

INVESTIGATING THE MACROPHAGE RESPONSE TO WEAR DEBRIS USING HIGH-THROUGHPUT PROTEIN CHIPS: INTENSE INFLAMMATORY REACTION TO TITANIUM-ALLOY PARTICLES

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INTRODUCTION

Particle-induced bone resorption represents a significant clinical problem in total joint arthroplasty, accounting for the majority of long-term implant failures (1;2). Submicron wear debris from both the metallic and polymeric prosthetic components is believed to stimulate resident macrophages to release a variety of inflammatory cytokines. These mediators recruit and activate inflammatory cells, orchestrating the formation of a peri-implant granuloma. This granuloma serves as a permanent source of cytokines, ultimately causing osteoclastic bone resorption and implant loosening.

In-vitro models using human macrophages remain the gold standard for investigating the acute response to wear debris (3;4). Researchers have classically relied on ELISA to identify individual proteins such as TNF- α , IL-1 and IL-6 as the primary drivers of osteolysis (5;6). This technique is simple, but labor intensive limiting studies to a handful of cytokines. Recent investigations using gene expression profiling demonstrate that particle stimulation of macrophages is incredibly complex and involves immediate expression of hundreds of macrophage genes(7). In this study, we utilized emerging high-throughput protein chips to characterize the short-term response of human macrophages to clinically relevant wear debris such as UHMWPE, TiAlV, CoCr and alumina particles. Protein chips permitted the simultaneous and precise quantification of 29 cytokines, chemokines and growth factors.

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Wear Debris	Mean (μm)	Median (μm)	Std. Dev.	Average # of particles per cell in culture
UHMWPE (PE)	0.48	0.37	0.4	853 particles
TiAlV	0.74	0.63	0.4	370 particles
CoCr	0.48	0.41	0.3	853 particles
Alumina	0.23	0.20	0.1	3732 particles
bPE	24.44	25.25	6.7	0.33 particles

Table I: Characteristics of Particles used in Human Macrophage Culture

METHODS

PARTICLE PREPARATION

Fine UHMWPE, and TiAlV debris were prepared as previously described (8;9). CoCr and Alumina particles were obtained from commercial sources (Zimmer, Inc and Polysciences). Particles were characterized using a JEOL 5910 scanning electron microscope 10,000 – 15000 x magnification. Approximately 300 particles were measured for each species (Table I).

CELL ISOLATION AND CULTURE

Human monocytes were isolated from peripheral blood donated by healthy volunteers (n=4) and purified by sequential discontinuous Percoll gradients (10). 2×10^6 cells/well were cultured in macrophage serum-free media (M-SFM) supplemented with antibiotics. After an overnight culture and washing to remove non-adherent cells, adherent macrophages were challenged with various particle species (see Table I) in 1 ml media at concentrations representing 2x the macrophage surface area (3). Controls included non-stimulated cells and lipopolysaccharide (LPS) endotoxin served as a positive control. After a 24h culture, macrophage conditioned media were harvested, aliquoted and stored at (-) 76°C.

HIGH-THROUGHPUT PROTEIN CHIPS AND ANALYSIS

Monocyte supernatants were analyzed using the protein profiling human Cytokine Biochip (Zyomyx, Hayward, CA). Each chip contained five replicates and one internal control. 40 μl of sample was analyzed for each determination of 29 cytokines in five replicates. Biochips were scanned on a fluorescent scanner with a 532 nm laser and parameters adjusted to provide the largest dynamic range with minimal feature saturation.

Protein concentrations were calculated based on a six-point calibration curve performed in each chip. Each reported value represents the average of five replicate measurements

Assessing Osteolysis Using High-throughput Protein Chips

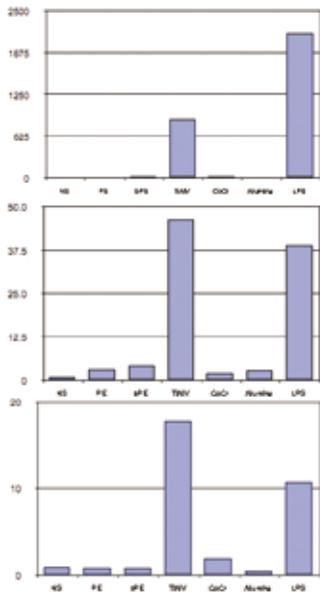


Figure 1: TNF- α , IL-1 β and IL-1 α released by macrophage after culture with Human monocytes for 24 h.. Note: Y-axis in all cases represents fold-increase over nonstimulated cells. TNF- α and IL-1 β are plotted on a log scale. All values represent mean + Std Error.

from each assay. Data is presented as the ratio of particle treatment over non-stimulated controls at time zero.

RESULTS

Data is presented and discussed in terms of fold-increase over non-stimulated (NS) macrophages from the same donor cultures (Figures 1 and 2). NS samples exhibited minimal cytokine activity, suggesting that they were not activated during the isolation procedure and thus validating the methodology. LPS used as a positive control in these studies because it provides the most robust stimulus to macrophages, attesting to their viability and responsiveness in culture.

Nine of twenty-nine (9/29) cytokines included on the chip were detected after the 24 h culture period. IL-1 α , TNF- α and IL-1 β stimulation was seen in select samples (Figure 1). TNF- α release was stimulated by TiAlV (2000-fold), CoCr (22-fold), bPE (22-fold) and alumina (5-fold). Similar trends were seen in IL-1 β expression and release as TiAlV elicited a 30-70 fold increase. CoCr and alumina particles elicited nominal increases of 2-fold and 3.1-fold increases over controls, respectively. Only TiAlV particles elicited IL-1 α , almost 20-fold higher than control macrophages. Submicron PE particles were surprisingly benign, causing only an ~ 3-fold increase in IL-1 β production with no significant IL-1 α or TNF- α stimulation.

IL-8, IL-6, MCP-1 (Figure 2) were also detected. IL-6 was stimulated only by TiAlV at levels ~ 20-fold higher than control macrophages. TiAlV and bPE particles stimulated macrophages to release nearly 5-fold and 2-fold higher level of IL-8. CoCr and PE particles again elicited minimal increases relative to NS cells. TiAlV also stimulated the highest levels of MCP-1 (18-fold increase) compared to CoCr (5-fold), bPE (5-fold) and alumina (3-fold) particles. GM-CSF and IL-10 were stimulated only by TiAlV particles.

DISCUSSION

Wear debris-stimulated macrophages have long been focused on as the primary driver of peri-implant loosening. Our recent gene array studies portrayed a complex macrophage

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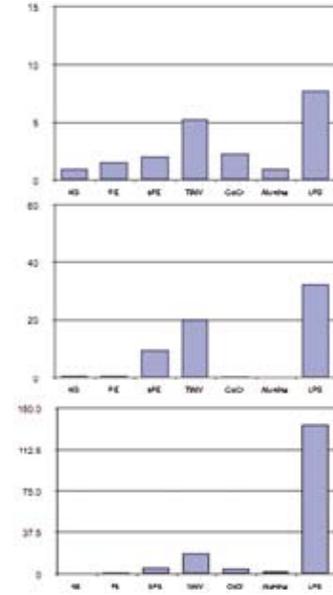


Figure 2: IL-8, IL-6 and MCP-1 released by macrophage after culture with Human monocytes for 24 h.. Note: Y-axis in all cases represents fold-increase over nonstimulated cells. MCP-1 values are plotted on a log scale. All values represent mean + Std Error.

response, as transcriptional alterations were made in hundreds of genes immediately following interaction with wear debris (7). The use of protein arrays in this study allowed us to simultaneously and precisely quantify 29 protein products of these genes, providing an accurate portrait of the acute response of human macrophages to wear debris.

Of the 29 cytokines targeted, 9 were detected consistently in the macrophage cultures. The absence of T-cell derived mediators (IL-2, IL-3, IL-4, IL-4, IL-12p40, IL-15, IL-13, SCD95, SCD23, IFN γ) is not surprising and alludes to the purity of isolated macrophages. IP-10 and MIG, are present in osteolytic tissues (11), requires T-cell release of IFN γ , not present in our model here.

A surprising finding in this study was the intense inflammatory response documented following macrophage challenge with TiAlV particles. TiAlV was the only particle species to cause a significant increase in GM-CSF, suggesting that the acute exposure to macrophages alone is enough to generate a systemic inflammatory response. TiAlV particles universally elicited the highest levels of cytokines and the expression of the classic osteolytic mediators TNF- α , IL-1 α and IL-1 β , it rivaled that of the positive control LPS. CoCr, alumina and bPE wear caused inflammation of intermediate intensity. The reaction to the cauliflower-shaped, 25 μ m bPE confirms that inflammation can occur independent of phagocytosis. The relatively brisk reaction to TiAlV has been documented in the literature. Comparing the macrophage response to CoCr and TiAlV, Haynes et al reported that while CoCr particles released basal levels of mediators, TiAlV particles elicited very high levels of several mediators including PGE $_2$, IL-1, TNF and IL-6 (12). In an earlier study using ELISA, Shanbhag et al reported that human macrophages increase secretion of no more than 2-3-fold after TiAlV stimulation (10). Compared to this body of literature, results from our current studies are consistent with an unprecedented response to TiAlV wear debris.

There are several possible explanations for this increased

response. As we have recently demonstrated, TiAlV disks also stimulate macrophages and elicit high levels of TNF- α , IL-6 and IL-1 β (13). This suggests the TiAlV surface chemistry and texture are stimulatory, independent of phagocytosis (13). The morphology of TiAlV debris is typically globular with some rod-shaped particles and flakes (14;15). In contrast, PE wear debris are predominantly spherical with occasional fibrils interconnecting debris aggregates (14).

TiAlV particles represent only 5% of wear released in vivo while PE represents the majority, over 70-95% (14). As such, engineering efforts to improve implants have focused on creating wear resistant polymers rather than preventing metallic debris. The results of this study suggest that PE debris is

relatively benign, eliciting cytokine levels only slightly higher than controls. In contrast, the macrophage response to TiAlV rivaled that of LPS and was generally more than 100 times as stimulatory as PE. Metallic wear can also decrease healing through metabolic alterations, osteoblastic inhibition (16), chemical carcinogenesis (17), decreased mineralization (18) and immunological interactions as hapten formation and anti-chemotactic action (19). It is possible that these rare, but stimulatory TiAlV particles could play a role equivalent to the abundant but benign PE particles in peri-implant loosening. While the emphasis on PE has resulted in developing wear resistant UHMWPE, a similar effort needs to be expended to reduce all sources of metal debris at the implant site.

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