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Polymorphonuclear Leukocyte Inhibition of Monocytes/Macrophages in the Foreign Body Reaction

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Abstract

The effect of polymorphonuclear leukocytes (PMNs) on the subsequent chronic phase macrophage-mediated foreign body reaction has not been previously investigated. Furthermore, while monocyte/macrophage-produced cytokines such as GM-CSF, G-CSF, or IL-1 β have been shown to increase PMN survival *in vitro*, few studies have examined the impact of directly co-cultured monocytes/macrophages on PMN viability. To this end, we used our established *in vitro* system of interleukin (IL)-4-induced monocyte-derived macrophage fusion to examine the role of PMNs in the subsequent foreign body reaction. Monocytes were directly cultured with PMNs for 3 days prior to the addition of IL-4 to induce monocyte-derived macrophage fusion to facilitate foreign body giant cell (FBGC) formation by Day 7 and Day 10 of culture. Optical microscopy was used to quantitatively determine adherent monocyte density, percent macrophage fusion, and FBGC density. A colorimetric MTT assay was used to assess PMN viability for direct co-cultures of monocytes/macrophages and PMNs. Our results strongly suggest that the presence of PMNs inhibit IL-4-induced macrophage fusion and FBGC formation. Additionally, our findings demonstrate that co-cultures containing PMNs and monocytes/macrophages increases PMN survival with respect to PMN-only cultures *in vitro*.

Keywords

foreign body reaction; polymorphonuclear leukocytes; monocytes; macrophages; foreign body giant cells

Introduction

The foreign body reaction to biomaterial implantation is characterized by the presence of mature macrophages and foreign body giant cells (FBGCs) at the surface of the implant. Upon activation, monocyte-derived macrophages direct the foreign body reaction through the production of inflammatory cytokines and chemokines. Polymorphonuclear leukocytes (PMNs), neutrophils, are the characteristic cell of the acute inflammatory response following biomaterial implantation¹. Despite the presence of both PMNs and monocytes at the site of injury, there has been little investigation into interactions between these cell types.

The primary role of PMNs in the innate immune system is phagocytosis during the early stages of infection². At the site of injury, activated PMNs also produce cytokines and chemokines that comprise the milieu of soluble factors and signaling molecules that guide acute inflammation. PMNs are short-lived, typically undergoing apoptosis during the first

24-hours during the inflammatory response. However, some biomaterials, such as chitosan, recruit additional neutrophils to the wound site by inducing PMN production of interleukin-8 (IL-8)³. Therefore, PMNs modulate both the intensity and duration of the acute inflammatory response through phagocytosis, apoptosis, and cytokine/chemokine release.

Activated macrophages produce inflammatory cytokines such as granulocyte macrophage colony-stimulating factor (GM-CSF), granulocyte-colony stimulating factor (G-CSF), and interleukin-1-beta (IL-1 β)¹. The production of GM-CSF and G-CSF, in particular, recruits additional macrophages to the wound site. While PMNs appear only transiently at the implant site, PMN “priming” with GM-CSF, G-CSF, or IL-1 β has been reported to prolong the survival of PMNs *in vitro*⁴.

The goals of our investigation were to study the effects of acute-phase PMNs on monocyte adhesion, macrophage maturation, and subsequent FBGC formation and the effect of monocytes on PMN survival. To study these interactions, we placed monocytes and PMNs in direct co-culture. A novel finding in our studies was the inhibition of monocyte/macrophage development in the presence of PMNs *in vitro*. The results from this research suggest that PMNs have the potential to inhibit the foreign body reaction by delaying monocyte/macrophage development *in vitro*.

Materials/Methods

Isolation and culture of PMNs and monocytes to determine PMN viability

Monocytes (MC) and PMNs were isolated from venous blood of non-medicated donors by methods previously documented^{5,6}. Isolated MC and PMN cell populations were suspended in separate sterile 50mL polypropylene tubes (Falcon). Both cell populations were plated in solutions containing serum-free medium for macrophages (SFM) (Invitrogen; Grand Island, NY) supplemented with antibiotics, antimycotics, L-glutamine, and 20% autologous human serum (AS).

Prior to cell culture, 96-well tissue-culture polystyrene (TCPS) plates were coated with 25 μ g per mL of fibronectin-like RGD peptide (Sigma; St Louis, MO), or human plasma vitronectin (Sigma), or were left uncoated. Initially, monocytes were plated at a density of 5×10^5 per well and allowed to adhere for 2 hours. Non-adherent cells were removed by washing with phosphate-buffered saline containing calcium and magnesium (PBS⁺⁺), prewarmed to 37°C. Polymorphonuclear leukocytes (PMNs) were then plated at a density of 1×10^6 PMN per well in SFM containing 10% AS. In an effort to prolong PMN viability at later time points as per Colotta *et al*⁴, human recombinant granulocyte macrophage colony-stimulating factor (GM-CSF, R&D Systems; Minneapolis, MN) was added at a concentration of 5ng per mL.

The TACS MTT assay (R&D Systems; Minneapolis, MN) evaluates cell viability through the addition of the tetrazolium compound MTT (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide) to cells in culture. Viable cells are able to metabolize MTT to form purple formazan dye crystals. At 0 (2 hours), 1, 2, and 3 days of incubation at 37°C in a humidified atmosphere of 5% CO₂ and 95% air, wells were washed with warm PBS⁺⁺ to remove non-adherent cells prior to the addition of 10 μ L MTT stock solution in 100 μ L of PBS⁺⁺ to each well per the manufacturer's directions. After incubating for two hours at 37°C, detergent provided by the manufacturer was used to solubilize the formazan dye crystals. After an overnight incubation, absorbance levels were read at 570nm using an EL-808 microplate reader (Bio-Tek Instruments, Inc).

Monocyte and PMN culture to determine adherent monocyte density, macrophage fusion, and FBGC formation

Human monocytes and PMNs were isolated as described above by previously published methods^{5,6}. TCPS plates were coated with the RGD peptide (25µg per mL) prior to cell seeding. Monocytes (2×10^5 per well) were plated on RGD-modified plates in SFM containing 20% AS for 2 hours at 37°C. Non-adherent cells were removed by washing with warm PBS⁺⁺, followed by the addition of SFM containing PMNs (2.5×10^5 per well) with or without 5ng per mL GM-CSF. The co-cultures were then incubated at 37°C. At both Day 3 and Day 7 time points, wells were washed with PBS⁺⁺ to remove non-adherent cells and either terminated or continued after the addition of 5% heat-treated (56°C for 1 hour) AS in SFM (100µL per well) with or without 15ng per mL human recombinant IL-4 (R&D Systems).

Determination of PMN viability

At two hours of culture, all adherent PMNs and MCs were assumed to be viable. Therefore, the 2-hour absorbance values were used to determine the percentage of viable PMNs at each subsequent time point. The absorbance readings from MC-only cultures were subtracted from the overall absorbance reading from PMN/MC co-cultures to determine the percentage of viable PMN in those cultures.

Determination of adherent monocyte density, macrophage fusion, and FBGC formation

At 0 (2 hours), 3, 7, and 10 days, wells were washed with warm PBS⁺⁺ and cultures were terminated by methanol fixation for 5 minutes. To quantify adherent monocytes, macrophages, and FBGCs, cultures were stained with May-Grunwald/Giemsa. Cells were stained with the May-Grunwald reagent for 5 minutes, washed briefly with PBS⁺⁺, and then stained with Giemsa for 15 minutes. Finally, all wells were washed with distilled water twice and allowed to dry overnight. Optical microscopy (Olympus BH-2) was used to quantitatively determine the extent of monocyte adhesion (cells per mm²) by counting three fields using a 20× objective for each well. FBGCs were defined as cells containing at least three distinct nuclei. The numbers of adherent monocyte nuclei and FBGCs were used to determine the percentage of macrophages fused to form FBGCs as well as the FBGC density for each well (FBGCs per mm²).

Statistical Analysis

All culture conditions were performed in triplicate for each experiment. For PMN viability, the results are presented as the average of each triplicate $\pm$ the standard deviation (SD). For monocyte adhesion, macrophage maturation, and FBGC formation, culture conditions were established in triplicate for three experiments (n=3) from different donors. The results are presented as the mean $\pm$ the standard error of the mean (SEM). For all data, statistical analysis was completed with computer software to perform an ANOVA and Tukey's *post hoc* test (Minitab; State College, PA).

Results

To analyze the response of monocytes/macrophages cultured in the presence of PMNs *in vitro*, our first aim was to determine the viability of isolated human PMNs under various culture conditions. We have previously demonstrated that macrophage maturation, fusion, and FBGC formation is enhanced by the adsorption of selected proteins to material surfaces^{7,8}. In an attempt to obtain a viable population of PMNs, while providing a substrate that facilitates adequate FBGC formation, we investigated the effects of surface-adsorbed vitronectin and the fibronectin-like RGD peptide on PMN survival. Results from

preliminary experiments indicated that PMN viability was greater on RGD-modified TCPS than on vitronectin-adsorbed TCPS at later time points in our culture system. Based on this preliminary data, we employed RGD-coated TCPS plates to investigate the effect of acute-phase PMNs on the chronic-phase monocyte/macrophage maturation and foreign body giant cell formation.

PMN viability

Because it was previously reported that PMN “priming” with the cytokine GM-CSF extends PMN viability *in vitro*⁴, we added exogenous GM-CSF to PMN-only cultures in an attempt to improve PMN survival after 4 days of culture. As suggested in Table 1, the addition of exogenous GM-CSF marginally increased PMN survival (9.9% at Day 4) when compared to PMN-only cultures (2.9% at Day 4).

However, as shown in Figure 1, this approach was not nearly as successful in maintaining PMN viability as the presence of monocytes/macrophages in the PMN/MC co-cultures. While not significantly different at 24 hours, the difference between PMN percent survival for PMN/MC co-cultures and PMN-only cultures increased over time (Figure 1). By Day 4, PMN/MC co-cultures exhibited a 13-fold increase in PMN survival. Differences in PMN survival between PMN/MC co-cultures and PMN-only cultures were significant ($p < 0.05$) at Days 2, 3, and 4.

Monocyte Adhesion, macrophage fusion, FBGC formation

Using light microscopy, levels of monocyte adhesion, macrophage fusion, and FBGC formation were determined. Both PMN/MC and MC-only cultures exhibited expected levels of monocyte adhesion at Day 0 (two hours post-culture). PMN/MC cultures showed decreased monocyte adhesion compared to MC-only cultures at later time points as shown in Figure 2. Results at Day 7 and Day 10 reported statistically significant differences in macrophage adhesion, indicating that co-culturing monocytes and PMNs inhibited monocyte/macrophage adhesion at later time points ($p < 0.05$). Specifically, at Day 10 PMN/MC cultures indicated a 45% decrease relative to MC-only cultures. Day 3 results are included but, as many MCs were washed away prior to staining, these data are less reliable than data for later time points. This susceptibility to washing is normal at this time point and likely reflects alterations in adhesion mechanisms as monocyte-to-macrophage development progresses *in vitro*⁹.

Monocyte-derived macrophage fusion was induced through the addition of IL-4 to all cultures at Day 3. As shown in Figure 3 prior to the addition of IL-4 to the culture wells on Day 3, there is essentially no macrophage fusion or FBGC formation. However, at later time points MC-only cultures had a higher percentage of macrophage fusion with respect to PMN/MC cultures. At Day 10, differences in macrophage fusion were statistically significant ($p < 0.01$). Furthermore, Day 10 macrophage fusion in PMN/MC cultures was reduced by over 40% from MC-only cultures. These results suggest that the presence of PMNs in direct co-culture with monocytes inhibits macrophage maturation and subsequent macrophage fusion.

On Day 3 of culture and prior to the addition of IL-4, there is no FBGC formation. The FBGC density per well was similar to percent macrophage fusion in that MC-only cultures exhibited the highest levels of FBGC formation at later time points. IL-4-induced FBGC formation at both Day 7 and Day 10 demonstrated statistically significant differences between PMN/MC and MC-only cultures ($p < 0.05$). FBGC density was reduced by approximately 41% at Day 7 and 59% at Day 10 for PMN/MC cultures when compared to MC-only cultures.

Discussion

During the acute inflammatory response to biomaterial implantation (0 to 24 hours), PMNs are the predominant cell type at the implant site¹. Despite their presence during acute inflammation, there has been little investigation into the effects of PMNs on the subsequent macrophage-mediated foreign body reaction at the cell-biomaterial interface. Furthermore, PMNs elaborate multiple cytokines and signaling molecules that dictate the duration and intensity of the acute inflammatory response². We hypothesized that the presence of acute inflammatory PMNs would affect monocyte/macrophage development and maturation including monocyte adhesion, macrophage fusion, and FBGC formation. The objective of this research was to investigate the role of PMNs in the foreign body reaction when placed in direct co-culture with monocytes/macrophages *in vitro*. To study the effects of these acute interactions on the subsequent chronic phase response, we incorporated PMNs in the initial 3-day period of our established 10-day monocyte/macrophage and IL-4-induced FBGC culture system.

Initially, PMNs were cultured alone as well as directly with monocytes to investigate the effect of surface adsorbed proteins on PMN survival over the course of 4 days. After one day of culture, the percentage of viable PMNs was statistically the same for both PMN-only and direct PMN/MC co-cultures. At Day 3 and Day 4, the direct co-cultures showed a 2-fold and 13-fold increase in PMN percent survival over the PMN-only cultures, respectively. These results demonstrate that PMN survival is generally increased in the presence of monocytes/macrophages *in vitro*. Over time, interactions with monocytes/macrophages maintained viable PMNs significantly longer than PMN-only cultures.

This monocyte/macrophage-mediated increase in PMN survival may have been due to cytokine production, i.e. GM-CSF, by adherent macrophages. However, culturing PMNs with exogenous human recombinant GM-CSF alone did not significantly increase PMN survival in our culture system. In addition to GM-CSF, monocytes/macrophages can produce G-CSF and IL-1 β which have also been shown to increase PMN viability *in vitro*⁴, and may act synergistically with GM-CSF and/or other factors to support PMN survival over time.

At Day 7 and Day 10, PMN/MC direct co-cultures showed a 30% and 45% decrease, respectively, in levels of adherent monocytes/macrophages when compared to MC-only cultures. The presence of PMNs in direct co-culture with monocytes appears to delay the maturation and development of monocyte-derived macrophages. PMNs have been shown to produce antimicrobial vesicles, known as ectosomes, during degranulation¹⁰. Ectosomes have been reported to interfere with macrophage development and maturation¹¹. The present results suggest a similar inhibitory phenomenon by PMNs in direct co-culture with monocytes/macrophages.

The effects of acute phase PMNs on subsequent macrophage fusion leading to FBGC formation have not been previously investigated. At Day 10, PMN/MC co-cultures exhibited a 41% decrease in the percentage of macrophages fusing to form FBGCs from MC-only cultures. These findings suggest that direct co-culture between PMNs and monocytes/macrophages decreases subsequent IL-4-induced macrophage fusion.

The process of macrophage fusion to form FBGCs shares characteristics with the process of phagocytosis including actin polymerization, intact microtubules, and the expression of mannose receptors at the surface of the macrophage¹². Previous studies have shown that PMN-derived ectosomes down-regulate the phagocytic activity of monocyte-derived dendritic cells¹¹. These previously published findings are extended by our data, which suggests that PMNs decrease macrophage fusion and IL-4-induced FBGC formation.

Consistent with our macrophage fusion data, FBGC density for PMN/MC co-cultures was significantly lower than MC-only cultures at Day 7 and Day 10 ($p < 0.05$). Because PMNs appear to delay or inhibit macrophage development, we would expect to see decreased FBGC density at both Day 7 and Day 10.

In summary, we were able to culture acute-phase PMNs with monocytes and maintain 40% PMN viability over the course of 4 days *in vitro*. Moreover, we observed an inhibitory effect of the early presence of PMNs on IL-4-induced macrophage fusion over the subsequent 10 days of *in vitro* culture. Monocyte/macrophage maturation, characterized by monocyte adhesion, macrophage fusion, and FBGC density, was negatively influenced by the presence of PMNs in direct co-culture with monocytes. While PMN survival was increased in the presence of monocytes in our culture system, we observed a decrease in macrophage development and IL-4-induced FBGC formation. These results support further investigation into the cellular and molecular mechanisms involved in PMN/MC interactions.

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Figure 1. PMN percent survival: effect of monocytes on PMN viability at 1, 2, 3, and 4 days of direct co-culture. Data presented as the average $\pm$ SD. **Significantly different from PMN-only culture ($p < 0.01$)

Figure 2.

Adherent monocyte/macrophage density: effect of PMNs on monocyte/macrophage adhesion at 0 (2hr), 3, 7, and 10 days of direct co-culture. Data presented as the average $\pm$ SEM, $n=3$. *Significantly different from the MC-only culture ($p < 0.05$). **Significantly different from the MC-only culture ($p < 0.01$).

Figure 3. Macrophage percent fusion: effect of PMNs on IL-4-induced macrophage fusion at 3, 7, and 10 days of direct co-culture. Data presented as the average $\pm$ SEM, $n=3$. **Significantly different from the MC-only culture ($p < 0.01$).

Figure 4. FBGC density: effect of PMNs on IL-4-induced FBGC formation at 3, 7, and 10 days of direct co-culture. Data presented as the average $\pm$ SEM, $n=3$. *Significantly different from the MC-only culture ($p < 0.05$). **Significantly different from the MC-only culture ($p < 0.01$).

Table 1PMN % Survival for various culture conditions over time (Average $\pm$ SD)

Time	PMN only	PMNCSF
Day 1	49.2 $\pm$ 2.2	44.1 $\pm$ 1.1
Day 2	21.5 $\pm$ 0.8	21.1 $\pm$ 6.0
Day 3	11.1 $\pm$ 1.5	13.8 $\pm$ 1.9
Day 4	2.9 $\pm$ 0.6	9.9 $\pm$ 3.2