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## Implant debris particle size affects serum protein adsorption which may contribute to particle size-based bioreactivity differences

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### Abstract

Biologic reactivity to orthopedic implant debris mediates long-term clinical performance of total joint arthroplasty implants. However, why some facets of implant debris are more pro-inflammatory remains controversial such as particle size, shape, base material etc. This precludes accurate prediction and optimal design of modern total joint replacements. We hypothesized that debris particle size can influence adsorbed protein film composition and affect subsequent bioreactivity. We measured size-dependent protein film-adsorption, and adsorbed protein film-dependent cytokine release using equal surface areas of different sized cobalt-chromium-alloy (CoCr-alloy) particle and *in vitro* challenge of human macrophages (THP-1 and human primary). Smaller 5 $\mu$ m vs 70 $\mu$ m sized particles preferentially adsorbed more serum protein in general ( $p < 0.03$ ), where higher molecular weight serum proteins consistent with IgG were identified. Additionally, 5 $\mu$ m CoCr-alloy particles pre-coated with different protein biofilms (IgG vs albumin) resulted in differential cytokine expression where albumin-coated particles induced more TNF- $\alpha$  and IgG-coated particles induced more IL-1 $\beta$  release from human monocyte/macrophages. In these preliminary *in vitro* studies we demonstrated the capability of equal surface areas of different particle sizes to influence adsorbed protein composition and that adsorbed protein differences on identical particles can translate into complex differences in bioreactivity. Together this suggests adsorbed protein differences on different sized particles of the same material may be a contributing mechanism by which different sized particles induce differences in reactivity.

### Keywords

Cobalt Alloy; Debris; Particle; Adsorbed protein film; Macrophage; IgG; TNF; IL-1b

### INTRODUCTION

Biological reactivity (inflammation, fibrous tissue formation, bone loss etc.) to implant debris has been well established to limit long-term implant performance. While metal-on-metal alternatives to traditional metal-on-polymer articulations in total joint replacements reduce the total amount of gravimetric wear, the degree of inflammation generated from

debris of these newer designs may differ depending on the type and size of debris [1-4]. If these alternative bearing surfaces produce less gravimetric (volumetric or mass) wear but greater numbers (doses) of smaller particles, the benefits of reduced gravimetric wear may be nullified.

Previous studies have shown that biomaterial type, size and shape influence subsequent tissue bioreactivity [4-10]. While all implant debris induce some degree of biologic reactivity, there is consensus that the more relevant debris to inflammation is less than 10  $\mu\text{m}$  in size, because it is readily phagocytosed leading to inflammatory reactions [11,12]. In addition, metallic debris may be more pro-inflammatory than polymeric debris [13], where macrophage activation and cytokine production have been used as proxies of *in vivo* inflammatory reactivity. Macrophage cytokines TNF- $\alpha$ , IL-6 and IL-1 $\beta$  are the hallmarks of *in vivo* inflammation implicated in implant loosening [13]. Recently particle size related reactivity, which has traditionally been reported as inversely proportional to particle size [4-10], has been revisited. New studies indicate that on a particle to particle basis smaller nanometer particles do not induce as much of a response as larger particles in the micron ranges [14]. Furthermore, studies on large vs small and smooth vs rough particles have shown that larger and rougher particles disrupt internal cell lysosomal compartments and thus cause more danger signaling (inflammation, IL-1 $\beta$ ) than smaller or smoother particles [15]. However the relationship between particle size and biologic reactivity is complex and likely many faceted, where the interactions of cell-to-particle recognition, adhesion and phagocytosis play a role, as well.

Immediately upon contact, biomaterial surfaces are coated with serum proteins forming a proteinaceous film, referred to in this study as the adsorbed protein film. The composition of adsorbed proteins and subsequent cell-material interactions have been shown to be determined by the physicochemical properties of the biomaterial [16-20]. It is also important to point out that the process of protein adsorption onto the surface of biomaterials is a dynamic one. The biologic reactivity associated with metallic implant degradation can be affected by protein adsorbed protein films (surface and particle) as well as the underlying type of implant material [21,22].

There remains an incomplete understanding of how differences in particle size are translated into differences in reactivity and inflammation *in vivo*. Does particle size, alone, influence protein binding? We hypothesized that different sized particles of the same biomaterial will adsorb different serum proteins, and these different protein films will mediate subsequent inflammatory responses. We tested our hypothesis by analyzing differential serum protein adsorption on smaller (5 $\mu\text{m}$ ) and larger (70 $\mu\text{m}$ ) spherical CoCr-alloy particles and evaluated the differential *in vitro* reactivity of serum protein adsorption on phagocytosable CoCr alloy particles.

## MATERIALS AND METHODS

### Particle preparation for adsorbed protein film adsorption analysis

For adsorbed protein film analysis of adsorption kinetics on large and small particles, smooth spherical Cobalt-Chromium-Molybdenum-alloy (ASTM F-75) (CoCr-alloy) of an

average mean size of 5 $\mu$ m and 70 $\mu$ m diameter particles (Starmet Corp.; Concord, MA) were used. >99% of 5 $\mu$ m particles were <10 $\mu$ m and >99% of 70 $\mu$ m particles were <100 $\mu$ m. Particle sizes were confirmed by scanning electron microscopy SEM, (Hitachi SN-5000), see Figure 1. For differential serum adsorption experimentation, differences in particle size were selected to maximize the chance of preliminary hypothesis testing on widely different sized particles with verifiably similar surface morphologies (smooth and spherical) while at the same time using large enough particles to accurately account for equal exposure areas to serum using weight calculations of particle number (i.e. the larger the smallest size particle the better). For protein adsorption, stock amounts of 5  $\mu$ m and 70  $\mu$ m particles were aliquoted to approximate equal surface areas of 600 cm<sup>2</sup>. For cell reactivity testing only phagocytosable CoCr-alloy 5 $\mu$ m diameter spherical particles were used to test the bioreactivity relevance of different adsorbed proteins. Particles were cleaned using a 3 step process: 1) metal-safe detergent (Alconox), 2) sonication in 70% ethanol for 1 hr followed by a 24 hour 70% ethanol soak at room temperature (25°C), and 3) a 24 hour soak in Pyroclean<sup>TM</sup> (a detergent for endotoxin removal). Each step was followed by triple washing with deionized water. Vacuum dried particles were autoclaved at the end of the cleansing process and tested to verify that they were Lipopolysaccharide (LPS) free using a quantitative limulus assay analysis, Kinetic QCL: <0.05Eu (Pyrogen 5000, Lonza).

### Serum adsorbed protein film analysis

Human serum samples were obtained from peripheral blood obtained intravenously from n=6 healthy subject volunteers using Rush IRB approved informed consent (4 males and 2 females, ages 23–54). CoCr-alloy particles of 5 $\mu$ m and 70 $\mu$ m diameter were incubated in serum at 37°C for 7 days with continuous gentle side-to-side (see-saw) rocking, at 1Hz (Labquake). After 168 hours of incubation time to allow for steady state protein adsorption-desorption kinetics, i.e. Vroman effects [23,24], the serum protein-coated particles were transferred to a 50mL centrifuge tube and washed 3 times with sterile, cold PBS to remove loose non-adherent proteins. Greater than 95% of adsorbed protein film proteins were eluted from the alloy particles for analysis with 500= L 8M urea and 40mM Tris-HCl, as verified by secondary 2%SDS elution at 100°C and SDS-Page. The eluted proteins were then desalted with 1500 $\mu$ L cold acetone. The eluent was centrifuged at 13,500g and air-dried in a vacuum to ultimately form a protein pellet. An electrophoretic analysis of the adsorbed protein layer was then performed. 50 $\mu$ L of a 2% SDS sample buffer was added to each sample pellet. Pellets were analyzed using 10-20% polyacrylamide gels (SDS-PAGE; BioRad, Hercules, CA). 40 $\mu$ L of sample from each material surface and 7.2ng (BioRad) of calibration marker was loaded into 1-D polyacrylamide gels. PAGE gels were subsequently fixed and stained with GelCode (Pierce, Rockford, IL). Gel densitometry was conducted with a transparency adapted scanner (420oe, Periferal Dynamics Inc., Plymouth Meeting, PA) and Scion Image (PC-NIH Image; NIH, Bethesda MD) image processing software was used for determination of total and peak protein amounts.

To determine IgG levels, pellets were rehydrated with PBS and a 40mM chaps solution. 100  $\mu$ L of sample was placed in a maxisorp plate in triplicate. Total IgG was determined using manufacturer's instructions. Briefly, sample adsorption to the plate was allowed to take place over 72 hours on an orbital shaker at room temperature. Horse-radish peroxidase

bound anti-IgG (R&D Systems) was then placed in each well and incubated for 2 hours after blocking with bovine albumin. Amounts of IgG were then measured with an Elisa plate reader at 450nm 20 minutes following TMB-substrate addition. Standards of IgG (R&D Systems) were also performed to determine approximate IgG concentrations of original serum samples. Original serum samples were also measured for amounts of IgG (ELISA) after being diluted 1:32 with PBS. IgG and total protein amounts were determined using a protein bioassay kit following manufacturer's protocols (Pierce\* BCA Protein Assay).

### **THP-1 macrophage cell line**

A THP-1 (ATCC) human monocyte cell line was used for dose testing. Approximately 200,000 cells/well were plated in 48-well plates in 350 $\mu$ L DMEM medium/well, 10% AB serum. Cells were treated with a phorbol ester (12-O-tetradecanoylphorbol-13-acetate, TPA) (200nM final concentration) for 48h to stimulate differentiation into adherent macrophages. Cell supernatants were collected at 24 hours and assayed for TNF- $\alpha$  and IL-1 $\beta$  production as an indicator of bioreactivity (R&D Systems, Minneapolis, MN) following manufacturer's protocols.

### **Primary Human Monocytes/Macrophages**

Blood samples were obtained from healthy volunteers with Institutional Review Board approved, informed consent. Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll gradient and CD14<sup>+</sup> monocytes were isolated from PBMCs by negative selection using magnetic bead antibody cocktail for CD3, CD7, CD16, CD19, CD56, CD123 and Glycophorin A (Miltenyi Biotec). Isolated human primary monocytes were assessed for >90% purity using FACS.

Isolated human monocytes from n=3 subjects were collected and isolated and allowed to differentiate into attached monocyte/macrophages (CD14<sup>+</sup>) for 48 hours (with human M-CSF at a working concentration of 50ng/mL). Cells were challenged with 5 $\mu$ m sized particles at doses of 1 particle/cell and 5 particles/cell. Monocytes/Macrophages were also challenged with protein coated particles, where CoCr-alloy 5 $\mu$ m spherical particles were incubated with different adsorbed proteins: human AB serum (Gembio Bioproducts), human albumin (Lee Biosolutions), IgG (Sigma), and fibronectin (American Diagnostica). These three proteins were selected as proxies of major components in human serum; 1) albumin represents a bulk less bioactive protein of serum protein, 2) immunoglobulins are key components of immuno-reactive serum protein and 3) fibronectin represents components of adhesion serum proteins [25]. Particles 5 $\mu$ m in size were incubated with 200ug of each protein in a 50  $\mu$ L PBS solution for 48 hours at 37 $^{\circ}$  C. Subsequently, particles were gently washed with sterile PBS to remove non-adherent protein then both differentiated human THP-1 macrophages and isolated primary human monocytes/macrophages were challenged with 5 $\mu$ m-sized particles at a concentration of 5 particles/cell . Supernatants were collected at 1 hour and 24 hours and Luminex multiplex assays were performed to determine secreted TNF- $\alpha$ , IL-6 and IL-1 $\beta$  (Millipore) production using manufacturer instructions.

All reactivity testing was conducted in triplicate. Statistical analysis between groups was performed for parametric data using one way ANOVA with Newman-Keuls Multiple

comparison test of pairs at a significance level of  $p=0.05$ . And non-parametric data Kruskal-Wallis testing with post-test Dunn's comparison of groups was performed at a  $p<0.05$  confidence level (Graphpad Prism).

## RESULTS

### Adsorbed protein film analysis

Adsorbed serum protein films demonstrated subject-dependent compositional differences on  $5\mu\text{m}$  and  $70\mu\text{m}$  diameter Co-Cr alloy particles. Electrophoresis results of protein elution from different size particles show that there were approximately 20 general ranges of proteins (prominent protein bands) detected on the surfaces of the metal alloys tested. Densitometric analysis of the 20 adsorbed protein bands revealed that among the 6 subjects tested, the only statistically significant difference in the intensity of protein(s) binding was within the range of approximately 150 kDa molecular weight ( $p < 0.05$ ) when normalized to the total amount of protein adsorbed for each subject, Figure 2a and 2b. Data was normalized to total protein to account for differences in total protein in each individuals serum.

Total protein adsorption concentrations for  $5\mu\text{m}$  and  $70\mu\text{m}$  diameter CoCr-alloy particles were determined by electrophoresis using known protein standards for extrapolation. Equal surface areas of different particle sizes ( $5\mu\text{m}$  vs  $70\mu\text{m}$  diameter) did not result in any statistically significant total adsorbed protein differences (Figure 2c).

### Adsorbed Particle IgG analysis

Total serum IgG for each subject as determined by ELISA ranged from  $378\ \mu\text{g/ml}$  to  $552\ \mu\text{g/ml}$  with an average of  $456\pm 63\ \mu\text{g/mL}$ . However, the total serum protein measurements did not vary significantly between subjects.

Differential adsorption of IgG onto  $5\mu\text{m}$  and  $70\mu\text{m}$  diameter spherical CoCr-alloy particles was measured after incubation in serum for 168 hours at  $37^\circ\text{C}$  as previously described. Our results show statistically significant amounts of IgG ( $p<0.02$ ) adsorbed by the smaller size ( $5\mu\text{m}$ ) CoCr-alloy particles compared to the large size ( $70\mu\text{m}$ ) CoCr-alloy particles, Figure 3. Adsorption of IgG was approximately 4 times greater for the  $5\mu\text{m}$  smaller sized particles compared to their  $70\mu\text{m}$  large-sized particles. Our findings were qualitatively similar to the results obtained using densitometry of electrophoresis gel results, which showed a higher amount of protein bound to smaller sized particles with respect to higher molecular weight ranges associated with IgG,  $p<0.05$ , Figure 2b vs Figure 3.

### THP-1 macrophage responses

To determine the optimal particle dose for subsequent particle bioreactivity of adsorbed protein films we challenged human THP-1 macrophages with increasing concentration of particles for 24 hours using IL- $1\beta$  and TNF- $\alpha$  as measures of inflammatory reactivity (Figure 4). There was a maximal IL- $1\beta$  dose response to  $5\mu\text{m}$  particles at 10 particles per cell (24 hours) and 5 particles per cell for TNF- $\alpha$  (1 hour). Confocal microscopy coupled with transmission light microscopy was used to determine that THP-1 macrophages were

able to phagocytose particles as early as 1-2 hours post challenge. 5 $\mu$ m spheres demonstrated that both active phagocytosis and lysosome activity (fluorescently labeled OVA) were associated with CoCr-alloy phagocytosis at 1 hour time post challenge. These responses are limited to particle dose comparison and positive controls with LPS alone and LPS biofilm only compared using primary human cells.

The viability of THP-1 macrophages was assessed using flow cytometry analysis of 7AAD (7-Aminoactinomycin D) and Annexin V. 7-AAD passes through disrupted cell membranes and stains DNA and is a measure of cell viability while Annexin V is used to detect cell surface phosphatidylserine which is expressed during apoptosis and other forms of cell death. A dose of 10 particles per cell decreased the viability of THP-1 macrophages to 84%, Figure 5. Thus a challenge concentration below 10 particles per cell was used for further testing of protein adsorption bioreactivity.

### Primary macrophages response

Primary human monocyte/macrophages (CD14+) TNF- $\alpha$  and IL-1 $\beta$  cytokine responses to 5 $\mu$ m CoCr-alloy particles were measured in n=3 subjects, Figure 6. The cytokine response of each subject varied in magnitude. However, the pattern of results was similar in that an increased dose of 5 $\mu$ m sized particles at 5 particles/cell produced a greater TNF- $\alpha$  and IL-1 $\beta$  than a dose of 1 particle per cell, p<0.05, Figure 6. Larger particles of 70 $\mu$ m diameter were not tested in culture because the particle size was too large to enable phagocytosis by single cells *in vitro*.

### Primary macrophages and reactivity to specific adsorbed protein films

To determine if observed adsorbed protein film and reactivity differences can be produced by using a single size of CoCr-alloy particle (i.e. 5 $\mu$ m diameter particle) coated with different adsorbed protein films, i.e. AB serum, albumin, IgG, fibronectin or LPS (positive control) 5 $\mu$ m sized CoCr-alloy particles were incubated in these respective common human serum proteins for 48 hours and then used to challenge primary human monocytes/macrophages (CD14+, n=3 subjects). A time course was used to establish the most effective time to detect TNF- $\alpha$  released from primary (non-primed) human monocytes/macrophages *in vitro*, Figure 7. TNF- $\alpha$  and IL-1 $\beta$  release was assessed at 1 hour and 24 hours respectively at a dose of 5 particles/cell (5 $\mu$ m dia). The greatest TNF- $\alpha$  response at 1 hour was to albumin and LPS particle films, Figure 8. At 24 hours the greatest IL-1 $\beta$  response was to particles with adsorbed human AB serum (i.e. a complex milieu of human serum proteins). While this implicates IgG in reactivity differences of different size, given that IgG adsorbed protein films induced significantly higher IL-1 $\beta$  than albumin, it also demonstrates the highest IL-1 $\beta$  response to particles was when coated with multiple serum proteins. These results of primary cells show that TNF- $\alpha$  and IL-1 $\beta$  released from primary human monocytes/macrophages can vary significantly with type of adsorbed protein film.

## DISCUSSION

In support of our original hypothesis, we found that particle size can influence protein binding characteristics and that different adsorbed proteins on the same type of particle can

induce variable inflammatory responses (i.e. serum albumin adsorbed protein film increased TNF- $\alpha$  and IgG increased IL-1 $\beta$ ). While compositional differences in the adsorbed protein films associated with polymeric and metal implant materials have been previously reported[26], this is the first report of experimentally determined bulk constitutive differences between the adsorbed serum proteins formed on differing sizes of a single material. Overall, total amounts of adsorbed serum protein on particles of different sizes (but same surface areas) did not vary between subjects (Figure 5a). Thus, surface properties affected by metal particle size are likely a significant determinant of adsorbed protein film composition. Factors related to the particle size and material such as surface chemistry, surface charge, and microstructure variation likely contribute to differences in the protein binding properties.

Differential amounts of IgG eluted from the adsorbed serum protein film of 5 $\mu$ m and 70 $\mu$ m CoCr-alloy particles further illustrate the influence of particle size on adsorbed protein film properties. Smaller 5 $\mu$ m sized particles bound larger proteins particularly in the W150kD range with significantly greater affinity than the larger particles. These differences may be due in part to changes in opsonized particle surface charge [27]. Gel electrophoresis and ELISA testing confirmed that particle size influences adsorption kinetics of specific proteins such as IgG and cell culture testing of IgG adsorbed protein film increased the reactivity of CoCr-alloy particles. It is likely that IgG proteins play a role in the *in vivo* reactivity of CoCr-alloy particles. Past investigations have shown that opsonization of particles with serum proteins (i.e.Immunoglobulins) compared to un-opsonized particles leads to an increase in inflammatory responses [21,28]; however, it remained unknown to what degree, if any, changes in the kinds of proteins in this layer effects subsequent bioreactivity of particulate implant debris.

Low dose particle challenge was used in an attempt to more closely model the subtle innate immune response that has been long established as the primary cause of implant inflammation and failure over the long term[29-31]. It is likely that if macrophages (and other cells) reacted to aseptic low dose particle challenge *in vivo*, with highly high amounts of inflammatory cytokines (e.g. >1000's pg/mL TNF- $\alpha$  and IL-1 $\beta$ ), then total joint replacement implants would not last very long (<1yr) without causing extreme inflammation. In this investigation we attempted to determine if (in a simplified model) particle size affects biofilm composition and if that composition change can translate into differences in reactivity at lower doses. Our preliminary evidence shows that particle size differences can affect human serum protein adsorption kinetics and that altering this protein "biofilm" has the potential to change the bioreactivity of particulate debris, but further study is required to determine if this is important clinically. However there are several limitations and caveats to these findings. First, the particles used in this study were made of implant grade alloy, but were larger than typical metal implant debris. Metal and ceramic particles have generally been characterized as an order of magnitude smaller than polymer particles (at approximately <0.05 $\mu$ m in diameter, i.e. in the nanometer range)[32-35]. But some CoCr-alloy corrosion products (e.g. chromium-phosphate ) range in size from sub-micron to aggregates of particles up to 500 micrometers [36,37]. Additionally the particles used in this investigation were cleaned with acid (endotoxin cleaning) and thus were fully surface-

oxidized. It is unclear to what degree particles produced by wear and corrosion *in vivo* are oxidized prior to protein adsorption, if at all. While SEM-EDS analysis did not indicate any demonstrable chemical differences in alloy make-up between the large and small particles, it is possible that chemical/microstructure differences in the CoCr-alloy particles of different sizes were present and mediate protein adsorption. Thus the clinical applicability of our findings have yet to be established and further work must be conducted to determine if the same phenomenon takes place *in vivo* and with particles in the submicron and nanometer size ranges. However, at smaller size ranges controlling for surface oxidation, shape and surface roughness is technically challenging and may be experimentally prohibitive.

In this study we demonstrated that adsorbed serum proteins can be dependent on particle size and that differences in adsorbed protein films can produce differences in macrophage inflammatory reactivity. The exact mechanism(s) by which this size-related adsorption phenomena occur are unknown at present, but are likely due to changes in surface charge inherent to different sized particles that have been previously shown to play a role in protein film adsorption kinetics [23,38]. CoCr-alloy particle opsonization with IgG produced more IL-1 $\beta$  and albumin opsonization produced more TNF- $\alpha$ , respectively ( $p < 0.05$ ). This suggests there may be a complex relationship between adsorbed proteins and resultant inflammatory responses that modulate intracellular reactivity where there can be differential pathogen recognition (PAMP) associated TNF reactivity vs more danger signaling (DAMP) IL-1  $\beta$  associated inflammatory responses. The mechanism(s) associated with how different sized particles and different adsorbed proteins elicit different kinds of inflammatory responses (DAMP/inflammasome IL-1 $\beta$  vs PAMP/NF $\kappa$ B TNF- $\alpha$ ) are currently under further investigation.

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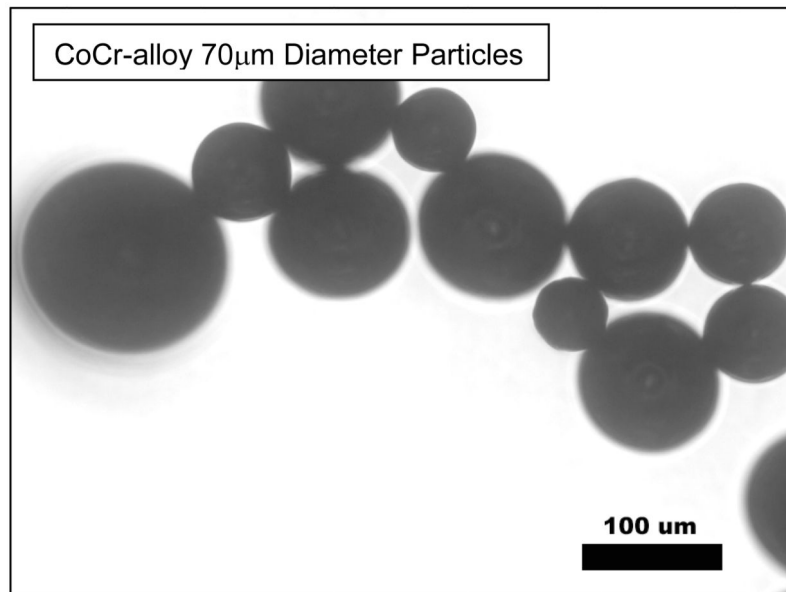
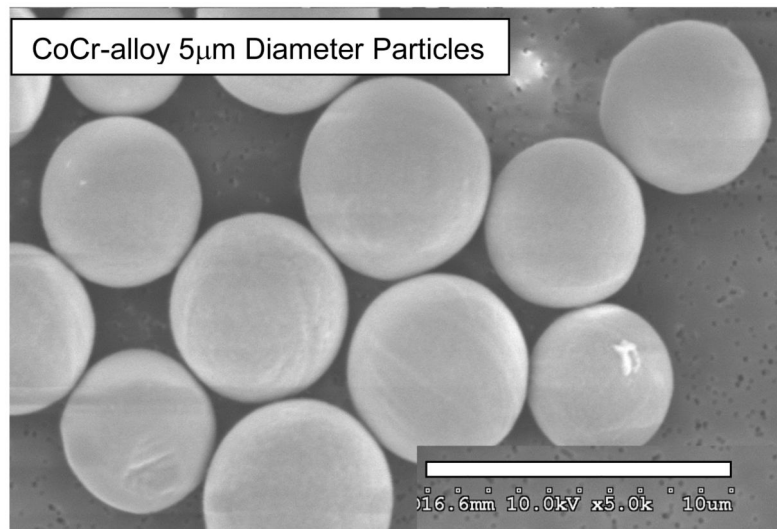
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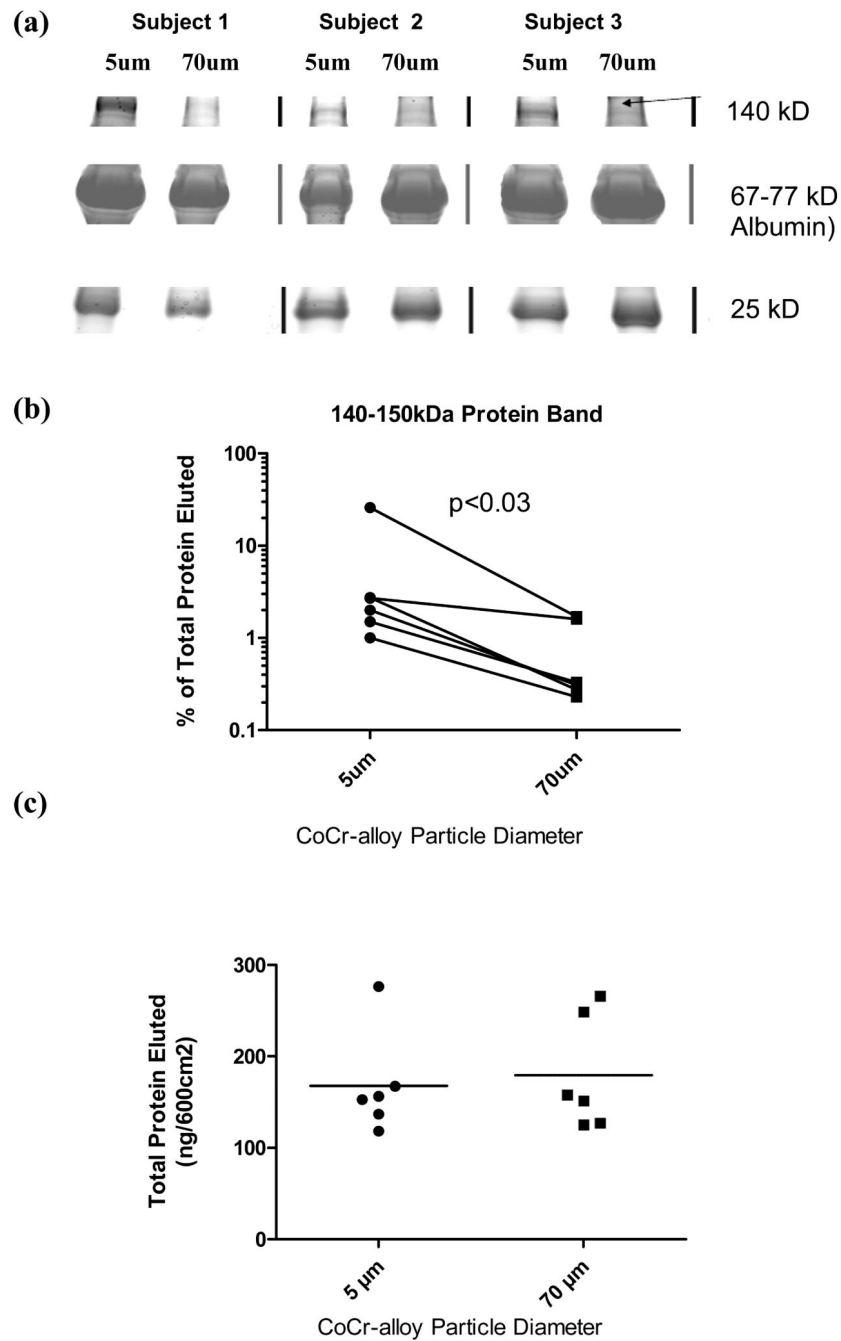


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**Figure 1.** Scanning electron micrographs (SEM) of (a) 5µm Co-alloy spherical particles and (b) Transmission light microscopy images of 70µm diameter Co-alloy spherical particles, where light microscopy was used in real time to view particle migration and motion and confirm that particles were not stuck together.



**Figure 2.** Analysis of eluted serum proteins from n= 6 subjects adsorbed onto 5um and 70um Co-alloy particles demonstrated (a) an example of eluted serum proteins from 5um and 70um particles from 3 of 6 subjects, SDS-PAGE, showing a higher weight (140-150kD) serum protein preferentially bound by smaller-sized (5 μm) CoCr-alloy particles compared to other protein that did not vary. (b) There was a statistically decreased amount of 140-150 kD sized proteins on 70um sized CoCr-alloy particles compared to 5um (p<0.03), Wilcoxon matched

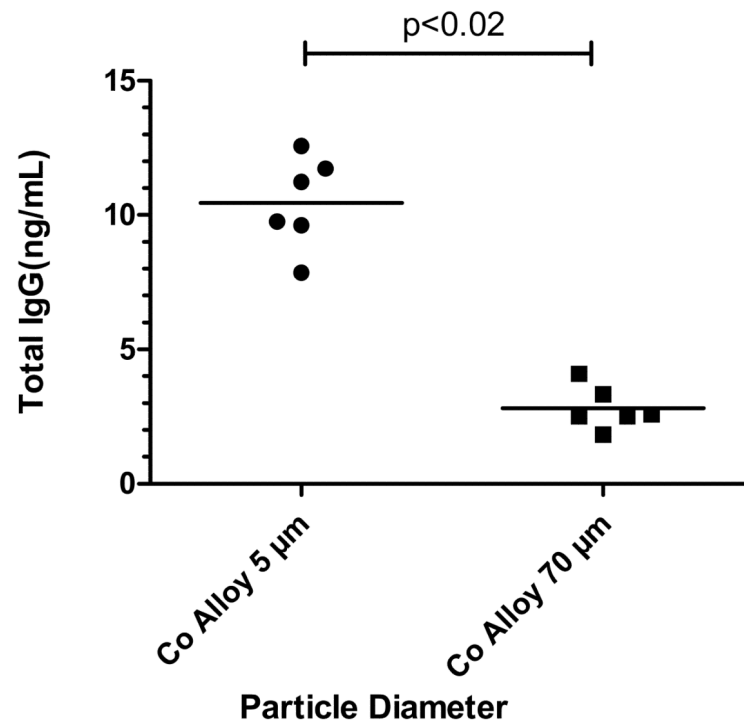
pairs test for non-parametric data. (c) However, there was no significant difference in the total amount of adsorbed protein.

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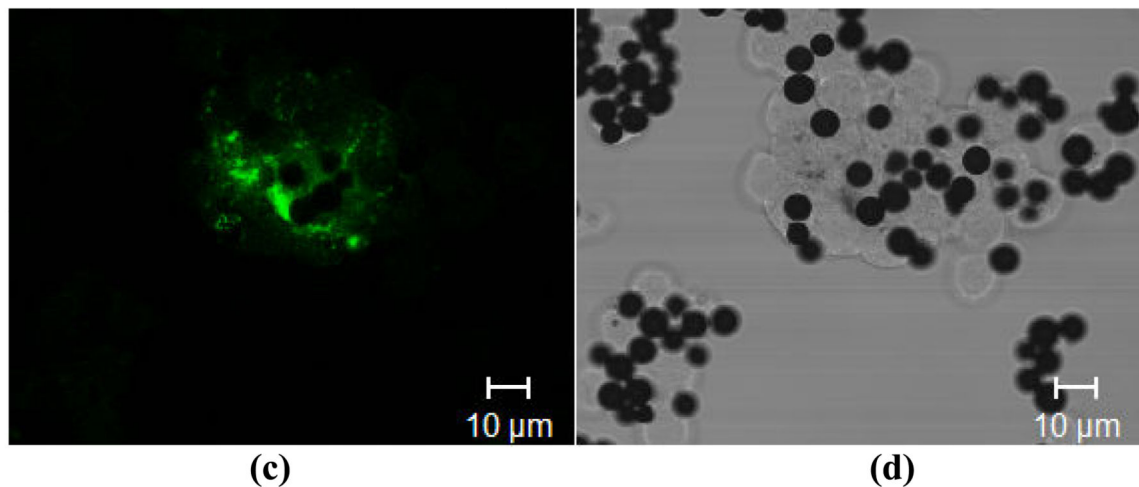
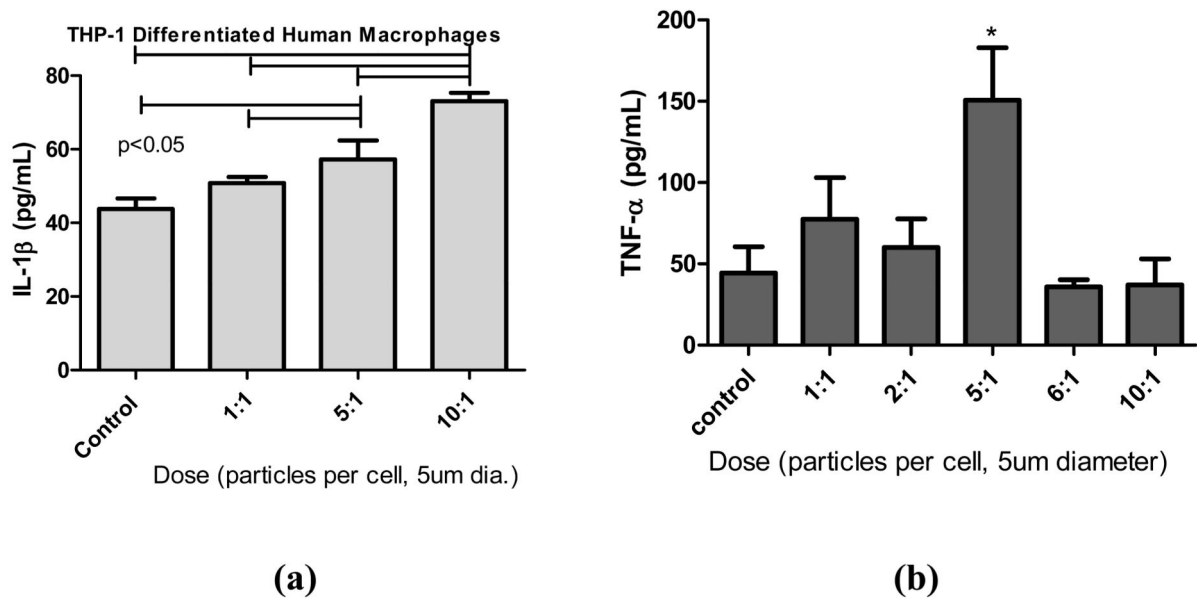
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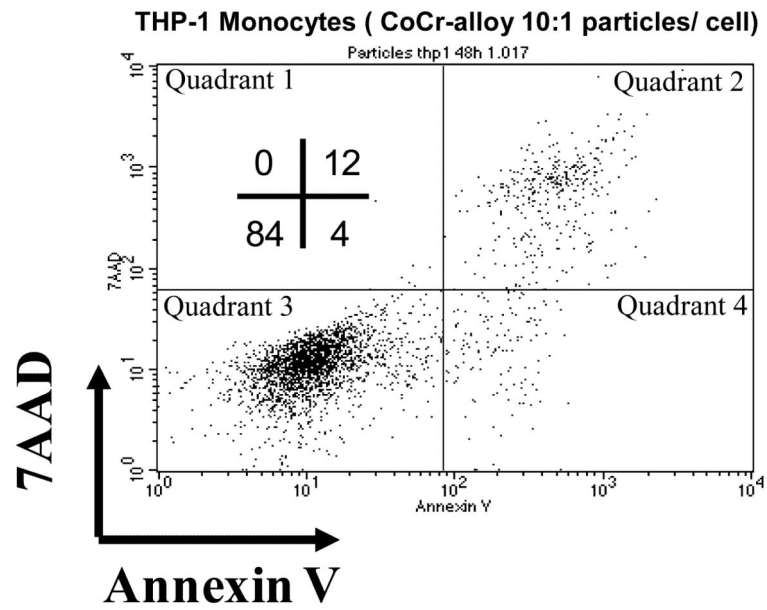


**Figure 3.** Quantitative determination of total eluted IgG ( $\text{ng}/\text{cm}^2$ ) adsorbed onto CoCr-alloy beads of 70 $\mu\text{m}$  and 5 $\mu\text{m}$  diameter in size were measured with ELISA. These results were comparable to relative amounts of 150kD proteins shown in Fig 5, where 5 $\mu\text{m}$  beads preferentially adsorbed IgG,  $p < 0.02$  t-test, unpaired data.



**Figure 4.**

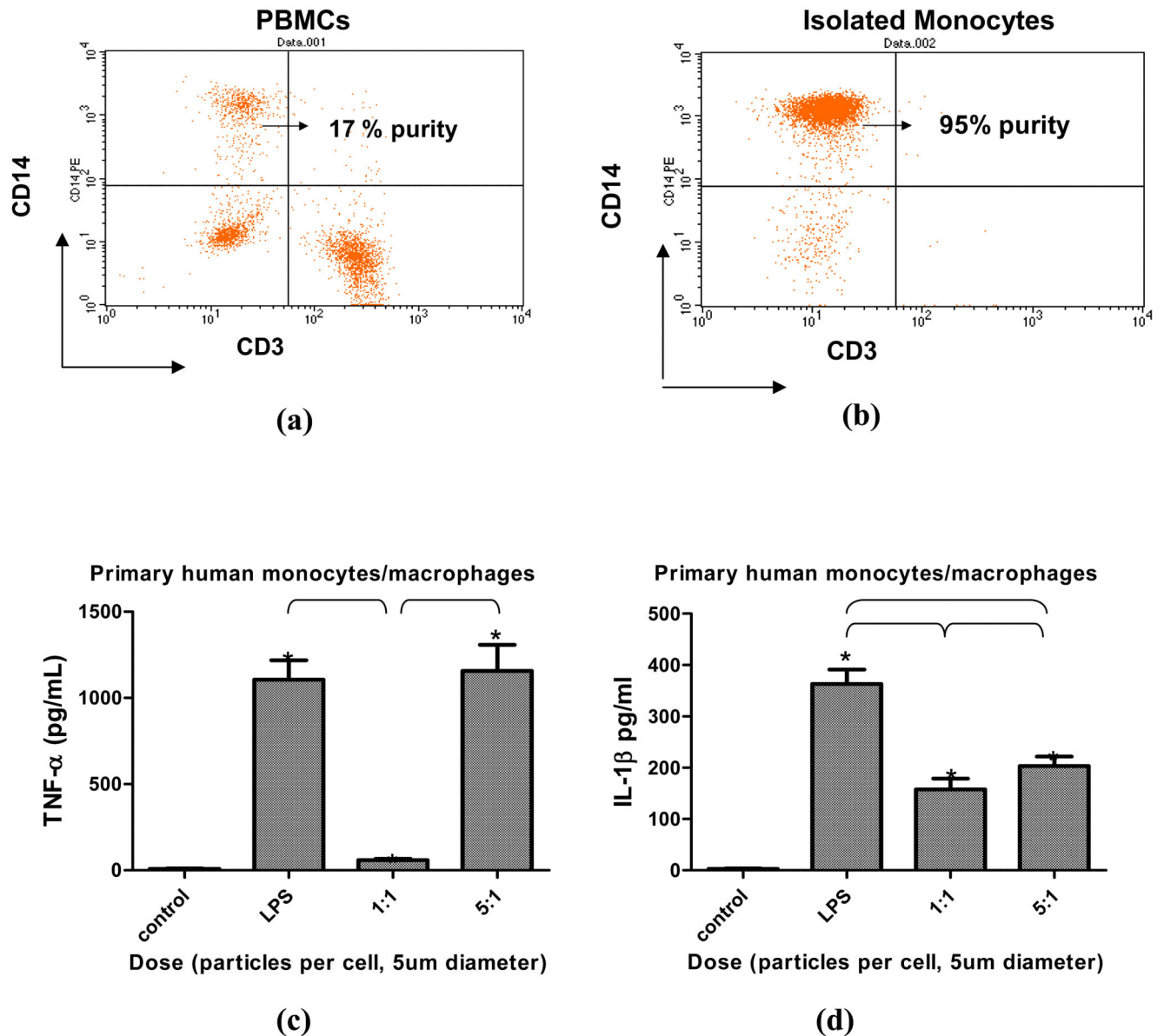
(a). IL-1b responses of differentiated THP-1 human macrophages over 24 hours when challenged with increasing particle dose of 5µm particles, where maximal stimulation is shown to occur at 10 particles/cell. Note  $p < 0.001$  where indicated by bars, one way ANOVA with Newman-Keuls Multiple comparison test of pairs. (b) TNF- α responses of differentiated THP-1 human macrophages at 1 hour post challenge with 5µm diameter particles, where maximal stimulation is shown to occur at 5 particles/cell. Note  $p < 0.05$  where indicated by bars, one way ANOVA with Newman-Keuls Multiple comparison test of pairs. (c) Fluorescent confocal microscopy and (d) transmission light microscopy of the same field of THP-1 macrophages after 2 hours of challenge with 5µm sized spheres showing active phagocytosis and lysosome activation (fluorescently labeled OVA) associated with particle phagocytosis.



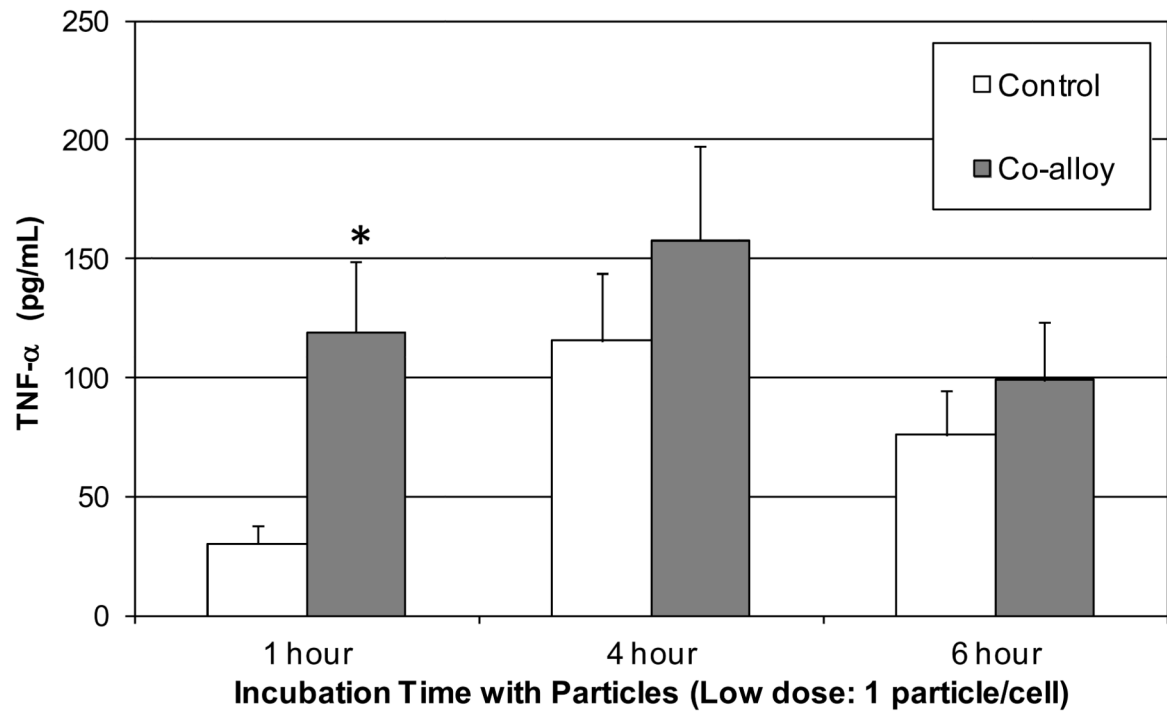
**Figure 5.**

THP-1 were 84% viable after treatment with CoCr alloy particles at a dose of 10:1 where apoptosis was determined by 7AAD uptake and Annexin V surface expression analyzed by FACS and expressed as percent of cells positive for either marker 48 hours after treatment. Data are representative results of one of three independent experiments.



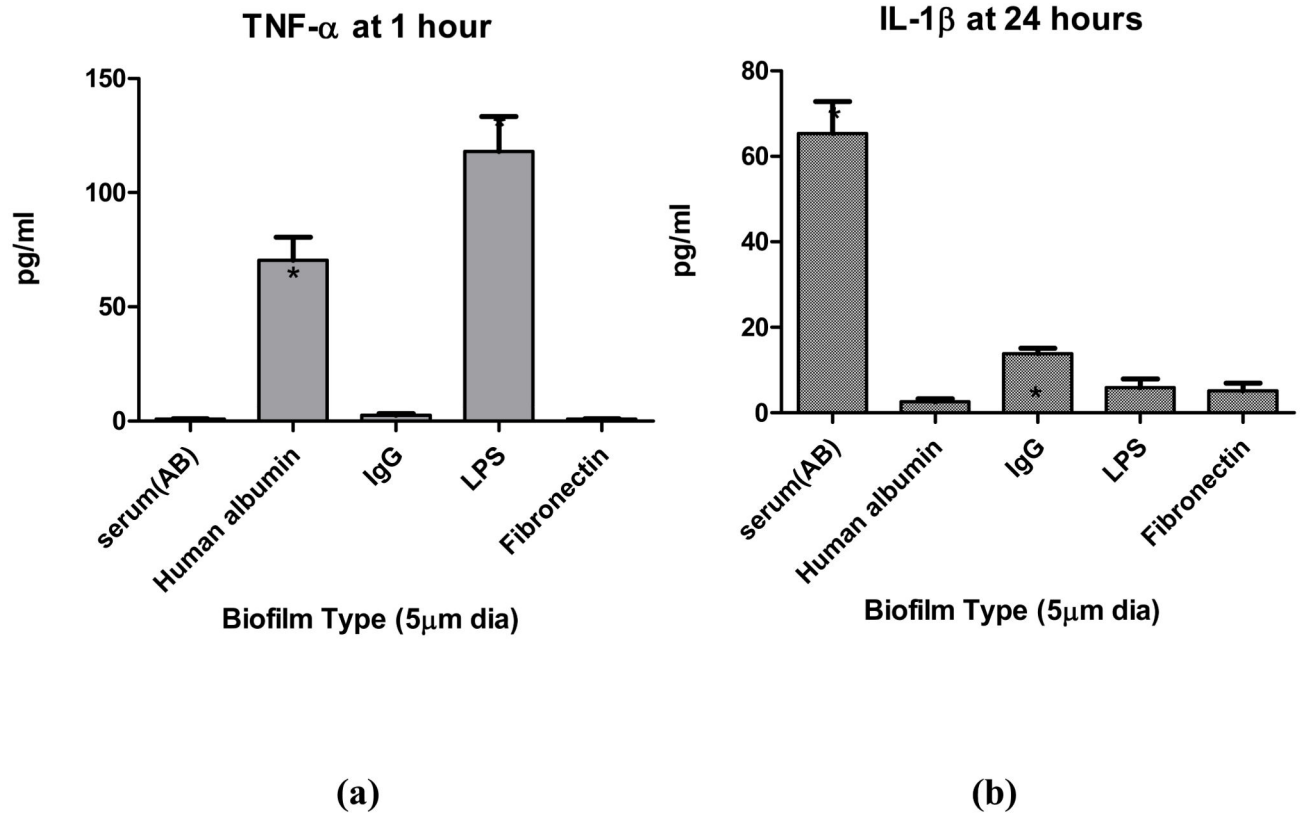
**Figure 6.**

(a) Flow cytometry of Ficoll separated human PBMCs labeled with CD14 and CD3 demonstrated 17% of primary human PBMCs were CD14<sup>+</sup> monocytes. (b) After isolation using magnetic bead separation (AutoMacs, Milteni) the purity of CD14<sup>+</sup> monocytes was increased to 95% and used for subsequent testing. (c) TNF- $\alpha$  and (d) IL-1 $\beta$  responses of isolated differentiated primary human monocyte/macrophages challenged with 5  $\mu$ m sized particles after 1hr (TNF- $\alpha$ ) and 24 hrs (IL-1 $\beta$ ), (n=3 subjects). Maximal stimulation occurred at a dose of 5 particles/cell. Note p<0.05 where indicated by bars or by \* indicating comparison to controls using one way ANOVA with Newman-Keuls Multiple comparison test of pairs.



**Figure 7.**

Time Course: Human primary monocytes (n=3) dosed with a low concentration of particles (1 particle per cell) demonstrated maximal TNF- $\alpha$  response difference with untreated controls at 1 hour (5  $\mu$ m diameter CoCr-alloy spherical particles). Note \* =  $p < 0.05$  in comparison to all other groups using one way ANOVA with Newman-Keuls Multiple comparison test of pairs.



**Figure 8.** Adsorbed protein film dependent differences in (a) TNF- $\alpha$  and (b) IL-1 $\beta$  responses of differentiated primary human monocyte/macrophages (n=3 subjects) using 5  $\mu$ m diameter CoCr-alloy spherical particles. These preliminary results of adsorbed protein film differences were produced by incubation in different serum proteins for 48 hours. Serum albumin and LPS produced the greatest amount of TNF- $\alpha$  whereas AB human serum and IgG adsorbed protein film produced the greatest amount of IL-1 $\beta$ . Note \* = p<0.05 in comparison to all other groups using one way ANOVA with Newman-Keuls Multiple comparison test of pairs.