

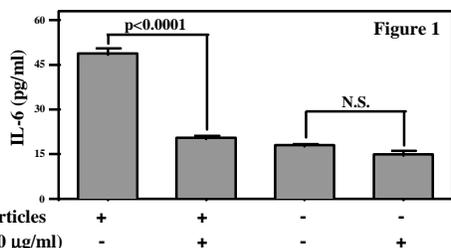
THE ROLE OF ADHERENT ENDOTOXIN IN STIMULATION OF OSTEOCLAST DIFFERENTIATION BY ORTHOPAEDIC WEAR PARTICLES

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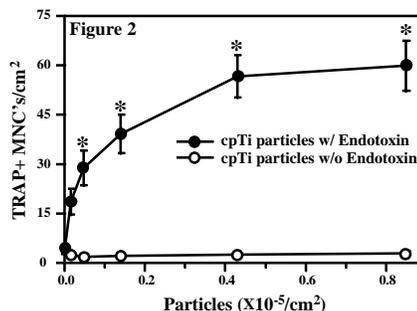
INTRODUCTION: Wear particles stimulate aseptic loosening by increasing production of bone resorptive cytokines. Previous studies found that wear particles primarily stimulate osteoclast differentiation rather than osteoclast activity [1], both commercially pure titanium (cpTi) particles as well as cpTi and titanium alloy (Ti-6Al-4V) implant surfaces contain adherent endotoxin [2], and endotoxin removal eliminates the ability of the particles to stimulate cytokine production without affecting their size or shape [3]. We, therefore, hypothesized that adherent endotoxin is also responsible for stimulation of osteoclast differentiation.

METHODS: Nucleated marrow cells were obtained from 6-12 week old C57Bl/6 mice and cultured in petri dishes in the presence or absence of cpTi particles (1-3 μm , Johnson Matthey) for 24 hours as we have previously described [1]. Conditioned media were harvested, centrifuged (1800g x 25 min), sterile-filtered, and stored at -80°C . The IL-6 levels in the conditioned media were measured by ELISA (Endogen Minikit). The effect on osteoclast differentiation of the conditioned media from mouse bone marrow cells incubated with particles before or after endotoxin removal was assessed. For this purpose, the number of tartrate-resistant acid phosphatase-positive multinucleated cells (TRAP+ MNCs) was counted in co-cultures of murine spleen cells and CIMC-4 mesenchymal support cells, an assay system that is stimulated by all of the resorptive cytokines that have been tested (IL-1, IL-6, IL-11 and TNF). To further confirm the osteoclast phenotype, formation of resorption lacunae was measured in selected osteoclast differentiation assays by performing the assays on slices of elephant ivory. The number of TRAP+ MNCs and the extent of resorption on the ivory slices was determined as previously described [4]. All data are presented as means \pm SEM. Statistical analyses were by ANOVA with Fisher's protected LSD (Fig. 1) and Bonferroni/Dunn (control) (Fig. 2 and 3) post hoc tests.

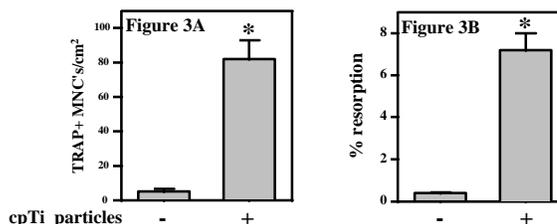
RESULTS: We initially attempted to confirm that elimination of the ability of the cpTi particles to stimulate cytokine production by endotoxin removal is due to endotoxin absence rather than other physico-chemical changes that may be induced in the particles during endotoxin removal. We, therefore, measured the effect of a specific endotoxin inhibitor. Polymyxin B (PmB, 50 $\mu\text{g}/\text{ml}$) completely blocked the stimulation of IL-6 induced by ($p < 0.0001$) particles with adherent endotoxin without affecting basal IL-6 production (Fig. 1, $n=3$).



Having confirmed that adherent endotoxin is required for stimulation of cytokine production, we tested the hypothesis that it is also required for stimulation of osteoclast differentiation. Figure 2 shows the effect on the number of TRAP+ MNCs of 25% conditioned media from marrow cells incubated with various cpTi doses. Conditioned media from marrow cells incubated with cpTi particles containing adherent endotoxin potently stimulate osteoclast differentiation. Thus, maximal stimulation is 23-fold compared to conditioned media without particles and all concentrations $> 0.04 \times 10^5$ particles/ cm^2 are $p < 0.0001$ ($n=6$). In contrast, conditioned media from marrow cells incubated with cpTi particles lacking endotoxin have no significant effect on osteoclast differentiation (Fig. 2, $n=6$).



To confirm that the TRAP+ MNCs that are generated in response to cpTi particles are authentic osteoclasts, we assessed their ability to form resorption lacunae on slices of elephant ivory. TRAP+ MNCs were found closely associated with resorption lacunae. Moreover, quantitative histomorphometry demonstrated that conditioned media from marrow cells incubated with cpTi particles significantly increase formation of resorption lacunae in parallel with the increase in the number of TRAP+ MNCs. Thus, the increase in number of TRAP+ MNCs (Fig. 3A, $n=6$) and formation of resorption lacunae (Fig. 3B, $n=6$) by conditioned media from marrow cells incubated with cpTi particles was 15-fold ($p < 0.0001$) and 20-fold ($p < 0.0001$), respectively, compared to cultures with conditioned media from marrow cells incubated without cpTi particles.



DISCUSSION: Our results demonstrate that adherent endotoxin is responsible for the ability of cpTi particles to stimulate both the production of cytokines and the formation of TRAP+ MNCs. Since the TRAP+ MNCs that are induced by the particles form abundant resorption lacunae on elephant ivory, we conclude that they represent bona fide osteoclasts. Taken together, these results demonstrate that adherent endotoxin is responsible for in vitro induction of osteoclast differentiation by cpTi particles. Moreover, wear particles that are generated in patients with orthopaedic implants likely contain significant levels of adherent endotoxin, since implant surfaces also contain adherent endotoxin [2], endotoxin derived from minor infections and gut flora exist systemically [5], bacteria can be found on many revised implants [6], endotoxin is very adherent to most surfaces [7], including titanium [8], and orthopaedic wear particles have enormous surface areas [2]. In conclusion, these findings suggest that adherent endotoxin may contribute to aseptic loosening in patients.

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