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Phenotypic Changes in Bone Marrow Derived Murine Macrophages cultured on PEG-based Hydrogels and Activated by Lipopolysaccharide

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Abstract

Macrophages are phenotypically diverse cells performing a number of functions involved in immunity, inflammation, wound healing, tissue homeostasis, and the foreign body reaction. In the latter, the type of biomaterial and the surrounding environment likely impacts macrophage phenotype and subsequently the severity of the reaction. The objectives for this study were to characterize the phenotype of bone marrow derived murine macrophages in response to poly(ethylene glycol) (PEG)-based hydrogels, a promising class of materials for cell delivery. Gene expression was used as a measure of phenotype and characterized by *IL-1 β* , *TNF- α* , *iNOS*, *IL-12 β* , *arginase*, *VEGF-A*, and *IL-10*. Macrophages were cultured on PEG hydrogels, PEG hydrogels with RGD tethers, and medical grade silicone rubber, a well-characterized biomaterial, up to 96 hours in the absence and presence of lipopolysaccharide (LPS) to simulate an inflammatory environment. Macrophage interrogation led to immediate upregulations (10x) in *IL-1 β* and *TNF- α* , within 4 hours followed by an increase in *IL-10/IL-12 β* and a subsequent concomitant decrease in the pro-inflammatory genes by 96 hours suggesting a shift from classically activated to a regulatory phenotype. LPS stimulation led to a stronger early upregulation of proinflammatory genes (e.g., 20-30x for *IL-1 β* and *TNF- α*) followed by upregulations (4-6x) in *arginase* suggesting a shift from an elevated classically activated to a wound healing phenotype. Material type played a significant role in regulating proinflammatory genes, which was most pronounced in PEG-only. Overall, our findings indicate that macrophages undergo similar phenotypic changes for the materials tested, but the magnitudes of these responses are highly material-dependent.

Keywords

Macrophage; Phenotype; In Vitro; poly(ethylene glycol); RGD

1 Introduction

Macrophages are one of the most phenotypically diverse cells in the body. They perform a number of roles in immunity, inflammation, wound healing and tissue homeostasis. Macrophages also play a key role in orchestrating the foreign body reaction [1,2], a series of

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events that are initiated when a synthetic biomaterial is implanted into the body [3-5]. The functions of macrophages are carried out through the secretion of cytokines, growth factors and small molecules and these cells are found in one form or another in virtually every tissue in the body. They have long been considered to fall into one of two states upon activation: classical or alternative. As knowledge about macrophage function is gained, this simplified classification scheme has become inadequate. Mosser [6] has suggested a more complex system for understanding macrophage responses to stimuli and their subsequent behavior. Recently, Anderson [7] also suggested that, in the understanding of macrophage interactions with biomaterials, neither classical nor alternative activation adequately describes their role.

Nominally, activation of macrophages can be classified as classical, wound healing or regulatory. Under classical activation, macrophages respond to Th1 cytokines, such as interferon gamma or interleukin 1 β (IL-1 β), and to bacteria by the secretion of proinflammatory cytokines and reactive oxygen and nitrogen species [8]. If this response is elicited upon implantation of a biomaterial or a cell-laden tissue engineering construct, unanticipated oxidative biodegradation of the material [9] or significant damage to the cells and/or developing tissue may occur. Under wound healing activation, macrophages respond to Th2 cytokines, such as interleukin-4 or 13, by enhanced arginase activity and decreased production of proinflammatory cytokines and reactive oxygen and nitrogen species, which lead to extracellular matrix production and overall wound healing [10]. Finally, regulatory activation is achieved by stimulation with immune complexes or interleukin 10 (IL-10) and is often characterized by the secretion of anti-inflammatory cytokines, such as IL-10 [11]. Recently, Mosser and Edwards [12] proposed that macrophage activation be described as a continuum between these three activation states rather than distinct and separate phenotypes.

The majority of studies have focused on the classically activated macrophage phenotype in response to synthetic biomaterials because of the three primary activation states, classical activation is the most detrimental. *In vitro*, macrophage activation has been shown to be largely dictated by surface chemistry. For example, several studies have shown that hydrophobic surfaces lead to increased expression of classically activated markers. For example, Naim *et al.* [13] found that surfaces which were more hydrophobic promoted a more severe classical macrophage activation state as evidenced by increased expressions of tumor necrosis factor alpha (TNF- α), interleukin-6 (IL-6), and IL-1 β when compared to surfaces that were less hydrophobic. It was found that the severity of the response was modulated by the type of protein adsorbed to the surfaces suggesting that macrophage activation is at least partly mediated by the proteins present at the surface. In a separate study, macrophages cultured on anionic surfaces (e.g., poly(acrylic acid)) had significantly lower expression of interleukin 8, a chemokine associated with classical activation, when compared to hydrophobic surfaces, suggesting a reduced classically activated state [14]. On the contrary, our previous work has shown that macrophages cultured on a hydrogel, crosslinked poly(ethylene glycol) (PEG), led to a strong upregulation in gene expression of proinflammatory cytokines when compared to the more hydrophobic material of medical grade silicone [15]. While the focus has primarily been on classical activation, a few studies have explored markers for other phenotypes. For example, Brodbeck *et al.* [14] described that macrophages cultured on hydrophilic surfaces (e.g., polyacrylamide) exhibited a regulatory macrophage activation phenotype as evidenced by high levels of IL-10 secretion when compared to hydrophobic surfaces (e.g., poly(ethylene terephthalate)). Taken together, these studies and others suggest that macrophage activation in response to biomaterials is highly variable and may indeed span a continuum space between the activation states, warranting further investigation.

From a tissue engineering perspective where scaffolds are placed *in vivo*, the macrophage response will likely impact the development of the engineered tissue and the overall integration of the engineered tissue into the host tissue. Recent studies from our group demonstrated that

PEG hydrogels, commonly used for cell encapsulation in tissue engineering strategies [16-18], elicit a strong inflammatory response during *in vitro* culture of bone marrow derived murine macrophages and when implanted into subcutaneous pockets in mice [15]. By simply incorporating a cell adhesion ligand, RGD, into the PEG hydrogels, the inflammatory response was reduced, but not eliminated. *In vivo*, the foreign body reaction to PEG hydrogels did not resolve after 28 days as indicated by a large presence of inflammatory cells at the implant surface. However, resolution of the foreign body reaction to PEG with RGD was more evident after 28 days with the formation of a fibrous capsule. Since macrophages are involved in both inflammation and wound healing and are thought to be the cells largely responsible for the foreign body reaction, a better understanding of macrophage phenotype in response to biomaterials may help to design and/or select materials for improved *in vivo* success.

The overall goal of this study was to characterize the phenotype of activated macrophages within the spectrum of activation states (i.e., classical, wound healing, and regulatory states [12]) when cultured on PEG-based hydrogels in the absence and presence of a proinflammatory molecule. Specifically, macrophage phenotype was evaluated through the expression of a series of genes including: i) *IL-1 β* , *TNF- α* , *inducible nitric oxide synthase (iNOS)*, *IL-12 β* , which are closely associated with inflammation, ii) *arginase* and *VEGF*, which are closely associated with wound healing, and iii) *IL-10*, an immunoregulatory cytokine that has anti-inflammatory functions. The specific aims of this study were two-fold: i) to elucidate the role of the biomaterial in directing macrophage phenotype and ii) to elucidate the combined role of the biomaterial and a proinflammatory stimulant in an effort to mimic better the initial acute inflammatory reaction immediately post-implantation. Since both infection and injury (such as would occur when a material is implanted) initiate inflammation, exogenous delivery of lipopolysaccharide (LPS), which is derived from the cell wall of bacteria, was chosen as a simple method for simulating an inflammatory environment *in vitro* [19,20]. While LPS stimulation may not be involved in the foreign body reaction, the pathway by which LPS stimulates macrophages (i.e., toll-like receptors) [21] has also been shown to be activated by biomaterials *in vitro* and *in vivo* [22-24]. Bone marrow derived murine macrophages were cultured on three biomaterials: PEG-only hydrogels, PEG hydrogels containing RGD tethers, and medical grade silicone rubber, which served as a reference for a typical foreign body reaction. Macrophage phenotype was assessed via real-time RT-PCR *in vitro* in the absence and presence of LPS.

2 Materials and Methods

2.1 Macromer synthesis and hydrogel formation

Poly(ethylene glycol) diacrylate (PEG-dA) macromers were synthesized by dropwise addition of acryloyl chloride (Sigma Aldrich) into dry toluene containing dissolved 3000 Da poly(ethylene glycol) (PEG) (Fluka) and triethylamine (Sigma) to a final molar ratio of 4:1:4.2. The reaction was allowed to proceed protected from light overnight at room temperature and under constant agitation. The PEG-dA product was purified by precipitation in cold diethyl ether, dried and stored at 4°C under vacuum until use. Acrylation percentage was determined by ¹H NMR to be above 92%. The cell adhesion oligopeptide YRGDS (tyrosine, arginine, glycine, aspartic acid, serine, Genscript) was conjugated to 3400 molecular weight monoacrylated PEG succinimide (Laysan Bio Inc.) at a 1.1:1 molar ratio in a sodium bicarbonate buffer (50mM) at a pH of 8.4 for 2 hours. The monoacrylated PEG YRGDS macromer was purified by dialysis, lyophilized and characterized by ¹H-NMR.

Macromer solutions contained 20% (w/w) PEG-dA, 0.0125% (w/w) photoinitiator 1-[4-(2-Hydroxyethoxy)-phenyl]-2-hydroxy-2-methyl-1-propane-1-one (PI2959, Ciba) with or without 5mM monoacrylated PEG YRGDS. Hydrogels were formed via photopolymerization (365nm, 5-10mW/cm²) into sheets between two glass slides using 0.8mm thick Teflon spacers.

Disks of medical grade silicone (Invotec, SIL), poly(ethylene glycol) (PEG-only) and poly(ethylene glycol) with 5mM monoacrylated PEG YRGDS (PEG-RGD) were punched from sheets using 5mM biopsy punches. All hydrogels were prepared under sterile conditions, rinsed 3-4 times in sterile filtered 70% ethanol, followed by 4-5 rinses in endotoxin free PBS over the course of several days. Sample hydrogels were tested using the LAL assay (Invitrogen) and determined to be endotoxin-free. Sterile disks were secured to tissue culture treated poly(styrene) plates using a small amount of vacuum grease. Prior to macrophage seeding materials were soaked in 100% fetal bovine serum (FBS) for 1 hour.

2.2 Macrophage isolation, culture, and lipopolysaccharide (LPS) stimulation

Primary murine macrophages were obtained from 6 week old c57bl/6 male mice as described by Jay et al. [25]. In brief, bone marrow isolates were resuspended in Iscove's Modified Dulbecco's Medium (IMDM, Invitrogen) + 10% fetal bovine serum (FBS) and PSF (penicillin, streptomycin, and fungizone). The cell suspension was layered over Lympholyte M (Accurate Chemical) and centrifuged per manufacturer and the fractionate containing mononuclear cells collected. The cells were resuspended at 10^6 cells/ml in expansion medium (IMDM+20% FBS, 2mM L-glutamine, PSF, 1.5 ng/ml human macrophage colony stimulating factor (R&D systems) and 100 ng/ml huFLT-3), plated at 1.7×10^5 cells/cm² in 100 mm Petri dishes, and cultured to confluency (~ 10 days) prior to use in the experiments. Macrophages were confirmed by positive staining for F4/80, a membrane bound glycoprotein specific to murine macrophages, through immunocytochemistry and were determined to be >90% of the total cell population. Cells were seeded onto the three material surfaces at a density of 5×10^4 cells/construct in media (IMDM supplemented with 25% FBS, 2mM L-glutamine, penicillin, streptomycin and fungizone) and allowed to adhere for 24 hours. After 24 hours of culture, medium was exchanged with either fresh medium (described above) or medium supplemented with 1µg/mL of lipopolysaccharide from *salmonella enterica* serotype enteritidis (Sigma Aldrich). This concentration of LPS was chosen because it has been previously shown to stimulate proinflammatory cytokine expression in macrophages *in vitro* [26,27]. Samples were removed from culture and prepared for analysis prior to, and at 4, 8 24, 48, 72 and 96 hours after the initial media exchange.

2.3 Real-time reverse transcription polymerase chain reaction

Cells adhered to material disks (n=4 per condition) were lysed using TRK lysis buffer (Omega) and RNA was isolated using E.Z.N.A. microelute kit (Omega) following the manufacturers protocol. Purified RNA was reversely transcribed to cDNA using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) and samples were stored at -80 °C until PCR analysis was performed. Real time PCR was performed with Fast SYBR Green Master Mix (Applied Biosystems) with the 7500 Fast system (Applied Biosystems). Custom primers (Table 1) were designed using Primer Express 3.0 software (Applied Biosystems) and validated for both efficiency and stability of the housekeeping gene *L32*, a mitochondrial ribosomal protein. Data are presented as [28]:

$$\text{Normalized Expression (NE)} = \frac{E_{HKG}^{C_{t(HKG, \text{calibrator})} - C_{t(HKG, \text{sample})}}}{E_{GOI}^{C_{t(GOI, \text{calibrator})} - C_{t(GOI, \text{sample})}}}$$

where E is the primer efficiency, HKG is the house keeping gene, GOI is the gene of interest and C_t is the cycle number where the fluorescence crosses the threshold. Calibrator is the average C_t value for the 0 hour time point for the material of interest. Additionally, data in Fig. 3 are presented as fold change where the NE for LPS treated samples is divided by the NE of corresponding untreated samples.

2.4 Statistical analysis

ANOVAs were run on all data using the General Linear Model within MiniTAB 15. Factors analyzed were material type and culture time for NE of each gene as the response. All pair wise comparisons were analyzed with material type and culture time as models. An alpha value of 0.05 was used to determine statistical significance. All data are presented as mean $\pm$ standard deviation.

2.5 Animal Use

All animal protocols used in this study were approved by the University of Colorado at Boulder animal use committee.

3 Results

Macrophage phenotype was assessed by RT-PCR for i) macrophages cultured on PEG-only hydrogels, PEG containing RGD (PEG-RGD) hydrogels, and medical grade silicone (SIL) (Fig. 1), ii) macrophages cultured on each biomaterial but in the presence of lipopolysaccharide (LPS) (Fig. 2), and iii) a synergistic effect between biomaterial and LPS stimulation by normalizing the LPS-stimulated response to the biomaterial response (Fig. 3). Results from two-way ANOVAs for material type and culture time are presented in Tables 2, 3, and 4, respectively.

3.1 Macrophage response to biomaterial

Initially, genes closely associated with inflammation were evaluated where culture time and material type were significant factors affecting *IL-1 β* (Fig. 1a), *TNF- α* (Fig. 1b), and *IL-12 β* (Fig. 1d) expressions, but only culture time affected *iNOS* (Fig. 1c) expression. The temporal expression pattern for *IL-1 β* and *TNF- α* , were similar peaking early at 4 hours followed by a significant decrease in expression to values that were below the initial levels by 96 hours. The highest *IL-1 β* expression occurred in response PEG-only, which was ~9-fold higher compared to initial expression levels. The highest *TNF- α* expression was similar in PEG-only and SIL, which was significantly higher by ~3-fold compared to PEG-RGD. The temporal expression pattern for *IL-12 β* on all materials showed increased expression with the highest levels seen in macrophages cultured on PEG-only, by ~4-fold, followed by a significant down-regulation at 48 hours and thereafter. *iNOS* showed cyclical expression patterns with time with values increasing/decreasing relative to initial levels throughout the course of the experiment for the PEG-only and SIL materials. In response to PEG-RGD, *iNOS* levels decreased initially followed by a late up-regulation at 48 hours, which continued to increase resulting in the highest level of expression at 96 hours and which was highest among all materials.

Next, two genes closely associated with wound healing were assessed where culture time, but not material type was a significant factor affecting *arginase* Type I (Fig. 1e) and *vascular endothelial growth factor A (VEGF-A)* (Fig. 1f) expressions. For all materials, *arginase* expression was down-regulated after 4 hours, but returned to initial levels by 24 hours. *Arginase* expression for macrophages cultured on PEG-RGD hydrogels remained at initial levels over the course of the culture, but was down regulated on PEG-only hydrogels and SIL to levels that were 10-25% of initial levels by 96 hours. *VEGF-A* expression was significantly and similarly down-regulated with culture time for all materials.

Finally, the immunoregulatory cytokine gene, *IL-10*, (Fig. 1g) and the ratio of *IL-10/IL-12 β* (Fig. 1h) were evaluated where culture time, but not material type ($p=0.051$ for the ratio) was a significant factor. For all materials, *IL-10* expression was down-regulated initially at 4 hours, followed by a return to initial levels by 24 hours and then a final down regulation by 96 hours. The temporal expression pattern of *IL-10/IL-12 β* was similar for all materials showing a down

regulation at 4 hours, followed by a steady increase in expression over the course of the study. By 96 hours, *IL-10/IL-12 β* values were highest by 4-9 fold in response to PEG-only compared to PEG-RGD and SIL.

3.2 Macrophage response to biomaterials when stimulated with LPS

Initially, genes closely associated with inflammation were evaluated where culture time and material type were significant factors affecting expression of *IL-1 β* (Fig. 2a), *TNF- α* (Fig. 2b), *iNOS* (Fig. 2c) and *IL-12 β* (Fig. 2d). The temporal pattern of expression was similar for *IL-1 β* and *TNF- α* peaking early by 4 hours followed by subsequent downregulations in their expression with culture time. The highest *IL-1 β* expression occurred in response to the PEG-only materials with values that were ~2-fold higher compared to the other materials. *TNF- α* expression was similarly high for PEG-only, but only significant over PEG-RGD by ~1.7 fold. *iNOS* expression steadily increased with culture time for all materials with the highest expression occurring by 48 hours and in response to PEG-RGD (~200 $\times$ higher than initial levels). *IL-12 β* temporal expression patterns were similar to *IL-1 β* with the highest expression levels occurring in response to PEG-only, but the levels were not as dramatic.

For the two genes closely associated with wound healing, *arginase type I* (Fig. 2e) and *VEGF-A* (Fig. 2f) were affected by culture time but not material type. LPS stimulation led to late upregulations in *arginase* expression on all materials by 24 or 48 hours, which were 4-6 $\times$ higher than initial levels. Small, but significant, cyclical expression patterns were observed with *VEGF-A* expression as a function of culture time for all materials but were most evident for PEG-only and SIL.

The immunoregulatory cytokine gene, *IL-10* (Fig. 2g) and the ratio of *IL-10/IL-12 β* (Fig. 2h) were affected by culture time and material type. *IL-10* expression was similar for macrophages cultured on both PEG-only and PEG-RGD, but was generally lower for SIL. The ratio of *IL-10:IL-12 β* expression was significantly decreased for macrophages cultured on both PEG-only and SIL from 4-48 hours with a return to initial levels after 96 hours of culture. Macrophages on PEG-RGD materials exhibited a cyclical expression pattern with culture time of downregulations and returns to initial levels throughout the culture time.

3.3 Synergistic effect of biomaterial and LPS stimulation in macrophage response

For the inflammatory genes, culture time and material type were significant factors affecting their gene expression. In general, fold change in their expression with LPS treatment compared to unstimulated controls increased with culture time. *IL-1 β* (Fig. 3a) and *IL-12 β* (Fig. 3d) had large increases in their fold change with their highest level occurring by 48 and 96 hours, respectively, on all materials and which was most significant in response to PEG-only. The magnitude of fold change for *TNF- α* (Fig. 3b) was not as dramatic, but was generally higher on PEG-only materials. Fold changes in *iNOS* (Fig. 3c) expression were the highest throughout the culture period when compared to the other genes and most significant in response to PEG-RGD (490 $\times$ from initial levels at 48 hours).

Fold changes in expression for the two genes closely associated with wound healing, *arginase type I* (Fig. 3e) and *VEGF-A* (Fig. 3f), were affected by culture time but only *arginase* was affected by material type. Fold changes in *arginase I* were highest in macrophages on PEG-only compared to PEG-RGD and SIL. Across all materials, fold changes in *arginase* expression increased with culture time with the exception of PEG-RGD which peaked after 48 hours followed by a down-regulation. Fold changes in *VEGF-A* increased significantly at late times over initial levels with a similar temporal pattern of expression across all materials.

The immunoregulatory cytokine gene, *IL-10* (Fig. 3g), and the ratio of *IL-10/IL-12 β* (Fig. 3h) were affected by culture time but material type was only significant in the ratio of *IL-10/IL-12 β* . For *IL-10* fold changes, the temporal pattern of expression exhibited a bimodal response for all materials peaking early at 4 hours, followed by a rapid decrease at 24 hours and then a subsequent second, but more moderate, increase. The temporal expression pattern for fold change in the ratio was similar among all materials with an up-regulation early between 4 and 8 hours followed by a subsequent decrease to sub-initial levels (e.g. 3%, 10% and 20% for PEG-only, SIL, and PEG-RGD, respectively). The ratio was generally highest in response to PEG-RGD materials.

4 Discussion

The work presented in this study demonstrates that PEG-based hydrogels, a biomaterial that is being investigated for tissue engineering applications led to unique gene expression profiles in macrophages adhered to their surfaces. This response was not defined by one specific phenotype (i.e., classically activated, wound healing or regulatory), but rather defined by a broad spectrum of phenotypes, which was dynamic in time. The spectrum of activation was also largely dependent on the type of material to which macrophages were adhered and further altered by culturing in an inflammatory environment (via the addition of LPS).

Characteristics of classically activated macrophages include the secretion of a whole host of proinflammatory molecules including cytokines *IL-1 β* and *TNF- α* . *IL-12 β* is also secreted by classically activated macrophages and is part of a positive feedback loop involving *Th*₁ maturation and secretion of interferon gamma leading to further macrophage activation [10]. While *IL-12 β* does not act directly on macrophages, its upregulation is indicative of the macrophage potential to sustain inflammation and could lead to enhanced activation of the adaptive immune response. In this study, upregulation of *IL-1 β* , *TNF- α* and *IL-12 β* in response to a biomaterial was most pronounced early with the largest increases observed consistently in macrophages adhered to PEG-only. As expected, stimulation with LPS, led to significant and immediate upregulations in all three genes on all materials with an extended and overall stronger response to PEG-only. These findings are in agreement with our previous work [15] demonstrating that PEG-only hydrogels up-regulate expression of proinflammatory cytokines, while the presence of RGD attenuates this inflammatory response.

In vivo, *iNOS* expression is considered a traditional marker of inflammation where *iNOS* converts arginine into reactive nitrogen species including nitric oxide (NO). While *iNOS* expression was not overall dependent on material type in the absence of LPS, a few differences are worth noting. Expression of *iNOS* was lowest in the PEG-RGD hydrogels at early time points indicating that the presence of RGD tethers initially mitigates high *iNOS* expression. More interesting is the fact that *iNOS* expression is then up-regulated due to the presence of RGD at later times and even more so in the presence of LPS. NO can have very different roles in inflammation. While NO serves as a signaling molecule involved in apoptosis, upregulation of matrix degrading enzymes, and inhibition of tissue synthesis, it is also an important signaling molecule in vasodilation and neovascularization suggesting that the late increases in *iNOS* expression (like those seen on PEG-RGD, albeit only after 96 hrs) may potentially have beneficial effects at the implant site [29] or may be important for resolution of inflammation and ultimately result in wound healing [30]. Although, the role of NO in the foreign body reaction is not well known.

Arginase type I and *VEGF-A* are typically up-regulated during wound healing. Arginase acts on arginine, the same substrate as *iNOS*, but converting it into L-ornithine, a precursor for proline and synthesis of collagen that is essential in wound healing. *VEGF-A* is a potent regulator of vasodilation and is a critical early mediator of wound healing. However, their roles

in the resolution stage of biomaterials are less clear. There was no overall dependence on material type for *arginase* and *VEGF-A* expression and in general a down-regulation in their expression was observed at later time points with one notable exception. In the PEG-RGD gels, mean *arginase* expression steadily increased with culture time. These findings suggest biomaterial activation by PEG-only of macrophages inhibits a wound healing phenotype, while the presence of RGD is capable of rescuing this phenotype in unstimulated samples. Additionally interesting is that a proinflammatory environment created by LPS not only stimulates proinflammatory gene expressions, but also up-regulates wound healing genes, in the presence of all materials. This transition may possibly set the stage for resolution and capsule formation as commonly seen during the FBR *in vivo*.

IL-10 is a potent anti-inflammatory cytokine, which acts to reduce secretion of $\text{TNF-}\alpha$ among other cytokines. When *IL-10* is upregulated compared to *IL-12\beta*, the macrophage is considered to be regulatory in nature acting to reduce Th_1 type responses. While there was no overall dependence on material type for *IL-10* or *IL-10:IL-12\beta*, both *IL-10* and *IL-12\beta* decreased with time but the regulatory gene decreased to a lesser degree indicating a shift towards a regulatory phenotype. This observation is consistent with recent studies showing that *IL-10* and other immunosuppressive signals are localized in tissues near implanted foreign bodies [31]. When macrophages were stimulated with LPS, *IL-10* was upregulated for the majority of the culture. However, the *IL-10:IL-12\beta* ratio decreased significantly over time indicating that LPS stimulation of proinflammatory cytokines was more pronounced and hence may dominate the macrophage phenotype.

In an effort to summarize our main findings and to begin to elucidate a macrophage phenotype (at least based on gene expression and the specific series of genes selected), we integrated our results into the context of a spectrum of activation [12]. By accounting for differences in expression levels among all the genes that span a range of biological functions (inflammation, wound healing, and immunoregulation), we can begin to gain insights into the activation state of the macrophages in response to a biomaterial. Interestingly our findings indicate that for the materials tested in this study, the overall phenotypic state of the macrophage was similar, but the magnitude was different. Fig. 4 summarizes the activation states of the macrophages as a function of material and time in the absence (white dots) and presence (black dots) of LPS. With regard to biomaterial activation of macrophages, initial expression levels were high for the proinflammatory cytokines, but low for the wound healing proteins and the immunoregulatory cytokine suggesting a classically activated phenotype. With time, there was an overall change in expression resulting in increased levels of immunoregulatory genes with a concomitant decrease in the proinflammatory cytokines suggesting a shift towards a regulatory phenotype. In the presence of an inflammatory stimulant (i.e. LPS), the response was largely different. Initially, expressions for proinflammatory cytokines and proteins were high and expressions for wound healing and immunoregulatory proteins were low suggesting an overall elevated classically activated phenotype. With time, there was a shift towards a wound healing phenotype as evidenced by high expressions of *arginase* and a concomitant decrease in expression of proinflammatory cytokines with minimal changes in immunoregulatory cytokine. These overall findings indicate that temporal changes in the macrophage phenotype in response to a biomaterial appear to be dependent on their initial environment. Interestingly, an inflammatory environment appears to trigger a late wound healing response, while the biomaterial itself, although initiating a classically activated phenotype, does not appear to be sufficient to shift into a wound healing phenotype and instead shifts towards a regulatory phenotype.

By removing the effects of biomaterial activation, our data confirms that macrophage phenotypic response to LPS is dependent on the surface to which macrophages are adhered suggesting a synergistic response between biomaterial and an inflammatory environment rather

than an additive response. Most interesting is that PEG-RGD and SIL exhibited similar responses, but were largely different from PEG-only. The primary mechanism by which macrophages interact with biomaterials is through adsorbed proteins. Protein adsorption onto the material surface may occur from the serum *in vitro* or from the exudate during implantation *in vivo* and is highly dependent on the surface characteristics of the material [32]. While hydrophilic and neutral materials typically resist protein adsorption, our previous study confirmed that macrophages indeed adhere to PEG-only hydrogels in similar numbers as they adhere to PEG-RGD and SIL suggesting that some degree of protein adsorption occurs to the PEG-only materials [15]. This observation is likely due to the fact that the structure and chemical composition of crosslinked PEG confers some degree of hydrophobicity as a result of the polyacrylate chains, which are crosslinked by PEG. Interestingly, the incorporating RGD into the PEG hydrogels significantly altered the macrophage phenotypic response and in general led to a decreased proinflammatory response. While the mechanisms of action by RGD is not clear, the presence of RGD may act on macrophages either through the presentation of specific cell adhesion sites for which macrophages have integrins that recognize RGD [33] or may act indirectly through proteins that adsorb as a result of the RGD. Several studies have shown that when macrophages bind to RGD their behavior is altered and in some cases can lead to a reduced inflammatory reaction [34]. Additionally, the type of protein adsorbed to a surface can affect how macrophages respond to LPS and in some cases leading to a reduced inflammatory reaction [35]. This study and our previous study [15] further confirm that incorporating RGD tethers into PEG hydrogels attenuates the strong inflammatory response observed on PEG-only. Studies are on-going to elucidate the mechanisms by which RGD modulates macrophage response to PEG-based hydrogels.

It is important to note the limitations of this study. In particular, macrophage phenotype was assessed solely by gene expression. While gene expression provides insight into the nature and phenotype of the macrophage, it does not necessarily predict protein production and hence the function of macrophages. In the context of the severity of the foreign body reaction, the degree to which this reaction is detrimental to a biomaterial and/or cell-laden tissue engineering scaffold will indeed depend on the type and magnitude of the cytokines produced by the macrophages. Schmidt *et al.* [36] have demonstrated that human macrophages when cultured on PEG-based hydrogels with and without grafted RGD and PHSRN secrete proinflammatory cytokines including TNF- α and IL-1 β . This study focused on gene expression as a measure of macrophage phenotype and future studies are planned to evaluate macrophage function by assaying for proteins produced under different culture conditions and in response to PEG-based hydrogels. It is also important to note that this study describes the phenotypic response of bone marrow derived macrophages in an isolated and controlled *in vitro* environment. *In vivo*, macrophages involved in the FBR are not only derived from monocytes in the circulating blood but are also derived from the tissue (i.e., tissue derived macrophages), whereby the phenotypic responses may differ. In addition, macrophage phenotype will be impacted not only by an inflammatory environment and the biomaterial, but also by the presence of other cell types (e.g., fibroblasts, endothelial cells), which are involved in the FBR.

In summary, these results suggest that as macrophages begin interrogating a biomaterial they respond with an initial classical activation phenotype followed by an immunoregulatory response at least for the materials investigated here. On the other hand, a simulated proinflammatory environment initiates a classically activated phenotype appears to signal the cells to transition into their wound healing state and possibly may set the stage for capsule formation as commonly seen *in vivo* after the initial strong classical response. For tissue engineering constructs, ideally a shift in macrophage phenotype toward wound healing to improve integration would be necessary for long-term success.

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Fig. 1.

Gene expression in bone marrow derived murine macrophages in response to biomaterial interrogation. Classical (a-*Interleukin 1 beta*, b-*tumor necrosis factor alpha*, c-*inducible nitric oxide synthase*, d-*interleukin 12 beta*), wound healing (e-*arginase type I*, f-*vascular endothelial growth factor A*) and regulatory (g-*interleukin 10*, h-ratio of *interleukin 10* to *interleukin 12 beta*) genes were measured as a function of culture time. Relative expression was normalized to day 0 values. Bar above material denotes statistically significant difference from one of the other two materials; * - $p < 0.05$, ** - $p < 0.01$ indicates difference from PEG-only. Note: Log scale (h).

Fig. 2.

Gene expression in bone marrow derived murine macrophages in response to biomaterial interrogation and stimulation with lipopolysaccharide. Classical (a-*Interleukin 1 beta*, b-*tumor necrosis factor alpha*, c-*inducible nitric oxide synthase*, d-*interleukin 12 beta*), wound healing (e-*arginase type I*, f-*vascular endothelial growth factor A*) and regulatory (g-*interleukin 10*, h-ratio of *interleukin 10* to *interleukin 12 beta*) genes were measured as a function of culture time. Bar above material denotes statistically significant difference from one of the other two materials; * - $p < 0.05$, ***- $p < 0.001$ indicates difference from PEG-only; #- $p < 0.05$ indicates difference from PEG-RGD. Note: Log scale (c).

Fig. 3.

Fold change in the gene expression in bone marrow derived murine macrophages is represented by gene expression in samples which were stimulated with lipopolysaccharide normalized to the gene expression for untreated samples for a given material and culture time. Classical (a- *Interleukin 1 beta*, b- *tumor necrosis factor alpha*, c- *inducible nitric oxide synthase*, d- *interleukin 12 beta*), wound healing (e- *arginase type I*, f- *vascular endothelial growth factor A*) and regulatory (g- *interleukin 10*, h- *ratio of interleukin 10 to interleukin 12 beta*) genes were measured as a function of culture time. Bar above material denotes statistically significant difference from one of the other two materials; * - $p < 0.05$, ** - $p < 0.01$ indicates difference from PEG-only. Note: Log scale (c,d,e).

Fig. 4.

Proposed model describing macrophage activation on a-PEG-only, b-PEG-RGD and c-SIL materials. White dots represent biomaterial activation while black dots represent the combination of biomaterial activation and lipopolysaccharide stimulation over the culture period. Dots closer to the edge of the circle represent a more elevated responses in one of the three primary activation states and are indicative an activation phenotype. The dots represent the change in activation phenotype from the 4 hour time point to the 96 hour time point. Adapted by permission from Macmillan Publishing Ltd: Nature Reviews Immunology [12], copyright 2008.

Table 1

Primers used for real-time RT-PCR

Gene	Primer Sequence	Efficiency	Accession #
<i>L32</i>	F: 5'-CCATCTGTTTACGGCATCATG-3' R: 5'-TGAACCTCTTGGTCCTCTTTTGA-3'	2.06	NM_172086
<i>TNF-α</i>	F: 5'-AAGGGATGAGAAGTTCCCAA-3' R: 5'-CCACTTGGTGGTTTGCTACGA-3'	1.90	NM_013693
<i>IL-1β</i>	F: 5'-CAGGTCGCTCAGGGTCACA-3' R: 5'-TCAGAGGCAAGGAGGAAAACA-3'	1.91	NM_008361
<i>IL-10</i>	F: 5'-CAGAGAAGCATGGGCCAGAA-3' R: 5'-CCACTGCCTTGCTCTATTTC-3'	1.98	NM_010548
<i>IL-12β</i>	F: 5'-CATCAGGGACATCAAAACC-3' R: 5'-CAAAGAACTTGAGGGAGAAGT-3'	1.85	NM_008352
<i>Arginase</i>	F: 5'-TGTGTCATTGGGTGGATGCT-3' R: 5'-TGGTACATCTGGGAACCTTCC-3'	2.01	NM_007482
<i>iNOS</i>	F: 5'-AAGGCCACATCGGATTAC-3' R: 5'-GTTGATGAACTCAATGGCATG-3'	1.92	NM_010927
<i>VEGF-A</i>	F: 5'-CC TGT GTG CCG CTG ATG-3' R: 5'- CGC ATG ATC TGC ATG GTG AT-3'	2.05	NM_001025250

F – forward primer, R – reverse primer

Table 2

Two-way ANOVA results for untreated controls

Gene of Interest	2-way ANOVA Factor	
	Culture Time	Material Type
<i>Interleukin-1β</i>	p < 0.001	p = 0.072
<i>Tumor Necrosis Factor-α</i>	0.001	0.021
<i>Inducible Nitric Oxide Synthase</i>	= 0.011	0.566
<i>Interleukin-12β</i>	0.001	0.007
<i>Arginase type I</i>	0.001	0.471
<i>Vascular Endothelial Growth Factor-A</i>	0.001	0.123
<i>Interleukin-10</i>	0.001	0.304
<i>Interleukin-10: Interleukin-12β</i>	0.001	0.051

Table 3

Two-way ANOVA results with addition of Lipopolysaccharide

Gene of Interest	2-way ANOVA Factor	
	Culture Time	Material Type
<i>Interleukin-1β</i>	p < 0.001	p = 0.002
<i>Tumor Necrosis Factor-α</i>	0.001	0.034
<i>Inducible Nitric Oxide Synthase</i>	0.001	0.003
<i>Interleukin-12β</i>	0.001	< 0.001
<i>Arginase</i>	0.001	0.803
<i>Vascular Endothelial Growth Factor-A</i>	= 0.046	0.206
<i>Interleukin-10</i>	= 0.004	0.017
<i>Interleukin-10: Interleukin-12β</i>	0.001	0.010

Table 4

Two-way ANOVA results for Fold Change from Lipopolysaccharide treated compared to untreated controls

Gene of Interest	2-way ANOVA Factor	
	Culture Time	Material Type
<i>Interleukin-1β</i>	p < 0.001	p = 0.001
<i>Tumor Necrosis Factor-α</i>	0.001	0.004
<i>Inducible Nitric Oxide Synthase</i>	0.001	0.025
<i>Interleukin-12β</i>	0.001	0.003
<i>Arginase</i>	0.001	0.006
<i>Vascular Endothelial Growth Factor-A</i>	0.001	0.571
<i>Interleukin-10</i>	0.001	0.363
<i>Interleukin-10: Interleukin-12β</i>	0.001	0.022