IL-4, IL-5, IL-10, and IL-13. B cells contribute to the immune response by generating antibodies that are specific to the antigen. NK cells are capable of mediating cell death as well as secreting cytokines such as IFN- γ . The cell surface marker CD56 differentiates NK cells from the other white blood cells. All of these cell types circulate in the peripheral blood surveying the body for foreign invaders.

IFN- γ , IL-4, and IL-13 are lymphokines secreted upon activation and are capable of modulating macrophage responses. IFN- γ is known to activate macrophages and polarize them towards a pro-inflammatory state which is reflected by their upregulation in capability to present antigen, phagocytose, and produce pro-inflammatory cytokines and effector molecules.⁸⁻¹¹ IL-4 and IL-13, on the other hand, induce macrophage activation to promote a downregulatory response which is shown by enhanced production of anti-inflammatory IL-10 and expression of non-opsonic receptors such as the mannose receptor.^{8,11}

Lymphocytes are capable of adhering onto surfaces composed of synthetic materials and are affected by surface properties. The use of hemodialysers composed of different synthetic polymers showed differential lymphocyte elution profiles after hemodialysis.^{12,13} Similarly, various polymers have shown the capability to retain specific subtypes of lymphocytes in the context of *in vitro* column separation methods.^{14,15} Additionally, lymphocyte adhesion on materials pre-adsorbed with proteins such as fibronectin and vitronectin showed the differential effects of biomaterial and adsorbed protein.¹⁶⁻¹⁸ More recently, Ito et al. demonstrated that an electrically charged polymer could control lymphocyte adhesion.¹⁹ However, these results provide limited information on lymphocyte adhesion behavior on biomaterial surfaces after implantation.

In this study, lymphocyte adhesion behavior and activation from interactions with adherent macrophages and FBGCs on biomaterial surfaces was investigated. Lymphocyte activation was measured by production of lymphokine IFN- γ . We hypothesized that lymphocytes adhere to biomaterial surface and that adhesion is enhanced by the presence of adherent macrophages. Additionally, lymphocytes are activated by interactions with adherent macrophages and FBGCs. To explore this, human peripheral blood lymphocytes were cultured alone and in co-culture with biomaterial-adherent monocytes, macrophages, and FBGCs and exposed to different biomaterial surfaces *in vitro*. We quantitatively evaluated and characterized the effect of adherent macrophages and biomaterial surface chemistries on lymphocyte subsets on the surfaces after a specified culture period and production of IFN- γ . This study provides information in regard to lymphocyte behavior at biomaterial surfaces and the effects biomaterial surface characteristics have on cell-cell and cell-material interactions.

Materials and Methods

Materials

24-well tissue culture polystyrene (TCPS) plates were acquired from Fisher Scientific (Pittsburgh, PA). Macrophage serum-free medium (SFM) was obtained from Invitrogen, Grand Island, NY. Mouse anti-human CD14 (clone M5E2) and mouse anti-human CD8 (clone UCHT4) were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), mouse anti-human CD56 (clone 123C3) from Abcam, Inc. (Cambridge, MA), goat anti-human CD4 from R&D Systems (Minneapolis, MN), and normal goat and mouse control IgG from Santa Cruz Biotechnology, Inc. Other reagents were obtained as follows: RNase A from EMD Biosciences, Inc. (La Jolla, CA), Alexa Fluor 594 donkey anti-mouse and anti-goat IgG from Invitrogen, YO-YO-1 from Molecular Probes (Eugene, OR), donkey serum from Sigma (St. Louis, MO), and Gel/Mount from Biomed (Foster City, CA).

Preparation of Biomaterial Surfaces

Mylar® PET surfaces were modified as described previously.²⁰ PET surfaces were coated with poly(benzyl N,N-diethyldithiocarbamate-co-styrene) (BDEDTC) to provide an additional hydrophobic surface. Subsequent UV polymerization to form polyacrylamide (PAAm), sodium salt of polyacrylic acid (PAA⁻Na), and methiodide of poly(dimethylaminopropylacrylamide) (DMA⁺PAAmMeI) provided material substrates with distinct hydrophilic/neutral, hydrophilic/anionic, and hydrophilic/cationic surface chemistries, respectively. The PET-based photograft copolymerized surfaces were cut into 1.5 cm diameter disks for sterilization by 100% ethanol prior to insertion into 24-well TCPS plates. Rings cut from silicone tubing of O.D. 5/8 and I.D. 3/8 (Cole Parmer, Vernon Hills, IL) were sonicated for 5 minutes in 100% ethanol, autoclave sterilized, and used to secure the material surfaces at the bottom of the well. Phosphate-buffered saline containing magnesium chloride and calcium chloride (Invitrogen) (PBS⁺⁺) was utilized to wash and remove any residual ethanol on the material surfaces.

Monocyte and Lymphocyte Cell Culture

Monocytes and lymphocytes were isolated from peripheral blood of healthy non-medicated donors by a non-adherent method as previously described²¹ and suspended separately in SFM containing L-glutamine, antibiotics, antimycotics, and 20% autologous serum (AS). As determined by flow cytometry and shown previously,²² the isolated monocyte population contains, on average, 60.5% $\pm$ 9.1% monocytes and 39.5 $\pm$ 9.1% contaminating lymphocytes while the isolated lymphocyte population consists of, on average, 51.3% $\pm$ 4.1% CD4⁺ T lymphocytes, 30.8% $\pm$ 5.2% CD8⁺ T lymphocytes, 10.7% $\pm$ 1.1% NK cells, 5.6% $\pm$ 0.5% B lymphocytes, and 1.6% $\pm$ 0.1% contaminating monocytes.

For analysis of lymphocyte adhesion, monocytes were plated onto the PET, PAAm, and PAAmNa surfaces at 5×10^5 (100%), 2.5×10^5 (50%), and 0.5×10^5 (10%) cells in 0.25 mL SFM with 20% AS, and allowed to incubate for 2 hours at 37°C and 5% CO₂. After a subsequent PBS⁺⁺ wash to remove non-adherent cells, 1.5×10^6 lymphocytes (100%) in 0.5 mL SFM with 20% AS were added to each well with lymphocyte/monocyte ratios designated as 100%/100% (1/1.0), 100%/50% (1/0.5), and 100%/10% (1/0.1). Additionally, 1.5×10^6 lymphocytes in 0.5 mL SFM with 20% AS were plated onto PET surfaces (1/0). After 3 days, adherent cells were washed twice with PBS⁺⁺ and fixed with acetone for 2 minutes at -20°C.

For quantification of lymphokine production, 5×10^5 monocytes in 0.25 mL SFM with 20% AS were plated onto PET, BDEDTC, PAAm, PAAmNa, and DMAPAAmMeI surfaces and allowed to incubate for 2 hours at 37°C and 5% CO₂. After the 2 hour incubation, non-adherent cells were removed by a PBS⁺⁺ wash leaving adherent monocytes which were then cultured with and without 1.5×10^6 lymphocytes. The 1.5×10^6 lymphocytes in 0.5 mL SFM with 20% AS were plated alone, co-cultured directly with adherent monocytes on the 5 biomaterial surfaces, and co-cultured indirectly with adherent monocytes via 0.02 µm membrane pore transwell inserts (Nunc, Naperville, IL). Supernatants were collected after 3, 7, and 10 days of culture, centrifuged at 3000 rpm to remove cells/debris, and cell-free supernatant stored at -20°C for cytokine analysis.

Lymphocyte Subtype Identification by Immunofluorescence

Adherent cells on material surfaces were prepared for immunofluorescent staining by fixation with acetone for 2 minutes at -20°C and air-dried. Samples were stored at 4°C prior to staining. PBS⁺⁺ was utilized as the diluent and wash buffer in all required staining steps. Samples were first treated with RNase A (100 µg/mL) for 1.5 hours at 37°C and then washed 3 times for 5 minutes each. Next, nonspecific sites were blocked with 10% donkey serum for 1 hour at 37°C. Then, primary detecting antibodies for CD14, CD8, CD4, CD56 and isotype-matched control IgG were applied at 15 µg/mL diluted with 1% donkey blocking serum for 1 hour at 37°C. After 4 washes at 5 minutes each, a secondary staining solution of YO-YO-1 (0.1 µmol/L diluted at 1:10,000 in PBS⁺⁺) and either Alexa Fluor 594 donkey anti-mouse or anti-goat IgG (20 µg/mL diluted at 1:100 in PBS⁺⁺) was added for 1 hour at 37°C. Samples were then washed 3 times for 10 minutes each. Surfaces were removed from the 24-well plates, transferred to glass slides, and mounted under glass coverslips using Gel/Mount. Samples were imaged by fluorescence microscopy (Olympus IX71) using the Olympus Microsuite software with settings that blacken residual background fluorescence from corresponding nonspecific control antibodies. Positively detected cells were quantified in 10 fields under 40X objective. Utilizing YO-YO-1 to visualize nuclei and phase contrast imaging of the same field, localization and physical interactions of identified cells were evaluated.

Quantification of Lymphokine Production

Production of IFN-γ, IL-4, and IL-13 were quantified by ELISA (R&D Systems, Minneapolis, MN), performed according to manufacturer's instructions, and measured by an EL808 ultra microplate reader with KC Junior software (Bio-Tek Instruments, Inc., Winooski, Vermont).

Statistical Analysis

Multiple human donors were utilized to account for donor variability. All results were presented as an average ± the standard error of the mean (SEM) (n = 3). Statistical analysis was performed utilizing Minitab statistical software (Minitab Inc., State College, PA) and

statistically significant differences were determined by ANOVA and the Tukey post hoc test.

Results

Differential Effects on Lymphocyte Adhesion

We utilized an immunocytochemical staining approach with fluorescent microscopy to quantitatively evaluate lymphocyte adhesion on model biomaterial surfaces. All surfaces were examined after a period of 3 days sufficient for monocyte to macrophage development with possible foreign body giant cell development. Anti-CD4 antibodies were used to identify T helper cells, anti-CD8 to identify T suppressor cells, and anti-CD56 to identify NK cells. Because B cells were such a small percentage of the isolated lymphocytes, they were not pursued.

Examination of the total adherent lymphocyte density in the different co-cultures on each of the biomaterials showed significantly higher (> 4.5 fold) lymphocyte adhesion in lymphocyte/monocyte co-culture ratios of 1/0.5 and 1/1.0 on the hydrophilic/anionic (PAANa) surface compared to the other surfaces (Figure 1A). However, the PAANa surface was similar to the PAAm surface in that both evoked 10 fold increases in adherent lymphocyte density from 1/0.1 to 1/1.0 co-cultures while PET elicited an increase of less than 2 fold. In co-cultures with initial low monocyte levels, very few lymphocytes were adherent ($< 50/\text{mm}^2$). These adherent lymphocytes were interacting with adherent macrophages and FBGCs in densities shown in Figure 1B. The ratios of adherent lymphocyte densities to adherent macrophage and FBGC densities (Table 1) further illustrate the biomaterial differences. PAANa evoked the highest ratios, but PAAm also had similar higher ratios. PET ratios were extremely low (> 3 fold decrease compared to the other two surfaces).

Monocytes and macrophages are known to readily adhere to biomaterial surfaces and were identified by anti-CD14 antibodies. As expected in co-cultures of 1/0 and 1/0.1 lymphocyte/monocyte ratios, the number of adherent macrophages after 3 days was minimal or below $50/\text{mm}^2$. Despite the increase in the initial plating of monocytes, PAAm evoked the lowest level of adherent macrophage and FBGC density after 3 days illustrated most clearly in 1/0.5 and 1/1.0 co-culture concentration ratios (> 5 fold decrease relative to other surfaces). As the initial plating of monocytes increased, the PET and PAAm surfaces increased adherent densities ~ 10 fold over 1/0.1 to 1/1.0 co-cultures while PAANa evoked a 70 fold increase in macrophage and FBGC adherence. PET and PAANa surfaces both promoted macrophage/FBGC adhesion. The 3 fold difference in adhesion seen in 1/0.5 co-cultures can at least be partially explained by the induction of macrophage fusion on PAANa. As Figure 1B shows, FBGC formation occurred more on hydrophilic surfaces (PAANa and PAAm) but minimally on PET ($< 5\%$). Although the percentage of FBGCs ($\sim 20\%$ in 1/0.5 and $\sim 15\%$ in 1/1.0 co-culture) was similar between PAANa and PAAm, fusion occurred at the highest level on PAANa in terms of number and size of FBGCs.

Figure 2 illustrates how the adhesion of lymphocytes depends on the actual density of adherent macrophages and FBGCs on the biomaterial surface. Adherent lymphocyte density increased to a limit as the adherent macrophage/FBGC density increased to a density of 100 macrophages and FBGCs/ mm^2 .

Adherent Lymphocyte Subtypes and Interactions

Adherent lymphocytes included CD4+ and CD8+ T cells and CD56+ natural killer cells (Figure 3A). CD4+ and CD8+ T cells were found on all surfaces while CD56+ cells were only found on PET and PAANa surfaces. Although CD56+ cells were capable of adhering

and associating with macrophages, they accounted for less than 5% of the detected adherent lymphocytes which was over 2 fold lower than the percentage of CD56+ NK cells initially plated (Figure 3B). The majority (> 95%) of the adherent lymphocytes were CD4+ and CD8+ T cells. The percentages of CD4+ and CD8+ T lymphocytes on the PAANa surface were essentially the same as those from the original lymphocyte isolation, whereas on the hydrophilic/neutral PAAm surface, the percentage of CD8+ cells was less than half that of the lymphocyte isolation that was initially plated. PET showed less than half the lymphocyte isolation percentage of CD8+ T cells in co-cultures with low levels of adherent macrophages. However, as the adherent macrophage density increased, the level of CD8+ T lymphocytes increased 2 fold over the percentage in the lymphocyte isolation while CD4+ T lymphocytes decreased 2 fold from the lymphocyte isolation.

After identifying the lymphocyte subtypes on the biomaterial surfaces, we examined whether they were adhering to adherent macrophages and FBGCs or interacting solely with the biomaterial surface. Utilizing the nucleic acid stain YO-YO-1 to visualize nuclei and overlaid phase contrast images of the same field to visualize the cells and their morphology, identified lymphocytes could be localized. Representative results are shown in Figure 4 in co-cultures after a period of monocyte to macrophage development and potential FBGC development (3 days). On all surfaces where the monocyte isolation was plated, there were initial 2 hour incubations for monocyte adhesion. Visualization of PET surfaces after the initial two hour incubation for the monocyte isolation showed that although lymphocytes were present as contaminants in the monocyte isolation and during the initial exposure to the PET surfaces, these lymphocytes were not adherent to monocytes or the surface (data not shown).

After 3 days of culture, lymphocytes exposed to the biomaterial surfaces with or without the presence of monocytes, macrophages, or FBGCs were found adhering directly and solely to the biomaterial surface (i.e. biomaterial-adherent). However, as Figure 5A shows, direct biomaterial-adherent lymphocytes were low (< 50 lymphs/mm²) regardless of the monocyte concentration plated. Lymphocytes at the biomaterial surface predominantly adhered to macrophages or FBGCs (> 90%) when there were significant numbers of adherent macrophages and FBGCs in co-culture (Figure 5B). When levels of adherent macrophages and FBGCs were low, the percentage of biomaterial-adherent lymphocytes was higher. This was further supported by the PAAm surface at higher monocyte concentration co-cultures. Despite abundant monocytes exposed to the PAAm surface (e.g. 1/0.5 and 1/1.0 co-cultures), macrophage adhesion was inhibited compared to the other surfaces (Figure 1B). The percentage of biomaterial-adherent lymphocytes was highest on PAAm compared to the other two monocyte, macrophage, and FBGC adhesion promoting surfaces. For instance, there were approximately 40% biomaterial-adherent lymphocytes in the 1/0.5 co-cultures.

Lymphocytes were capable of adhering to both adherent macrophages as well as adherent FBGCs as shown in Figure 5. Cell-associated lymphocytes on hydrophobic (PET) and hydrophilic/neutral (PAAm) surfaces were virtually all adhering to macrophages since these surfaces do not readily facilitate FBGC formation. Although FBGCs do form on PAAm, the numbers are few. On the other hand, the hydrophilic/anionic surface (PAANa) evoked abundant FBGC formation. On PAANa, not only did the lymphocytes interact with macrophages, they also were adhering to FBGCs. The number and percentage of FBGC-adherent lymphocytes increased with the number of monocytes initially plated in co-culture. Up to 40% of the adherent lymphocytes on PAANa were observed to be interacting with FBGCs despite FBGCs accounting for < 25% of the adherent macrophages and FBGC. On PAAm, < 5% of adherent lymphocytes were observed to be interacting with FBGCs despite FBGCs accounting for 15-25% of adherent macrophages and FBGCs.

Table 2 shows the adherent lymphocyte population normalized to the number of respective adherent FBGC or macrophage populations. On the PAANa surface, the normalized FBGC-adherent lymphocyte population was more than 2.5 fold greater than the normalized macrophage-adherent lymphocyte population in the 1/0.5 co-culture ratio and more than 5 fold greater in the high initial monocyte co-culture ratio. On the PET and PAAm surfaces, the normalized macrophage-adherent lymphocytes were greater than normalized FBGC-adherent lymphocytes.

Lymphokine Production

Lymphokines IFN- γ , IL-4, and IL-13 were assayed in order to evaluate lymphocyte activation in the response to biomaterials. IFN- γ production over 3, 7, and 10 days from lymphocytes and monocytes in individual as well as co-cultures on PET-based photograft copolymerized surfaces was quantified and shown in Figure 6. IL-4 and IL-13 were not detected in any cultures by ELISA with detection sensitivity limits of less than 10 and 32 pg/mL, respectively.

Over 10 days of culture, virtually no IFN- γ was produced in lymphocyte and monocyte-only cultures while the co-cultures elicited IFN- γ . Indirect lymphocyte co-cultures with adherent monocytes evoked a relatively steady production of IFN- γ near or below 100 pg/mL over time and showed no material dependence or trends.

Lymphocytes over 3, 7, and 10 days in direct co-culture with adherent macrophages and FBGCs produced approximately 2-2.5 fold (PET), 1.5-2.5 fold (BDEDTC), 0.8-2.5 fold (PAAm), 3.5-5 fold (PAANa), and 1.3-1.5 fold (DMAPAAmMeI) greater IFN- γ than lymphocytes in indirect co-cultures. Thus, direct co-cultures elicited IFN- γ production which varied with material surface chemistry. Differences in hydrophilicity and hydrophobicity as well as charge affected the production of IFN- γ . The hydrophilic/anionic surface (PAANa) demonstrated the highest level of production showing a greater than 5 fold increase compared to the hydrophilic/neutral surface (PAAm) which evoked the least IFN- γ and greater than 2.8 fold increase relative to the level on the hydrophilic/cationic surface (DMAPAAmMeI) over all time points. PET and BDEDTC were both hydrophobic surfaces and showed similar levels of IFN- γ production (< 1.4 fold differences). The levels on these hydrophobic surfaces were lower (1.4 – 2.3 fold) than on the hydrophilic/anionic surface but greater than the amount of IFN- γ elicited (1.4 – 2 fold) on the two other hydrophilic surfaces, PAAm (neutral) and DMAPAAmMeI (cationic). Except for the hydrophilic/anionic surface (PAANa), the hydrophobic surfaces showed slightly higher IFN- γ levels than the hydrophilic surfaces. The levels of IFN- γ increased slightly after 3 days.

Discussion

Previous studies have demonstrated that lymphocytes are capable of modulating adherent macrophage and FBGC behavior^{2,3}; however, the reverse effects and the details of the lymphocyte response to biomaterial surfaces are currently unclear. This study investigated lymphocyte adhesion at the surface of biomaterials *in vitro* and lymphocyte activation as a result of interactions with adherent macrophages and foreign body giant cells. We showed that lymphocytes are capable of adhering directly to biomaterial surfaces, but to a limited extent. The majority of adherent lymphocytes were found to be interacting with adherent macrophages and FBGCs and the number, type of lymphocyte interactions, and extent of IFN- γ production was surface chemistry dependent.

Regardless of biomaterial surface chemistry or presence of adherent macrophages and FBGCs, lymphocytes predominantly adhered to adherent macrophages and foreign body giant cells rather than directly to the biomaterial surface. Groth et al, however, found that

surface wettability could influence direct lymphocyte adhesion on biomaterials.¹⁶ The disparate results are most likely to be due to differences in the adsorbed protein layer that the lymphocytes were exposed to on the surface of the biomaterial. Groth et al pre-adsorbed specific proteins such as fibrinogen, fibronectin, and vitronectin while we utilized autologous serum containing biological proteins that the biomaterial would encounter upon introduction into the body.

In this study we demonstrate the production of IFN- γ over 3, 7, and 10 days in lymphocyte and monocyte co-cultures suggesting that induction of activation occurs in at least a subset of the lymphocyte population. IFN- γ is secreted by CD4⁺ Th1 cells, CD8⁺ Tc1 cells, and NK1 cells.²³ The lack of production in lymphocyte- and monocyte-only cultures indicated lymphocyte interactions with biomaterial-adherent macrophages and FBGCs were required. However, because IFN- γ was induced in indirect co-cultures, direct lymphocyte and macrophage cell-cell interactions were not necessary. The results suggest that the lymphocyte population is activated via non-contact mechanisms. This is consistent with previous findings where induction of lymphocyte proliferation occurred through macrophage-derived soluble factors (i.e. indirect paracrine interactions).^{2,24} The specific proliferating lymphocyte population in these *in vitro* cultures, however, was not identified. Rodriguez has shown (unpublished data) that lymphocytes in a population of mononuclear cells cultured on biomaterial surfaces did not proliferate utilizing a more specific assay, and T cells specifically did not express activation cell surface markers (CD69 and CD25). There was donor variability as not all donors showed production of IL-2 and IFN- γ . Differences in cell culture ratios and conditions perhaps explain these inconsistent studies. Brodbeck et al. found that there were optimum co-culture ratios for examining lymphocyte/macrophage interactions.²⁴ Additionally, the dissociation between activation (e.g. cytokine secretion, calcium flux, and surface marker expression), and proliferation has also been demonstrated by a number of studies as alveolar macrophages have shown the ability to selectively inhibit lymphocyte proliferation despite evidence of activation.²⁵⁻²⁷ Furthermore, Cantrell et al. demonstrated that extent of activation is important as T cell proliferation requires higher levels of activation for extended periods of time.²⁸

In the immune response, T cells and NK cells are generally activated through contact with antigen; however, lymphocytes can also be activated via cytokine stimulation (i.e. antigen-independent mechanisms). Cytokines capable of activating T and NK cells to secrete IFN- γ independent of T cell receptor (TCR) ligation include a combination of IL-2, TNF- α , and IL-6 along with IL-12, IL-15, IL-18, and IL-21.²⁹⁻³⁰ IL-2 and IL-21 are products of activated lymphocytes while IL-12, IL-15, IL-18 can be produced by activated macrophages. The lymphocyte population in this study consisted of primarily T lymphocytes in addition to NK cells; both are capable of interacting with adherent macrophages and FBGCs and thus, both could contribute to IFN- γ production.

Synthetic materials utilized in clinical applications have shown evidence of lymphocyte activation. Katzin et al. found that lymphocytes in the fluid and tissue surrounding silicone gel breast implants were predominantly T cells, and relative to peripheral blood, a greater percentage of the T cells were HLA-DR⁺ and CD29⁺ indicating a state of immune activation.⁵ Additionally, left ventricular assist device (LVAD) patients have shown the development of B cell hyperreactivity and immune dysfunction leading to heightened risk of infection and potential autoimmune disorders due to biomaterial-activated T cells.⁵⁻⁷ Schuster et al. demonstrated the elevated presence of anti-HLA antibodies and soluble CD40L in LVAD patients indicating B cell activation.⁶ The results here are consistent in showing that lymphocytes can be activated by exposure to biomaterials through interactions with macrophages and FBGCs.

Biomaterial surface properties made a significant impact on the interactions occurring on the surface. The hydrophilic/anionic surface (PAANa) evoked the highest level of adherent lymphocytes, the majority of which were adherent to macrophages and FBGCs. In comparing the densities of adherent macrophages and FBGC density as well as densities of adherent lymphocytes on the hydrophilic/anionic PAANa, hydrophobic PET, and hydrophilic/neutral PAAm surfaces, we find that lymphocyte adhesion is not simply a function of the number of adherent macrophages or FBGCs. The results indicate that adherent macrophage and FBGC phenotype and secreted soluble products play a role. We demonstrated previously that the PAAm and PAANa surfaces are highly activating surfaces in terms of macrophage cytokine and chemokine production.³ These inflammatory mediators can lead to increased avidity and affinity of integrins for stronger or more stable lymphocyte interactions with adherent macrophages.³¹⁻³⁴ In addition to increased production of inflammatory cytokines and chemokines, macrophage activation also leads to upregulation of cell surface molecules (e.g. chemokine receptors and MHC) with enhanced capability for antigen presentation and interactions with lymphocytes.⁸

CD4⁺ and CD8⁺ T lymphocytes were the predominant lymphocyte subtypes adhering directly to biomaterial surfaces and to macrophages and foreign body giant cells. The paucity of CD56⁺ NK cells on the biomaterial surfaces does not rule out the fact that NK cells may be interacting at the surface. The few NK cells found on the surfaces were all interacting with adherent macrophages. These interactions could be merely transient or non-specific. Other studies have shown a predilection for CD3⁺ T lymphocytes to adhere onto surfaces pre-adsorbed with particular extracellular matrix proteins.¹⁷ The biomaterial surfaces utilized in this study demonstrated differences in terms of the percentage of CD4⁺ and CD8⁺ T lymphocytes interacting with adherent macrophages and FBGCs. The PAANa surface was not selective for CD4⁺ and CD8⁺ T lymphocytes as the percentages of each were similar to those from the lymphocyte isolation. PAAm, on the other hand, was less selective for CD8⁺ T cells as the percentage of these CD8⁺ T cells found on the surface were less than half that of the lymphocyte isolation we plated initially. PET showed greater selectivity for CD8⁺ T lymphocytes interactions. In previous studies involving column cell separation and dialysis, various material surfaces were also capable of selectively retaining lymphocyte subpopulations.¹³⁻¹⁵ Again, this could be the result of differences in adherent macrophage responses on the different biomaterial surfaces. The different activation levels and cytokine profiles evoked from adherent macrophages and FBGCs on each of the surfaces can result in different cellular targets, behavior, and interactions.³⁵

The level of IFN- γ production was found to vary on the different biomaterial surface chemistries suggesting that lymphocytes were differentially activated. This dependency on biomaterial surfaces is in agreement with many studies evaluating cellular responses to biomaterials. The hydrophilic/anionic (PAANa) surfaces have been shown to promote macrophage activation,³ fusion to form giant cells,^{22,36} and lymphocyte interactions with macrophages and FBGCs. In this study, we found that this surface also evoked a higher level of IFN- γ relative to the other surfaces. This result makes sense when we consider that the production of IFN- γ is induced by mediators produced by activated cells such as lymphocytes and macrophages, and PAANa was found to be an activating surface for macrophages. Additionally, IFN- γ was found to be significantly enhanced when lymphocytes were permitted to engage in direct cell-cell contact with adherent macrophages and giant cells on the PAANa surface more so than on all other surfaces investigated. This implies that the phenotypes of the lymphocytes, macrophages, and FBGCs on PAANa in regards to surface molecules may be distinct relative to the phenotypes on the other surfaces. For instance, lymphocyte surface molecules such as integrins can change in conformation as well as localization based on direct cell-cell interaction with macrophages and indirect interactions via cytokines and chemokines.³¹⁻³⁴ Additional studies are required to

investigate lymphocyte cell surface changes that occur upon interactions at biomaterial surfaces.

One of the primary differences in cellular behavior observed on PAANa compared to the other surfaces is its induction of macrophage fusion. Lymphocytes were observed to adhere to FBGCs in addition to macrophages primarily on this fusion promoting hydrophilic/anionic surface. We currently do not have a full understanding of the functional capabilities of foreign body giant cells. In addition to promoting degradation of biomaterial surfaces,³⁷ FBGCs have been shown to produce cytokines,³⁸ and display a wide range of cell surface antigens similar to macrophages.³⁹ FBGCs display many cell surface molecules such as major histocompatibility complex molecules (e.g. HLA-DR) capable of providing potential molecular interactions with lymphocytes.³⁹ It is possible that the FBGCs could possess enhanced lymphocyte activating capabilities. Therefore, future studies must continue to investigate FBGC functions, particularly in relation to lymphocyte interactions at implant sites.

Although synthetic biomaterials have already been utilized as biomedical implants, the full capability of biomaterials as components in therapeutic applications such as tissue engineering, regenerative medicine, or biomedical devices has yet to be determined. We are still in the process of gaining a complete mechanistic understanding of the complex signaling, cellular behavior and interactions that occur when these materials enter the body. During the foreign body reaction, lymphocytes have the opportunity to engage in direct interactions with both biomaterial and the adherent macrophages and FBGCs. In this study, all surfaces showed limited direct biomaterial-adherent lymphocytes regardless of the density of macrophages or FBGCs. Instead, lymphocytes predominantly adhered to macrophages and FBGCs. These cellular interactions lead to lymphocyte activation. The number, type of lymphocyte interactions with macrophages and/or FBGCs, and extent of lymphocyte activation were dependent on biomaterial surfaces. The results from this investigation broaden our understanding of the interactions that occur at surfaces of biomaterials and how biomaterials influence cell-cell and cell-material interactions as well as prompt future investigation into the specific molecular mechanisms involved in these interactions. The goal is that this information will provide us with tools for modulating biomaterial-mediated responses as well as potentially directing biological responses.

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References

1. Anderson JM. Biological responses to materials. *Annu Rev Mater Res*. 2001; 31:81–110.
2. Brodbeck WG, Macewan M, Colton E, Meyerson H, Anderson JM. Lymphocytes and the foreign body response: lymphocyte enhancement of macrophage adhesion and fusion. *J Biomed Mater Res A*. 2005; 74(2):222–9. [PubMed: 15948198]
3. Chang DT, Colton E, Anderson JM. Paracrine and juxtacrine lymphocyte enhancement of adherent macrophage and foreign body giant cell activation. *J Biomed Mater Res A*. 2008
4. Prantl L, Fichtner-Feigl S, Hofstaedter F, Lenich A, Eisenmann-Klein M, Schreml S. Flow cytometric analysis of peripheral blood lymphocyte subsets in patients with silicone breast implants. *Plast Reconstr Surg*. 2008; 121(1):25–30. [PubMed: 18176202]
5. Katzin WE, Feng LJ, Abbuhl M, Klein MA. Phenotype of lymphocytes associated with the inflammatory reaction to silicone gel breast implants. *Clin Diagn Lab Immunol*. 1996; 3(2):156–61. [PubMed: 8991629]

6. Schuster M, Kocher A, John R, Hoffman M, Ankersmit J, Lietz K, Edwards N, Oz M, Itescu S. B-cell activation and allosensitization after left ventricular assist device implantation is due to T-cell activation and CD40 ligand expression. *Hum Immunol.* 2002; 63(3):211–20. [PubMed: 11872239]
7. Itescu S, John R. Interactions between the recipient immune system and the left ventricular assist device surface: immunological and clinical implications. *Ann Thorac Surg.* 2003; 75(6 Suppl):S58–65. [PubMed: 12820736]
8. Mantovani A, Sica A, Sozzani S, Allavena P, Vecchi A, Locati M. The chemokine system in diverse forms of macrophage activation and polarization. *Trends Immunol.* 2004; 25(12):677–86. [PubMed: 15530839]
9. Maher SG, Romero-Weaver AL, Scarzello AJ, Gamero AM. Interferon: cellular executioner or white knight? *Curr Med Chem.* 2007; 14(12):1279–89. [PubMed: 17504213]
10. Enelow RI, Sullivan GW, Carper HT, Mandell GL. Induction of multinucleated giant cell formation from in vitro culture of human monocytes with interleukin-3 and interferon-gamma: comparison with other stimulating factors. *Am J Respir Cell Mol Biol.* 1992; 6(1):57–62. [PubMed: 1728295]
11. McNally AK. Interleukin-4 induces foreign body giant cells from human monocytes/macrophages. Differential lymphokine regulation of macrophage fusion leads to morphological variants of multinucleated giant cells. *Am J Pathol.* 1995; 147(5):1487–99. [PubMed: 7485411]
12. Castiglione A, Pagliaro P, Romagnoni M, Beccari M, Cavaliere G, Veneroni G, Sorgato G. Flow cytometric analysis of leukocytes eluted from haemodialysers. *Nephrol Dial Transplant.* 1991; 6(Suppl 2):31–5. [PubMed: 1866065]
13. Grooteman MP, Nube MJ, van Limbeek J, Schoorl M, van Houte AJ. Lymphocyte subsets in dialyser eluates: a new parameter of bioincompatibility? *Nephrol Dial Transplant.* 1996; 11(6):1073–8. [PubMed: 8671971]
14. Yokoyama M, Nakahashi T, Nishimura T, Maeda M, Inoue S, Kataoka K, Sakurai Y. Adhesion behavior of rat lymphocytes to poly(ether)-poly(amino acid) block and graft copolymers. *J Biomed Mater Res.* 1986; 20(7):867–78. [PubMed: 3760003]
15. Maeda M, Kimura M, Inoue S, Kataoka K, Okano T, Sakurai Y. Adhesion behavior of rat lymphocyte subpopulations (B cell and T cell) on the surface of polystyrene/polypeptide graft copolymer. *J Biomed Mater Res.* 1986; 20(1):25–35. [PubMed: 3485099]
16. Groth T, Altankov G, Klosz K. Adhesion of human peripheral blood lymphocytes is dependent on surface wettability and protein preadsorption. *Biomaterials.* 1994; 15(6):423–8. [PubMed: 8080932]
17. Groth T, Zlatanov I, Altankov G. Adhesion of human peripheral lymphocytes on biomaterials preadsorbed with fibronectin and vitronectin. *J Biomater Sci Polym Ed.* 1994; 6(8):729–39. [PubMed: 7535560]
18. Bergman AJ, Zygorakis K. Migration of lymphocytes on fibronectin-coated surfaces: temporal evolution of migratory parameters. *Biomaterials.* 1999; 20(23-24):2235–44. [PubMed: 10614930]
19. Ito T, Iwasaki Y, Narita T, Akiyoshi K, Ishihara K. Controlled adhesion of human lymphocytes on electrically charged polymer surface having phosphorylcholine moiety. *Science and Technology of Advanced Materials.* 2003; 4(2):99–104.
20. Nakayama Y, Anderson JM, Matsuda T. Laboratory-scale mass production of a multi-micropatterned grafted surface with different polymer regions. *J Biomed Mater Res.* 2000; 53(5):584–91. [PubMed: 10984708]
21. McNally AK, Anderson JM. Complement C3 participation in monocyte adhesion to different surfaces. *Proc Natl Acad Sci USA.* 1994; 91(21):10119–23. [PubMed: 7937848]
22. Chang DT, Jones JA, Meyerson H, Colton E, Kwon IK, Matsuda T, Anderson JM. Lymphocyte/macrophage interactions: Biomaterial surface-dependent cytokine, chemokine, and matrix protein production. *J Biomed Mater Res A.* 2008
23. Santana MA, Rosenstein Y. What it takes to become an effector T cell: the process, the cells involved, and the mechanisms. *J Cell Physiol.* 2003; 195(3):392–401. [PubMed: 12704648]
24. MacEwan MR, Brodbeck WG, Matsuda T, Anderson JM. Student Research Award in the Undergraduate Degree Candidate category, 30th Annual Meeting of the Society for Biomaterials, Memphis, Tennessee, April 27-30, 2005. Monocyte/lymphocyte interactions and the foreign body

- response: in vitro effects of biomaterial surface chemistry. *J Biomed Mater Res A*. 2005; 74(3): 285–93. [PubMed: 16124082]
25. Upham JW, Strickland DH, Bilyk N, Robinson BW, Holt PG. Alveolar macrophages from humans and rodents selectively inhibit T-cell proliferation but permit T-cell activation and cytokine secretion. *Immunology*. 1995; 84(1):142–7. [PubMed: 7534261]
 26. Strickland DH, Kees UR, Holt PG. Suppression of T-cell activation by pulmonary alveolar macrophages: dissociation of effects on TcR, IL-2R expression, and proliferation. *Eur Respir J*. 1994; 7(12):2124–30. [PubMed: 7713193]
 27. Upham JW, Strickland DH, Robinson BW, Holt PG. Selective inhibition of T cell proliferation but not expression of effector function by human alveolar macrophages. *Thorax*. 1997; 52(9):786–95. [PubMed: 9371209]
 28. Cantrell DA, Collins MK, Crumpton MJ. Autocrine regulation of T-lymphocyte proliferation: differential induction of IL-2 and IL-2 receptor. *Immunology*. 1988; 65(3):343–9. [PubMed: 3264805]
 29. Monaco C, Andreaskos E, Kiriakidis S, Feldmann M, Paleolog E. T-cell-mediated signalling in immune, inflammatory and angiogenic processes: the cascade of events leading to inflammatory diseases. *Curr Drug Targets Inflamm Allergy*. 2004; 3(1):35–42. [PubMed: 15032640]
 30. Hallett WH, Murphy WJ. Positive and negative regulation of Natural Killer cells: therapeutic implications. *Semin Cancer Biol*. 2006; 16(5):367–82. [PubMed: 16934486]
 31. Ley K, Laudanna C, Cybulsky MI, Nourshargh S. Getting to the site of inflammation: the leukocyte adhesion cascade updated. *Nat Rev Immunol*. 2007; 7(9):678–89. [PubMed: 17717539]
 32. von Andrian UH, Mackay CR. T-cell function and migration. Two sides of the same coin. *N Engl J Med*. 2000; 343(14):1020–34. [PubMed: 11018170]
 33. Miyamoto YJ, Andruss BF, Mitchell JS, Billard MJ, McIntyre BW. Diverse roles of integrins in human T lymphocyte biology. *Immunol Res*. 2003; 27(1):71–84. [PubMed: 12637769]
 34. Hogg N, Laschinger M, Giles K, McDowall A. T-cell integrins: more than just sticking points. *J Cell Sci*. 2003; 116(Pt 23):4695–705. [PubMed: 14600256]
 35. Luttkhuizen DT, Harmsen MC, Van Luyn MJ. Cellular and molecular dynamics in the foreign body reaction. *Tissue Eng*. 2006; 12(7):1955–70. [PubMed: 16889525]
 36. Jones JA, Chang DT, Meyerson H, Colton E, Kwon IK, Matsuda T, Anderson JM. Proteomic analysis and quantification of cytokines and chemokines from biomaterial surface-adherent macrophages and foreign body giant cells. *J Biomed Mater Res A*. 2007; 83(3):585–96. [PubMed: 17503526]
 37. Zhao Q, Topham N, Anderson JM, Hiltner A, Lodoen G, Payet CR. Foreign-body giant cells and polyurethane biostability: in vivo correlation of cell adhesion and surface cracking. *J Biomed Mater Res*. 1991; 25(2):177–83. [PubMed: 2055915]
 38. Hernandez-Pando R, Bornstein QL, Leon D Aguilar, Orozco EH, Madrigal VK, Cordero E Martinez. Inflammatory cytokine production by immunological and foreign body multinucleated giant cells. *Immunology*. 2000; 100(3):352–8. [PubMed: 10929057]
 39. Athanasou NA, Quinn J. Immunophenotypic differences between osteoclasts and macrophage polykaryons: immunohistological distinction and implications for osteoclast ontogeny and function. *J Clin Pathol*. 1990; 43(12):997–1003. [PubMed: 2266187]

Figure 1.

Adherent lymphocyte density (A) and adherent macrophage density (B) as a function of the ratio of lymphocyte/monocyte co-culture concentration after 3 days. Lymphocytes were co-cultured with different monocyte concentration for 3 days on each of the biomaterial surfaces. Results are expressed as the mean of 3 experiments $\pm$ SEM. *Significant difference relative to other surfaces ($p < 0.05$). **Significance relative to other surfaces and 1/0.1 co-culture ratio. ($p < 0.05$).

Figure 2.

Adherent lymphocyte density as a function of the adherent macrophage and FBGC density after 3 days of culture. Data represents the mean $\pm$ SEM ($n = 3$). *Significant increase compared to the other two surfaces and 1/0.1 co-culture ($p < 0.05$).

Figure 3.

Types of adherent lymphocytes on the biomaterial surfaces as a function of the ratio of lymphocyte/macrophage co-culture concentrations. A) Total numbers of adherent CD4+ T helper cells, CD8+ T suppressor cells, and CD56+ NK cells and B) percentages of each subtype contributing to the total lymphocyte population on the biomaterial surfaces.

Figure 4.

Immunofluorescent identification of lymphocyte subtypes and their interactions on the biomaterial surfaces for A) CD14+ monocytes/macrophages, B) CD8+ T suppressor cells, C) CD4+ T helper cells, and D) CD56+ natural killer cells after 3 days of co-culture. Cells were identified by staining with anti-CD14 antibodies for monocytes/macrophages, anti-CD8 antibodies for T suppressor cells, anti-CD4 antibodies for T helper cells, and anti-CD56 antibodies for natural killer cells. Utilizing YO-YO-1 (green, nuclei) and an overlaid phase contrast image, identified cells (red) were analyzed for their interactions with macrophages or FBGCs. Representative images are from the hydrophilic/anionic surface (PAANa) where both macrophages and FBGCs are present. Solid arrows indicate macrophage- or FBGC-adherent lymphocytes while dashed arrows indicate biomaterial-adherent lymphocytes. Scale bars, 50 μm.

Figure 5.

Types of adherent lymphocyte interactions at the surface of the biomaterial as a function of the ratio of lymphocyte/monocyte co-culture concentrations. A) Total macrophage-, FBGC-, or direct biomaterial-adherent lymphocyte densities and the B) percentages of each of the types of adherent lymphocyte interactions. Data represents mean $\pm$ SEM ($n = 3$).

*Significance compared to the two other surfaces ($p < 0.05$).

Figure 6. Quantification of IFN- γ production from lymphocyte and monocyte single and co-cultures over A) 3, B) 7, and C) 10 days. Data represents average $\pm$ SEM (n=3). *p < 0.01

Table 1

Ratio of Adherent Lymphocyte Densities to Adherent Macrophage/FBGC Densities on PET-based Surfaces

Surface	1/0.1	1/0.5	1/1.0
PET	0.8	0.1	0.1
PAANa	3.0	1.5	0.4
PAAm	2.7	0.5	0.3

Table 2
FBGC- or Macrophage-Adherent lymphocytes Normalized to the Respective Adherent FBGC or Macrophage Population

	1/0.5				1/1.0			
	PET	PAANa	PAAm	PET	PAANa	PAAm	PET	PAANa
FBGC-adherent lymphocytes:FBGCs	0.0	2.5	0.0	0.0	1.4	0.1		
Macrophage-adherent lymphocytes:Macrophages	0.1	0.9	0.4	0.1	0.3	0.3		