Understanding Macrophage Activation in Response to LPS-stimulation When Cultured on PEG-RGD Hydrogels

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Statement of Purpose: Chronic inflammation results from the implantation of synthetic biomaterials through a process termed the foreign body reaction (FBR). Because activated macrophages are the predominant leukocytes involved in the FBR, efforts to understand the mechanisms mediating secretion of pro-inflammatory cytokines and enzymes have intensified. This research aims to better understand macrophage activation in response to poly(ethylene glycol) (PEG)-based hydrogels, which are being explored for cell encapsulations and tissue engineering. To simulate the *in vivo* inflammatory environment in an *in vitro* culture system, lipopolysaccharide (LPS) was delivered exogenously in the culture medium.[1] Macrophages were cultured directly onto PEG-RGD hydrogels to simulate macrophage interrogation *in vivo*, and activation was evaluated via gene and protein expression for pro-inflammatory molecules. Efforts are underway in our laboratory to begin to elucidate the mechanisms involved in macrophage activation, specifically investigating the role of hydrogen peroxide.

Methods: PEG Hydrogel Fabrication- Diacrylated PEG_{3000} (PEG-dA) was synthesized and hydrogels were formed by photopolymerization of 10wt% PEG-dA with 5mM monoacrylated-PEG_{3400}-YRGDS in PBS with a photoinitiator (Irgacure 2959).

Methods: MΦ Culture, Treatment, & Evaluation– RAW 264.7 macrophages were seeded onto PEG-RGD gels at 250,000 cells/cm^2 or seeded onto TC-treated 12 well plates. Macrophages on the hydrogels were stimulated with LPS. At specified times, the culture medium was collected and saved and macrophages were lysed and RNA extracted for quantitative RT-PCR. Custom primers were created to monitor gene expression of TNF-α, IL1-β and L32 (housekeeping gene). Protein secretion for IL1-β was evaluated using a commercially available ELISA. In a separate study, macrophages cultured on TCPS were treated with DMEM, as a control, LPS, or hydrogen peroxide for 1 hour after which the cells were washed 2X with PBS, then lysed and snap-frozen. Protein was extracted and probed for the presence of iNOS and TNF-α through western blot analysis.

Results: Macrophages when cultured on PEG-RGD and in the presence of LPS exhibited a classically activated phenotype. Gene expression for pro-inflammatory molecules, interleukin-β (IL- β) and tumor necrosis factor-α (TNF-α) were significantly elevated by 200- and 2-fold, respectively ($p<0.05$) (Fig 1A). In addition, the protein interleukin-β had accumulated in the culture medium at a concentration of 10.5±3.4 pg/ml by 48 hours. One mechanism by which macrophages can perpetuate an inflammatory response is through autocrine signaling. It is known that macrophages secrete hydrogen peroxide (H_2O_2), which can lead to an up-regulation in pro-inflammatory cytokines mediated through inducible nitric oxide synthase (iNOS), including IL- β and TNF-α . While we have not yet confirmed that macrophages when cultured on PEG-RGD secrete hydrogen peroxide, we wanted to first confirm that macrophages were indeed responsive to hydrogen peroxide. Macrophages were treated with LPS, hydrogen peroxide or left untreated. iNOS was present in untreated macrophages and similarly in macrophages treated with LPS. However, macrophages treated with H_2O_2 had higher levels of iNOS (Fig 1B). TNF-α was not detected in any of the three conditions (data not shown).

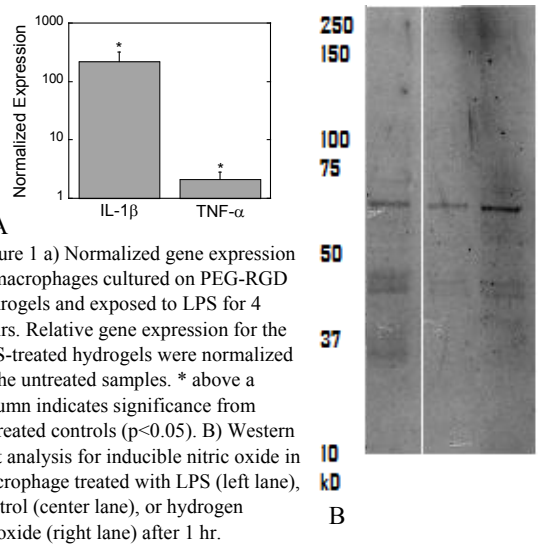

Figure 1 a) Normalized gene expression in macrophages cultured on PEG-RGD hydrogels and exposed to LPS for 4 hours. Relative gene expression for the LPS-treated hydrogels were normalized to the untreated samples. * above a column indicates significance from untreated controls ($p<0.05$). B) Western blot analysis for inducible nitric oxide in macrophage treated with LPS (left lane), control (center lane), or hydrogen peroxide (right lane) after 1 hr.

Conclusions: Overall, these findings indicate that macrophages are activated when cultured on PEG-RGD and treated with LPS, mimicking an inflammatory environment, as evidenced by increased gene and protein expression levels for pro-inflammatory cytokines. One mechanism by which pro-inflammatory cytokines may be upregulated is through H_2O_2-mediated events. We confirm that H_2O_2 induced production of iNOS at levels exceeding those of LPS supporting the explanation that H_2O_2 may be responsible for the production of pro-inflammatory mediators, possible through the activation of transcription factor NfκB.[1,2] Studies are ongoing to determine whether H_2O_2 is indeed playing a role in macrophage activation when cultured on PEG-RGD hydrogels. Further studies will explore whether treatment with antioxidants before or after treatment with H_2O_2 and LPS reduces secretion of inflammatory proteins as a means to attenuate macrophage activation.

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References: (1) Kaul N Forman HJ. Free Rad Bio Med. 1996. 21: 401-405 (2) Genestra M. Cell Sig. 2007. 19 (9) 1807-19.