In vitro implant debris-induced inflammatory responses between monocytes and osteoclasts

BY

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THESIS

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This thesis is dedicated to Dr. Nadim Hallab, my research advisor, for believing in me, and supporting me throughout the process.

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I would like to thank my family and friends for supporting me through my ups and downs and pushing me onwards.

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List of Abbreviations

PBMCs	Peripheral Blood Mononuclear Cells
M-CSF	Macrophage Colony-Stimulating Factor
RANKL	Receptor Activator of Nuclear factor-Kappa-B Ligand
TNF-α	Tumor Necrosis Factor-alpha
IL-1β	Interleukin-1 beta
LPS	Lipopolysaccharide
PMMA	Poly(methyl methacrylate)
ELISA	Enzyme-Linked Immunosorbent Assay
TRAP	Tartrate-Resistant Acid Phosphatase
OD	Optical Density
DAMP	Danger-Associated Molecular Patterns
PAMP	Pathogen-Associate Molecular Patterns

Summary

Aseptic failure of joint replacement is attributed to implant debris induced osteolysis, or local resorption of bone surrounding the implant. Monocytes and osteoclasts, when in presence of implant debris (metal particles and ions), release pro-inflammatory cytokines such as IL-1β, TNF-α, and IL-6, which in turn facilitate osteolysis. The degree to which metal implant debris affects monocytes/macrophages versus osteoclasts directly is unknown. We investigated monocyte versus osteoclast responses to metal implant debris (particles and ions) to determine the relative inflammatory and osteoclastogenic effects of metal debris on each cell type (e.g. released IL-1β and TNF-α). Our results show that osteoclasts have a highly reduced inflammatory response, than monocytes, in terms of the amount of cytokine released, indicating that as monocytes differentiate into osteoclasts, they lose some monocyte characteristics and functionalities and become more role-specific. Specifically for monocytes, over 10,000 pg/ml of IL-1β is secreted and less than 5000 pg/ml of TNF-α is produced. For osteoclasts, the number reduce to less than 150 pg/ml for IL-1β and less than 600 pg/ml for osteoclasts. Osteoclast precursors challenged by supernatants from activated monocytes and osteoclasts challenged directly exhibit relatively the same amount of TRAP positivity, but greater than negative controls.

Introduction

Clinical Problem

When certain body parts face severe dysfunction due to injury or age, artificial implants are fixed in place to restore function and alleviate discomfort. Total joint replacement, where a damaged joint is replaced, is becoming a more common practice over the years [1]. While greater than 99% of total joint replacement implants do well over the short term [7, 11] after 10-15 years patients with total joint replacements often require revision surgery caused by the aseptic loosening of their implants. This loosening is caused by local osteolysis, i.e. resorption of bone surrounding the implant [7]. As a result of this, revision surgery is done, which not only increases the costs for the patients but also increases chances of morbidity.

Background

Research over the past two decades has shed some light on osteolysis and that wear debris from the articulation between implant and bone stimulates inflammatory cells to inhibit osteoblast, bone forming cell, activity, and promote osteoclastogenesis and subsequent bone resorption. Osteoclasts and osteoblasts together play an integral role in maintaining bone homeostatis. Inflammatory cells such as monocytes and macrophages, when activated by particulate debris secrete cytokines, are known to induce osteolysis by promoting the activity of bone-resorbing cells, i.e. osteoclasts [11, 24, 25] but the direct effects of implant debris on osteoclasts are still somewhat unclear. The presence and activation of macrophages to implant debris in periprosthetic tissues and their subsequent role in osteolysis has been well established [11, 26]. While activated macrophages and monocytes are involved in osteoclastogenesis and osteoclast activation [11] the degree to which implant debris impact osteoclasts directly versus indirectly through inflammatory cells such as monocytes remains uncertain [24]. Some pro-inflammatory cytokines typically released by monocytes/macrophages cells are TNF-α, IL-1β, and IL-6 [26]. TNF-α has been established as a fundamentally critical cytokine in osteoclastogenesis [6, 18, 27]. IL-1β has also been found to influence osteoclasts formation, and play an

important role in osteolysis as well [14, 17]. It is unclear to what degree osteolysis occurs due to wear debris effects on osteoclasts directly or whether macrophage induced inflammatory responses mediate this resorptive process on an equivalent cell number comparative basis. The results of this study develop a framework for better engineering of implants by understanding reactivity to biomaterials.

Approach

Given that osteoclasts precursors are also macrophage precursor cells, the question remains: are they equivalently immuno-reactive to implant debris? To address this question we studied the individual inflammatory reactivity of monocytes versus osteoclasts to implant debris and the role of implant debris-induced monocyte driven osteoclastogenesis versus osteoclasts alone. We aimed to find out if monocytes and osteoclasts react similarly to implant debris via their cytokine release profile, and if implant-debris-activated monocytes induce more osteoclastogenesis than implant debris challenged osteoclast precursors. Our hypothesis was that monocytes will produce a greater proinflammatory effect than will osteoclasts when exposed to implant debris even though they are from the same lineage, and that this response by monocytes will play a greater role in osteoclast formation than implant debris alone. We tested this hypothesis by challenging monocytes and osteoclasts with an array of metal particles and ions and analyzing resulting differences in cytokine production and by also evaluating the effects of direct and indirect metal implant debris exposure on osteoclastogenesis as measured by TRAP staining. We used TNF-α and IL-1β as signature cytokine of innate immune responses, shown previously to have a direct effect on bone resorption [9, 17, 25, 27].

Materials and Methods

Cell Culture

Peripheral blood mononuclear cells (PBMCs) were obtained from human whole blood by layering blood mixed with 1X PBS over Lymphocyte Separation Medium (Lonza) and centrifuging on high speed for 30 minutes. The monocytes were isolated from the PBMCs using the Monocyte Isolation Kit II (Miltenyi Biotech) and the autoMACS Pro Separator. For testing the effect of implant debris on osteoclasts, the monocytes were incubated in media (RPMI-1640 with 10% human AB serum), 100 ng/ml receptor activator or NF-κB ligand (RANKL) (R&D Systems), and 50ng/ml macrophage colony-stimulating factor (M-CSF) (R&D Systems), for 6-7 days, at 37°C and 5% CO_2 in a 48-well plate, at 300,000 cells/well, with new media every other day, and supernatant collected on day 8.

To test the effect of activated monocytes on osteoclastogenesis, on day one, one portion of the monocytes were plated in a 96-well plate in media with 10% human AB serum and stimulated overnight with the challenge agents. The second portion was plated separately in a 96-well plate in media with 10% human AB serum, 100 ng/ml RANKL and 50 ng/ml M-CSF, to become into osteoclasts. The monocyte supernatants were collected on day two, and replaced the media for the osteoclasts on day three. On the sixth day, the osteoclasts are TRAP stained (Sigma-Aldrich) and analyzed on an ELISA plate reader at 450 nm, for their optical density (OD) values.

Metal Challenge

The cells were stimulated with the appropriate challenge agents: Co particles (0.9 μm, 10:1=particles:cell), Ti particles (1.2 μm, 10:1=particles:cell), Co ions (0.01-0.1 mM), Ni ions (0.01-0.1 mM), PMMA (1.8 μm, 10:1=particles:cell), and positive controls, Alum (350 ug/ml) and Nigericin (10 uM).The primed portions of the osteoclasts plated in the 48-well plate were stimulated with LPS (50 ng/ml) for 2-3 hours before adding the challenge agents.

TRAP Staining and Analysis

Our study followed a 6-day design, starting with isolation of monocytes from human whole blood. One portion of the monocytes is cultured in a plate with the different challenge agents overnight, while the second portion is cultured separately, with RANKL and M-CSF, to become osteoclasts. The supernatants from these monocytes are collected and replace the media of the osteoclasts on the third day, for the last 3 days of the study. On the sixth day, TRAP staining is performed to determine the amount of TRAP positive cells, which is used as an indicator of osteoclastogenesis. The first study is done in n=8 subjects and the second study is performed on n=3 subjects. Osteoclastogenesis is analyzed by reading the TRAP stains on an ELISA plate reader at 450 nm for optical density (OD) values and the data is analyzed on GraphPad Prism 5.

Cytokine Analysis

The supernatants were analyzed via ELISA (R&D Systems) or Luminex (Millipore) assay for IL-1β and TNF-α.

Statistical Analysis

Statistical analysis was determined by ANOVA and Mann-Whitney test to determine the variance between compared groups.

Results

Characterization of osteoclasts

To verify osteoclast differentiation, images were taken during the differentiation process. Monocytes at day 1, immediately after they have been isolated and plated, were small, round, adherent, and nearly confluent (Figure 1A). After a week of incubation with RPMI-1640, M-CSF, and RANK-L, and replacing this media every other day, the monocytes fused to become large multi-nucleated osteoclasts (Figure 1B). Osteoclasts were verified not only by the appearance of multinucleated cells, but also by TRAP-positive staining. Figure 1D shows that osteoclasts were TRAP positive by day 7. The qualitative similarity of osteoclast-like monocytes exposed to Co particles was also demonstrated (Figure 1C).

To determine the cytokine profile of the inflammatory response of monocytes and osteoclasts to implant debris, these cells are challenged with particles and ions overnight, and their cytokine profile analyzed via ELISA the next day. Unchallenged cells are used as negative controls, and Alum- and Ng-challenged cells as positive controls. IL-6 results (data not shown) were similar to TNF-α.

Effect of implant debris on unprimed osteoclasts vs. unprimed monocytes (Fig 2A, 3A)

Overall the IL-1β and TNF-α secretion significantly increased for monocytes than for osteoclasts. Generally, osteoclasts secreted more TNF-α than IL-1β, and monocytes secreted more IL-1β than TNF-α. IL-1β values are significantly higher for all conditions in monocytes than in osteoclasts ($p < 0.05$), and TNF-α values are higher in monocytes especially for Co-alloy particles ($p = 0.0139$), Co ions ($p = 0.0057$), Ni ions ($p = 0.0195$), and PMMA ($p = 0.0139$). Metal ions induce higher cytokine secretion in monocytes than particles for both IL-1β and TNF-α ($p < 0.05$). PMMA also induces more cytokine than particles, though insignificant. For monocytes and osteoclasts both, Co ions and Ni ions produce more IL-1β than Ti-alloy or Co-alloy particles. Ng and Alum are positive

controls, but do not secret significant amounts of cytokines, possibly because the dosage used was not enough to cause cells to induce an adequate response.

Data shows that insignificant levels of IL-1β are also secreted for monocytes challenged with implant debris alone ($p > 0.05$). Monocytes generate a 2-fold increase in TNF-α secretion for Ti particles and Co-alloy particles alone, compared to control values, and insignificant changes in IL-1β. Insignificant levels of IL-1β are secreted for osteoclasts challenged with implant debris alone ($p > 0.05$) in comparison to monocytes. TNF-α increases are seen in monocytes that are challenged with Ti-alloy particles and Co-alloy particles, compared to the negative control, but are insignificant. However, TNF-α increases significantly in monocytes challenged with metal ions. Co ions, Ni ions, and PMMA alone induce nearly a 6-fold increase in TNF-α secretion in monocytes, and 5-6x more IL-1β, compared to control values. Monocytes have the highest TNF-α secretion for Co ions ($p = 0.0057$) and Ni ions ($p = 0.0195$). Osteoclasts barely secrete any IL-1β, but do secrete some TNF-α. For osteoclasts, a slight increase in TNF-α production takes place with Co-alloy particles ($p = 0.0284$) but Ni ions ($p = 0.0484$) and PMMA ($p = 0.0284$) produce the highest amount of TNF-α secretion.

Effect of implant debris on primed (+LPS) and unprimed (-LPS) osteoclasts and monocytes

Osteoclasts secrete less IL-1β and TNF-α than monocytes for both primed and unprimed sets of data. Similar to the unprimed conditions, primed osteoclasts secrete more TNF-α than IL-1β, and primed monocytes secrete more IL-1β than TNF-α. IL-1β and TNF-α secretion are both higher than controls for monocytes with particles and ions alone, and even higher for samples primed with LPS. Similar to monocytes, osteoclasts challenged with both LPS and implant debris also generally secreted much higher amounts of TNF-α when compared to osteoclasts challenged with implant debris alone. Insignificant levels of IL-1β are seen in unprimed osteoclasts. Priming cells with LPS alone induces monocytes and osteoclasts to secrete more IL-1β and TNF-α. Primed monocytes produce more IL-1β than primed osteoclasts, similar to the unprimed condition, especially those challenged with Ni ions secreting the largest amount ($p = 0.0091$). Similarly, primed

monocytes also produce more TNF-α than primed osteoclasts with especially significant variance shown for Co-alloy particles ($p = 0.0095$) and Co ions ($p = 0.0057$) and Ni ions ($p= 0.0195$).

Primed monocytes do secrete more IL-1β than unprimed monocytes, though the values are not statistically significant. However, a statistically significant increase is seen for TNF-α from unprimed to primed monocytes when challenged with Co-alloy particles ($p = 0.0286$). Primed osteoclasts do not show significant increases in IL-1β secretion, but produce slightly more IL-1β with LPS only ($p = 0.04$), Co-alloy particles ($p = 0.0142$), and PMMA ($p = 0.0142$) relative to negative controls. With LPS only, TNF-α secretion in osteoclasts jumps up significantly relative to the negative control ($p = 0.0028$). Ti-alloy particles, Co-alloy particles, and PMMA show similar amounts of TNF-α secretion in osteoclasts. Primed osteoclasts do secrete more TNF-α than unprimed osteoclasts, especially when challenged with Co-alloy particles ($p = 0.005$), Co ions ($p = 0.0028$), and PMMA ($p = 0.0304$).

Implant debris and osteoclastogenesis

Slightly higher TRAP staining is seen in cells that are directly challenged with particles and ions, than in controls, especially in donors 1 and 2. Only in donor 3, the positive control, monocytes incubated with media, RANKL and M-CSF, show significantly larger amount of TRAP than the negative control. Donor 1 shows significantly higher TRAP than controls for Ni ions. Donor 2 shows highest TRAP for Co ions, Co ions supernatant, and Ni ions, but virtually no increase in the positive control. There is evidently high donor variability. Metal ions show to induce more TRAP than controls, whereas metal particles (Co particles) and LPS do not increase TRAP activity by a significant amount. One-way ANOVA, Dunnett's test was performed on each donor and all their conditions relative to the negative control. Co ions and Ni ions showed significance in inducing TRAP.

Monocyte activation and osteoclastogenesis

Supernatants from debris-challenged monocytes induce similar amount of osteoclastogenesis as osteoclast precursors challenged directly with implant debris. Supernatant from Co ion-challenged monocytes induce the same amount of TRAP as monocytes directly challenged with Co ions, which are similar in levels to the positive control as well. Supernatant from Co-alloy particle-challenged monocytes induce the same amount of TRAP as monocytes directly challenged with Co-alloy particles. Supernatants from Ni ion-challenged monocytes induced less TRAP activity than monocytes challenged directly with Ni ions. The One-Way ANOVA test determines a value of $p = 0.9888$ for the mean data between the three donors, estimating the variance between results as significant.

Discussion

The results of this study support the first part of our hypothesis because we find that monocytes show a much stronger inflammatory response via release of high amounts of pro-inflammatory cytokines IL-1β and TNF-α. They partially support the second part of our hypothesis because we find that monocytes in presence of osteoclasts precursors encourage osteoclasts formation, which is confirmed via increased TRAP positive staining. However between direct challenge by particles and challenge by monocyte-activated supernatants, the TRAP positive quantification is relatively the same indicating that the conditioned media from cells challenged with particles induce relatively the same amount of osteoclast formation on precursor cells as direct particles do.

In the first part of this investigation, we compare the inflammatory responses of the osteoclasts and monocytes to particles and ions, both with and without LPS, in terms of the amounts of cytokines IL-1β and TNF-α produced. Osteoclasts are cells that are derived from the monocytes/macrophage lineage and may have some functional similarities. However, the primary roles of monocytes and osteoclasts are different, so we attempt to understand their inflammatory response to implant wear debris. IL-1β and TNF-α are both established in various studies as pro-inflammatory cytokines that play a significant role in bone resorption [6, 9, 14, 25, 27]. Cytokine IL-1β is monitored as a proxy of inflammasome-mediated DAMP responses, and cytokines TNF-α and IL-6 are monitored as proxies of NF-κB-mediated PAMP responses. Monocytes showed overall much higher cytokine secretion than osteoclasts, implying that as monocytes differentiate into osteoclasts, they lose their ability to induce a strong inflammatory response probably due to role specialization. Monocyte polarization is the first step in any immune response and these results are also in line with the main function of monocytes, which is to produce the appropriate signals to combat the specific attack at the immune system, hence the significantly stronger cytokine induction than osteoclasts. For both monocytes and osteoclasts, values higher than negative controls are observed for all challenge agents, this data being consistent with previous studies [25], indicating that metal particle

and ion wear enhances production of bone-resorbing cytokines. A previous study by Stea et al. also demonstrated a positive correlation between wear debris and the cytokines IL-1β, IL-6, and TNF-α, and osteolysis [22]. Osteoclasts here secrete more TNF-α than IL-1β and monocytes secrete more IL-1β than TNF-α, which is in support of the studies that identify the NF-kb pathway as a potential mediator of osteoclastogenesis, as well as osteolysis [5, 6]. Specifically that more TNF-α is seen in monocyte cultures than in osteoclast cultures is in line with studies that state that TNF-α is a fundamental cytokine in "mediating particle-driven osteoclastogenesis and osteolysis" [6] which may explain the need for more TNF-α once osteoclasts are formed. This also suggests a more direct role of TNF-α on bone resorption. Earlier studies have also confirmed the positive correlation of TNF-α, over IL-1β, in osteolysis [22]. IL-1β has been shown to have different effects on osteoclastogenesis. Early presence of IL-1β shows inhibitory effects of osteoclast formation but synergizing with RANKL later on to promote differentiation [16] and this may clarify why more TNF-α and less IL-1β is seen as monocytes are differentiated into osteoclasts. Possibly this time-dependent balancing mechanism of IL-1β may explain the reduced amounts of IL-1β seen once osteoclasts had been formed. Metal ions showed high TNF-α secretion, specifically Ni ions, which are known as the most common sensitizers [10].

Lipopolysaccharide (LPS, i.e. endotoxin) comes from the outer membrane of Gram-negative bacteria and is a known pathogen associated with bone loss [12], a known inducer of a strong inflammatory response in monocytes through CD14 induced toll-like receptor 4 (TLR 4) activation and its role in osteoclast formation and osteolysis has also been reported [13, 18]. There is controversy between studies about the role endotoxins play in the biological activity due to implant debris [20] and that endotoxin contamination augments the cytokine production in pre-osteoclasts [11]. To test the supplementary effects of LPS on the pro-inflammatory response of monocytes and osteoclasts, both are stimulated with challenge agents, with (primed) or without LPS (unprimed). In general, both monocytes and osteoclasts, unprimed and primed with LPS, and challenged with implant debris secrete high levels of TNF-α, suggesting some PAMP response in either case, with monocytes generally secreting more cytokine than

osteoclasts, as in the unprimed portion of cells. This supports Pearl et al.'s results that demonstrate TNF-α production occurs even in the absence of LPS [20]. However, they do not conduct a side study to test the effects of challenging *with* LPS for comparison. In our results, we find that while TNF-α secretion is greater in the presence of LPS, it is still secreted in significant amounts even without LPS. For primed monocytes and osteoclasts, LPS alone produces a significantly high jump in TNF-α, suggesting that the TNF-α levels for the rest of the primed monocytes might possibly be only the effects of LPS, though only primed monocytes show an increase in IL-1β secretion. These results contrast those of another study where it is shows that osteoclasts produce insignificant levels of TNF-α and IL-1β with or without LPS [13]. This difference in their results could be easily due to the environment because their osteoclasts were formed in cocultures with osteoblasts, which could expose the osteoclasts to various cytokines that may counterbalance to limit the inflammatory response of the osteoclasts [21]. IL-1β and TNF-α secretion are both higher than controls for monocytes with particles and ions alone, and even higher for samples conditioned with LPS. This confirms that not only is there a significant inflammatory response with metal debris alone, but that the presence of endotoxins like LPS can heighten this response by a great deal. Partially in sync with the results of Itoh et al. [13], we find that osteoclasts overall secrete insignificant levels of IL-1β with and without LPS, suggesting minimal DAMP activity. Monocytes, on the other hand, produce high levels of IL-1β with particles and ions alone, and even higher levels with LPS addition, though this effect is not synergistic. Monocytes release high amounts of TNF-α for Co and Ni ions specifically, and much higher for all cells primed with LPS, though again the effect is not synergistic.

The second part of this study focuses on the role of monocytes in the formation of osteoclasts. There is evidence of particle-directed osteoclastogenesis in osteoclast precursor cells [5]. One article talks about how titanium particles induce bone resorption by osteoclast developments [3] and another article states that titanium ions released by implant corrosion directly induces osteoclast formation [4]. Similarly, one more study discusses how Co and Cr ions mildly promote osteoclast formation [2]. Additionally, our previous work has also shown that Co-alloy particles induce osteoclast activity,

characterized by analyzing resorption levels [23]. For this study, monocytes are portioned into two parts, the first of which is directly challenged with particles and ions. The second portion is incubated for 3 days with RANKL and M-CSF and challenged on the 3rd day with supernatants from the first set of monocytes, to see the effect of supernatants released from monocyte activation on osteoclastogenesis, which is measured by TRAP positive staining. In this part of this study, we utilized an innovative method to quantify TRAP positive staining, by reading the stains on an ELISA plate reader at 450 nm. This method proved useful in assessing the amount of TRAP positive activity seen on the 96-well plates. Day 3 osteoclasts that are directly challenged with implant debris show higher TRAP than control values; supporting our hypothesis that exposure to implant debris does encourage osteoclastogenesis [5]. Specifically, Co ions and Ni ions are key stimulants and induce higher TRAP values, as seen in donors 1 and 2. Metal ions were seen to induce more TRAP than particles. Monocytes that are stimulated with the supernatants of implant-debris-activated monocytes show similar optical density for TRAP stains than monocytes that are directly challenged with implant debris, indicating that both forms of stimulations near equally encourage formation of osteoclasts. In contrast to others' findings of where LPS is shown to encourage differentiation of pre-osteoclast cell lines to bone-resorbing osteoclasts [12, 18], we find that LPS in fact does not induce any more osteoclast formation than the control conditions, and this is consistent in all three donors.

Combining the results of these two investigations, we conclude that the tissues surrounding implants contain, among other cells, monocytes which upon stimulation by wear particles and ions, release TNF-α and IL-1β, both of which facilitate osteoclastogenesis in the same way direct particles/ions to monocyte interaction does. Even though priming with LPS induces a stronger inflammatory response in terms of cytokine release, it does not significantly have an effect on osteoclastogenesis. We can conclude that Co ions and Ni ions are relatively the highest induces of inflammation and osteoclastogenesis. Various studies have demonstrated that TNF-α in culture with pre-osteoclasts promotes TRAP positive cells [12, 18, 27] via a RANK independent mechanism [15] which is supported by our results which show that higher TNF-α is seen

in debris-stimulated monocytes, which may then be using TNF-α to signal osteoclast formation in our M-CSF- and RANKL-primed monocytes. We also observe that IL-1β increases are significant in the inflammatory response and that in future investigations, IL-1β responses will be key targets to study. The next steps to follow up with this study would be to find the cytokine composition of the secretions from the activated monocytes, and to test the effects of the individual cytokines on osteoclastogenesis. The same study can be done to observe bone resorption as indication of osteoclast activity by challenging osteoclast precursors with implant debris, versus by supernatants of activated monocytes. The role of the NF-kb pathway in particle-[6, 19] and LPS-induced osteoclastogenesis [12] is reported and further work with inflammasome inhibitors like zVAD and NF-kB inhibitors like TPCK and CPI [6] can be used to elucidate the pathway that TNF-α and IL-1β are a part of to get the observed inflammatory response. Conditional osteolysis potential can be tested via hydroxyapatite bone plates or calcium phosphate films. While attempts at reduction of wear debris generation should be continued, controlling the explosive immune system response via the direct players of osteolysis is also an important alternative. The information from this study is useful to current pharmacologic strategies of reducing osteolysis by reducing osteoclastogenesis.

The outliers in each set of data can be explained by the variability seen in working with human primary cells and donor variability. Every subject has a different response system to wear debris. Previous studies used cell lines [18, 25] to obtain a high purity of monocytes and to have reproducibility, but different cells lines react differently to the same microenvironmental signals [8]. We have used monocytes derived from human peripheral blood mononuclear cells (PBMCs) to obtain results that will more closely simulate *in vivo* conditions. Human primary cells are difficult to work with because they do not last outside their natural environment as long as cells from cell lines do. This posed a challenge because osteoclasts were seen to not survive after 7-8 days in culture in the given conditions unlike in other studies where they have outlived 2 weeks [18, 28]. Aside from the inter-donor variability, using primary cells also contributed to a great deal of variation in the data [9].

The first study was performed in n=7-8 subjects, and the second study was performed in n=3 subjects. The data are expressed as the mean ± SEM. The Mann-Whitney test was used to determine statistical differences within the same conditions in monocytes versus osteoclasts, and within the same cell type between different conditions. TRAP stained data was analyzed with one-way ANOVA with overall significance of $p < 0.05$ for each donor. Within each donor set, each condition was compared to negative control values using the Dunnett's Test.

Figures

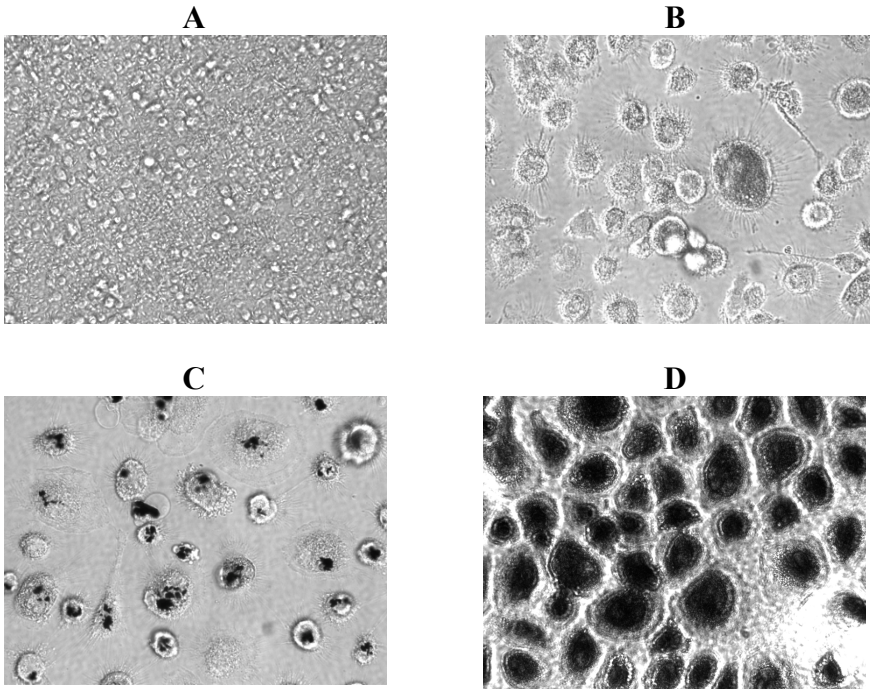

Figure 1: Light microscopy is used to image monocytes and osteoclasts. Peripheral blood mononuclear cells (PBMCs) are obtained from human whole blood, and monocytes are isolated from the PBMCs and plated in 96-well plates. (A) Freshly isolated small circular monocytes at day 1, (B) Large multinucleated osteoclasts, formed by fusion, at day 6-7, (C) Osteoclasts exhibiting phagocytosis have ingested Co particles, (D) TRAP-positive stained osteoclasts, (200x).

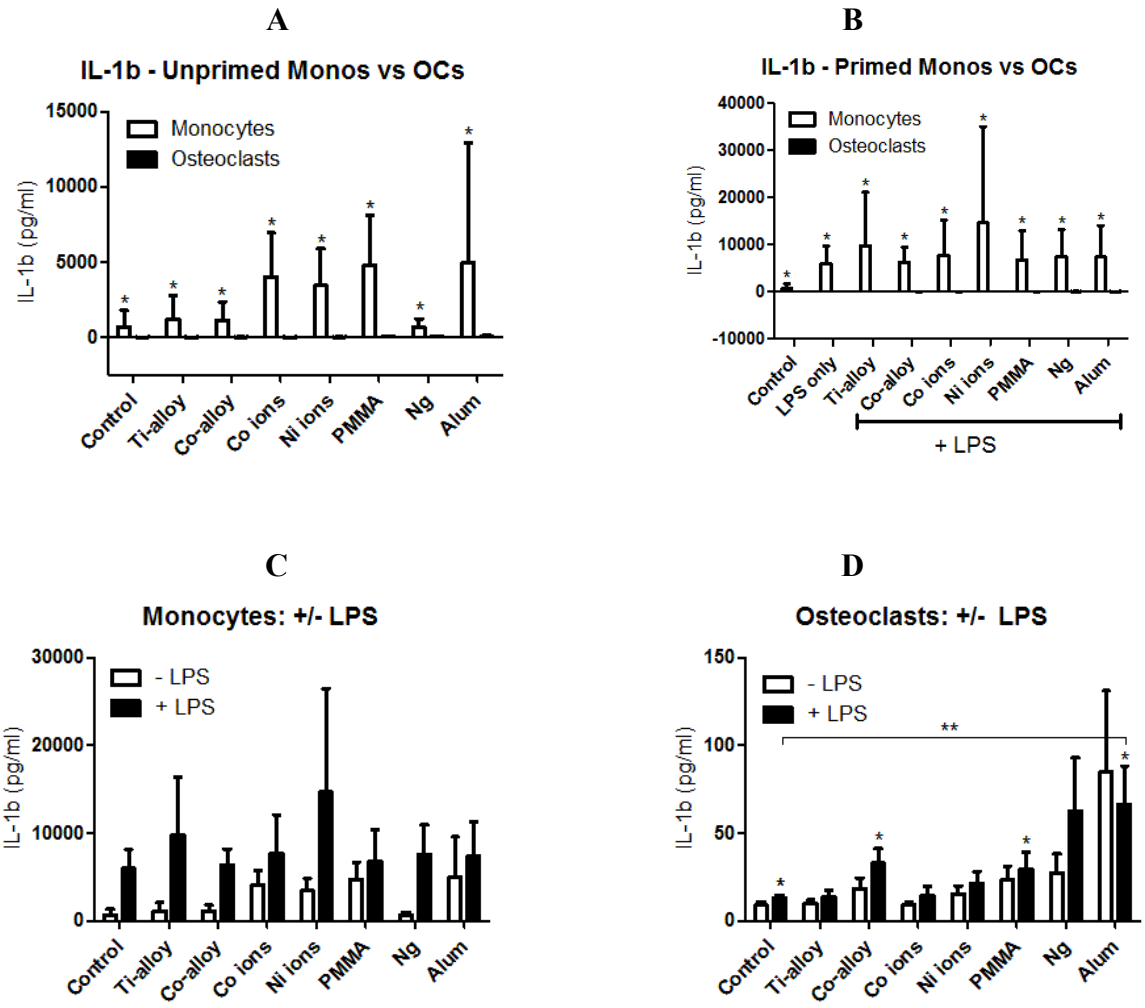

Figure 2: IL-1β secretion from monocytes (n=3-4) and osteoclasts (n=8), challenged with particles and ions with or without LPS for 24 hours. PBMCs are obtained from human whole blood, monocytes are isolated from PBMCs, and challenged with metal particles or ions, with and without LPS, for 24 hours. To form osteoclasts, monocytes are cultured with M-CSF (50 ng/ml) and RANKL (100 ng/ml) for 6-7 days, and challenged with metal particles or ions, with and without LPS, for 24 hours. 24 hours later, IL-1β cytokine secretion is assessed in supernatants of each condition. (A) Comparison between the cytokine secretion of unprimed monocytes and osteoclasts ($p = 0.0002$), (B) Comparison between the cytokine secretion of primed monocytes and osteoclasts ($p < 0.0001$), (C) Comparison between the cytokine secretion of primed and unprimed monocytes, and (D) Comparison between the cytokine secretion of primed and unprimed osteoclasts.

* = $p < 0.05$ comparing corresponding values between monocytes and osteoclasts (A and B) and comparing corresponding values between unprimed and primed (C and D)
** = $p < 0.05$ between the two highlighted conditions

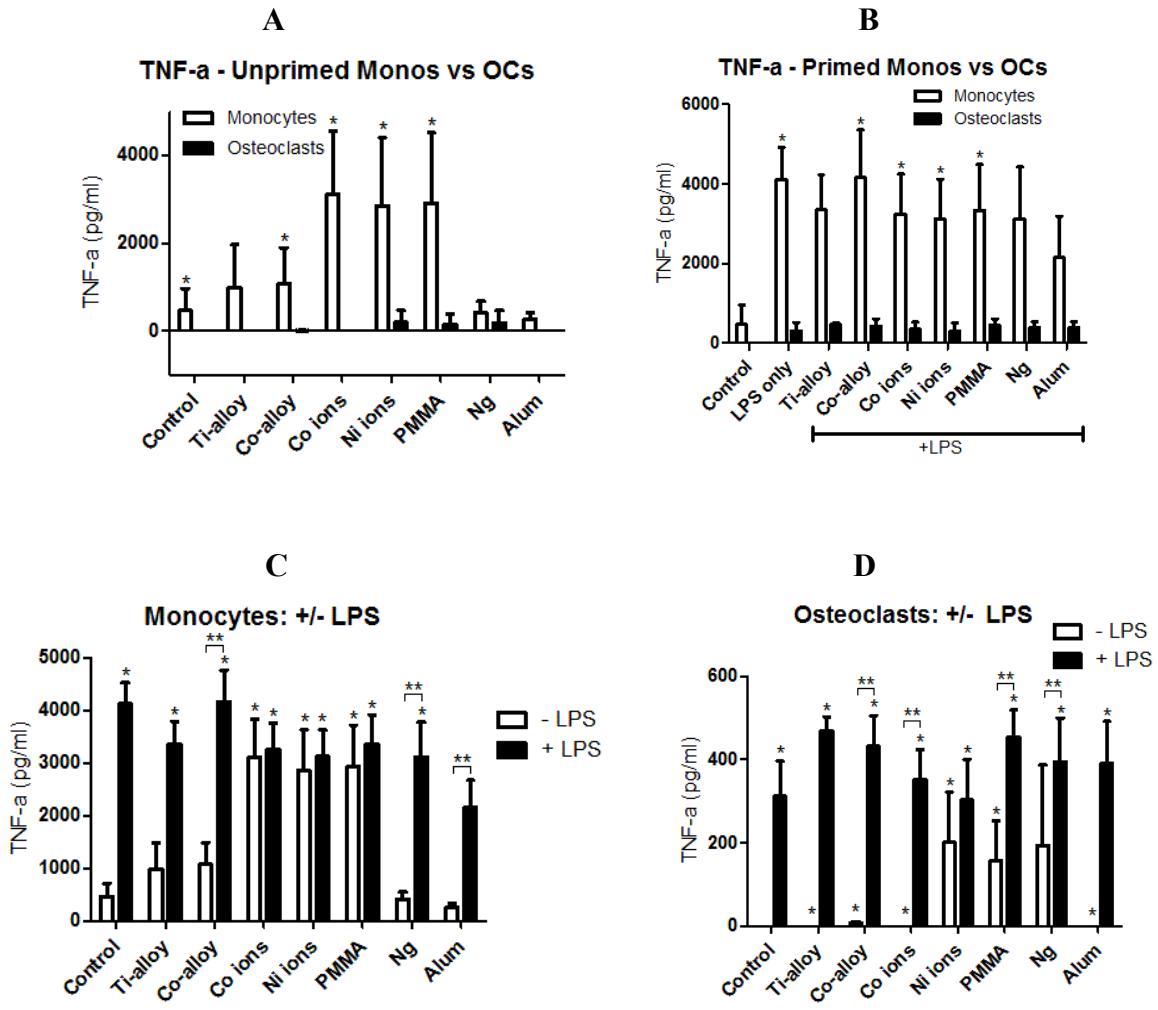

Figure 3: TNF-α secretion from monocytes (n=3-4) and osteoclasts (n=8), challenged with particles and ions with or without LPS for 24 hours. PBMCs are obtained from human whole blood, monocytes are isolated from PBMCs, and challenged with metal particles or ions, with and without LPS, for 24 hours. To form osteoclasts, monocytes are cultured with M-CSF (50 ng/ml) and RANKL (100 ng/ml) for 6-7 days, and challenged with metal particles or ions, with and without LPS, for 24 hours. 24 hours later, TNF-α cytokine secretion is assessed in supernatants of each condition. (A) Comparison between the cytokine secretion of unprimed monocytes and osteoclasts ($p = 0.0009$), (B) Comparison between the cytokine secretion of primed monocytes and osteoclasts ($p < 0.0001$), (C) Comparison between the cytokine secretion of primed and unprimed monocytes, and (D) Comparison between the cytokine secretion of primed and unprimed osteoclasts.

* = $p<0.05$ comparing corresponding values between monocytes and osteoclasts (A and B) and comparing corresponding values between unprimed and primed (C and D)
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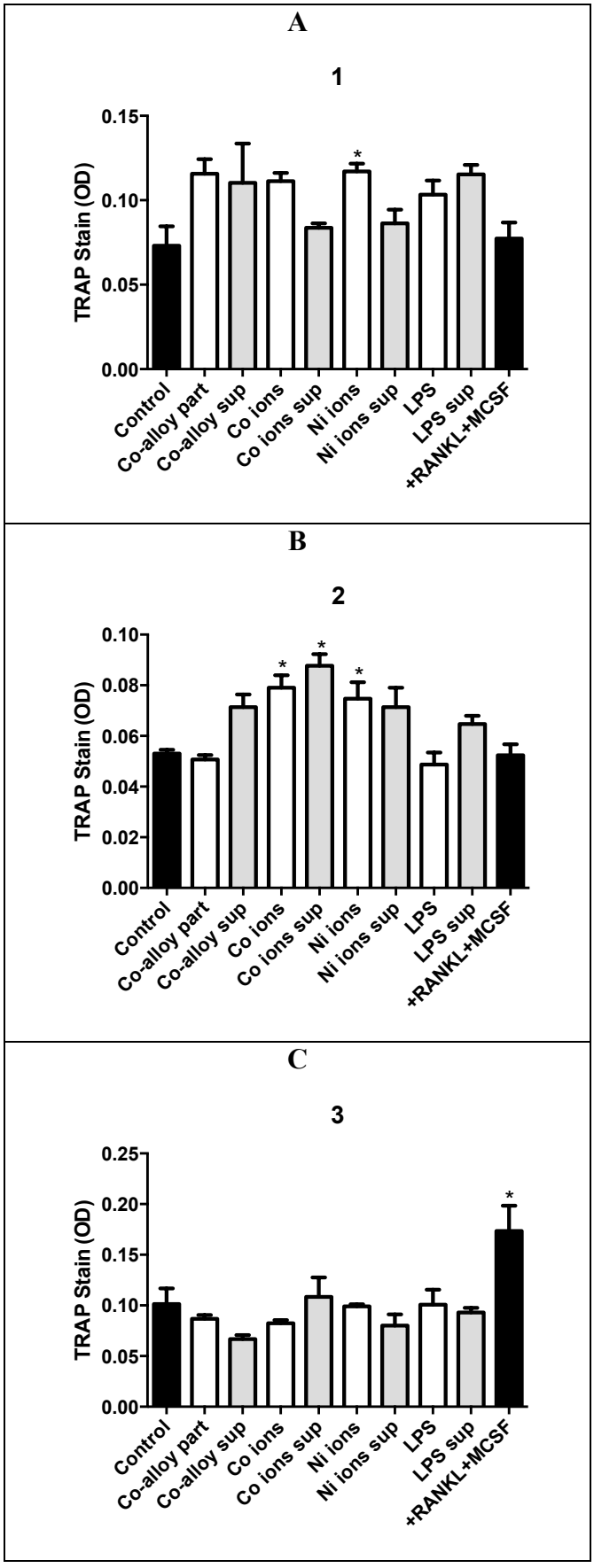

Figure 4: TRAP staining on monocytes stimulated with implant debris and monocytes stimulated with supernatants from implant-debris-activated-monocytes. Monocytes are isolated from human PBMCs and divided in two portions. One portion is challenged with metal particles and ions, and the second portion is differentiated into osteoclasts with M-CSF (50 ng/ml) and RANKL (100 ng/ml) for 3 days. On day 3, supernatants from the first portion accordingly replaced media of the osteoclasts, giving osteoclasts that were now challenged with supernatants from activated monocytes. The osteoclasts were challenged for 24 hours, and TRAP staining is measured via the optical density (OD) value. (A) Donor 1 data, (B) Donor 2 data, and (C) Donor 3 data.

Appendix

Monocytes vs. Osteoclasts (Mann-Whitney Test)					
For IL-1β			For TNF-α		
Compared Condition	**p value**	**Significant?**	**Compared Condition**	**p value**	**Significant?**
Control	0.0091	Yes	Control	0.0057	Yes
Ti-alloy	0.0238	Yes	Ti-alloy		No
Co-alloy	0.0091	Yes	Co-alloy	0.0139	Yes
Co ions	0.0091	Yes	Co ions	0.0057	Yes
Ni ions	0.0091	Yes	Ni ions	0.0195	Yes
PMMA	0.0091	Yes	PMMA	0.0139	Yes
Ng	0.0357	Yes	Ng		No
Alum	0.0357	Yes	Alum		No
LPS	0.0091	Yes	LPS	0.0095	Yes
Ti-alloy + LPS	0.0238	Yes	Ti-alloy + LPS		No
Co-alloy + LPS	0.0091	Yes	Co-alloy + LPS	0.0095	Yes
Co ions + LPS	0.0091	Yes	Co ions + LPS	0.0095	Yes
Ni ions + LPS	0.0091	Yes	Ni ions + LPS	0.0159	Yes
PMMA + LPS	0.0091	Yes	PMMA + LPS	0.0095	Yes
Ng + LPS	0.0357	Yes	Ng + LPS		No
Alum + LPS	0.0357	Yes	Alum + LPS		No

Table 1: Mann-Whitney Test performed on samples to check the variance between monocytes and osteoclasts for each condition and cytokine

TNF-α (Mann-Whitney)								
Monocytes					Osteoclasts			
Condition			p value	Significant?	Condition			p value	Significant?
Control	vs.	Ti-alloy		No	Control	vs.	Ti-alloy	<0.0001	Yes
Control	vs.	Co-alloy		No	Control	vs.	Co-alloy	0.0284	Yes
Control	vs.	Co ions	0.0286	Yes	Control	vs.	Co ions	<0.0001	Yes
Control	vs.	Ni ions	0.0286	Yes	Control	vs.	Ni ions	0.0484	Yes
Control	vs.	PMMA	0.0286	Yes	Control	vs.	PMMA	0.0284	Yes
Control	vs.	Ng		No	Control	vs.	Ng		No
Control	vs.	Alum		No	Control	vs.	Alum	<0.0001	Yes
Control	vs.	LPS	0.0286	Yes	Control	vs.	LPS	0.0028	Yes
Control	vs.	TI-alloy + LPS	0.0286	Yes	Control	vs.	TI-alloy + LPS	0.0164	Yes
Control	vs.	Co-alloy + LPS	0.0286	Yes	Control	vs.	Co-alloy + LPS	0.0028	Yes
Control	vs.	Co ions + LPS	0.0286	Yes	Control	vs.	Co ions + LPS	0.0028	Yes
Control	vs.	Ni ions + LPS	0.0286	Yes	Control	vs.	Ni ions + LPS	0.0039	Yes
Control	vs.	PMMA + LPS	0.0286	Yes	Control	vs.	PMMA + LPS	0.0028	Yes
Control	vs.	Ng + LPS	0.0286	Yes	Control	vs.	Ng + LPS	0.0164	Yes
Control	vs.	Alum + LPS		No	Control	vs.	Alum + LPS	0.0164	Yes
Ti-alloy	vs.	TI-alloy + LPS		No	Ti-alloy	vs.	TI-alloy + LPS		No
Co-alloy	vs.	Co-alloy + LPS	0.0286	Yes	Co-alloy	vs.	Co-alloy + LPS	0.005	Yes
Co ions	vs.	Co ions + LPS		No	Co ions	vs.	Co ions + LPS	0.0028	Yes
Ni ions	vs.	Ni ions + LPS		No	Ni ions	vs.	Ni ions + LPS		No
PMMA	vs.	PMMA + LPS		No	PMMA	vs.	PMMA + LPS	0.0304	Yes
Ng	vs.	Ng + LPS	0.0286	Yes	Ng	vs.	Ng + LPS	<0.0001	Yes
Alum	vs.	Alum + LPS	0.0286	Yes	Alum	vs.	Alum + LPS		No
Overall	ANOVA		<0.0001	Yes	Overall	ANOVA		<0.0001	Yes

Table 2: Mann-Whitney test performed on samples to check the TNF-α variance between each two conditions for monocytes and osteoclasts separately

IL-1β (Mann-Whitney)									
Monocytes					Osteoclasts				
Condition			p value	Significant?	Condition			p value	Significant?
Control	vs.	Ti-alloy		No	Control	vs.	Ti-alloy		No
Control	vs.	Co-alloy		No	Control	vs.	Co-alloy		No
Control	vs.	Co ions		No	Control	vs.	Co ions		No
Control	vs.	Ni ions		No	Control	vs.	Ni ions		No
Control	vs.	PMMA		No	Control	vs.	PMMA		No
Control	vs.	Ng		No	Control	vs.	Ng		No
Control	vs.	Alum		No	Control	vs.	Alum		No
Control	vs.	LPS		No	Control	vs.	LPS	0.04	Yes
Control	vs.	TI-alloy + LPS		No	Control	vs.	TI-alloy + LPS		No
Control	vs.	Co-alloy + LPS		No	Control	vs.	Co-alloy + LPS	0.0142	Yes
Control	vs.	Co ions + LPS		No	Control	vs.	Co ions + LPS		No
Control	vs.	Ni ions + LPS		No	Control	vs.	Ni ions + LPS		No
Control	vs.	PMMA + LPS		No	Control	vs.	PMMA + LPS	0.0142	Yes
Control	vs.	Ng + LPS		No	Control	vs.	Ng + LPS		No
Control	vs.	Alum + LPS		No	Control	vs.	Alum + LPS	0.001	Yes
Ti-alloy	vs.	TI-alloy + LPS		No	Ti-alloy	vs.	TI-alloy + LPS		No
Co-alloy	vs.	Co-alloy + LPS		No	Co-alloy	vs.	Co-alloy + LPS		No
Co ions	vs.	Co ions + LPS		No	Co ions	vs.	Co ions + LPS		No
Ni ions	vs.	Ni ions + LPS		No	Ni ions	vs.	Ni ions + LPS		No
PMMA	vs.	PMMA + LPS		No	PMMA	vs.	PMMA + LPS		No
Ng	vs.	Ng + LPS		No	Ng	vs.	Ng + LPS		No
Alum	vs.	Alum + LPS		No	Alum	vs.	Alum + LPS		No
Overall	ANOVA		0.6705	No	Overall	ANOVA		0.0003	Yes

Table 3: Mann-Whitney test performed on samples to check the TNF-α variance between each two conditions for monocytes and osteoclasts separately

TRAP Data (Dunnett's Test)					
			Donor 1	**Donor 2**	**Donor 3**
	Conditions				
Control	vs.	Co-alloy	ns	ns	ns
Control	vs.	Co-alloy sups	ns	ns	ns
Control	vs.	Co ions	ns	Yes	ns
Control	vs.	Co ions sups	ns	Yes	ns
Control	vs.	Ni ions	Yes	Yes	ns
Control	vs.	Ni ions sups	ns	ns	ns
Control	vs.	LPS	ns	ns	ns
Control	vs.	LPS sups	ns	ns	ns
Control	vs.	RANKL+MCSF	ns	ns	Yes
Overall		1-way ANOVA	Significant	Significant	Significant

Table 4: Dunnett's test performed on samples to check the TRAP positive staining variance between each conditions and the negative control for each donor separately

IRB Use Only
L99052722
IRB Approval Date: 11/28/2007
IRB Expiration Date: 11/28/2008

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Bioreactivity to Orthopedic Implant Debris
Sponsor: Rush University Medical Center, Zimmer Inc.

RUSH

Subject Information Sheet and Consent Document

Introduction

This form provides you with information so you can understand the possible risks and benefits of participating in this study; so that you can decide whether or not you want to be a part of this research study. Before deciding whether to participate in this study, you should read the information provided on this document and ask questions regarding this study. Once the study has been explained and you have had all your questions answered to your satisfaction, you will be asked to sign this form if you wish to participate.

Why are you invited to participate in this study?

You are invited to participate in the Metal Ion Migration, Metal Hypersensitivity and Pathologic Bone Resorption research studies under the direction of the orthopedic surgeons and faculty at Rush University Medical Center. Total joint replacements are made of various metals. Depending on the kind of implant one has, the metal will vary. Titanium and cobalt-chromium are the common materials used. We would like to determine whether or not any of these metals are present in the blood or urine of people with and without joint replacements. We would also like to determine if sensitivity to specific metal within the human body exists and if so, determine if this sensitivity affects interactions between the body and a metallic implant. We also would like to determine if there are substances in the blood that indicates the loss of bone or reactivity following joint replacement surgery.

Research studies include only people who choose to take part. Please take your time to make your decision and discuss it with your friends, family and/or physician. Remember that your participation is completely voluntary. There is no penalty if you decide not to take part in this study or decide later that you want to stop participating in this research study. Your care at Rush University Medical Center will not be affected if you decide not to participate.

What is the purpose of this study?

The purpose of this study is (1) to develop metal sensitivity testing, (2) to determine if this sensitivity plays any role after implantation of a metal prosthesis, and (3) to see if there are substances in blood that indicates the loss of bone or heighten

immune reactivity.

How many people are expected to take part in the study?

We will be recruiting approximately 500 people for this study. Group one will consist of people who have had total joint replacement surgery in the past. Group two will consist of people with osteoarthritis of the hip and without any metal implants in place that are about to undergo joint replacement surgery. Group three will consist of people without osteoarthritis of the hip and without any ***metal implants in place. Group four will consist of people with a history of metal sensitivity.***

What will you be asked to do?

After consent is obtained, we will ask you to do the following:

1. Have your blood drawn; we will take two ounces (60mL or 4 tablespoons) of your blood.
2. Complete metal exposure and medical history questionnaires, which will take approximately five to ten minutes.

How long will you be in the study?

Your participation will be limited to the collection of the above specimens, granting us access to your medical record and the completion of the above questionnaire.

You may be removed from this study without your consent for any of the following reasons: the study doctor decides that continued participation in the study will be harmful to you, you will need a treatment not allowed on the study, your disease becomes worse, you are unable to take the treatment as indicated, or the study is canceled.

What are the possible risks of the study?

Blood Draw: The risks involved with having blood drawn include, pain, bruising, infection and fainting. You should not participate in this study if you (1) tend to faint when your blood is taken, and (2) you have a history of excessive bruising or frequent bleeding from any area.

Privacy: The results of your research will not be placed in your medical records, unless you chose to do so. In addition the results of your test will coded and locked and the key to the code will kept in a separate locked file. Although every effort will be made to keep your participation confidential, the investigators cannot guarantee absolute confidentiality.

If you have any problems after having your blood drawn, call Dr Nadim Hallab at (312) 942-7079.

Are there benefits to taking part in the study?

There may be no direct benefit to you as a study participant. However, through this research we will be able to learn more about the interaction between orthopedic implants and the body and ultimately this may affect the design and composition of future implants.

What other options are there?

- The only alternative to participating in this study is to choose to not participate.

What about confidentiality of your information?

Records of participation in this research study will be maintained and kept confidential as required by law. The collection of medical information from this study will be accomplished with strict adherence to professional standards of confidentiality. Except for specific conditions spelled out in this consent document, your identity and identifying information in this study will remain confidential as required by law. Specific persons may have access to your medical records. These specific persons might include appropriate government agencies, the National Institute of Arthritis & Musculoskeletal & Skin Disease and the Rush Institutional Review Board (IRB). The IRB is a special committee that reviews human research to check that the rules and regulations are followed. If results from this study are ever published for scientific purposes, your name will remain confidential.

In order to conduct the study, the study doctor, (Dr Nadim James Hallab), will use and share personal health information about you. This includes information already in your medical record, as well as information created or collected during the study. Examples of the information that may be shared include your medical history, physical exam and laboratory test results. The study doctor will use this information about you to complete this research.

Confidentiality and disclosure of your personal information is further described in the attachment to this form. The attachment is entitled HIPAA Authorization to Share Personal Health Information in Research (2 pages).

Your identity will not be revealed on any report, publication, or at scientific meetings.

The Rush Institutional Review Board (IRB) will have access to your files as they pertain to this research study. The IRB is a special committee that reviews human research to check that the rules and regulations are followed.

What are the costs of your participation in this study?

There will be no costs to you for participation is this study and the examination of your blood specimens. There will be no additional expense for you to participate, as the research staff will meet you at your routine appointment with your surgeon.

This study is supported by Rush University Medical Center and Zimmer Inc. A portion of this money will go to Rush University Medical Center to compensate for other institutional research related costs.

Will you be paid?

You will receive no financial compensation for participation in this research study.

What happens if you experience a research related injury?

Rush University Medical Center has no program for financial compensation or other forms of compensation for injuries which you may incur as a result of participation in this study.

Whom do you call if you have questions or problems?

Questions are encouraged. If there are any questions about this research study or if you experience a research related injury, please contact: **Kyron McAllister at (312) 942-9723 or Dr. Nadim Hallab at (312) 942-7079 or Dr. Joshua Jacobs at (312) 942-5000.** Questions about the rights of research subjects may be addressed to the Rush Research & Clinical Trials Administration Office at 312-942-5498.

By signing below, you are consenting to participate in this research study. You have read the information given or someone has read it to you. You have had the opportunity to ask questions, which have been answered satisfactorily to you by the study personnel. You do not waive any of your legal rights by signing this consent document. You will be given a copy of the signed and dated consent document for your records.

SIGNATURE BY THE SUBJECT:

Name of Subject	Signature of Subject	Date of Signature

SIGNATURE BY THE WITNESS
I observed the signing of this consent document.

Signature of Witness	Date of Signature

SIGNATURE BY THE INVESTIGATOR/INDIVIDUAL OBTAINING CONSENT:
I attest that all the elements of informed consent described in this document have been discussed fully in non-technical terms with the subject. I further attest that all questions asked by the subject were answered to the best of my knowledge.

Signature of Individual Obtaining Consent	Date of Signature

Signature of the Principal Investigator	Date of Signature

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VITA

JESSICA YADAV

Education B.S., Chemistry, with distinction, University of Illinois at Urbana-Champaign, 2009
M.S., Bioengineering, University of Illinois at Chicago, 2012

Experience Dr. Nadim Hallab, Department of Orthopedic Surgery, Rush University Medical Center
Chicago, IL Jan 2010-Aug 2012
Investigated the pro-inflammatory reactivity of monocytes and osteoclasts due to implant debris and the role of monocytes in implant debris-mediated osteoclastogenesis

Dr. Irena Ivanovska, Merck Research Laboratories
Boston, MA Jun 2011 – Dec 2011
Explored primary CD4+ T cell differentiation to Th17 cells
Contributed to developing a Jurkat cell assay to understand TCR independent T cell activation

Publications
"Osteoclast inflammatory responses to implant metals are negligible compared to metal-induced monocyte/macrophage pro-inflammatory and osteoclastogenic effects." Yadav, J.; Steinberg, R.; Glivar, P.; McAllister, K.; Samelko, L.; Hallab, N. J. *Biomaterials*. (submitted)

"Oxygen Reduction Activity of a Copper Complex with 3,4-Diamino-1,2,4-triazole Supported on Carbon Black." Thorum, M.S.; Yadav, J.; Gewirth, A. A. *Angew. Chem.*

Abstracts
Orthopedic Research Society, 2012
Osteoclasts (versus monocytes) lose their ability to mount a strong inflammatory response to implant debris, in vitro**.**