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Quantitative *in vivo* Cytokine Analysis at Synthetic Biomaterial Implant Sites

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Abstract

In order to further elucidate the foreign body reaction, investigation of cytokines at biomaterial implant sites was carried out using a multiplex immunoassay and ELISA. Macrophage activation cytokines (IL-1β, IL-6, TNFα), cytokines important for macrophage fusion (IL-4, IL-13), anti-inflammatory cytokines (IL-10, TGFβ), chemokines (GRO/KC, MCP-1), and the T cell activation cytokine IL-2 were quantified at biomaterial implant sites. Empty cages (controls) or cages containing synthetic biomedical polymer (Elasthane 80A (PEU), Silicone rubber (SR), or polyethylene terephthalate (PET)) were implanted subcutaneously in Sprague Dawley rats for 4, 7, or 14 days, and cytokines in exudate supernatants and macrophage surface adhesion and fusion were quantified. The presence of a polymer implant did not affect the levels of IL-1β, TGFβ, and MCP-1 in comparison to the control group. IL-2 was not detected in virtually any of the samples. Although the levels of IL-4, IL-13, IL-10, and GRO/KC were affected by polymer implantation, but not dependent on a specific polymer, IL-6 and TNFα were significantly greater in those animals implanted with PEU and SR, materials that do not promote fusion. The results indicate differential material dependent cytokine profiles are produced by surface adherent macrophages and foreign body giant cells *in vivo*.

Keywords

cage implant system; multiplex immunoassay; cytokines; synthetic biomaterials

Introduction

Following biomaterial implantation, tissue injury leads to a series of inflammatory and wound healing events that ultimately results in the foreign body reaction and fibrous encapsulation[1, 2]. The inflammatory response to biomaterials, known as the foreign body reaction (FBR), is mediated by the complex interactions between inflammatory (i.e. neutrophils, macrophages, and lymphocytes) and wound healing (i.e. fibroblasts, endothelial cells, keratinocytes) cells[3]. These cells communicate with each other via signaling cytokines. Cytokines can influence cellular functions such as differentiation, proliferation, activation, induction of cytokine release, and apoptosis. Chemokines are a subgroup of

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cytokines that are responsible for leucocyte migration to sites of inflammation and injury and also play a central role in directing both innate and adaptive responses[4].

Macrophages and foreign body giant cells (FBGCs) are critical for determining the FBR. Human peripheral blood monocyte derived macrophages adhere to the protein coated biomaterial surface and can fuse to form FBGCs[5]. *In vitro* the cytokines IL-4 and IL-13 can induce the formation of FBGCs which are morphologically similar to those found on retrieved biomaterials[6, 7]. Blockade of IL-4 *in vivo* alters FBGC formation[8]. *In vitro* studies have shown that macrophage adhesion and fusion are dependent on material surface chemistry[9-11]. Activated macrophages are known to secrete a wide range of cytokines which include tumor necrosis factor α (TNFα), IL-1, IL-6, IL-12, transforming growth factor β (TGFβ) and chemokines such as IL-8, macrophage inflammatory protein-1α (MIP-1α), and monocyte chemoattractant protein-1 (MCP-1)[12, 13]. Secretion of cytokines, such as IL-1β, IL-6, IL-8, and TNFα, from *in vitro* surface adherent activated macrophages has been shown to be material dependent[14-16]. Material surface chemistry can induce differential IL-10, IL-1ra, and IL-8 mRNA expression in *in vitro* and in *in vivo* material adherent macrophages[17, 18]. Recently, our laboratory has begun using proteomic analyses to investigate cytokines/chemokines released *in vitro* by monocytes/macrophages[19] or co-cultured lymphocytes and monocytes[20] on synthetic biomaterials.

In contrast to *in vitro* studies, little investigation of cytokine release at *in vivo* biomaterial implant sites has been conducted. Leukocyte mRNA expression levels (i.e. IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-13) were semiquantified at implant sites using a mouse cage implant system[18]. However, mRNA levels do not necessarily reflect the functional effects of cytokines at the protein level in the *in vivo* environment. Quantitative analysis of IL-1α, IL-1β, and TNFα, up to 24h post implantation[21] and TNFα and IL-10 production up to 21 days post implantation[22] have been conducted using enzyme linked immunosorbent assays (ELISA) at the biomaterial implant site. The levels of cytokines detected were found to not be material dependent.

The assessment of cytokines *in vivo* is difficult due to the local nature of the effects and the complex interactions of wide array of cytokines at any given time point. Measurement of one or several cytokines does not give a representative idea of these varied parameters. Conventional ELISA methodology requires large amounts of sample to quantify multiple cytokines. High throughput immunoassays, such as multiplex systems, allow for the quantification of a wide range of cytokines simultaneously from minimal sample sizes[23, 24]. These multiplex assays have been used to quantify complex cytokine profiles in a variety of applications ranging from clinical diagnostic medicine[25] to immune responses to pathogens[26].

Using the well established rat cage implant system and a multiplex immunoassay, we quantified cytokines involved in macrophage activation (IL-1β, IL-6, and TNFα) and macrophage fusion (IL-4 and IL-13), a cytokine responsible for T cell proliferation (IL-2), anti-inflammatory cytokines (IL-10 and TGFβ), and chemokines (GRO/KC and MCP-1). By using the multiplex immunoassay system, we were able to quantify multiple cytokines in single small volume samples thereby eliminating continued sample processing. Cytokine profiles released at biomaterial implant sites in response to three clinically relevant non-biodegradable synthetic polymers were compared to implant sites containing no polymer.

Materials and Methods

Rat Cage Implant System

Elasthane 80A, a polyether urethane (PEU), was synthesized by Polymer Technology Group (Berkeley, CA, USA) and extruded by Medtronic (Minneapolis, MN, USA). Polyethylene terephthalate (PET, Mylar ®) (Toray Co., Japan) and a silicate resin filled, cross-linked polydimethylsiloxane (SR) (Dow Corning, Midland, MI) were also used. The polymers were cut into 1.5×0.8 cm rectangular sections, wiped with 100% ethanol, sonicated in 100% ethanol for 10 min, rinsed in distilled water and allowed to air dry.

Surfaces (1.5cm×0.8cm) were placed inside cylindrical stainless steel wire mesh cages measuring approximately 3.5cm long and 1.0cm in diameter. The cages were prepared as previously described[27]. All cages, with or without polymers, were sterilized with ethylene oxide by sterilization services at University Hospitals of Cleveland. Female 12 week-old Sprague-Dawley rats (Charles River Laboratories), weighing 200-250g, fed ad libitum on standard pellets and water were used (Animal Resource Center, Case Western Reserve University). NIH guidelines for the care and use of laboratory animals were observed. All protocols were approved by the Institutional Animal Care and Use Committee of Case Western Reserve University.

Cage implantations were performed as previously described[27]. Rats were anesthetized with a continuous stream of Aerrane® (Baxter Healthcare Corporation) and two cages, each containing the same biomaterial, were implanted subcutaneously in the lower back. Control rats were implanted with empty cages. Each time point is representative of data obtained from four rats (n=4).

Exudate Supernatant Collection

On days 4, 7, and 14, exudate was collected from the implanted cages as previously described[28]. Briefly, after anesthetizing the rat with Aerrane® (Baxter Healthcare Coporation), a heparinized tuberculin syringe (27.5 gauge needle) was inserted through the skin and into the stainless steel cage. 400-800μl of exudate was collected from each animal at each time point. Exudate was spun down (1000g, 10min) and supernatant was collected, aliquoted, and immediately stored at −80°C.

Multiplex Immunoassay

A multiplex cytokine immunoassay containing a rat cytokine panel (IL-1β, IL-2, IL-4, IL-6, IL-10, GRO/KC, MDC-1, TNFα) was purchased from Lincoplex (Millipore, Billerica, MA). Due to the multiplex technology, the cytokines in the rat panel were measured simultaneously in each sample. Exudate samples collected from days 4, 7, and 14 were run in duplicate. The multiplex immunoassay was run in accordance with manufacturer's instructions for serum or plasma samples. The plate was run on a Luminex® 200 Instrument using Bio-plex manager 4.1 standard software (Bio-Rad Laboratories, Hercules, CA). Raw fluorescence data was analyzed by the software using a 5 parameter logistic method. Minimum detection concentrations are listed in Table I.

TGFβ1 Immunoassay

A TGFβ1 anti-human/mouse/rat/porcine singleplex was purchased from Lincoplex (Millipore, Billerica, MA). Exudate samples collected from days 4, 7, and 14 were run in duplicate. Prior to running the assay, samples were acidified as directed by the manufacturer. Besides sample preparation, the TGFβ1 immunoassay was run as outlined above for the multiplex assay. Minimum detection concentration is listed in Table I.

IL-2 and IL-13 ELISA

Rat IL-2 and IL-13 ELISA kits were purchased from Biosource (Invitrogen, Carlsbad, CA). Samples collected from days 4, 7, and 14 exudate withdrawals were run in duplicate as per the manufacturer's instructions for serum or plasma samples. Samples were incubated overnight at 4°C. Absorbance at 450nm was measured by an EL808 ultra microplate reader with KC Junior software (Bio-Tek Instruments, Inc., Winooski, Vermont). Minimum detection concentrations are listed in Table I.

Adhesion and Fusion Analyses

On days 4,7, and 14, adherent cell densities and macrophage fusion were determined on the biomaterial surfaces obtained from explanted cages as described previously[29]. Surfaces were rinsed twice with phosphate buffered saline (PBS), subsequently fixed in methanol for 5min, and air dried. May Grunwald reagent was added to surfaces for 5min. Surfaces were rinsed in PBS twice and Giemsa reagent added for 15min immediately. Surfaces were rinsed with distilled water twice and allowed to air dry. Adherent cell densities were determined by counting the number of nuclei from 5 representative 20x fields for each sample and expressed as cells/mm^2. Percent fusion was determined by dividing the number of nuclei within foreign body giant cells (containing 3 or more nuclei as identified histologically) by the total number of nuclei in the field. No adherent lymphocytes to macrophages or biomedical polymers were identified with optical microscopy. Cell densities and percent fusion from the five fields were averaged for each sample from 4 animals in each group.

Statistical Analysis

Data were represented as the mean ± the standard error mean (n=4). Comparisons among groups were made using ANOVA and statistical significance between groups was determined using Tukey post-hoc test with a 95% confidence interval (Minitab, Inc., State College, PA).

Results

Macrophage Activation Cytokines (IL-1β, IL-6, and TNFα)

IL-1β levels decreased over the implantation period and were comparable at each respective time point. IL-6 levels were significantly higher in the PEU group compared to all other groups at all time points. Most impressively, on day 14, IL-6 levels in the PEU group were 47 times greater than that seen in any other implanted animal. For the control, SR, and PET groups, the low levels of IL-6 decreased over time to negligible levels at day 14, but high levels were maintained in PEU implanted animals at all time points (Figure 1).

TNFα levels in the biomaterial implanted groups were greater than the control group at all time points. TNFα levels were most potently stimulated by SR and PEU implants. At day 4, TNFα levels in the SR group were greater than all other groups. At days 7 and 14, the TNFα levels in the PEU and SR groups were 8 to 25 times greater than the control and PET groups. No significant differences could be observed in the TNFalpha levels between SR and PEU groups at these time points (Figure 1).

Macrophage Fusion Cytokines (IL-4 and IL-13)

IL-4 levels appeared higher in the control group than the biomaterial implanted groups on days 4 and 7 although there was variability observed in the animals. At day 14, the level of IL-4 in PEU implanted animals increased and was significantly higher than that observed in animals with SR and PET implants or controls.

IL-13 levels were higher in the biomaterial implanted rats in comparison to the control animals at all time points. At day 4, the PEU group had significantly higher levels of IL-13 in comparison to the control and PET groups but not SR. By day 14, IL-13 was not detectable in the control group and biomaterial implanted groups had comparable levels of IL-13 at the implant site (Figure 2).

Anti-inflammatory Cytokines (IL-10 and TGFβ)

For IL-10, the concentration increased over time for all groups. At day 4, the PEU group had significantly higher levels of IL-10 than all other groups. By day 14 the biomaterial implanted groups had higher concentrations of IL-10 than the control group although significant variability was observed. TGFβ levels increased from day 4 to day 14 although concentrations of TGFβ were comparable in all animals at all time points (Figure 3).

Chemokines (GRO/KC and MCP-1)

GRO/KC concentrations were higher in the biomaterial implanted groups than the control group at all time points. For all biomaterial implanted groups, the lowest concentration of GRO/KC was at day 14.

MCP-1 was comparable between all groups at all time points except at day 14 when the SR group had a statistically higher concentration of MCP-1 than the PET group. MCP-1 concentrations were lowest at day 7 for all groups (Figure 4).

T cell Activation Cytokine (IL-2)

IL-2 was not detected in most samples (data not shown). Although IL-2 was detected above minimal detection levels in 16% of all samples, it was detected in random individual animals at sporadic timepoints with no discernable pattern.

Adherent Cell Density and Percent Fusion

Adherent cell density was comparable between groups at all time points. Macrophage percent fusion was 0% for all materials at day 4. At days 7 and 14, the percent fusion trend was PEU<SR<PET. Percent fusion was significantly greater on the PET surface in comparison to the PEU and SR surfaces (Figure 5).

Discussion

In this study, we investigated various cytokines important in the foreign body reaction at biomaterial implants sites using the rat cage implant system. Few studies have quantified cytokines produced *in vivo* in response to implanted biomaterials[21, 22]. The *in vivo* environment is complex due to the potential for cytokines to be secreted from a wide variety of sources, possible cytokine degradation, and cytokine binding to cell membrane receptors[23]. In our study, surgical implantation of the biomaterials induced tissue injury which causes the release of inflammatory mediators including a wide range of cytokines. Therefore, we investigated cytokine profiles from biomaterial implanted animals in comparison to the empty cage control (or injury control). We also compared material groups in order to identify material dependent trends in the cytokine profiles.

Adherent macrophages/FBGCs are considered the main cell types that mediate the foreign body reaction to biomaterials. Activated macrophages secrete a wide range of cytokines and chemokines[13, 30]. The proinflammatory cytokines IL-1β, IL-6, and TNFα are just three of the plethora of cytokines secreted by monocytes/macrophages. It has been demonstrated *in vitro* that biomaterial adherent macrophages/ FBGCs secrete the pro-inflammatory cytokines IL-1β and IL-6 in a material dependent manner. In this study, IL-1β levels decreased with

time and were comparable between all groups (Figure 1). IL-6 levels were significantly higher in rats implanted with PEU compared to other material groups and correlated with significantly decreased macrophage fusion (Figures 1 and 5). These IL-6 data correlate with previous *in vitro* studies in our laboratory, in which adherent macrophages on surfaces that do not promote fusion secreted higher levels of IL-6 per cell than adherent macrophages/FBGCs on surfaces that do promote fusion[19].

IL-6 is a cytokine important for the proliferation and differentiation of B cells[31]. Patients implanted with polyurethanes used for left ventricular assist devices experience B-cell hyperreactivity and allosensitization[32]. In our study, the polyurethane group had increased levels of IL-6 at the biomaterial implant site, however previously we have failed to identify B cells in the exudate surrounding the PEU implants[33]. IL-6 also can chemoattract macrophages and fibroblasts and participate in inhibiting extracellular matrix breakdown by upregulating tissue inhibitors of metalloproteinases production[34].

TNFα production was also found to be increased in both the PEU and SR groups, surfaces that do not promote a high percentage of macrophage fusion (Figure 1 and Figure 5). TNFα is responsible for the apoptosis of biomaterial adherent macrophages[35]. Furthermore macrophage fusion is considered an escape mechanism in order to avoid apoptosis. Surfaces that do not promote fusion have a higher percentage of apoptotic adherent macrophages *in vitro* and *in vivo*[36, 37]. Therefore the apoptotic effect of TNFα may explain the correlation with the decrease in fusion seen on these surfaces. It is likely that adherent macrophages on these surfaces undergo apoptosis to a higher extent than cells on the PET surface and therefore forego the possibility of fusing into FBGCs although this needs to be formally validated.

The PEU surface promoted both high levels of IL-6 and TNFα whereas the SR surface promoted higher levels of TNFα. It is likely that these material dependent differences in IL-6 and TNFα are due to production from surface adherent macrophages. However, IL-6 and TNFα can also be secreted by a multitude of other cells which are present in the *in vivo* environment such as mast cells, fibroblasts, and lymphocytes[34]. Our comparison to cytokine levels in the empty cage control group, which contains no polymer, further implicates the possibility of surface adherent macrophages impacting cytokine levels in exudate supernatants. We have previously shown material dependent differences in mRNA expression, inclusive of IL-6, in *in vivo* adherent macrophages using the mouse cage implant system[18].

IL-4 and IL-13 are cytokines that mediate macrophage fusion[6-8]. However, no clear material dependent correlations between IL-4 and IL-13 levels and macrophage fusion could be made from these experimental data. The requirement for macrophage fusion is not simply binding IL-4 and IL-13 to induce phenotypic changes necessary for fusion. Macrophages not only require IL-4 induction but also a permissive surface in order to fuse[9, 38, 39]. Therefore material surface properties also impact the adherent cells' phenotype and influence the ability to fuse or not fuse. In our *in vitro* human monocyte culture system, IL-4 or IL-13 is added at a concentration of 15ng/ml at days 3 and 7[6, 11, 38, 40-44]. Despite cultured monocytes being exposed to the same amount of IL-4, macrophage adhesion and fusion is disparate on different surfaces. Therefore, it is difficult to correlate IL-4 and IL-13 levels to macrophage fusion considering material surface properties contribute to the fusion capabilities of adherent macrophages.

IL-2 is an important cytokine necessary for the maintenance of T cell activation. IL-2 and the IL-2 receptor alpha chain (CD25) are transcribed after appropriate stimulation[45, 46]. T cells expressing CD25 have been found at biomaterial implant sites[33]. However, IL-2 was

not detected *in vitro* when T cells and monocyte were co-cultured on biomaterials[20]. The lack of IL-2 production *in vitro* could be due to the absence of signals and/or cells necessary for activation that could potentially be present in the *in vivo* environment. We were not able to detect IL-2 in almost all our samples (data not shown). To ensure that our incapability to detect IL-2 was not due to cross-reactive binding to beads other than those containing IL-2 capture antibody, we utilized IL-2 ELISAs. We were also unable to detect IL-2 using this method (data not shown). Brodbeck et al. have identified IL-2 mRNA in leukocytes isolated from biomaterial implant sites[18]. IL-2 message is not transcribed in resting T cells, but only in activated T cells[47] indicating the presence of activated T cells at biomaterial implant sites. IL-2 is an autocrine cytokine; therefore T cells both secrete and bind IL-2. It is possible that activated T cells, which express the high affinity binding IL-2 receptor, secrete IL-2 and capture self-associated cytokine preventing its secretion in exudate. There is also evidence that an IL-2 reservoir on the secreting T cell surface membrane is present[48]. The presence of IL-2 reservoirs on the surfaces of T cells may make it difficult to measure IL-2 in the exudate supernatant. We have found CD25 expression *in vivo* at days 4, 7, and 14, indicating T cells at the biomaterial implant site may be activated. However, T cells in the exudate surrounding the empty cage control also had comparable levels of CD25 expression[33]. Therefore it is likely that activated IL-2 secreting T cells may be present at the implant site.

The concentration of the anti-inflammatory cytokines, IL-10 and TGFβ, increased from day 4 to day 14 for all groups (Figure 3). An increase in anti-inflammatory cytokines over time is expected as the phases of wound healing progress. IL-10 plays an important role in inhibiting pro-inflammatory cytokine synthesis[49] and TGFβ affects collagen matrix formation[34]. Comparable levels of TGFβ were present in the material implanted groups and control group. IL-10 levels were comparable at days 4 and 7 for the material implanted groups and the control group exclusive of the PEU group. At day 4, the PEU group had statistically higher levels of IL-10 than all other groups (Figure 3). We have previously reported an increase in CD25 T cell expression at day 4 at the PEU implant site suggesting an increase in regulatory T cells which are a potential source of IL-10[33]. However definitive characterization of these T cells has not yet been made.

The two chemokines GRO/KC and MCP-1 (CCL2) were also quantified at the biomaterial implant sites. GRO/KC is a murine homolog to IL-8. IL-8 is secreted by adherent macrophages/FBGCs *in vitro*[19]. This chemokine can recruit neutrophils and T cells[50]. GRO/KC concentrations were greater in biomaterial implanted groups than in the control group (Figure 4). This is most likely due to the contribution of surface adherent cells to GRO/KC production. In contrast, MCP-1 was comparable between material implanted groups and the control group (Figure 4). MCP-1 is a chemoattractant for monocytes, memory T cells, and NK cells[50]. MCP-1 has been found to participate in macrophage fusion into foreign body giant cells[51]. However, in this study correlations between MCP-1 concentration and macrophage fusion were not found.

In conclusion our most significant findings were the IL-6 and TNFα profiles. These material dependent profiles are likely due to material adherent macrophages and FBGCs that secrete these cytokines. *In vitro* IL-1β and IL-6 secreted from biomaterial adherent macrophages decreased with time[19]. However, *in vivo* IL-6 and TNFα levels did not decrease with time indicating that cytokine production from adherent macrophages may have a prolonged influence on the normal inflammatory and wound healing responses to injury. We will continue to investigate the possible downstream effects of differential cytokine profiles in the host response to biomaterials. This study is the first of its kind in quantifying an extensive array of cytokines *in vivo* in response to biomaterial implantation.

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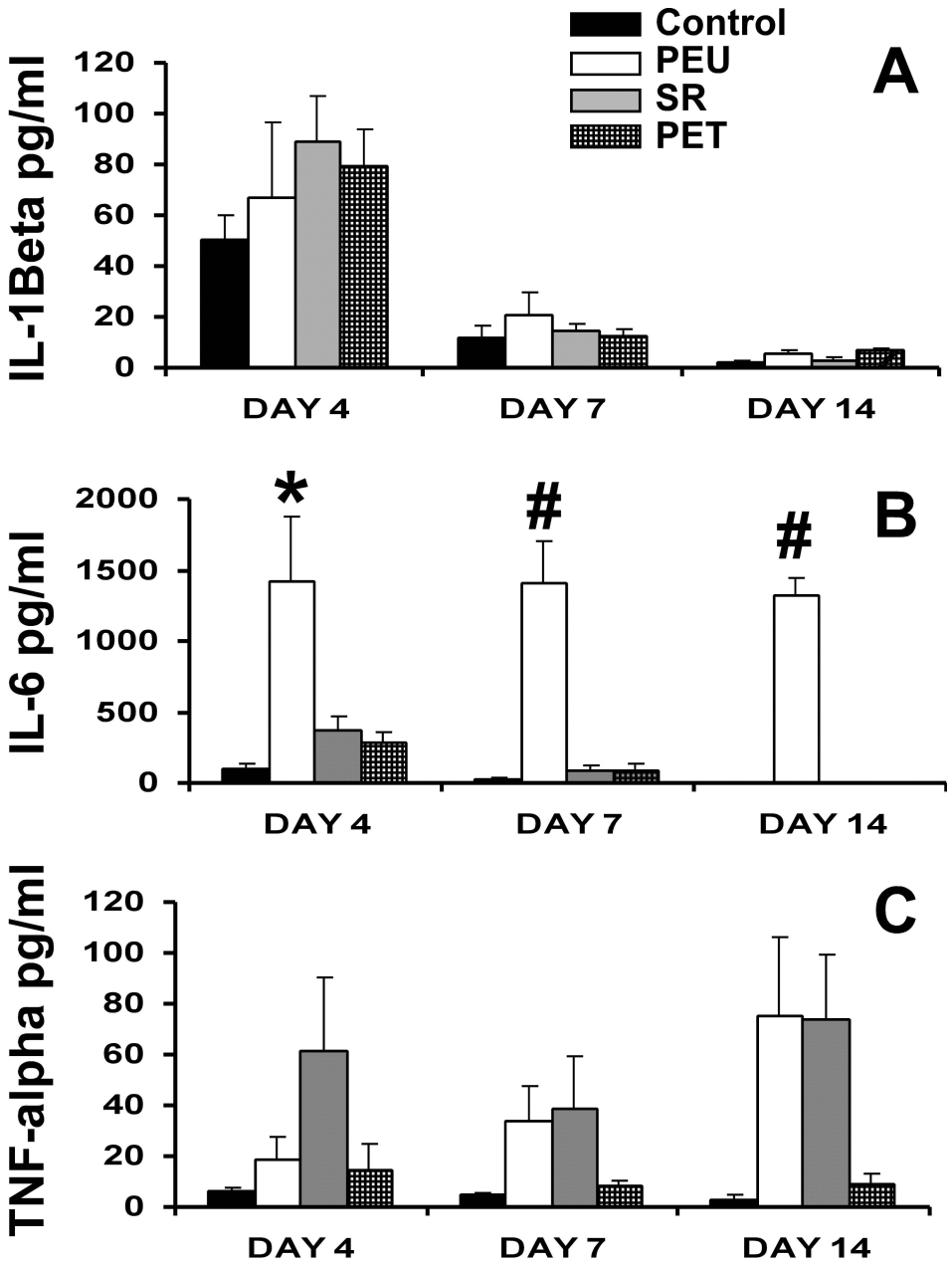

Figure 1.

Pro-inflammatory cytokine levels at days 4, 7, and 14 post implantation: IL-1B (A), IL-6 (B), and TNFa (C). Cytokine levels measured in exudate supernatants. Data represent mean ±SEM, n=4. * indicates statistical significance ($p<0.05$) in comparison to Control and PET. #- indicates statistical significance ($p<0.05$) in comparison to Control, SR, and PET.

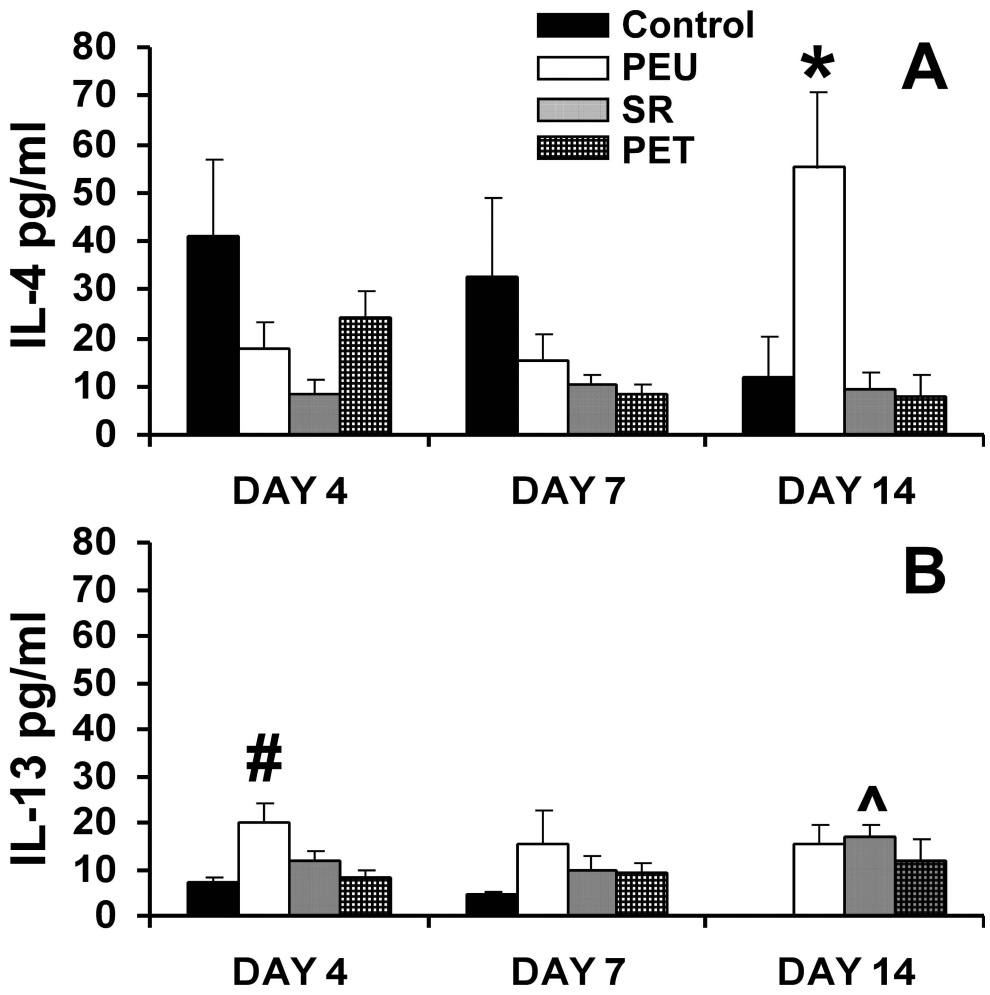

Figure 2.

Cytokines important in macrophage fusion at days 4, 7, and 14 post implantation: IL-4 (A), and IL-13 (B). Cytokine levels measured in exudate supernatants. Data represent mean ±SEM, n=4. * indicates statistical significance ($p<0.05$) in comparison to Control, SR, and PET. # indicates statistical significance ($p<0.05$) in comparison to Control and PET. ^ indicates statistical significance ($p<0.05$) in comparison to Control.

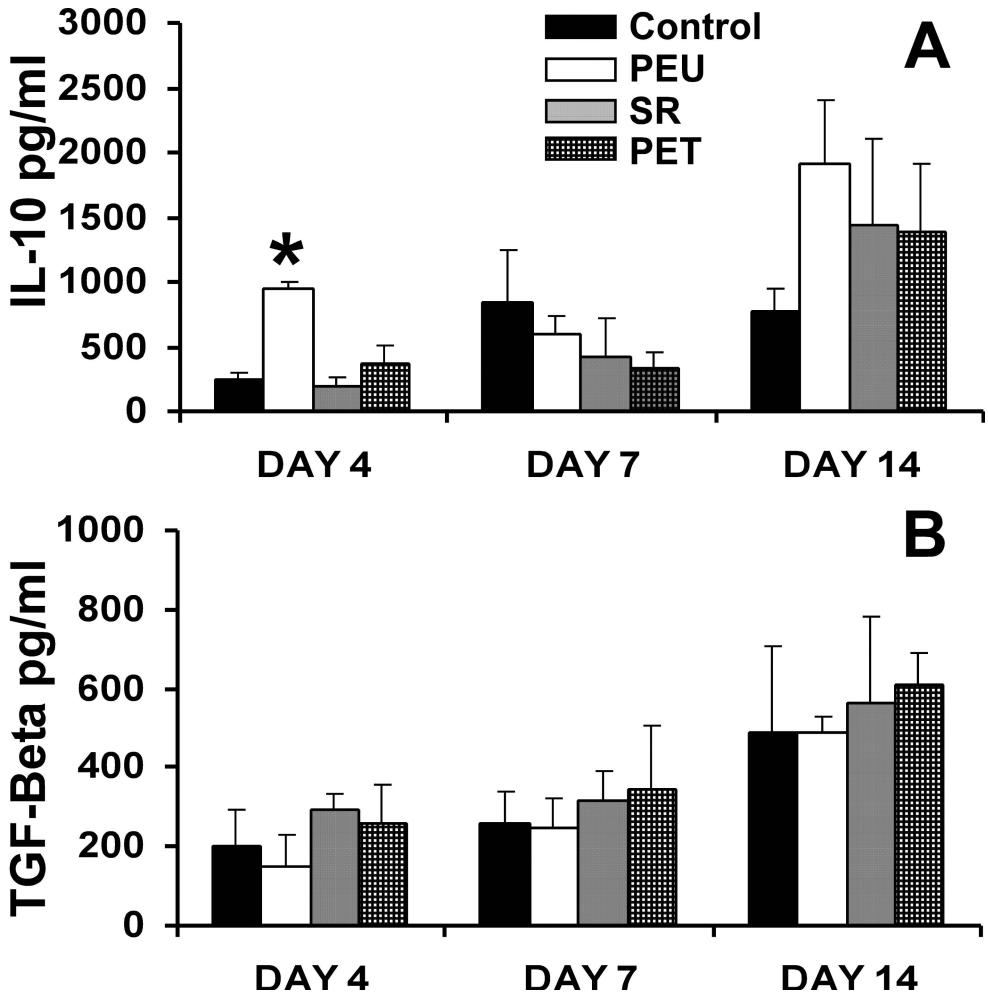

Figure 3.

Anti-inflammatory cytokine levels at days 4, 7, and 14 post implantation: IL-10(A), and TGF-B (B). Cytokine levels measured in exudate supernatants. Data represent mean±SEM, n=4. *-indicates statistical significance ($p<0.05$) in comparison to Control, SR, and PET.

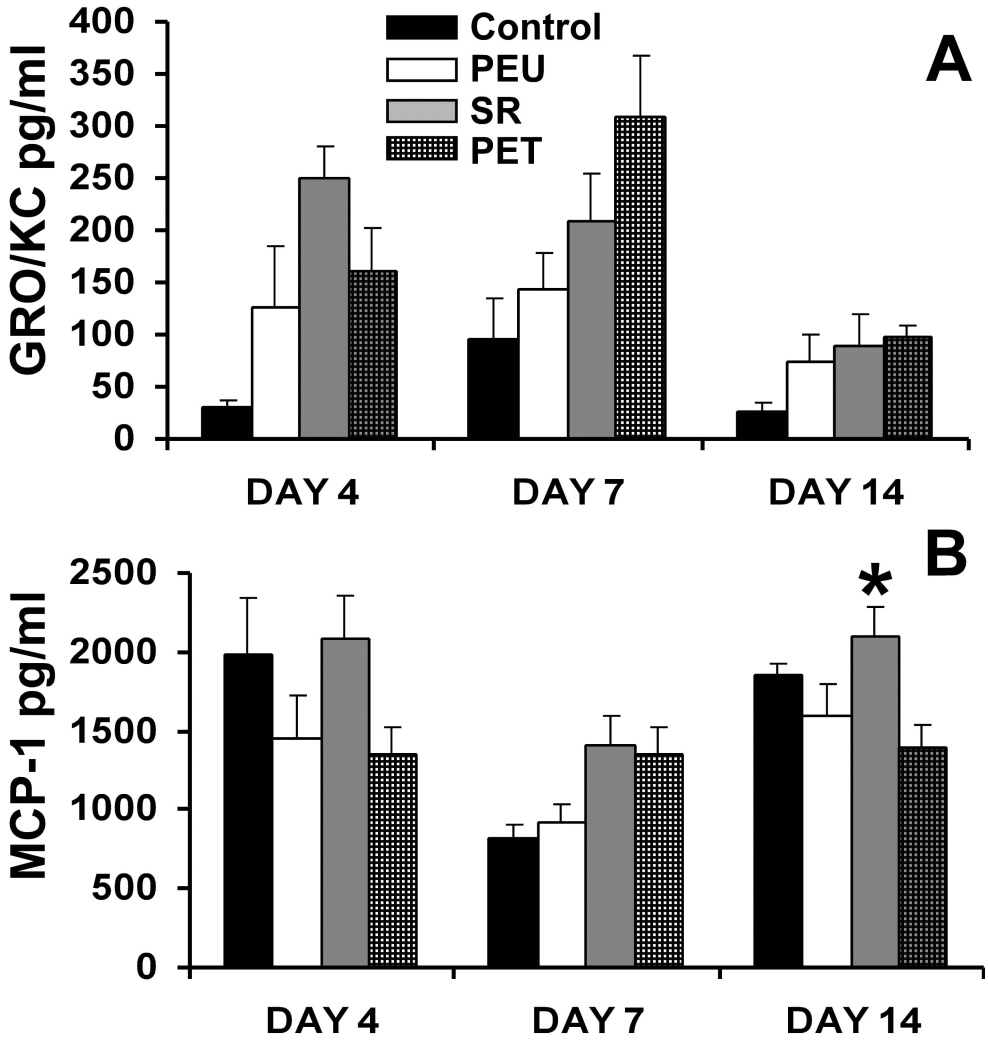

Figure 4.
Chemokine levels at days 4, 7, and 14 post implantation: GRO/KC(A), and MCP-1(B). Cytokine levels measured in exudate supernatants. Data represent mean±SEM, n=4. * indicates statistical significance ($p<0.05$) in comparison to PET.

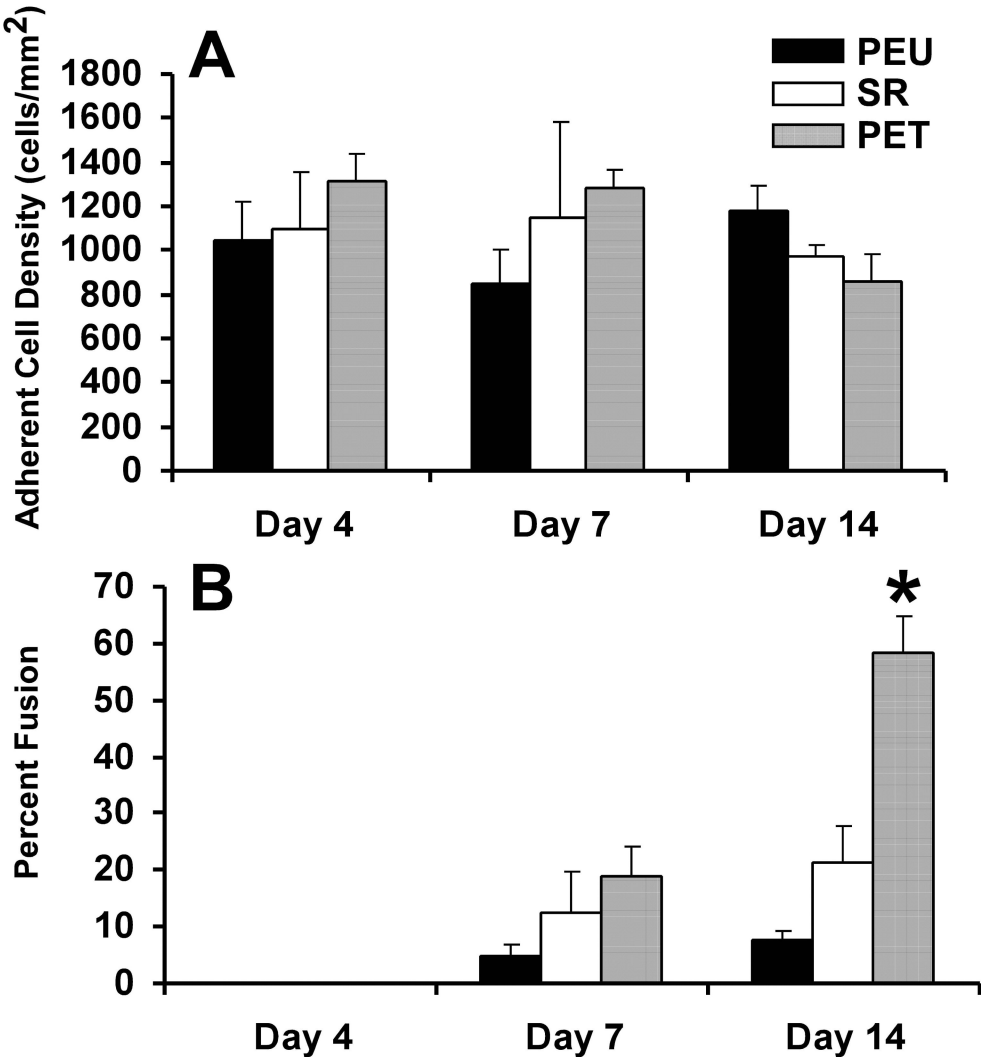

Figure 5.
Adherent cell density (A) and percent fusion (B) at days 4, 7, and 14. Data represent mean ±SEM, n=4. * indicate statistical significance ($p<0.05$) in comparison to PEU and SR.

Table I

Sensitivity of Cytokine Multiplex Detection Assay

	Minimum Detection Concentration (pg/ml)	
IL-1β	2.32	
IL-2	3.67	5.00*
IL-4	2.30	
IL-6	9.80	
IL-10	5.41	
IL-13	23.2	1.5*
GRO/KC	2.06	
MCP-1	3.81	
TNFα	4.44	
TGFβ	20	

* Biosource ELISA sensitivity level