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Pearson, Mark; Grover, Liam; Williams, Richard; Floyd, Hayley; Bodansky, David; Davis, Edward; Lord, Janet

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The effects of cobalt-chromium-molybdenum wear debris *in vitro* on serum cytokine profiles and T cell repertoire

Mark J. Pearson[1†], Richard L. Williams[2†], Hayley Floyd[2], David Bodansky[1], Liam M. Grover[2], Edward T. Davis[3‡], Janet M. Lord[1‡]

[1] MRC-ARUK Centre for Musculoskeletal Ageing Research, School of Immunity and Infection, University of Birmingham, Birmingham, B15 2TT, United Kingdom

[2] School of Chemical Engineering, University of Birmingham, Birmingham, B15 2TT

[3] The Royal Orthopaedic Hospital NHS Foundation Trust, Bristol Road South, Birmingham B31 2AP, United Kingdom

[†]These authors contributed equally to the manuscript

[‡]Joint senior authorship

Corresponding author:

Professor Janet M Lord

MRC-ARUK Centre for Musculoskeletal Ageing Research,

School of Immunity and Infection,

University of Birmingham, Birmingham, B15 2TT, UK

Tel: 0121 371 3234 Fax: 0121 371 3203 email: J.M.Lord@bham.ac.uk

Abstract

Cobalt-chromium-molybdenum (CoCrMo) alloy-based metal-on-metal prostheses have been the implant of choice for total hip replacement in younger patients. However 6.2% of patients require revision of their CoCrMo total hip replacement (THR) implant within five years of surgery and their use was restricted in 2013. We aimed to determine if there were individual differences in the immune response to wear debris that might indicate a poor outcome with a CoCrMo prosthesis.

Blood from 22 donors was incubated with CoCrMo particles (>99.9% less than 10μm diameter) generated by a wear simulator for 24h. T cell phenotype was assessed by immunostaining and secretion of 8 different pro- and anti-inflammatory cytokines was measured using multiplex technology. Clear differences were seen between individuals in the induction of Th17 and Th1 responses, with some donors showing pro-inflammatory responses (increased IL17 or IFNγ) and others showing anti-inflammatory responses (decreased IL17 or IFNγ). The only differences seen for gender and age related to increased IL-10 expression from T cells in females (p=0.008) and a trend towards decreased IL-6 expression systemically for older donors (p=0.058).

We conclude that individuals show differential responses to CoCrMo wear debris and that these responses could give early indications of the suitability of the patient for a metal-on-metal prosthesis.

Keywords: Metal-on-metal, total hip replacement, osteoarthritis, wear particles, wear particle characterisation

Introduction

The number of total hip replacement (THR) operations carried out in the UK in 2011 reached 80,314 (National Joint Registry of England and Wales, 9th Report 2012). It is expected that by 2030 this number will increase by 170% (1, 2) due to an ageing and increasingly obese population who are more at risk of developing osteoarthritis (OA)(1, 3). Currently, the most effective treatment for patients presenting with hip OA is THR surgery.

Surgery is carried out with varying rates of success dependent on several factors, including the choice of prosthesis, with some prosthesis lasting for only 5-7 years (1). Cobalt-chromium-molybdenum (CoCrMo) alloy-based metal-on-metal THR prostheses have been shown to have a 50% greater risk of failure two years post-primary surgery compared to metal-on-polyethylene or ceramic-on-ceramic THR (1). Recent clinical observations have shown that 6.2% of patients have an adverse reaction to their CoCrMo THR implant within the first two years after THR (1) and as a result the Medicines and Healthcare products Regulatory Agency (MHRA) in the UK issued guidelines on the use of these implants which has resulted in a number of NHS Trusts placing restrictions on their use..

However, it should be noted that the issues of early implant failure and wear with CoCrMo THR and CoCrMo Hip Resurfacing differ in their origin. Taper junction wear and misplacement of the joint are major contributors to failure in THR (4, 5) whereas poor design of the implant has been implicated with early failure in hip resurfacing (6). Certain designs of CoCrMo hip resurfacing have been shown to have excellent survivorship and have the additional benefit of preserving femoral bone and reducing the risk of dislocation. Therefore the ability to return to the use of some well designed CoCrMo hip resurfacing might enable patient to benefit from it's unique advantages.

The major factors contributing to the requirement for revision surgery in patients with CoCrMo implants are pain and aseptic joint loosening (7). As nano-scale wear debris is generated, it can

enter the peri-joint tissue leading to tissue necrosis and induce general inflammation (8-12) and it has been postulated that it is this combination of tissue damage and inflammation, which causes aseptic loosening. There is also the potential for torque to be a factor in loosening if the joint is not sufficiently lubricated by entrained fluid from around the joint (13).

Instances of aseptic loosening of the joint in patients with metal-on-metal prostheses are greater than with any other material combination (1) and there is evidence that wear particles from cobalt chromium metal-on-metal prostheses are contributing to joint failure. The size and shape of the wear particles generated are varied and are generally in the range of 0.1-100μm in diameter. It is thought that particles which are in the region of 10μm in diameter are of most immunological relevance as they are of a size which is readily ingested by phagocytic immune cells, primarily macrophages (10, 12, 14). In support of this, wear debris of different sizes were shown to elicit different immune responses with respect to increased inflammatory marker secretion from macrophages (9, 15) with debris ~5-10μm the most bioreactive. Furthermore, metal wear debris become coated in protein from intra-tissue fluids and serum and it is postulated that this coating, which would include immunomodulatory proteins such as complement, could be used by macrophages to recognise and internalise non-biological materials (14).

Beyond the role of macrophages, the mechanisms of how the immune system reacts to such wear particles and the degree of individual variability in the response are unknown. In particular immune function alters with age (16-19) and gender (20, 21) and thus the immune response to orthopaedic wear debris is also likely to differ between young and old patients and males and females. Understanding how different age and gender groups respond to debris is important if we are to stratify patients and provide the most suitable implant for specific patients.

This study aimed to characterise the size and size distribution of particulate wear debris and determine the impact of particulate wear debris on the immune system with particular reference

to T lymphocytes and the plasma cytokine/chemokine repertoire. There is potential for such characterisations to be used to form the basis of prognostic testing to determine the suitability of a patient to be able to benefit from the unique advantages that CoCrMo hip resurfacing provides.

Materials and Methods

Participants

Healthy volunteers aged between 21 and 80, with no known inflammatory diseases and no recent infections, took part in the study. All volunteers gave written informed consent and ethical approval for the study was received from the University of Birmingham's Ethical Review Committee (ref: ERN_12-1184).

Wear Debris

Generation

CoCrMo wear debris were a kind gift from Smith & Nephew (Warwick, UK). Debris was generated using a proprietary wear simulator following $2.5x10^5$ cycles on the simulator under adverse conditions (subluxation) and was collected in heat inactivated fetal calf serum (FCS). Debris was isolated from FCS by centrifugation at 80,000*x*g for 30 minutes. Debris was then resuspended in phosphate buffered saline (PBS) and sterilised with 100U/ml penicillin and 100μg/ml streptomycin for 24 hours. Sterility was confirmed through incubation of debris on an antibiotic-free agar plate for 24 hours at 37°C.

Characterisation

Particle size analysis was performed using a laser diffraction based Mastersizer 2000 (Malvern Instruments, Malvern, UK) with an automated liquid sample homogeniser and feed system. The system was flushed three times with fresh PBS (no particles) filtered beforehand with 0.22μm pore size syringe filter (Millipore) to remove potential contaminants in the size range of interest.

The particle-PBS solution was added to the sample feeder 1mL at a time and diluted when necessary to obtain a laser obscuration value of 3-5%, which was found to produce the most reliable measurements. The system was then flushed again with PBS before adding the final particle-PBS sample for analysis. The measurement integration time was 1 second with 10 repeat measurements performed on each sample. Wear debris was imaged using a Zeiss EVO MA19 scanning electron microscope (Carl Zeiss Ltd., UK).

Blood Samples

Peripheral venous blood, which contains on average $1x10^6$ peripheral blood mononuclear cells (PBMCs) was collected in to heparinised Vacutainer™ tubes (BD Bioscience). The blood was aliquoted into two separate bijoux tubes and CoCrMo wear debris at a concentration of 20 particles/PBMC were added to one tube as previously described (22) and the second was left unchallenged but included 10% FCS to account for any FCS protein forming the protein corona around the wear debris. HEPES (5% v/v) was added to buffer the blood to prevent coagulation and haemolysis. All tubes were incubated on a rotator at 37°C, 5% CO_2 for 24 hours.

T Cell and Cytokine Analysis

PBMC Isolation

PBMCs were isolated from whole blood by density gradient centrifugation using Ficoll-paque (GE Healthcare). Briefly, whole blood was layered onto Ficoll-paque and centrifuged at 400*xg* for 30 minutes. The buffy coat was aspirated and washed twice with PBS and the PBMCs were counted using a CASY® Cell Counter (Roche). Plasma was also aspirated, aliquoted and frozen until required for cytokine analysis.

T cell phenotype and cytokine secretion

T cell populations and cytokine secretion were analysed by immunostaining and flow cytometry using fluorochrome conjugated antibodies to CD3 (APC-Cy7), CD4 (FITC or Pacific Blue), CD25 (APC), CD45RA (APC), CCR7 (FITC), FoxP3 (PE), IL-10 (APC), IL-17 (PE), and IFNγ

(APC) to identify Tregs, T helper cell memory cell subsets, Th1, Th17, and plastic Th1 populations ($IFN\gamma^{+}IL\text{-}17^{+}$). All antibodies were from eBioscience and fluorescence staining was detected and analysed using a CyAn ADP flow cytometer (Beckton Dickinson) and analysed using GraphPad Prism 5 software.

Cytokine analysis

IL-1β, IL-6, IL-10, IL-13, GM-CSF and TNFα were measured in plasma taken from CoCrMo treated whole blood using a Bio-Plex Luminex assay (Bio-Rad). All plasma samples were diluted in accordance with the manufacturer's recommendations.

Statistical Analysis

SPSS® Statistics 20 (IBM®, UK) was used for the statistical analyses of raw data. The normality of the distribution of the data was assessed using the Sharpiro-Wilk test as $N < 50$; $p > 0.05$ indicated normality. An independent or paired T-test was applied - with natural log transformation if necessary - for normally distributed data, whilst Mann Whitney U or Wilcoxson Rank Sum test were used for unpaired and paired non-parametric data, respectively. $P<0.05$ was deemed statistically significant.

Results

Subject Demographics

22 subjects were recruited and split into two age groups with the 'young' group consisting of subjects aged 20-35 whilst the 'old' group consisted of subjects aged 45-65. 75% of subjects were in the young category. Gender was split reasonably evenly with 55% of subjects being female (Table 1).

Wear particle characterisation

Figure 1a shows the particle size distribution, in terms of total volume and number, of particle wear samples obtained directly from the wear simulator ('stock solution') and after isolating the

particles of interest (up to 10μm) from that stock solution. The contribution of sub-micron and micron (up to 10μm) are quantified in Table 2. In the stock sample, particle sizes between 10-200μm accounted for approximately 54% of the total volume (and, by extension, total mass) of the wear particles with a volume-weighted mean size of 27μm. However, the number% distribution data showed that this size range represented just 0.02% of the total number of particles, with those below 10μm by far the most prevalent in number (>99%) (Figure 1b). Scanning electron microscopy (SEM) images of the sample confirmed the prevalence of these sub-micron particles and revealed their highly rounded morphology (Figure 2a, b). The majority of the micron scale particles were found to be between 1-3 μm in size in both the particle sizing data (by number) and from analysis of the SEM images. In the fraction of debris isolated after centrifugation, the upper size limit of the wear particles was 60μm (Figure 1a), but close examination of the data revealed that wear-particles above 10μm in size contributed only 0.01% to the total number of particles in the sample (figure 1b). The number-percent distribution of the isolated fraction was still found to cover the 1-10μm range and hence the procedure successfully isolated the wear-particles of interest from the FCS solution.

Effect of wear debris on T cell populations

Hallab *et al* (2012) showed that T cells are activated as a result of increased wear debris and metal ions, but phenotypic and cytokine expression changes have not been characterised. To address this PBMCs from healthy donors were challenged with CoCrMo wear debris or 10% FCS for 24 hours. Immunostaining and flow cytometry data were analysed accounting for changes in $CD3^+CD4^+$ T cell phenotypes within the control (PBS) and wear debris (CoCrMo) groups. No significant differences between the control and CoCrMo groups were found with regard to T cell phenotype or T cell cytokine expression (Figure 3). However, differences between the control and CoCrMo treated cells became apparent once individual donor responses to debris challenge were analysed (Figure 4). The two cell populations showing the differential response between

subjects were the two pro-inflammatory cell types Th17 and Th1, with the most marked differences for Th17. For Th17 there was a clear split in the volunteer responses, with subjects either giving a clear pro-inflammatory response, increasing their IL17 expression, or an anti-inflammatory response with decreased IL-17 (Figure 4). For Th1, there was an even split in the population with 6 subjects displaying decreased numbers of Th1 cells (measured by reduced IFNγ expression in the $CD4^+$ population) and 6 subjects displaying an increase in the prevalence of Th1 cells.

Cytokine analysis

Plasma samples from each subject were analysed using a Bio-Plex Luminex cytokine panel to determine the total cytokine response to CoCrMo challenge, regardless of originating cell type. There was a very clear response to CoCrMo debris with 5 cytokines significantly elevated above baseline following CoCrMo challenge, with little individual variability (Figure 5).

Gender and age impact on immune response to wear debris

Data from the National Joint Registry of England and Wales shows greater incidence of aseptic loosening of CoCrMo hip implants in females compared to males (1). The reasons for this are unclear and therefore the possibility of an altered immune response to wear debris in women was investigated. Of all of the cytokines and T cell populations analysed, $CD4^+IL\text{-}10^+$ T cells were the only population found to differ with gender, with these anti-inflammatory cells significantly increased in female subjects following CoCrMo challenge (Figure 6a).

Hip replacements are more frequent in the elderly. The immune system changes with age and thus the response to pathogens or other foreign objects differs as we get older. Therefore, it was important to assess whether the immune response to CoCrMo wear debris also altered with age. We found a trend towards increased serum IL-6 in younger subjects compared with older subjects following CoCrMo challenge (Figure 6b), in line with previous reports of immunosenescence and reduced inflammatory response to challenge in older people (23, 24).

No significant differences could be found for other cytokines or T cell populations when comparing young vs old or male vs female subjects.

Discussion

Much attention has been focussed on the failure of CoCrMo bearings used in THR surgery in recent years (25-28). Aseptic loosening and subsequent joint failure is accompanied with peri-joint tissue necrosis and osteolysis leading to pain and reduced mobility for 6.2% of CoCrMo THR patients (1). However, for the remaining 85% of patients, these prostheses cause no unwanted pathology and have a longevity which matches and in some cases, surpasses that of other bearing types. Well designed CoCrMo hip resurfacing implants have shown excellent survivorship in certain groups with survivorship of 99.4% at 15 years in patients under 50 years of age with osteoarthritis (29). These devices have the added advantages of preservation of the femoral bone and a reduced rate of dislocation. However, due to the concern over the use of CoCrMo their use has declined.

In this study we aimed to determine whether there was a profile of inflammatory markers in blood from healthy donors challenged with CoCrMo wear debris, which would indicate a potentially adverse inflammatory response which could lead to accelerated bearing failure and aseptic loosening. We found that T cell populations and serum-derived cytokine expression differed greatly between each subject. Different combinations of pro- and anti-inflammatory cytokines were present following CoCrMo challenge in different subjects, with the Th17 cell response showing the most differential response. Approximately half of the donors increased expression of IL-17 in response to the wear debris, whilst half reduced expression.

Th17 cells, defined by their secretion of IL-17, play a crucial role in the initiation of inflammation, recruiting neutrophils and macrophages to sites of infection (29), or tissue damage in the case of sterile inflammation. It has become clear in recent years that aberrant regulation of

Th17 cells is involved in the pathogenesis of a range of chronic inflammatory conditions, including Rheumatoid Arthritis (30, 31). To determine whether the differential Th17 response, or the Th1 response which also showed a marked difference between donors, are associated with a poor response to a CoCrMo implant we will need to repeat the analysis in patients undergoing revision surgery for a failed implant. Equally, determining a mechanism of T cell activation, perhaps through interaction with macrophages as has previously been postulated (22) could be valuable for development of therapeutic tools for the management of patients who currently have a CoCrMo prosthesis.

It is known that the immune system becomes differentially regulated with age causing immune senescence and changes in systemic inflammatory status. Discharge data from the US Department for Health & Human Services (HHS) show a 2.2 fold increase in THR operations for people aged 45-55 between 2000 and 2010 (32). Therefore, given this trend towards younger patients receiving THR, we investigated whether the ageing immune system had a differential response to wear debris. Analysis of a healthy young and healthy old cohort in this study revealed that the healthy old subjects had a reduced pro-inflammatory IL-6 response to CoCrMo wear debris whilst no other marker differed significantly with age. The overall reduction in the IL-6 response in the older cohort suggests that older patients may produce a less severe inflammatory response to CoCrMo wear debris than younger patients.

Smith *et al* (2012) (1) identified that the failure rate of CoCrMo bearings was greater in females. There are possible anatomical and tribological reasons for this increased failure although differences in the immune response have not previously been investigated. To address this we analysed serum-cytokine levels in male and female patients. Females were found to have significantly increased levels of IL-10 following debris challenge. Although the anti-inflammatory nature of IL-10 could be thought of as being desirable, excess IL-10 is known to impair osteoclast formation and function (10, 12). This could lead to decreased bone remodelling

around the bone-bound surfaces of the implant and thus lead to loosening of the implant and subsequent accelerated failure over time.

We have demonstrated here that T cells are activated in response to wear debris from CoCrMo alloys. Our experiments using whole blood carried out over 24h have allowed for initial macrophage processing of the debris followed by downstream activation of $CD4^+$ T cells. This link was postulated by Hallab et al in 2010 (6) and although we are unable to show a direct relationship between macrophages and $CD4^+$ T cells, the fact that T cells become activated suggests that macrophages may instigate this either through presentation of debris as antigen or cytokine secretion. $CD4^+$ T cells do not have the capacity to respond to free debris thus suggesting upstream activation by another cell type.

Particle analysis confirmed that debris within the size range currently considered to be immunologically relevant (1-10 μm) were present in the isolated debris fraction used in the biological response experiments where T cell pro- and anti-inflammatory cytokine repertoire was reported. However, the 1-10μm particles only made up just over 50% of the total number of debris particles in the sample with most of the remaining fraction sized between 400nm and 1μm. The extent of the contribution from the sub-micron particles to the overall debris population was not evident at face value in the sizing data in its native volume% format. Furthermore, the prevalence of this 400nm-1μm fraction of particles made up 60% (by number) of the fluid obtained directly from the wear simulator (i.e. without any debris isolation post-processing). This in turn raises an interesting question as to what extent (and in what manner) do sub-micron particles contribute to the immune response and bone homeostasis. While it has already been established that individual debris particles of the order of 400nm may be internalised via endocytosis and provoke an inflammatory response (32), one could speculate that these sub-micron particles could aggregate to illicit the same response as the larger debris particles. This could occur via two processed: i) sub-micron sized debris aggregates outside of

the cell triggering phagocytic uptake as with the solid micron scale debris or, ii) aggregation of sub-micron sized debris within the cell, potentially to the extent where they can no longer be transported to and/or processed by the lysosomes. A recent early report from within our group (33) suggested that the latter process occurs with ceramic particles in osteoblast-like cultures and an investigation into this effect with MoM debris will form part of our future work.

This study does have limitations. The number of subjects used is relatively small, though it does support our original hypothesis that individuals show differential responses to wear debris. A much larger sample size will be needed however to define a distinct response profile and to confirm if this is related to the age and gender of the donor. The influences such as BMI should also be considered as these are known to influence the inflammatory status of an individual (33). The implants were wear tested under adverse conditions (subluxation), which explains the production of particles of 400nm or more in size while smaller particles are known to be produced under other wear conditions. While this wear condition alone may not fully represent the nature of the wear behind clinical cases, it does exclude the production of smaller particles from the surface and hence the findings from the immunological studies can be attributed to the micron scale particles with greater confidence. Additionally, this serves to highlight that correct positioning of the implant is of paramount importance. Finally, the CoCrMo debris from the wear simulator were generated in FCS and it is possible that FCS-derived proteins which make up the protein corona on the CoCrMo debris could contribute to the differential response to debris which we observe, however we have controlled for this by including 10% FCS in the control situation. It is possible that FCS proteins bound to the wear particles provide an element of immunogenicity, but pilot studies comparing PBS and 10% FCS effects on T cell populations found only modest effects on memory T cell subset distribution (decreased TEM and increased TEMRA, data not shown).

We have thus, for the first time, shown differential responses in individuals to wear debris from CoCrMo THR implants generated on a wear simulator. We believe that we have identified potential candidate markers which could form the basis of future clinical trials to evaluate such differential cytokine expression along with other parameters - such as age and gender - in order to determine the prognostic value of each.

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Author contributions

MJP, RLW, HF, and DB did the experimental work; MJP, RLW, ETD, LG and JML designed the study; MJP, RLW, ETD and JML wrote the manuscript.

Table 1 Demographic data showing age and gender breakdown

	n (%)	Mean age ± SD (years)
Young	16 (73%)	25.1 ± 4.27
Old	6 (27%)	58.3 ± 8.43
Male	10 (45%)	37.2 ± 16.9
Female	12 (55%)	31.8 ± 16.6

Table 2 Contribution of wear-particle size classes of interest to the total number and volume of wear particles before and after the isolation procedure.

	Wear particle stock solution from wear simulator		**Wear particles isolated from stock solution**	
Size range:	0.4µm-200µm		0.5µm-12µm*	
Size classes	**% by number**	**% by volume**	**% by number**	**% by volume**
0.40µm-0.99µm	59.62 (±0.15)	0.98 (±0.01)	48.57 (±1.21)	1.58 (±0.10)
1.00µm - 10µm	40.35 (±0.12)	44.81 (±0.33)	51.41 (±0.72)	72.13 (±0.42)
>10um	0.02 (<<0.01)	54.20 (±0.60)	0.01 (<<0.01)	26.29 (±0.39)
TOTAL	100	100	100	100

** particles up to 60µm were detected, but not repeatable in consecutive measurement runs.*

Figure Legends

Figure 1: Size distribution of wear-particles in FCS taken from a hip-wear simulator ('stock solution') and of the wear-particles isolated from the stock solution. Distributions are shown in terms of contribution to total volume of wear-particles analysed (a) and the total number of wear-particles analysed (b). VM = volume weighted, SD = standard deviation.

Figure 2: Scanning Electron Microscopy (SEM) images of the CoCrMo wear debris isolated from the bulk serum solution used during the wear simulation. The low magnification image (a) shows both the micron and the more prevalent sub-micron scale debris particles identified from the particle sizing data. The reduced contrast of the image relates to the serum protein coating around the particles, which would be the case for wear debris generated *in vivo*. The high magnification image (b) revealed the rounded morphology of the debris along with their propensity to aggregate without further sample processing.

Figure 3: Whole blood from 22 donors was exposed to wear debris or 10% FCS for 24h and T cell populations identified by immunostaining and flow cytometry. The prevalence of different T cell populations, Th1, Th17, Treg and plastic Th17 are shown for control cells or cells challenged with CoCrMo. Each data point represents the response of an individual subject and the horizontal bar represents the median response.

Figure 4: Data from figure 3 were re-plotted to show the response of individual subjects for the FCS control and CoCrMo exposure. For Th17 and Th1 there were marked differences and thus these data are shown as a pro- or anti-inflammatory response.

Figure 5: Plasma levels of pro-inflammatory and anti-inflammatory cytokines in FCS control and CoCrMo exposed cells were measured using multiplex technology. Each data point represents the response of an individual subject and the bar indicates the median value and *** is p=<0.0001.

Figure 6: The response to CrCrMo debris was analysed with respect to (a) Gender for the IL-10 response and (b)age for the IL-6 response. Each data point represents the response of an individual subject and the bar indicates the median value and actual *p* values are reported.

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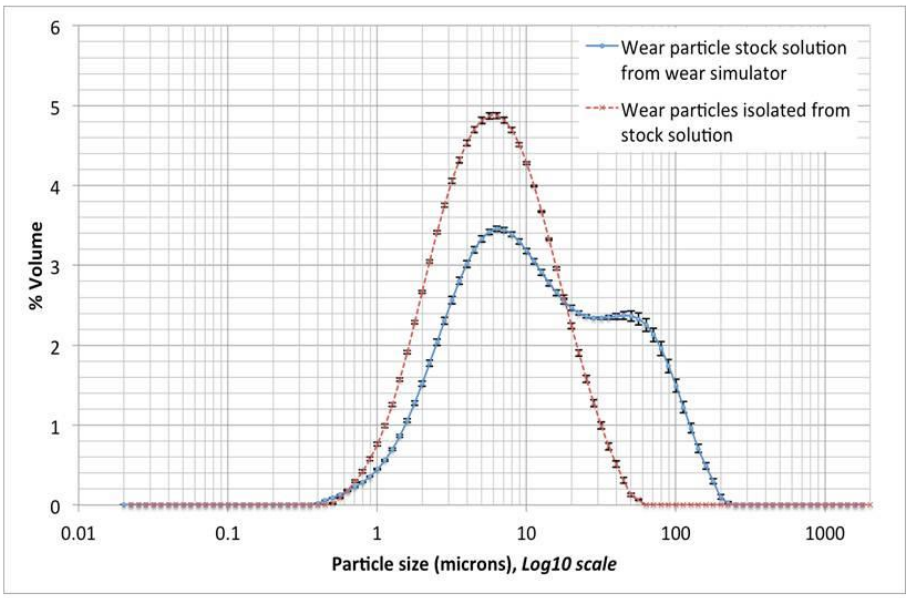
Figure 1

a


Wear particle stock solution from wear simulator
Wear particles isolated from stock solution
% Volume
Particle size (microns), Log10 scale
0
1
2
3
4
5
6
0.01
0.1
1
10
100
1000

b

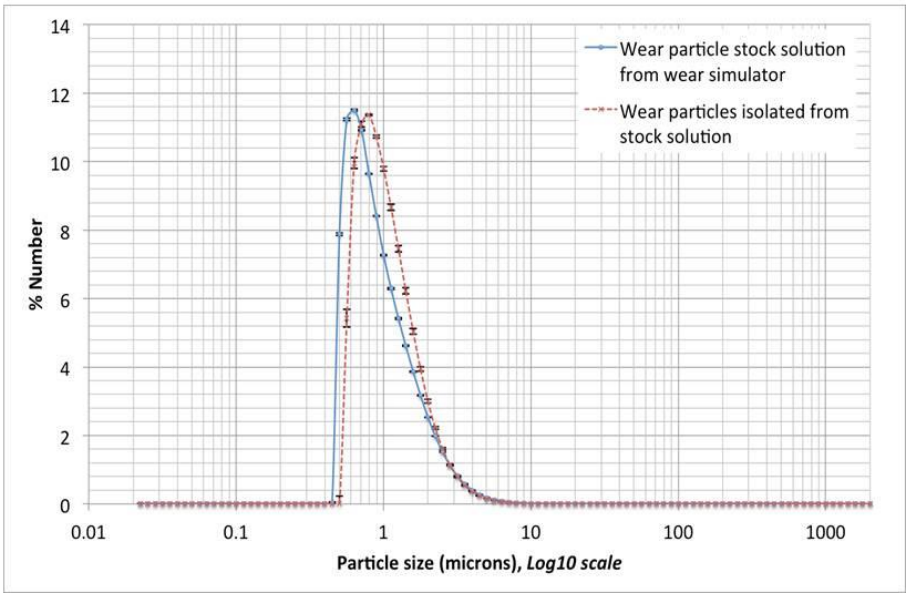

Wear particle stock solution from wear simulator
Wear particles isolated from stock solution
% Number
Particle size (microns), Log10 scale
0
2
4
6
8
10
12
14
0.01
0.1
1
10
100
1000

Figure 2

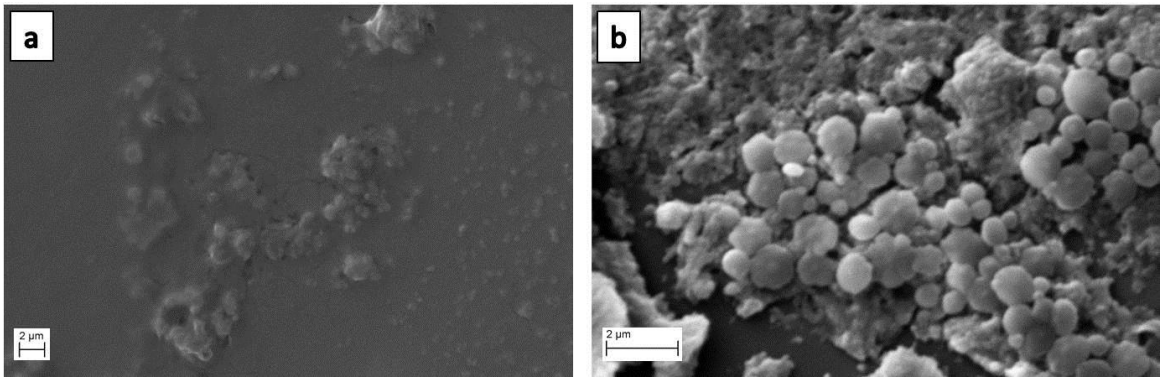


Figure 3

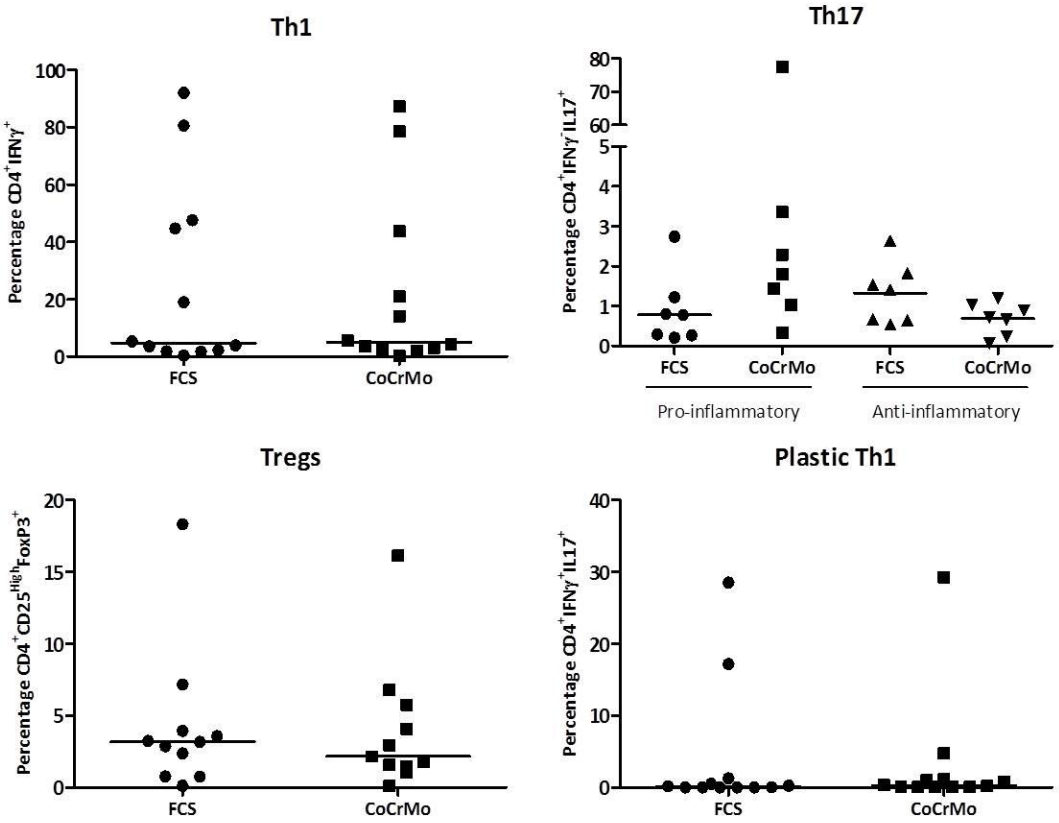

Th1
Percentage CD4+IFNγ+
100
80
60
40
20
0
FCS
CoCrMo
Th17
Percentage CD4+IFNγ-IL17+
80
70
60
5
4
3
2
1
0
FCS
CoCrMo
FCS
CoCrMo
Pro-inflammatory
Anti-inflammatory
Tregs
Percentage CD4+CD25HighFoxP3+
20
15
10
5
0
FCS
CoCrMo
Plastic Th1
Percentage CD4+IFNγ+IL17+
40
30
20
10
0
FCS
CoCrMo

Figure 4

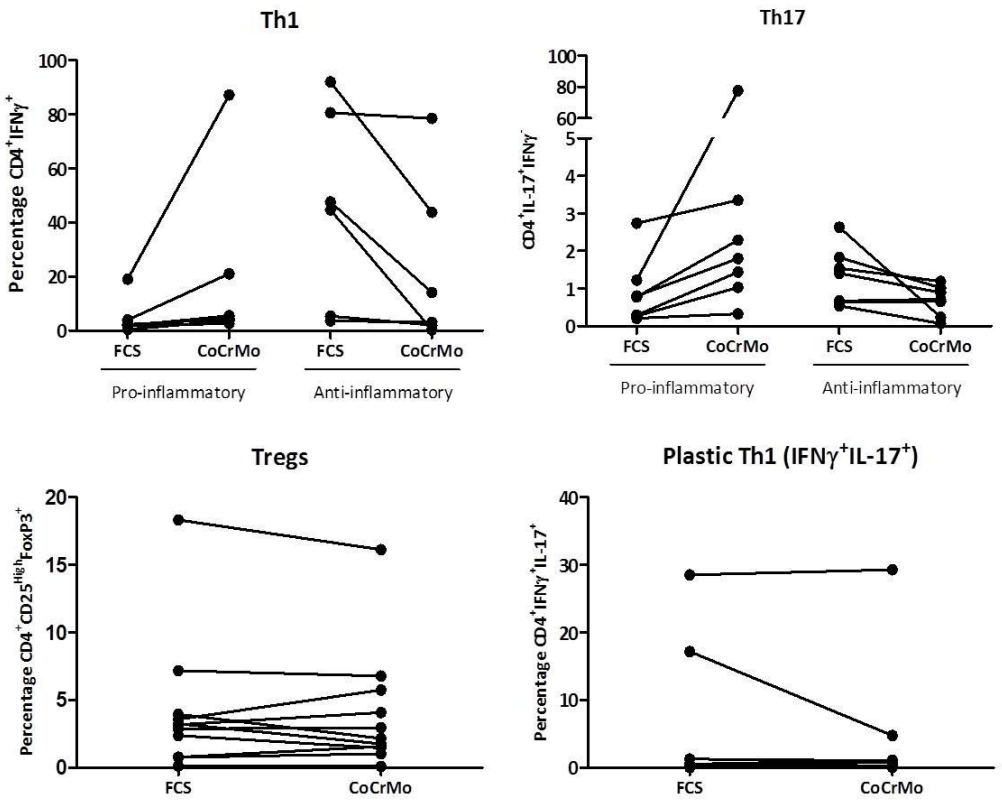

Th1
Percentage CD4+IFNγ+
100
80
60
40
20
0
FCS
CoCrMo
FCS
CoCrMo
Pro-inflammatory
Anti-inflammatory
Th17
CD4+IL-17+IFNγ-
100
80
60
5
4
3
2
1
0
FCS
CoCrMo
FCS
CoCrMo
Pro-inflammatory
Anti-inflammatory
Tregs
Percentage CD4+CD25HighFoxP3+
20
15
10
5
0
FCS
CoCrMo
Plastic Th1 (IFNγ+IL-17+)
Percentage CD4+IFNγ+IL-17+
40
30
20
10
0
FCS
CoCrMo

Figure 5

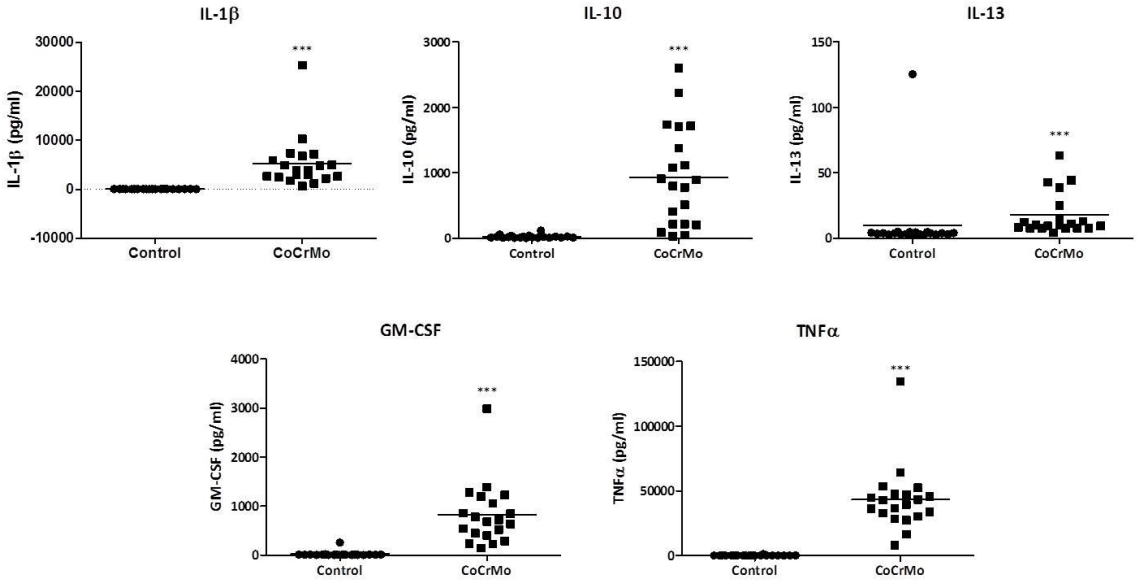
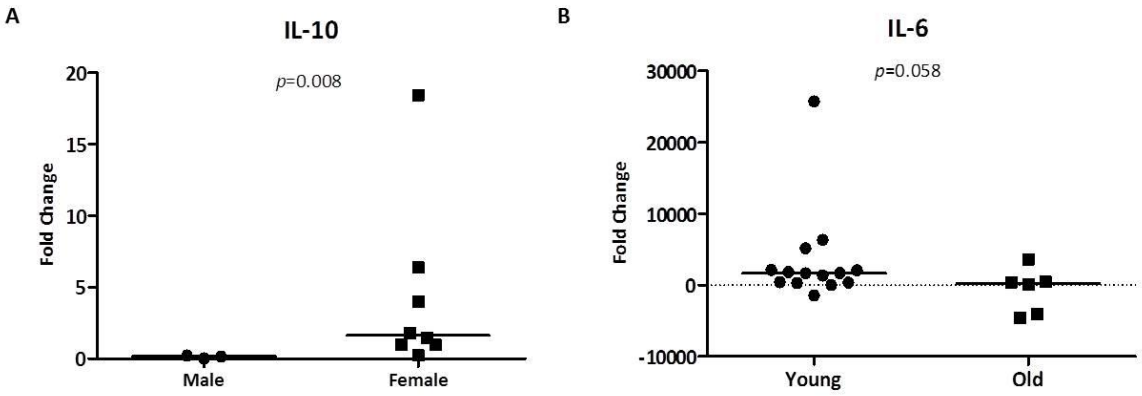

IL-1β
IL-1β (pg/ml)
30000
20000
10000
0
-10000
***
Control
CoCrMo
IL-10
IL-10 (pg/ml)
3000
2000
1000
0
***
Control
CoCrMo
IL-13
IL-13 (pg/ml)
150
100
50
0
***
Control
CoCrMo
GM-CSF
GM-CSF (pg/ml)
4000
3000
2000
1000
0
***
Control
CoCrMo
TNFα
TNFα (pg/ml)
150000
100000
50000
0
***
Control
CoCrMo

Figure 6


A
IL-10
p=0.008
Fold Change
20
15
10
5
0
Male
Female
B
IL-6
p=0.058
Fold Change
30000
20000
10000
0
-10000
Young
Old