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Integrin-Directed Modulation of Macrophage Responses to Biomaterials

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Abstract

Macrophages are the primary mediator of chronic inflammatory responses to implanted biomaterials, in cases when the material is either in particulate or bulk form. Chronic inflammation limits the performance and functional life of numerous implanted medical devices, and modulating macrophage interactions with biomaterials to mitigate this response would be beneficial. The integrin family of cell surface receptors mediates cell adhesion through binding to adhesive proteins nonspecifically adsorbed onto biomaterial surfaces. In this work, the roles of integrin Mac-1 ($\alpha_M\beta_2$) and RGD-binding integrins were investigated using model systems for both particulate and bulk biomaterials. Specifically, the macrophage functions of phagocytosis and inflammatory cytokine secretion in response to a model particulate material, polystyrene microparticles were investigated. Opsonizing proteins modulated microparticle uptake, and integrin Mac-1 and RGD-binding integrins were found to control microparticle uptake in an opsonin-dependent manner. The presence of adsorbed endotoxin did not affect microparticle uptake levels, but was required for the production of inflammatory cytokines in response to microparticles. Furthermore, it was demonstrated that integrin Mac-1 and RGD-binding integrins influence the *in vivo* foreign body response to a bulk biomaterial, subcutaneously implanted polyethylene terephthalate. A thinner foreign body capsule was formed when integrin Mac-1 was absent (~30% thinner) or when RGD-binding integrins were blocked by controlled release of a blocking peptide (~45% thinner). These findings indicate integrin Mac-1 and RGD-binding integrins are involved and may serve as therapeutic targets to mitigate macrophage inflammatory responses to both particulate and bulk biomaterials.

Introduction

The implantation of a biomaterial within the body initiates a series of host immune responses termed collectively as the foreign body response, which attempts to eliminate and/or isolate the implanted foreign body [1]. Macrophages play a major role in this foreign body response, being recruited to the site of biomaterial implantation and secreting cytokines and other signaling molecules [1]. If the implanted material is too large to

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phagocytose, macrophages fuse to form foreign body giant cells, while releasing reactive oxygen intermediates, degradative enzymes and acid, which mediate degradation of the biomaterial [2,3]. Furthermore, macrophages and foreign body giant cells recruit fibroblasts which direct the fibrous encapsulation of the implant [2]. This foreign body response limits the functional performance of numerous implanted biomaterial such as pacemaker leads [4], glucose sensors [5], sensor electrodes [6], drug delivery devices [7] and breast implants [8]. Macrophages also play a major role in the body's response to particulate materials. For example, the process of aseptic loosening of artificial joints results from activated macrophages secreting inflammatory cytokines after phagocytosing wear debris particles [9]. Because macrophages play such a key role in the response to implanted materials, various studies have explored modulating macrophage response to mitigate inflammatory response upon implantation of foreign materials. For instance, approaches such as modifying surface chemistry and roughness [10-14], coatings that release anti-inflammatory and angiogenic molecules such as dexamethasone [15] and heparin [16] have been investigated to modulate macrophage interactions with biomaterials. While demonstrating some influence, exploration of alternative approaches to modulate macrophage responses to biomaterials, are needed, as clinical impact has yet to be realized.

One approach is to target modulation of the initial adhesion of macrophages to implants. Adhesive interactions of macrophages with synthetic biomaterials are primarily mediated through the cell surface receptors known as integrins, which specifically bind to surface-adsorbed proteins on the material [2,17,18]. Macrophage integrins direct numerous cell functions including adhesion to extracellular matrix proteins, adhesion and signaling to other cell types, cell migration and spreading, as well as phagocytosis of particulate matter [19]. One adhesive protein of particular interest in this field is fibrinogen. A primary component of plasma, this extracellular matrix protein has been shown to be deposited on biomaterial surfaces, mediating acute inflammatory responses to the implanted material [20,21]. The integrin Mac-1 (CD11b/CD18) is a leukocyte integrin which binds to fibrinogen and is present on macrophages and neutrophils. Binding of Mac-1 to fibrinogen has been shown to direct macrophage adhesion and activation [22]. Integrin Mac-1 also mediates cell adhesion to a number of other proteins that adsorb from physiologic fluids onto synthetic materials including complement factor fragment C3bi, albumin, vitronectin, and fibronectin [23-26]. Mac-1 is also known to direct various inflammatory processes [27-29] and therefore warrants further investigation in the context of modulating macrophage response to biomaterials. Furthermore, it is a complex milieu of proteins which spontaneously adsorb onto biomaterial surfaces following implantation, such as fibrinogen, fibronectin and vitronectin [30]. These proteins and others involved in cellular adhesion are rich with the tripeptide sequence, Ar-Gly-Asp (RGD), which has been shown to bind to a number of integrins, including $\alpha_v\beta_3$, $\alpha_5\beta_1$ and $\alpha_v\beta_5$ [31]. Therefore, investigating the role of RGD-binding integrins in addition to integrin Mac-1 is of interest in order to identify potential targets to modify macrophage behavior in response to biomaterials.

Herein, we investigate the quantification of *in vitro* phagocytosis of polystyrene microparticles (MPs) and the subsequent inflammatory cytokine secretion by macrophages obtained from Mac-1 knockout (KO) mice compared to wild type (WT) controls. Parallel studies were performed to determine the role of RGD-binding integrins in macrophage phagocytosis of MPs using blocking RGD and RGD-containing peptides to disrupt RGD-binding integrin receptors. Furthermore, we also investigated the role of integrin Mac-1 and RGD-binding integrins in the *in vivo* foreign body response to implanted bulk (not particulate) biomaterial using a subcutaneous mouse implant model. Delineating the role of integrin receptors could suggest new therapeutic targets for control over macrophage-driven inflammatory responses to particulate and bulk biomaterials.

Materials and Methods

Macrophage generation

Bone marrow-derived macrophages were generated from 7-10 week-old C57BL6/J (wild type; WT) and Mac-1 KO mice using a 10 d culture protocol [32-34]. Animals were handled in accordance with protocol approved by the Institutional Animal Care and Use Committee (IACUC) at University of Florida. Briefly, mice were euthanized by CO₂ asphyxiation followed by cervical dislocation and tibias and femurs were harvested for isolating marrow cells. Marrow cells were obtained by flushing the shaft of the bones with a 25 G needle using wash media comprising of RPMI media (Hyclone Laboratories Inc, Logan, Utah) containing 1% fetal bovine serum (FBS) (Hyclone Laboratories Inc, Logan, Utah) and 1% penicillin-streptomycin-neomycin antibiotic mixture (Hyclone Laboratories Inc, Logan, Utah). The macrophages were cultured in a growth media comprised of Dulbecco's Modified Eagle's Medium (DMEM)/F12(1:1) (Cellgro, Herndon, VA) medium containing 1% penicillin-streptomycin, 1% L-glutamine (Lonza, Walkersville, MD), 1% non-essential amino acids (Lonza, Walkersville, MD), 1% sodium pyruvate (Lonza, Walkersville, MD), 10% fetal bovine serum (FBS) and 10% L-929 cell conditioned media (LCCM). The LCCM serves as an established source of macrophage colony stimulating factor, which pushes the differentiation of marrow cells towards the macrophage phenotype [35]. To produce LCCM, L-929 cells were grown to a confluent monolayer in 150 cm² tissue culture flasks. 50 mL media was added to each flask for 7 days after which all the media in the flask was replaced with fresh media for 7 additional days. The media collected at day 7 and 14 were pooled, sterile-filtered and stored at -20°C. From the isolated marrow cells, the red blood cells were lysed using ACK (Ammonium-Chloride-Potassium) lysis buffer (Lonza, Walkersville, MD). The precursor cells were isolated using centrifugation, resuspended in macrophage growth medium and then seeded in a tissue culture treated T-flask (day 0) to remove adherent cells including fibroblast and mature macrophages. After 48 h (day 2), the floating cells were collected, resuspended in fresh media and seeded on low attachment plates for 4 additional days. The cells in the low attachment plates were supplemented with 1 ng/mL IL-3 (Peprotech, Rocky Hill, NJ) for expansion of the macrophage precursor cells. Half of the media in the low attachment wells was exchanged on day 4 with fresh growth media. At the end of 6 days, cells were lifted from the low attachment wells by gentle pipetting, resuspended and seeded on tissue culture-treated polystyrene plates for 2 more days to allow macrophage adhesion and maturation. On day 8, all the media in the wells was replaced with fresh media and at day 10 of culture, the cells were ready for experiment. The purity of the macrophage culture was verified by staining for CD11b (α chain of Mac-1) and F4/80 murine macrophage markers and analyzed using flow cytometry [36]. Macrophages isolated from at least 4 separate mice were used for each type of experiment.

Microparticle preparation

For the particulate biomaterial system, we used commercially available fluorescent polystyrene particles Fluoresbrite® YG Microspheres (Polysciences Inc., Warrington, Pennsylvania). In order for the particles to have a known amount of endotoxin, the particles were coated with lipopolysaccharide (LPS) by incubating in a 0.7 - 1.2 μ g/mL (depending on the protein to be adsorbed subsequently) LPS solution for 3 h to obtain a final endotoxin concentration of 5 EU/ million MPs/mL. The LPS coated MPs were then protein coated by incubating with various extracellular matrix proteins such as human plasma-derived fibronectin (FN) (BD Bioscience), human plasma-derived vitronectin (VN) (BD Bioscience) and bovine plasma fibrinogen (FG) (MP Biomedicals) as well as with fetal bovine serum (Serum) and bovine serum albumin (BSA) (Fisher Bioreagents). Species- specific protein sequence homologies, as compared to murine, are as follows: FN – 92%, COL – 89%, VN – 76%, FG – 81% and BSA – 70%; determined by HomoloGene, an online resource made

available through the National Center for Biotechnology Information. The Serum coating condition represents a standard culture condition which allows non-specific protein adsorption from serum onto the MPs and serves as a reference. The protein solution concentrations used for coating MPs was 200 µg/mL. The particles were vortexed in the protein solution and then allowed to incubate with the protein overnight at 4°C. After incubation, the particles were separated from protein solution by filtration (pore size 0.22 µm) and re-suspended in PBS.

Endotoxin testing

Analysis of endotoxin on MPs was performed in duplicate with the Chromo-limulus amoebocyte lysate (Chromo-LAL) assay [37] (Associates of Cape Cod Incorporated, Falmouth, MA). Briefly, 50 µL of the Chromo-LAL substrate (Limulus Amebocyte Lysate co-lyophilized with chromogenic substrate) was mixed with 50 µL of endotoxin free water containing protein coated MPs (20 million/ml) in a 96 well plate. The 96 well plate was then read in a microplate reader measuring absorbance at 405 nm every 2 min for 2 h at room temperature. Based on the manufacturer's instructions, a threshold absorbance value of 0.2 was selected and the time point that the sample crossed the threshold value was used for calculating the endotoxin concentration with the help of a standard curve plotted with the control standard endotoxin provided by the manufacturer. The detection limit of the assay at 2 h is 0.04 EU/mL.

Macrophage phagocytosis of polystyrene microparticles

For *in vitro* phagocytosis experiments, macrophages (1×10^5 /well) were plated in 96 well tissue culture plates. Fluorescent polystyrene MPs were added to each well at either 10:1, 20:1 or 40:1, MP:cell ratios and incubated at 37°C, 5% CO₂ to allow phagocytosis. MP uptake by macrophages was evaluated at the end of 2, 3.5, 5 and 7.5 h incubation after two PBS washes to remove non-phagocytosed MPs and read in a fluorescent plate reader. The number of MPs phagocytosed was determined from a standard curve obtained by plotting relative fluorescence intensity versus MP number. The results are reported as MPs/cell and normalized for cell numbers by staining cell nuclei with DAPI and measuring fluorescence intensity per well. For the RGD blocking experiments, macrophages were incubated with 1 mM and 10 mM RGD peptide in macrophage media for 1 h prior to feeding the MPs and throughout the incubation time for quantifying MP uptake at 2 h and 24 h respectively. MP uptake at 2 h and 24 h after feeding 20 MPs per cell was quantified as described above.

Macrophage cytokine production upon phagocytosis of polystyrene microparticles

For quantification of macrophage cytokine production, macrophages (1×10^6 /well) were plated in 12 well tissue culture plates. MPs were added to each well to achieve a MP:cell ratio of 25:1. The well plates are incubated at 37°C, 5% CO₂ for 24 h to allow phagocytosis and secretion of cytokines. Untreated cells were setup as a negative control for the baseline cytokine secretion, whilst an additional control of cells incubated with 1 µg/mL soluble LPS was considered for activation by a strong inflammatory signal. After 24 h, the supernatant was collected and frozen at -20°C for cytokine analysis using sandwich ELISA. The supernatant was assayed for cytokines TNF-α and IL-6 using sandwich ELISA kits (R & D Systems) according to the manufacturer's directions.

For the RGD blocking experiments, macrophages (1×10^5 /well) plated in 96 well tissue culture plates were incubated with 10 mM RGD in macrophage media for 1 h prior to adding MPs and throughout the MPs incubation time for a MP:cell ratio of 20:1. After 24 h, the supernatant was collected and frozen at -20°C for quantifying TNF-α and IL-6 secretion using sandwich ELISA.

Biomaterial implantation and analysis

Discs (7 mm diameter, 0.5 mm thick) were cut from polyethylene terephthalate (PET) sheets, washed, and sterilized by washing in 70% ethanol for 48 h. At the end of 48 h the discs were soaked in 50 μ L endotoxin free water and the endotoxin level on the discs was determined using Chromo-LAL assay. The endotoxin levels on the disc were determined to be below the recommended maximum FDA level. (< 0.5 EU/mL)

Two discs per mouse were implanted subcutaneously in 4-7 animals, in the lateral dorsal area on the mice in accordance with protocol approved by the University of Florida IACUC committee. Incisions were closed with wound clips, which were removed 7 d after implantation. At 14 d, mice were sacrificed and PET discs were explanted, formalin-fixed and paraffin-embedded. Histological sections (5 m thick) were stained with hematoxylin eosin stain for nuclei (dark blue) and collagen (pink) and the thickness of the capsule surrounding the disc was determined by bright field microscopy [34,38]. During histology, microtome slicing often caused the sectioned disc to curl and separate from the paraffin for a number of the discs, reducing the number of intact discs from which the thickness of fibrous capsules could be measured to a minimum of 6 discs, (reported as n=6 samples/group). In order to examine the role of Mac-1 integrin, discs were implanted in Mac-1 KO mice and WT control mice. For the RGD blocking experiments, echistatin, an RGD mimetic, was loaded into ethylene vinyl acetate (EVA) polymer. Echistatin is a 49-residue peptide containing the RGD amino acid sequence, binding with higher affinity than just RGD peptide alone [39]. Polymer/ drug coatings were prepared from an emulsion of EVA polymer beads (5% by weight) and echistatin (final conc. = 50 μ g/mL) in a ratio of 9:1 (EVA: drug) were mixed with methylene chloride in a sealed glass vessels. This solution was agitated vigorously for 15 min followed by sonication in a water bath at 25°C for 15 min. Approximately 20 μ L of solution was then pipetted onto each PET disc and discs were allowed to dry overnight under mild vacuum to remove the solvent by evaporation. Control discs with only EVA coatings were prepared similarly. EVA coated discs were embedded in methyl methacrylate and stained with MacNeal tetrachrome stain (Polysciences, Warrington, PA) to visualize and quantify the fibrous capsule formed around the implanted disc in 14 days.

Foreign body giant cell formation

Fusion was induced in expanded macrophages obtained at the end of 10 day culture protocol as described above. The macrophages were lifted and re-plated onto non-tissue culture treated 24-well plates with 1×10^6 cells per well. Fusion was induced by addition of 10 ng/mL recombinant mouse GM-CSF (R&D Systems) and 10 ng/mL recombinant mouse IL-4 (R&D Systems) to macrophage growth medium depleted of LCCM [40]. Complete media in the wells was replaced on day 3 and 5 and fusion was analyzed on day 7. Cells with 3 or more nuclei were counted as foreign body giant cells.

Loading and release kinetics of echistatin from EVA coating around PET Discs

The loading efficiency of the echistatin into the EVA polymer was determined using a solvent extraction method. Briefly, PET discs were submersed in methylene chloride to dissolve the EVA coating. Water was added in order to extract the water-soluble echistatin into the aqueous phase and this solution was agitated vigorously for 15 min followed by sonication in a water bath at 25°C for 15 min. The mixture was then centrifuged at $10000 \times g$ for 10 min in order to separate out the oil and water phase of the emulsion. The aqueous layer was carefully collected and spectrophotometric analysis was used to determine the amount of echistatin encapsulated in the EVA around each disc.

To examine the release kinetics of the encapsulated echistatin from the prepared discs, discs were placed on a shaker in deionized water at 37°C. Every alternate day for 3 weeks, supernatants were collected and samples were replenished with fresh water. Using spectrophotometric analysis, the concentration of echistatin in the supernatant was determined and the release was plotted as a percentage of loaded echistatin over a span of 3 weeks.

Statistical Analysis

Statistical analyses were performed using general linear nested model ANOVA, in Systat (Version 12, Systat Software, Inc., San Jose, CA). Pair-wise comparisons were made between the different groups (Mac-1 KO vs. WT; and RGD blocked samples vs. control) across different protein coatings using Tukey's test with p-values of less than or equal to 0.05 considered to be significant.

Results

Macrophage culture purity

Generation of a relatively pure macrophage population from the bone marrow precursor cells after a 10 d culture protocol was verified, using immunostaining techniques and flow cytometry analysis. Greater than 87% of the cells from the wild type (WT) C57BL6/J mice stained for both macrophage markers CD11b and F4/80, indicating a high purity macrophage population (**Figure 1A**). The number of bone marrow-derived macrophages expressing CD11b (Mac-1) from the Mac-1 KO mice was also quantified (**Figure 1B**). Approximately 99% of the F4/80⁺ macrophages from the Mac-1 KO mice lacked CD11b. These results indicate that there was an effective knockdown of the Mac-1(CD11b) receptor in macrophages derived from Mac-1 knockout (KO) mice.

Role of Mac-1 and RGD-binding integrins in microparticle uptake

Macrophage inflammatory responses to particulates have been shown to depend on particle size, where the size range 0.5-5 μm has been reported to be most reactive [41,42]. Within this range, polystyrene microparticles (MPs) of 1 μm diameter were chosen as a model microparticle (MP) system. To gain better understanding of the process of phagocytosis mediated by macrophage integrins, the role of different ligands involved in protein-opsonized MP uptake was investigated. Microparticle groups were coated with a number of different opsonizing proteins: bovine serum albumin (BSA, as a reference), fibrinogen (Fg), fibronectin (FN), vitronectin (VN) and serum (Serum; also as a reference). These proteins have been shown to adsorb onto synthetic biomaterial surfaces from physiologic fluids upon implantation and mediate cell-biomaterial interactions [21,30]. Care was taken to ensure opsonized MPs contained negligible (non-activating) endotoxin levels (< 0.02 EU/million MPs/ml). However, while endotoxin-free polystyrene MPs resulted in phagocytic uptake, the macrophages produced undetectable levels of cytokines. In fact, this observation has been corroborated by multiple groups reporting a lack of macrophage inflammatory cytokine secretion after MP phagocytosis of endotoxin-free MPs, for various types of MPs [43-46]. Therefore, establishing a model system where uptake of polystyrene MPs resulted in associated inflammatory cytokine secretion required the presence of adsorbed endotoxin. Consequently, sequential adsorption of LPS followed by different opsonizing proteins was carried out to yield a normalized amount of LPS for each MP group (5 EU/million MPs/mL). Thus, differential responses across groups can be attributed to the opsonizing protein. Comparing groups with or without LPS coating, for each opsonin, no differences in MP uptake levels were found (**Figure 2A**), indicating that the presence of adsorbed LPS does not affect the number of polystyrene MPs phagocytosed by macrophages. However, large differences were found in cytokine secretion between groups of opsonized MPs with and

without pre-coated LPS for every opsonin group. Specifically, MPs without pre-coated LPS demonstrated undetectable levels (< 30 pg/mL lower detection limit) of inflammatory cytokines secreted, namely TNF- α (**Figure 2B**) and IL-6 (data not shown). Accordingly, for the remaining *in vitro* experiments, pre-coating with LPS followed by opsonizing proteins was carried out, establishing a model particulate system with which to investigate receptor-mediated macrophage responses of microparticle uptake and subsequent cytokine secretion.

Microparticle uptake was compared between the Mac-1 deficient and WT macrophages for various MP-to-cell ratios over a range of time points to determine the role of integrin Mac-1. The absence of Mac-1 resulted in decreased uptake of MPs opsonized with Fg, VN, FN and Ser with reduction ranging from 15-45% compared to WT control at a MP:cell ratio of 40:1, depending on the uptake time (**Figure 3A-D**). Microparticles opsonized with BSA, however, did not demonstrate Mac-1 dependent uptake. The influence of Mac-1 on uptake was consistent across all time points for the Fg-coated MPs, with the largest differences at 5 and 7.5 h. Results also suggest that macrophages differentially phagocytosed MPs depending on which opsonizing protein was adsorbed, with highest uptake levels for Fg-coated MPs. Additionally, experimental variables of the opsonizing protein and receptor group (Mac-1 KO vs WT) were found to simultaneously influence MP uptake, determined by the ANOVA interaction term p-value ($p = 0.05$). Similar results were also seen at other MP:cell ratios (10:1 and 20:1; data not shown).

Soluble RGD peptide [47] and the RGD-containing peptide, echistatin [48], were next utilized to block cell adhesion to RGD-containing opsonizing proteins in order to determine the role of RGD-binding integrins in MP uptake. At 2 h, MP uptake of RGD-blocked samples decreased by 20-70% for Fg, FN, Ser and VN coated MPs as compared to the unblocked control, at a MP:cell ratio of 20:1 (**Figure 4A**). The Fg coated MPs demonstrated the highest level of uptake blocking at 2 h. This MP uptake blocking became further amplified at 24 h for all opsonins, where uptake was reduced by 70-90% compared to the unblocked groups (**Figure 4B**). Microparticles opsonized with BSA did not demonstrate RGD-binding integrin-dependent uptake at 2 h. However, at 24 h, uptake of BSA opsonized MPs was blocked by ~85% by soluble RGD peptide. While this result may seem somewhat aberrant given that there is a history of using BSA as a means to block cell adhesion, anecdotally, the ability of BSA to block cell adhesion has varied widely in a cell type dependent manner (also in a substrate-dependent manner). In fact, the Latour group recently thoroughly demonstrated that an RGD-binding integrin on platelets can bind to surface adsorbed BSA; this binding was dependent on the degree of albumin unfolding, and could be blocked by RGD-blocking peptides.[49,50] Thus, this finding corroborates those of the Latour group. Finally, MP uptake was simultaneously dependent on the opsonizing protein and receptor group (RGD blocked vs unblocked controls), as determined by the ANOVA interaction term p-value ($p = 0.05$). Taken all together, these data demonstrate that both Mac-1 and RGD-binding integrins play a role in the adhesion to and uptake of these protein-opsonized MPs, with RGD-binding integrins demonstrating a larger role than Mac-1. This difference in modulation level is not surprising, however, given that Mac-1 is a single receptor, whereas there are multiple receptors in the class of RGD-binding integrins.

Role of Mac-1 and RGD-binding Integrins in Microparticle-Induced Inflammatory Cytokines

To investigate the role of Mac-1 in macrophage inflammatory cytokine secretion post MP uptake, TNF- α and IL-6 production levels were compared between Mac-1 KO macrophages and macrophages generated from WT mice. As before, MPs were pre-coated with LPS and opsonizing proteins. The absence of Mac-1 resulted in secreted IL-6 levels that were reduced by 30-50% for the opsonin groups Serum and VN, compared to WT control (**Figure 5A**). In contrast, IL-6 production post-uptake of Fg and FN opsonized MPs, was not Mac-1

dependent. A reduction was also observed in TNF- α secretion upon uptake of Serum and VN -opsonized MPs, with a 50-60% reduction compared to WT control (**Figure 5B**). Here also, TNF- α production was not Mac-1 dependent for Fg and FN opsonized MPs. Interestingly, IL-6 and TNF- α secretion by the Mac-1 KO macrophages was increased over the WT controls for the BSA coated MPs. This suggests the potential for albumin or an albumin-associated factor coordinating with Mac-1 in a manner which leads to suppression of these inflammatory cytokines. Cytokine production was negligible in the no MP controls. Notably, there was a large decrease in IL-6 and TNF- α secretion by Mac-1 KO macrophages when incubated with soluble LPS (used as a positive control for the assay), where cytokine secretion was reduced by 60-75% as compared to WT. This indicates that Mac-1 plays a role in the cytokine response both to soluble LPS, as well as to LPS-adsorbed, protein-opsonized MPs. This result is consistent with reports demonstrating that Mac-1, in coordination with the CD-14 and TLR-4 receptors has been shown to be necessary for signal transduction in response to LPS [51,52]. Finally, both the opsonizing protein and receptor group (Mac-1 KO vs WT) were found to influence cytokine secretion, as determined by the ANOVA interaction term p-value ($p = 0.05$).

The role of RGD-binding integrins in macrophage inflammatory cytokine secretion was also examined. Production of TNF- α and IL-6 by macrophages was quantified after 24 h of MP exposure either in the presence or absence of soluble blocking RGD peptide. The production of both IL-6 and TNF- α was almost completely abrogated when incubated with blocking RGD peptide for all opsonin groups. The decrease in IL-6 secretion was 90% for BSA coated MPs and greater than 98% for all the remaining proteins (**Figure 6A**). For TNF- α , levels of all RGD-blocked samples were below detection limits (30 pg/mL) of the assay (**Figure 6B**). Interestingly, in this case, cytokine response to soluble LPS was completely blocked for samples with the blocking RGD peptide. Thus, RGD binding integrins are demonstrated to also play a role in the macrophage response to soluble LPS. This result is consistent with an earlier study that has reported that blocking with soluble RGD peptide decreases ERK and JNK phosphorylation in a macrophage cell line and reduces TNF- α production in response to LPS [53].

Role of Mac-1 in Foreign Body Response to Implanted Biomaterial

In order to determine the role of Mac-1 in the foreign body response, *in vivo*, a commonly used biomaterial, polyethylene terephthalate (PET) was implanted. PET has application in medical devices such as vascular grafts [54], surgical mesh [55] and sutures [56]. PET discs were subcutaneously implanted in Mac-1 KO and WT mice and the thickness of fibrous capsule formed around the discs was measured after 14 d. Representative histological sections of the explanted discs with fibrous capsules are shown for Mac-1 KO (**Figure 7A**) and WT (**Figure 7B**). Quantitatively, the thickness of the capsule formed around the discs explanted from Mac-1 KO mice was found to be, on average, 27% thinner compared to WT control (**Figure 7C**), indicating that Mac-1 influences fibrous capsule formation. Because the formation of foreign body giant cells is thought to play a mechanistic role in the response to implanted materials [2], we investigated the role of Mac-1 integrin in the *in vitro* fusion of macrophages to form foreign body giant cells. Approximately 50% of the macrophages tested fused to form foreign body giant cells, with Mac-1 having statistically higher fusion than WT controls (**Figure 8**). This indicates that the presence of Mac-1 reduces macrophage fusion and the role of Mac-1 in foreign body capsule formation must operate through some mechanism other than modulation of FBGC formation.

Role of RGD-binding Integrins in Foreign Body Response to Implanted Biomaterial

In order to examine the role of RGD-binding integrins in the foreign body response to subcutaneously implanted biomaterial *in vivo*, PET discs coated with a drug eluting polymer

were utilized to provide controlled release of functionally-blocking soluble RGD-containing peptide. Echistatin is a 49-residue peptide containing the RGD amino acid sequence, binding with higher affinity than just RGD peptide alone [39]. Polyethylene terephthalate discs were coated with the commonly used drug-eluting polymer, EVA [57], formulated with or without encapsulated echistatin. The loading efficiency of echistatin into the EVA-coated disc was found to be 95%, with a 12 µg total dose of echistatin delivered with each disc. Release of echistatin from the coated disc was characterized over 3 weeks to estimate the amount that may be released into the implant site *in-vivo*. A small burst of echistatin from the discs was seen in the first 2 d, releasing about 20% of the encapsulated echistatin (**Figure 9**). This was followed by a gradual release of 7-10 % of the encapsulated echistatin every 2 d for next 10 d after which during the next 7 d the release fell to 3-5% every 2 d. Capsule formation after 14 d of subcutaneous implantation was visualized by tetrachrome staining (**Figure 10 A-B**) and quantified by image analysis (**Figure 10 C**). Capsule thickness around the echistatin-releasing discs was on average, 45% thinner compared to the unloaded control. This result indicates that RGD-binding integrins play a significant role in the foreign body response to implanted biomaterials.

Discussion

Macrophages act as sentinels of the body's response to implanted bulk biomaterials as well as the response to particulate products of wear of these biomaterials [2,9,58]. Macrophages interact with implanted biomaterials through the layer of proteins adsorbed on the biomaterial surface, and macrophage integrin receptors bind to the milieu of biomaterial-adsorbed adhesive proteins [2,18,59,60]. Binding of integrins initiates signaling pathways leading to macrophage activation and release of inflammatory cytokines such as TNF- α , IL-6, IL-1 β , prostaglandin-E [9,61-63] and chemotactic factors such as MIP-1 β and IL-8 [64]. For non-particulate biomaterials that are too large to be phagocytosed, macrophages can fuse together to form foreign body giant cells, in what has been reported to be an integrin-dependent process [65]. Because integrins are involved at the initial adhesion stages of material-macrophage interactions, they are potential therapeutic targets for reducing inflammatory responses to implanted materials in particulate and bulk form. To explore potential target integrins, we have examined the role of different macrophage integrins such as Mac-1 and RGD-binding integrins in the inflammatory response to particulate and bulk biomaterials using integrin knockout mice and integrin blocking strategies.

Macrophage inflammatory responses have been shown to depend on the size, shape, surface area and texture of wear particles [41,42]. In the aseptic peri-prosthetic osteolysis process, upon phagocytosing 0.5-5 µm particles, macrophages have been shown to release high levels of cytokines that trigger bone resorbing pathways; hence this size range is considered highly reactive [41,42]. To elucidate the influence of integrins on macrophage-biomaterial inflammatory responses, we utilized commercially available 1 µm fluorescent polystyrene particles, representing a highly homogeneous model MP system.

There are contradicting thoughts regarding the role of adsorbed endotoxin on the immunogenicity of the various wear debris particles. Some groups have demonstrated an inflammatory cytokine response to particulates with undetectable levels of endotoxin on polyethylene [66,67], cobalt alloy [68] and titanium microparticles [67], whereas others have reported a lack of inflammatory cytokine secretion in the absence of detectable endotoxin levels on titanium, polyethylene and Co-Cr-Mo alloy MPs [43,45,46]. However, often, reports claiming a cytokine response in the absence of endotoxin have not provided sufficient information regarding the endotoxin quantification procedure used [67,68]. Specifically, the chromo-LAL endotoxin assay is sensitive to the number of particles and the volume in which they are tested, both of which are typically not reported [67]. Some groups

also test only the supernatant used to incubate the microparticles without the actual particles which may produce erroneous results as shown by Ragab et al., because endotoxins are adsorbed to the particle surface [43]. The chromo-LAL is a chromogenic kinetic assay and the endotoxin quantification depends upon the time point the absorbance crosses a threshold value which is selected by the user from a range specified by the manufacturer. Hence a detailed description of the endotoxin quantification is necessary to ensure standardization.

On the other hand, *in-vivo* studies with well-documented endotoxin-free microparticles have been shown to induce osteolysis – possibly resulting from systemic endotoxin derived from intestinal flora, minor infections, or dental procedures [69]. Wear particles have been known to have high affinity for endotoxin [70,71] which may allow systemic endotoxin to bind to generated wear particles. Furthermore, endotoxin in subclinical concentrations of bacteria have been demonstrated to be present on implants and wear particles [43]. Notably, corroborating our results, phagocytosis of endotoxin-free polystyrene MPs failed to activate macrophage cell line J774.2 resulting in cytokine levels barely above the negative controls [72]. Similar effects were also seen with dendritic cell activation after phagocytosis of polystyrene MPs [73]. Considering the contention on this issue, we conducted initial studies to understand the role of adsorbed endotoxin in our experimental setup. We found that MP uptake did not depend on the level of endotoxin adsorbed on the MPs. However, macrophages that phagocytosed polystyrene MPs with undetectable levels of endotoxin had undetectable levels of cytokine secretion. Consistent with our results, it has been shown that adsorbed endotoxin did not have an effect on phagocytosis of titanium wear particles, but did increase TNF- α secretion by a macrophage cell line [74]. In our case, in order to establish an *in vitro* polystyrene MP model system providing a particle-associated inflammatory response, it was required to pre-adsorb LPS onto the MPs. Our results indicate both Mac-1 and RGD-binding integrins play a role in modulating macrophage uptake of protein opsonized MPs, as well as modulating macrophage response to soluble endotoxin.

Integrins bind to specific domains within their ligands, for instance, integrin $\alpha_v\beta_3$ binds the RGD sequence found in proteins such as fibronectin [75], and Mac-1 binds to the P1 and P2 binding sites on the γ chain of Fg [76]. Microparticles coated with different opsonizing proteins, each containing unique sets of integrin binding domains, target different sets of integrin-ligand interactions. To understand the role of Mac-1 in macrophage response to microparticles, we investigated MP uptake as well as subsequent cytokine secretion by macrophages generated from Mac-1 KO mice. Among the opsonizing proteins investigated, uptake of Fg opsonized MPs was significantly influenced by Mac-1. Fibrinogen is one of the primary components of plasma deposited on the biomaterial surface [20,21], and a known ligand for Mac-1 [26]. Furthermore, Mac-1 has been previously reported to play a role in macrophage phagocytosis [27,28]. Upon adsorption onto a hydrophobic biomaterial surface, Fg adopts an energetically favorable conformation thereby exposing the hidden epitopes P1 (γ 190-202) and P2 (γ 377-395) which are known to be binding sites for integrin Mac-1 [76]. Additionally, Mac-1 influenced uptake of Fn, VN and Serum-coated MPs. Fibronectin and vitronectin present in serum-containing solutions adsorb onto MPs, and both these adhesive proteins have been shown to be ligands for integrin Mac-1 [25,77].

The influence of Mac-1 on particle uptake, although small at some time points, may indicate a role for Mac-1 in ligand-specific kinetics of receptor-mediated macrophage phagocytosis. In fact, these differences did translate into substantial downstream effects of IL-6 and TNF- α cytokine secretion, although the ligand-specific relationship between MP uptake and cytokine production is unclear. Pearson's coefficient showed no correlation between cytokines and MP uptake, suggesting that while ligand-specific signals modulate cytokine production, this response is independent of the number of MPs phagocytosed and correspondingly not correlated with differential LPS exposure (where LPS was adsorbed on

the MPs). Additionally, for the soluble LPS control groups, our results show that IL-6 and TNF- α levels from Mac-1 KO macrophages is lower than WT for the positive control. This observation is in agreement with work done by Vogel *et al.*, where they found that integrin Mac-1 complexes with the CD-14 receptor, which does not have a transmembrane component [78], and TLR-4 in response to LPS, and that the complex is required for expression of the whole spectrum of inflammatory genes [51]. It was also demonstrated that LPS-induced NF- κ B translocation and MAPK activation are down-regulated in Mac-1 deficient mice [52].

In order to investigate the role of RGD-binding integrins in MP uptake, we blocked RGD receptors using soluble RGD peptide in the culture media. The use of functionally-blocking soluble RGD containing peptide inhibits cell adhesion [47,79]. The RGD motif is the integrin binding site present in several proteins such as fibronectin, fibrinogen, vitronectin and laminin [31] known to adsorb on to biomaterial surfaces. Blocking the macrophage RGD-binding integrin receptors such as $\alpha_5\beta_1$, $\alpha_v\beta_3$ and $\alpha_v\beta_5$, was hypothesized to prevent interaction and binding to protein-opsonized MPs thus resulting in a significant reduction in MP uptake as well as subsequent cytokine secretion, and our results support this. Interestingly, there was also a complete down regulation in cytokine secretion from the soluble LPS positive controls when soluble RGD was present. This finding is corroborated by literature showing that integrin α_v subunit is involved in LPS-induced TLR4 signaling pathways leading to macrophage adhesion and cytokine release [80]. It has also been shown that RGD peptide interferes with increased adherence seen with LPS-treated cells as well as decreases ERK and JNK phosphorylation which are linked to LPS-induced TNF- α production [53]. A cell type closely related to macrophages, dendritic cells, which are also adhesion dependent antigen presenting cells, have also demonstrated ligand-dependent modulation of adhesion and activation by engaging different integrins [36,81]. This ligand-specific differential activation of dendritic cells is being explored for the potential to modulate immune responses [82,83], including through the targeting of RGD-binding integrins [84].

The role of Mac-1 and RGD-binding integrins in the *in-vivo* foreign body response to subcutaneously implanted biomaterials was examined by measuring the fibrous capsule formed around implanted materials. There was a 27% reduction in the thickness of capsule formed around discs implanted in Mac-1 KO mice compared to WT. Based on current paradigms [1], macrophages are likely the key cell type in the influence of Mac-1 on the foreign body response. Based on our *in vitro* results with MPs, it can be speculated that the *in vivo* loss of Mac-1 may have resulted in a dampened cytokine response to the implanted biomaterial. Fibrinogen, a large component of plasma, is likely a primary Mac-1 ligand driving this effect, as it has previously been linked to biomaterial-associated inflammation [21,23,76]. However, Mac-1 is also present on a range of other leukocytes [85-87]. For instance, the granulocytes, neutrophils or eosinophils, possess Mac-1 and could be involved. Additionally, Mac-1 binds complement factors known to be involved in complement-mediated inflammation to implanted biomaterials [24,88,89]. Given that foreign body giant cells have been thought to be linked to fibrous capsule formation, the role of Mac-1 integrin in FBGC formation was investigated. However, Mac-1 had no significant effect on FBGC formation. This suggests that *in vivo*, other factors drive fibrous capsule formation. Notably, it has been recently reported that signaling through the adaptor protein DAP12 plays a major role in macrophage fusion, with the β_2 integrins (such as Mac-1) only having a minor role in [90].

Blocking the receptor class of RGD-binding integrins through the controlled release of echistatin, a high affinity RGD peptide, resulted in a 45% reduction in fibrous capsule thickness formed around implants. This relatively larger modulation may be rationalized,

given that RGD-binding integrins comprise multiple receptors present on leukocytes (such as $\alpha_5\beta_1$, $\alpha_v\beta_3$ and $\alpha_v\beta_5$), whereas Mac-1 represents a single leukocyte receptor. As before, macrophages likely play a primary role in the RGD-binding integrin-directed modulation of the foreign body reaction, but a role for other cell types cannot be ruled out here either, such as neutrophils or others. For example, integrin $\alpha_{IIb}\beta_3$ binding to the RGD domain in Fg is linked to platelet activation [91], the blocking of which could lead to altered transitional matrix formation. We speculate that the controlled release of echistatin served to reduce leukocyte adhesion to proteins adsorbed on the implant surface. Our results demonstrate that RGD-binding integrins play a major role in the macrophage response to particulate biomaterials, where almost complete blocking of cytokine production was effected. A similar blocking of the cytokine response may be expected in the macrophage response to bulk biomaterials. Cytokine secretion by macrophages at the implant site plays a major role in recruitment of other cell types that participate in capsule formation [2].

Conclusion

The integrin Mac-1 and RGD-binding integrins were found to play a role in the *in vitro* response to particulate biomaterials and the *in vivo* fibrous capsule formation around subcutaneously implanted biomaterials. These results add to the understanding of receptor-ligand interactions in these processes and will help in the further exploration for anti-integrin therapeutic targets to mitigate inflammatory responses to implanted biomaterials. Redundancy is expected in integrin function hence, the effects seen in the paper ranged from modest for the single receptor, Mac-1, to considerably larger, for the family of RGD-binding integrins. Approaches targeting multiple integrins need to be explored to determine if further mitigation of macrophage responses is possible, and to attain clinical relevance.

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Figure 1.

A.) Purity of C57BL6/J bone marrow-derived macrophages was determined to be > 87% by flow cytometry analysis of F4/80 and CD11b, murine macrophage markers. **B.)** Deficiency of the Mac-1 integrin receptor on bone marrow-derived macrophages from the Mac-1 KO mouse was verified by immunofluorescence staining for CD11b (the α chain of integrin Mac-1). The percentage of F4/80⁺ cells expressing CD11b was determined to be ~1%.

Figure 2.

The presence of surface-adsorbed lipopolysaccharide (LPS) endotoxin on polystyrene microparticle (MP) does not influence uptake by C57BL/6/J bone marrow-derived macrophages, but is required for cytokine production. **A.)** Microparticles opsonized with various proteins (BSA: bovine serum albumin; Fg: fibrinogen; FN: fibronectin; Serum: fetal bovine serum; VN: vitronectin) were either tested and found to be endotoxin-free (white bars), or were coated with LPS to a level of 5 EU/million MPs/mL (grey bars). After incubating 7.5 h at the MP:cell ratio of 20:1, MP uptake was quantified by pooling data from at least 9 samples from 3 separate runs, and the mean and standard error were plotted. No statistical differences were found when comparing MPs with and without LPS coating for each protein coating. **B.)** Secretion of the inflammatory cytokine, TNF- α , was quantified after 24 h exposure to MPs with LPS coating (grey bars) or without LPS coating (white bars). Cytokine concentrations were quantified by pooling data from 6 samples from 2 separate runs. Plotted are mean and standard error. Pair-wise significant difference between MPs coated with or without LPS for each protein coating (by ANOVA and Tukey significance test) is denoted by the * symbol (p value < 0.05).

Figure 3.

Integrin Mac-1 modulates macrophage phagocytosis of protein-opsonized polystyrene microparticles (MPs). Microparticles pre-coated with LPS (5 EU/million MPs/mL) and an opsonizing protein were incubated with macrophages at a 40:1 ratio of MP:cell, and uptake was quantified at: **A.**) 2 h **B.**) 3.5 h **C.**) 5 h, and **D.**) 7.5 h. Results comparing Mac-1 KO macrophages to WT control are shown. The average number of MPs per cell taken up was quantified by pooling data from at least 15 samples from 5 separate runs. Plotted are mean and standard error. Pair-wise significant differences between Mac-1 KO and WT control samples (by ANOVA and Tukey's test) for each protein coating is denoted by the * symbol (p value < 0.05).

Figure 4.

Phagocytosis of protein-opsonized microparticles by macrophages is modulated by blocking RGD-binding integrins. Microparticles pre-coated with LPS (5 EU/million MPs/mL) and an opsonizing protein were incubated with macrophages at a 40:1 ratio of MP:cell, and uptake was quantified. **A.)** Uptake at 2 h, blocking with 1 mM RGD peptide. The average number of MPs taken up by macrophages was quantified by pooling data from 18 samples from 6 separate runs. **B.)** Uptake at 24 h, blocking with 10 mM RGD peptide. The average number of MPs taken up by macrophages was quantified by pooling data from 8 samples from 2 separate runs. Plotted are mean and standard error. Pair-wise significant differences between RGD blocked and control samples (by ANOVA and Tukey's test) for each protein coating is denoted by the * symbol (p value < 0.05).

Figure 5.

Integrin Mac-1 modulates macrophage cytokine secretion in response to protein-opsonized, LPS-coated polystyrene microparticles (MPs). Secreted cytokines: **A.)** IL-6, and **B.)** TNF- α , from Mac-1 KO and WT macrophages upon 24 h exposure to protein-opsonized, LPS-coated (5 EU/million MPs/mL) MPs. Cytokine concentrations were quantified by pooling data from 30 samples from 5 separate runs. Plotted are mean and standard error. Pair-wise significant difference between Mac-1 KO and WT control samples for each protein coating (by ANOVA and Tukey significance test) is denoted by the * symbol (p value < 0.05).

Figure 6.

Blocking RGD-binding integrins modulates macrophage cytokine secretion in response to protein-opsionized, LPS-coated polystyrene microparticles (MPs). Secreted cytokines: **A.)** IL-6, and **B.)** TNF- α , from macrophages with or without RGD blocking with 10 mM soluble peptide in response to 24 h exposure to protein-opsionized, LPS-coated (5 EU/million MPs/mL) MPs. Cytokine concentrations were quantified by pooling data from 6 samples from 2 separate runs. Plotted are mean and standard error. Pairwise significant difference between Mac-1 KO and WT control samples for each protein coating (by ANOVA and Tukey significance test) is denoted by the * symbol (p value < 0.05).

A. **B.** **C.** **Figure 7.**

Integrin Mac-1 modulates the foreign body response to subcutaneously implanted biomaterials. Hematoxylin and eosin stained sections (protein, typically primarily collagenous, stains pink; cell nuclei stain dark blue) of the fibrous capsule surrounding PET discs explanted at 14 d from: **A.)** Mac-1 KO mice, and **B.)** WT control, indicating loss of Mac-1 results in an attenuated foreign body response. **C.)** Capsules are 27% thinner around implants in the Mac-1 KO compared to WT, quantified by pooling data from 8 samples. Plotted are mean and standard error. Significant difference between Mac-1 KO and WT control samples (by Student's *t*-test) is denoted by the * symbol (*p* value < 0.05).

Figure 8.

Integrin Mac-1 does not play a role in the fusion of macrophages to form foreign body giant cells. Macrophage fusion was quantified as the percentage of giant cell nuclei relative to the total number of nuclei. Data was quantified by pooling data from 12 samples from 3 separate runs. Plotted are mean and standard error. No significant difference between Mac-1 KO and WT control samples (by Student's *t*-test) was found.

Figure 9. Release kinetics of encapsulated echistatin from ethylene vinyl acetate polymer coating on PET discs. Samples were incubated at 37°C in deionized water with continuous shaking. Each point represents the mean and standard deviation of 3 samples.

Figure 10. Blocking RGD-binding integrins modulates the foreign body response to subcutaneously implanted biomaterials. Tetrachrome stained sections of the fibrous capsule surrounding PET discs explanted at 14 d from: **A.)** echistatin-releasing discs, and **B.)** unloaded control discs, indicate blocking RGD-binding integrins results in an attenuated foreign body response. **C.)** Capsules are 45% thinner around the echistatin loaded implants than the unloaded control, quantified by pooling data from 6-7 samples. Plotted are mean and standard error. Significant difference between echistatin loaded and unloaded control samples (by Student's *t*-test) is denoted by the * symbol (*p* value < 0.05).