

2018 Selected Proceedings of SOMOS

Do Battlefield Injury-acquired Indwelling Metal Fragments Induce Metal Immunogenicity?

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Received: 8 March 2019 / Accepted: 13 August 2019 / Published online: 0 xxxx
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Abstract

Background A battlefield-related injury results in increased local and systemic innate immune inflammatory responses, resulting in wound-specific complications and

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an increased incidence of osteoarthritis. However, little is known about whether severe injuries affect long-term systemic homeostasis, for example, immune function. Moreover, it also remains unknown whether battlefield-acquired metal fragments retained over the long term result in residual systemic effects such as altered immune reactivity to metals.

Questions/purposes Does a retained metal fragment from a battlefield injury contribute to increased (1) adaptive metal-specific immune responses, (2) systemically elevated metal ion serum levels, and (3) serum immunoglobulin levels compared with combat injuries that did not result in a retained metal fragment?

Methods In this pilot study, we analyzed metal-immunogenicity in injured military personnel and non-injured control participants using lymphocyte transformation testing (LTT, lymphocyte proliferation responses to cobalt, chromium and nickel challenge at 0.001, 0.01 and 0.1-mM concentrations in triplicate for each participant), serum metal ion analysis (ICP-mass spectroscopy), and serum immunoglobulin analysis (IgE, IgG, IgA, and IgM). Military personnel with a battlefield-sustained injury self-recruited without any exclusion for sex, age, degree of injury. Those with battlefield injury resulting in retained metal fragments (INJ-FRAG, n = 20 male, mean time since injury ± SD was 12 ± 10 years) were compared with those with a battlefield injury but without retained metal fragments (INJ-NO-FRAG, n = 12 male, mean time since injury ± SD was 13 ± 12 years). A control group comprised of male noninjured participants was used to compare measured immunogenicity metrics (n = 11, males were selected to match battlefield injury group demographics).

Results Military participants with sustained metal fragments had increased levels of metal-induced lymphocyte

responses. The lymphocyte stimulation index among military participants with metal fragments was higher than in those with nonretained metal fragments (stimulation index = 4.2 ± 6.0 versus stimulation index = 2.1 ± 1.2 (mean difference 2.1 ± 1.4 [95% CI 5.1 to 0.8]; $p = 0.07$) and an average stimulation index = 2 ± 1 in noninjured controls. Four of 20 participants injured with retained fragments had a lymphocyte proliferation index greater than 2 to cobalt compared with 0 in the group without a retained metal fragment or 0 in the control participants. However, with the numbers available, military personnel with retained metal fragments did not have higher serum metal ion levels than military participants without retained metal fragment-related injuries or control participants. Military personnel with retained metal fragments had lower serum immunoglobulin levels (IgG, IgA, and IgM) than military personnel without retained metal fragments and noninjured controls, except for IgE. Individuals who were metal-reactive positive (that is, a stimulation index > 2) with retained metal fragments had higher median IgE serum levels than participants who metal-reactive with non-metal injuries (1198 ± 383 IU/mL versus 171 ± 67 IU/mL, mean difference 1027 ± 477 IU/mL [95% CI 209 to 25]; $p = 0.02$).

Conclusions We found that males with retained metal fragments after a battlefield-related injury had altered adaptive immune responses compared with battlefield-injured military personnel without indwelling metal fragments. Military participants with a retained metal fragment had an increased proportion of group members and increased average lymphocyte reactivity to common implant metals such as nickel and cobalt. Further studies are needed to determine a causal association between exposure to amounts of retained metal fragments, type of injury, personnel demographics and general immune function/reactivity that may affect personal health or future metal implant performance.

Level of Evidence Level IV, therapeutic study.

Introduction

Battlefield-related injuries inflict an enormous physiologic insult, disturbing soft tissue and bone and possibly leading to dysregulated local and systemic inflammatory responses [18, 19, 32]. Additionally, injuries from a gunshot, improvised explosive device, blast, or other battlefield-related injury sustained during combat operations may result in retained metallic fragments. These fragments are largely considered to be biologically inert [43, 63] and published recommendations urge leaving them in vivo, and to close wounds as soon as possible to reduce fibrosis and the progression of ectopic mineralization [43, 63]. However, it is unlikely these metal fragments are biologically inert

since all metals in contact with biological systems corrode, and released metal ions may activate the immune system by forming complexes with native proteins as has been well-associated with orthopaedic implant debris [11, 17, 24, 29, 36, 52]. Consequently, these metal-protein complexes are candidate antigens for eliciting innate and adaptive immune responses. The pharmacodynamics and bio-availability of circulating metal degradation products in vivo during the short- and long-term remains largely uncharacterized.

Little is known about systemic pathologies that may result from a metal fragment acquired during a battlefield-related injury. There have only been a few case reports about lead-containing metal fragments (buckshot) in soft tissue causing systemic metal toxicity [3, 13, 15, 46, 56]. Indwelling metal fragments and implant surfaces continuously release chemically active metal ions into the surrounding tissue. Metal ions bind to serum proteins, reside locally, and are transported via the bloodstream and the lymphatics to remote organs and tissues. There is increasing concern regarding the known potential toxicities of metals used in modern orthopaedic implant alloys and indwelling fragments of metals such as aluminum, chromium, cobalt, copper, lead, nickel, titanium, and vanadium [5, 10, 31, 51, 53]. Metal toxicity may alter cell or tissue metabolism, host and parasite interactions, and immunologic interactions, and induce chemical carcinogenesis [4, 6, 21, 30, 38]. However, metal-related toxicity has only been identified in patients with extreme environmental exposure (for example, by inhalation). No referable systemic toxicity has been connected to well-performing orthopaedic implants or from a retained metal fragment. To date, the only well-established systemic effect induced in a small subset of people with orthopaedic implants was an increased incidence and level of metal sensitivity responses (adaptive immune responses) [29]. Cohort studies have indicated a correlation between metal exposure, implant wear/performance and metal-induced lymphocyte responses [12, 25, 26, 35, 59].

The most common metal sensitivity among the general population is sensitivity to nickel, followed by cobalt and chromium, with an incidence of approximately 10% to 15% [23]. However, the proportions of metal sensitivity among individuals with asymptomatic orthopaedic implants is approximately 25% and is approximately 60% among individuals awaiting total joint arthroplasty revision for aseptic pain/loosening/osteolysis [23]. Cohort studies have indicated a strong correlation between metal implant-related aseptic pain, implant performance, and the incidence of metal sensitivity [7, 23] as measured by lymphocyte transformation testing (LTT), which measures the proliferative responses of peripheral blood lymphocytes after exposure to metal or other potential immunogens. Long-term study about how the

adaptive immune system is affected by an injury with indwelling metal fragments is needed to determine what, if any, are the pathogenic consequences, for example inflammaging (chronically elevated inflammation associated with aging), and metal sensitivity that may then affect metal implant outcomes [40, 49, 50]. Metal fragments acquired during a battlefield-related traumatic injury may have a similar effect as some forms of orthopaedic implant debris, where increased metal exposure over time has been associated with the development of metal-specific adaptive immune responses [12, 25, 26, 29, 35, 59]. We theorize that retained metal fragments from a battlefield-related injury can alter long-term adaptive immune responses, particularly metal reactivity.

We therefore asked, does a retained metal fragment from a battlefield injury contribute to increased (1) adaptive metal-specific immune responses, (2) systemically elevated metal ion serum levels, and (3) serum immunoglobulin levels compared with combat injuries that did not result in a retained metal fragment? Each of these three question areas was tested using a specific clinically available assay technique for human diagnostic testing (Table 1).

Patients and Methods

Participant Groups and Parameters

After the study was approved by the institutional review boards of the Brooke Army Medical Center and Rush University Medical Center, all participants provided consent to participate and were self-recruited without exclusion criteria for sex, age, degree of injury, smoking, immune disease. Only one participant in the injured, nonretained metal fragment group reported an immune disease, which was rheumatoid arthritis. Male noninjured participants were recruited to match military personnel sex demographics only, and not for age, height and weight (controls: n = 11) (Table 2).

Military participants with a retained metal fragment acquired during a battlefield-related injury were recruited from the Brooke Army Medical Center and called the INJ-FRAG group. There were 20 military participants in the INJ-FRAG group; 10 of 20 patients had an upper extremity injury, six had a lower extremity injury, four had both upper and lower extremity injuries, and six had osteoarthritis, as determined by self-reported questionnaire (Table 2).

Battlefield-injured military participants without penetrating wounds or retained metal fragments were also recruited from the Brooke Army Medical Center and called the INJ-NO-FRAG group (n = 14) (Table 2). Of these 14 military participants, one had an upper extremity injury, four had a lower extremity injury, one had both an upper and lower extremity injury, eight had no reported site of injury, two had osteoarthritis, and one had rheumatoid arthritis (Table 2). Eleven of the participants had

Table 1. Summary of the three clinically available human diagnostic assays used in this study along with method, analyte and outcome measure

Type of blood test	Method	Analyte	Outcome measure
Metal ion level: The amount of trace metal ions in blood that may be increased by retained metal fragments and have systemic impact, that is, most likely reflected in the adaptive immune system (that is, T-cell and B-cell responses).	Inductively coupled plasma-mass spectrometer	Serum	Aluminum, chromium, cobalt lead, nickel, titanium, vanadium (parts per billion, ppb or ng/mL)
Lymphocyte reactivity: Lymphocyte reactivity to a challenge agent is measured for proliferation responses to metals that may be released by metal fragments, primarily T-cell.	Lymphocyte transformation test (LTT)	Peripheral blood mononuclear cells	Stimulation index
Immunoglobulin levels: The amount of circulating antibodies was measured for each subtype as an indicator of overall adaptive immune responses, primarily B-cell.	Luminex assay	Serum	IgG, IgA, IgM, IgE (ng/mL)

IgG = immunoglobulin G; IgA = immunoglobulin A; IgM = immunoglobulin M; IgE = immunoglobulin E.

Table 2. This table shows demographic and injury data of the participants in this study.

Factor	Control	Injured-retained metal-fragment(s) (INJ-FRAG)	Injured-nonretained metal-fragment(s) (INJ-NO-FRAG)
Group size	11	20	14
Mean age (years)	35 ± 11	41 ± 13	42 ± 13
Osteoarthritis	0	6	2
Mechanism of injury			
Gunshot	0	5	0
Blast	0	15	14
Location of wound from improvised explosive device/blast or gunshot			
Upper extremity	0	10	1
Lower extremity	0	6	4
Upper and lower extremity	0	4	1
Mean time of retained metal- fragments (years)	0	12 ± 10	0
Mean time of nonretained metal fragment-related injury (years)	0	0	13 ± 12

nonpenetrating blast injuries, and three patients had metal penetrating injuries with all fragments removed, as determined by self-reported questionnaire. Participant involvement was limited to a one-time 60 mL-blood draw.

Specimen Collection and Lymphocyte Transformation Testing (LTT)

Whole blood was collected from the participants by venipuncture. A specialized blood collection kit (Orthopedic Analysis LLC, Chicago IL, USA) was used to ensure the quality of the blood draw materials, and the temperature of the samples was kept consistent and stable during transport. All samples were transported to Rush University Medical Center by priority overnight and processed within 24 hours of the initial blood collection. Mononuclear cells were collected from 30 mL of peripheral blood using standard Ficoll gradient separation (control group, $n = 11$; INJ-FRAG group, $n = 20$; INJ-NO-FRAG group, $n = 11$). An insufficient blood draw of less than 20 mL resulted in inadequate isolated peripheral blood mononuclear cell counts for cell culture and reduced the number of assays. Peripheral blood mononuclear cells were washed in a sterile phosphate buffer solution and resuspended in RPMI-1640 media with 10% autologous serum and were cocultured with soluble nickel, cobalt, chromium, iron, zirconium, vanadium, molybdenum, and aluminum at 0.001 mM, 0.01 mM, and 0.1 mM concentrations each in separate wells in triplicate for each metal challenge agent. Peripheral blood mononuclear cells were also cocultured with endotoxin-free titanium alloy particles and cobalt-

chromium-molybdenum particles (cobalt alloy) at 10 particles:1 cell concentration. Phytohemagglutinin was used as a positive control; that is, it was used as a nonspecific mitogen stimulus to induce lymphocyte proliferation in triplicate. Peripheral blood mononuclear cells were cultured for 6 days. On Day 5 of cell culturing, 3H-thymidine was added. On Day 6, 3H-thymidine incorporation was analyzed with a beta scintillation counter at a CLIA approved human diagnostic laboratory for metal-LTT (Orthopedic Analysis LLC). A stimulation index value of metal reactivity was calculated by dividing scintillation counts per minute of metal-challenged cells by nonchallenged cells from the same individual (nonchallenged wells). Patients with a stimulation index of less than 2 were considered nonreactive, and a stimulation index of at least 2 indicated metal reactivity based on well-established thresholds [8, 9, 16, 41, 45, 60]. We assessed whether an indwelling metal fragment from a battlefield-related injury (INJ-FRAG group) affected metal lymphocyte reactivity using the highest person-specific lymphocyte stimulation index value for metal sensitivity at any tested concentration of 0.001 mM to 0.1 mM.

Metal Ion Analysis

Metal ion content was measured using an inductively coupled plasma mass spectrometry analysis of serum using previously detailed methods [35]. All vessels and utensils used for collecting specimens were verified to be metal ion-free. Methods to reduce contamination by trace metal were used as previously described [35]. Serum from the control ($n = 10$), INJ-FRAG ($n = 19$), and INJ-NO-FRAG ($n = 14$)

groups was separated within 24 hours of collection and frozen at -80°C until analysis. The inductively coupled plasma-mass spectrometry detection limits for metals were 1 ng/mL or parts per billion for aluminum, 0.3 ng/mL or parts per billion for titanium, 0.02 ng/mL or parts per billion for vanadium, 0.1 ng/mL or parts per billion for chromium, 0.15 ng/mL or parts per billion for cobalt, and 0.2 ng/mL or parts per billion for nickel. All preparations were conducted in a Class II biological safety cabinet.

Serum Immunoglobulin Analysis

We assessed if serum antibody levels differed among the groups and thus, in part, contributed to observed differences in adaptive immune system reactivity. We used Millipore's milliplex human immunoglobulin isotyping panel (Millipore Sigma, Burlington, MA, USA) to measure immunoglobulin (IgE, IgA, IgM, and IgG1-4) in serum from the control ($n = 8$), INJ-FRAG ($n = 18$), and INJ-NO-FRAG groups ($n = 13$). We performed the assay in triplicate per sample, and we used the mean value per sample for data analysis of the total immunoglobulin serum concentration.

Statistical Analysis, Study Size

Metal reactivity among groups, as determined by metal LTT, serum metal ion concentrations, and serum immunoglobulin levels, was determined to be non-normally distributed using D'Agostino and Pearson's omnibus normality testing (Graphpad Prism 6.0, San Diego, CA, USA). Thus, statistical differences were determined using a Kruskal-Wallis test followed by a Mann-Whitney test for nonparametric data. Groups were considered different if p was < 0.05 ; 95% CIs are reported. We expected those with retained fragments to have twofold increases in lymphocyte reactivity and metal ion content as has been shown in previous study of people with metal debris [29]. Using a priori power analysis with F-test family and ANOVA: fixed effect, omnibus one way a power of 80% and an expected difference of stimulation index = 2 in INJ-FRAG compared with stimulation index = 1 INJ-NO-FRAG with an alpha of 0.05 the total sample size was determined to be 15 (actual power calculated 86%) (G*power, Universität Düsseldorf, Germany).

Results

Lymphocyte Adaptive Immune Responses to Metal

A retained metal fragment contributed to increased lymphocyte adaptive immune responses to metals. Patients in

the INJ-FRAG group had a greater sensitivity to nickel than participants in the other groups, at all tested concentrations, as demonstrated by higher stimulation indexes in the INJ-FRAG group (stimulation index = 3.6 ± 6.0 versus stimulation index = 1.0 ± 0.5 from INJ-NO-FRAG; mean difference 2.6 ± 1.4 [95% CI 5.5 to 0.2]; $p = 0.03$) (Fig. 1A). Participants in the INJ-FRAG group also had more severe lymphocyte metal reactivity to nickel, cobalt, or chromium than those in the INJ-NO-FRAG (stimulation index = 4.2 ± 6 versus stimulation index = 2.1 ± 1.2 (mean difference 2.1 ± 1.4 [95% CI 5.1 to 0.8]; $p = 0.07$) and an average stimulation index = 2 ± 1 in noninjured controls (Fig. 1B). Control participants did not have increased lymphocyte metal reactivity to the most common metal sensitizers tested (stimulation index = 1.8 ± 1.5) (Fig. 1B). Lymphocyte reactivity to a nonspecific, positive mitogen stimulus such as phytohemagglutinin was increased in participants in all groups compared with the respective control values (Fig. 1C), indicating prototypical lymphocyte responses were not impaired in any of the groups. The participants were characterized as "metal reactive" based on well-established threshold levels of a stimulation index of greater than 2 (stimulation index ≥ 2) [35, 44]. The proportion of metal reactive participants (that is, stimulation index ≥ 2 to nickel, cobalt, or chromium) in INJ-FRAG (seven of 20) was higher than in the INJ-NO-FRAG group (three of 11). Additionally, the level of metal reactivity was higher for INJ-FRAG group participants with a gunshot wound ($n = 5$) than for those with retained metal fragments from a blast/IED injury ($n = 15$) (stimulation index = 9.7 ± 4.7 versus stimulation index = 2.4 ± 0.5 from INJ-NO-FRAG; mean difference 7.3 ± 4.7 [95% CI 20.3 to 5.6]; $p = 0.09$) (Fig. 2A). However, there was no difference in the metal reactivity levels of participants in the INJ-FRAG group with osteoarthritis versus those without osteoarthritis (Fig. 2B).

Serum Metal Ion Levels

We found no difference in circulating metal ion levels between the INJ-FRAG group and the INJ-NO-FRAG group (Fig. 3A-C). Surprisingly, people in the INJ-FRAG group had elevated metal ion levels only for vanadium (INJ-FRAG 0.08 ± 0.05 ppb V versus control 0.05 ± 0.01 ppb V; mean difference 0.03 ± 0.01 [95% CI 0.003 to 0.05]; $p = 0.02$), aluminum (INJ-FRAG 4.2 ± 1.9 ppb Al versus control 1.7 ± 0.5 ppb Al; mean difference 2.4 ± 0.5 [95% CI 1.4 to 3.4]; $p < 0.0001$), and titanium (INJ-FRAG 1.1 ± 2.3 ppb Ti versus control 0.1 ± 0 ppb Ti; mean difference 1.0 ± 0.5 [95% CI 0.1 to 2.1]; $p = 0.001$) compared with people in the control group (Fig. 3A-C). Despite this increase, the median serum metal ion levels were within normal ranges for

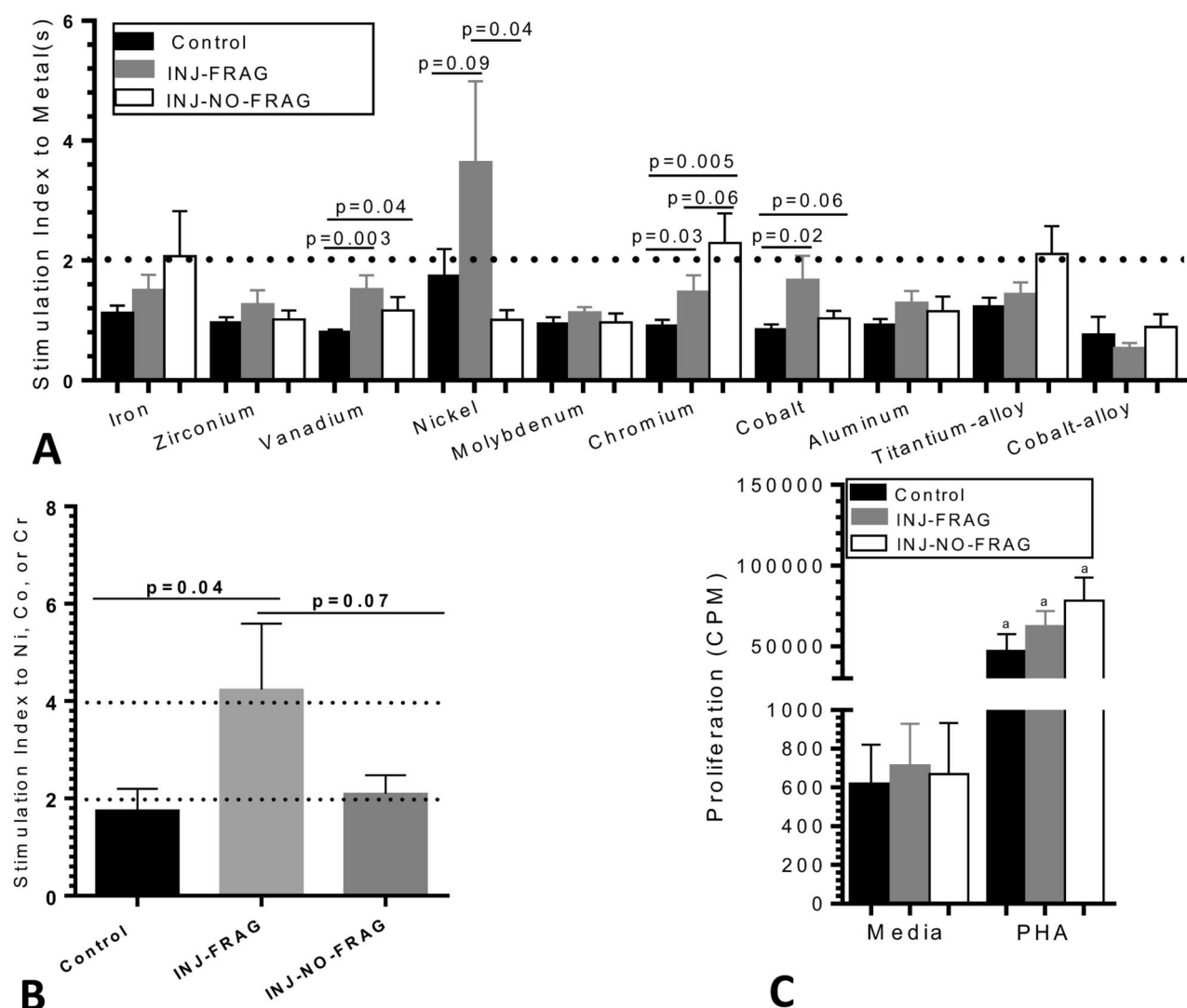


Fig. 1 A-C We determined in vitro lymphocyte metal reactivity with lymphocyte transformation testing. **(A)** The lymphocyte stimulation index of reactivity at any concentration for each metal was determined and graphed as the mean stimulation index with standard error of the mean. The dotted line represents mild metal reactivity, with a sensitivity index of at least 2. **(B)** The lymphocyte stimulation index of reactivity to nickel, cobalt, or chromium, whichever was the highest, at any challenge concentration, was determined and graphed as the mean stimulation index with standard error of the mean. The dotted lines represent mild metal reactivity with a stimulation index of 2 to 4 and reactive with a stimulation index of at least 4. **(C)** There was no difference between the groups for lymphocyte proliferation to media, negative control, or to phytohemagglutinin, positive mitogen control (graphed as counts per minute, cpm) ^a $p \leq 0.05$ for control: $n = 11$; INJ-FRAG: $n = 20$; and INJ-NO-FRAG: $n = 11$.

these same metals: < 0.01 ng/mL for vanadium, < 10 ng/mL for aluminum, and < 4.1 ng/mL for titanium. When metal ion levels were compared among metal-reactive individuals in the INJ-FRAG and INJ-NO-FRAG groups using a stimulation index of ≥ 2 , there remained no difference between serum metal ion levels (Fig. 4A-D). Overall, the median metal ion levels for each group were within normal ranges of human serum metal levels. Serum lead ions were undetectable in all participants in all groups (that is, the levels were below the detection limit of 0.5 parts per billion).

Immunoglobulin (IgE, IgG) Serum Levels

A retained metal fragment or fragments in the INJ-FRAG group contributed to an altered immunoglobulin profile, where IgE was elevated (Fig. 5). IgE, which is involved in Type I hypersensitivity responses and inflammatory diseases, was increased among INJ-FRAG participants compared with INJ-NO-FRAG patients (1037 ± 1432 IU/mL versus 142.6 ± 136.4 IU/mL; mean difference 894.6 ± 339.7 IU/mL [95% CI 1610 to 179.1]; $p = 0.0009$) (Fig. 5A). Additionally, participants who were metal-reactive in

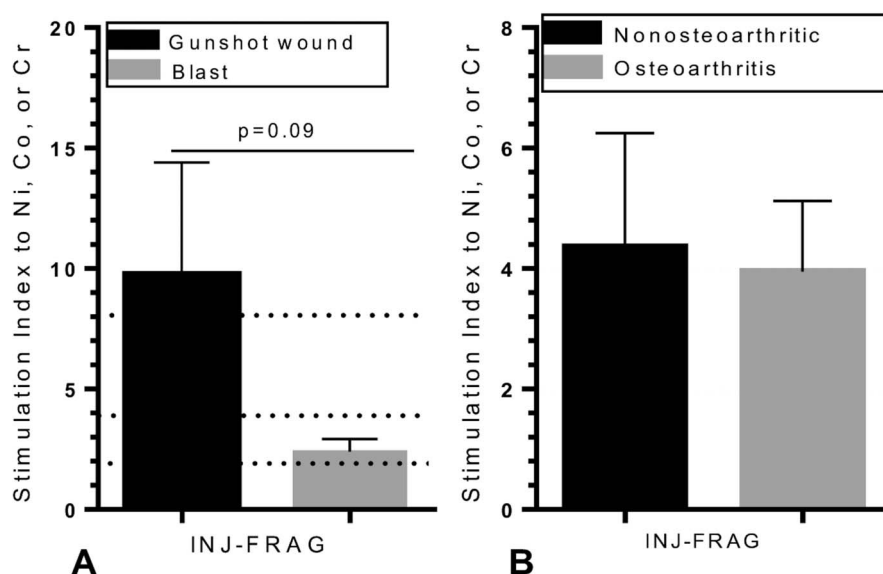


Fig. 2 A-B This figure shows our determination of lymphocyte metal reactivity to either nickel, cobalt or chromium among INJ-FRAG group (**A**) based on either a gunshot wound ($n = 5$) versus blast-related injury ($n = 15$) or (**B**) based on either participants without osteoarthritis ($n = 14$) versus participants with osteoarthritis ($n = 6$). The dotted lines represent mild metal reactivity with a stimulation index of 2 to 4 and reactive with a stimulation index of at least 4.

the INJ-FRAG group had higher median IgE serum levels than metal-reactive INJ-NO-FRAG, as determined by metal LTT assays (1198 ± 383.3 IU/mL versus 171.2 ± 67.4 IU/mL, mean difference 1027 ± 477 IU/mL [95% CI

2029 to 25.31]; $p = 0.02$) (Fig. 5B) [20]. Additionally, we found that IgE levels were higher for personnel with osteoarthritis compared with those without osteoarthritis in the INJ-FRAG group (osteoarthritis = 2101 ± 989 IU/mL

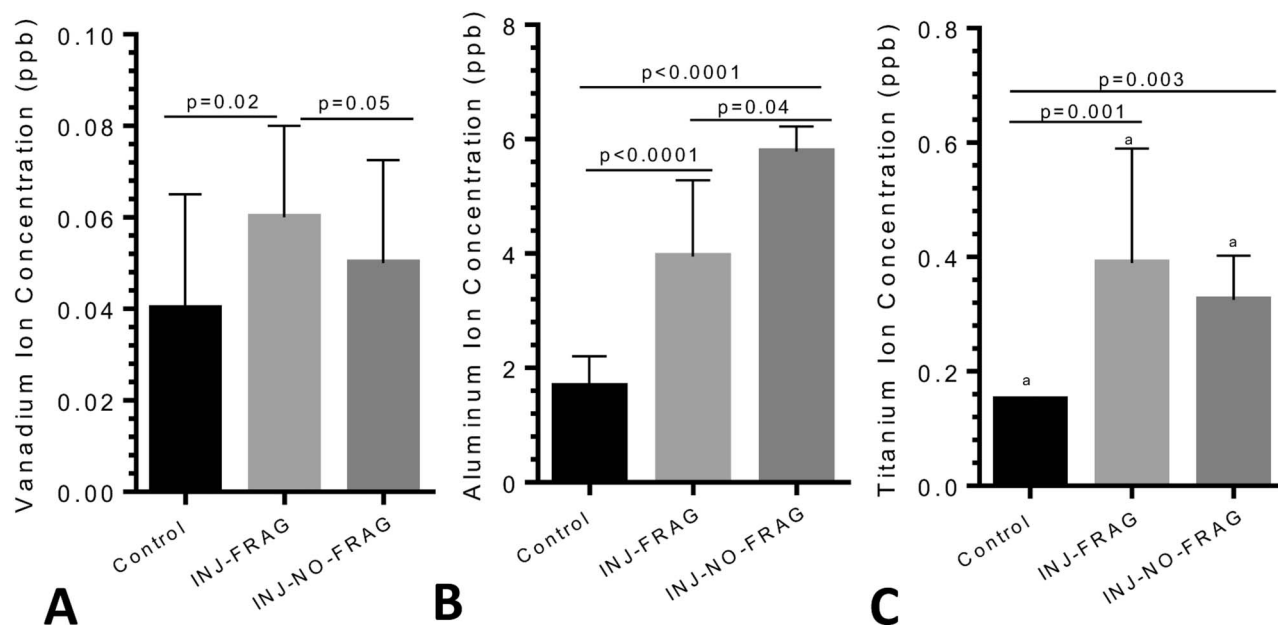


Fig. 3 A-C This figure shows our determination of systemic serum metal ion levels for (**A**) vanadium, (**B**) aluminum, and (**C**) titanium in the control ($n = 10$), INJ-FRAG ($n = 19$), and INJ-NO-FRAG groups ($n = 14$). Data are shown as the median with interquartile range, and statistical significance was determined with a nonparametric Mann-Whitney test. ^aData in the dataset were below the detection limit, and half of the detection limit was used for statistical analyses of titanium = 0.15.

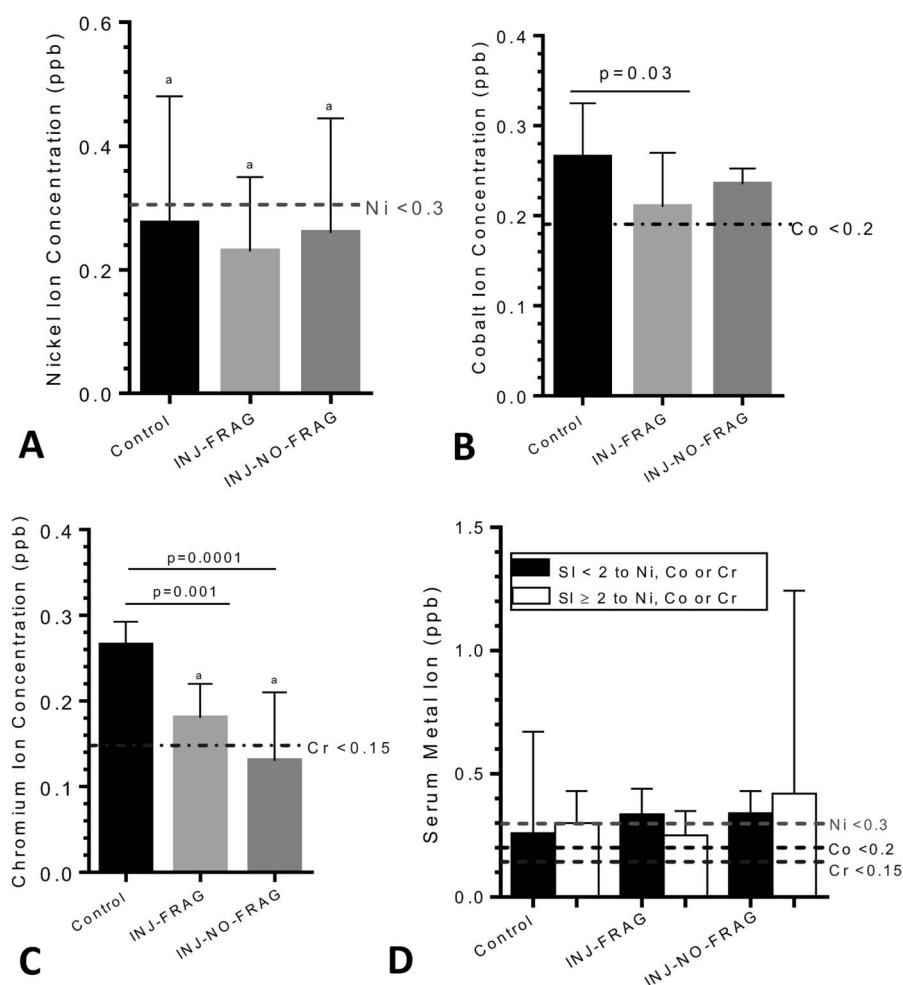


Fig. 4 A-D This figure shows our determination of systemic serum metal ion levels for (A) nickel, (B) cobalt, and (C) chromium in the control (n = 10), INJ-FRAG (n = 19), and INJ-NO-FRAG groups (n = 14). (D) Serum metal ion levels did not correlate with the level of metal reactivity (stimulation index ≥ 2 to nickel, cobalt, or chromium) among any of the groups tested: control stimulation index < 2 (n = 7), control stimulation index ≥ 2 (n = 3), INJ-FRAG stimulation index < 2 (n = 10), INJ-FRAG stimulation index ≥ 2 (n = 7), INJ-NO-FRAG stimulation index < 2 (n = 7), and INJ-NO-FRAG stimulation index ≥ 2 (n = 4). Data are shown as the median with interquartile range, and statistical significance was determined with a nonparametric Mann-Whitney test. ^aData in that dataset were below the detection limit and half of the detection limit was used for statistical analyses of chromium = 0.05 and nickel = 0.1. The dotted lines indicate the accepted reference values of metal ion levels in normal human serum.

versus no osteoarthritis = 733.3 ± 1506 IU/mL; mean difference 1367 ± 451 IU/mL [95% CI 418 to 2317]; $p = 0.003$) (Fig. 5C) (INJ-NO-FRAG only had two participants with osteoarthritis). In contrast to IgE, both IgG1 and IgA serum levels were higher in patients in the INJ-NO-FRAG group than in those in the INJ-FRAG group (Fig. 6A-E). Similar to IgE, IgM was higher in metal reactive individuals (stimulation index > 2) compared with nonmetal reactive individuals in the INJ-FRAG group (Fig. 7A-D).

Discussion

Given the conditions of battlefield injuries and the complexity of trauma surgery, indwelling metal fragments are often unavoidable. However, there are currently no studies of which we are aware that have investigated the long-term immunologic effects of an indwelling metal fragment or fragments. To partially address this in this pilot study, we assessed the long-term effect of retained battlefield metal

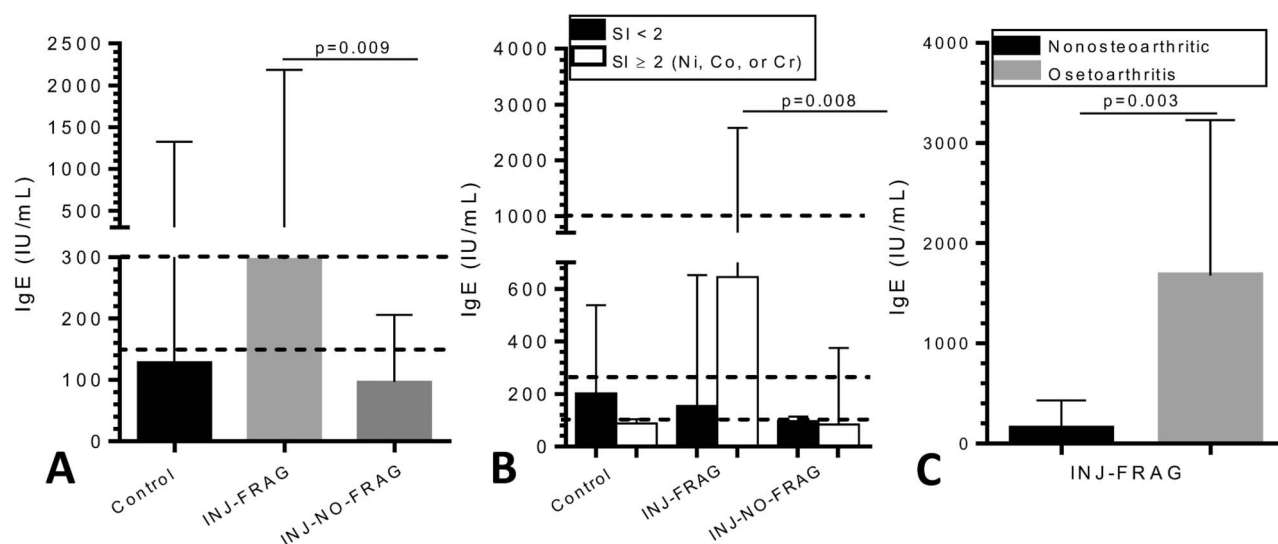


Fig. 5 A-C This figure shows our determination of serum immunoglobulin levels of (A) IgE from the control (n = 8), INJ-FRAG (n = 18), and INJ-NO-FRAG groups (n = 13) and (B) IgE levels among metal-reactive individuals with a lymphocyte stimulation index of at least 2 for sensitivity to nickel, cobalt, or chromium. Control stimulation index was < 2 (n = 7), control stimulation index ≥ 2 (n = 1), INJ-FRAG stimulation index < 2 (n = 9), INJ-FRAG stimulation index ≥ 2 (n = 7), INJ-NO-FRAG stimulation index < 2 (n = 6), and INJ-NO-FRAG stimulation index ≥ 2 (n = 4), and (C) IgE levels from INJ-FRAG with osteoarthritis (n = 4) versus participants without arthritis (n = 14). Data are shown as the median with interquartile range, and statistical significance was determined with a non-parametric Mann-Whitney test; SI = stimulation index.

fragments in military participants on adaptive immune responses and compared these individuals with injured military participants without a sustained metal fragment and noninjured control participants. Individuals with a long-term retained metal fragment had a higher proportion and severity of lymphocyte metal hypersensitivity-type reactivity to metals such as cobalt and nickel, which are commonly found in orthopaedic implants. These results indicate that metal fragments are not likely biologically inert and potentially affect the immune system over the long term. However, it remains unknown whether there is a consequent health risk of this reactivity and to what extent surgeons should take extra risks to remove all metal fragments. These findings demonstrate that removing all metal fragments from an injured patient is likely to avoid metal-related immune system alterations that could be detrimental.

Limitations

There are important limitations to this pilot investigation. First, the group sizes were limited. We did not control for factors such as age, sex, weight, time from injury, personal history, and type of metal fragment, due to practical limitations of recruitment and a one-time blood draw (without the possibility of repeated blood draws or follow-up). As a result, this limited the number of assays that could be performed to assess profiles of immune function. Despite

these confounding factors, we identified elevated metal reactivity and circulating immunoglobulin levels. The clinical relevancy of this with regard to general immunologic health or comorbidity with metallic orthopaedic implants remains unknown. However, it is unlikely that given these results that metal fragments can be regarded as inert and without any consequence if left in situ.

A second important limitation to this pilot study was that participants in the injured military personnel group who self-recruited via flier were male. Additional studies should include female participants, in whom the effects of indwelling metal fragments sustained during battlefield injuries may be more pronounced. This is pertinent given reports that pain related to metal sensitivity may be more severe in females with metal-debris from orthopaedic implants [7, 47].

A third important limitation to this study was heterogeneity of the participant populations with regard to injury extent and location as well as the inability to determine the size of the retained fragments and how this may affect consequent immunogenicity. The presence and location of retained metal fragments were self-reported, and so we could not analyze by size and anatomic location. Future study would benefit from objective fragment size determination perhaps using radiographic analysis. It may be that a threshold size or amount of fragments are required to produce elevated serum metal levels and/or induce adaptive immune responses, as is suggested by the higher

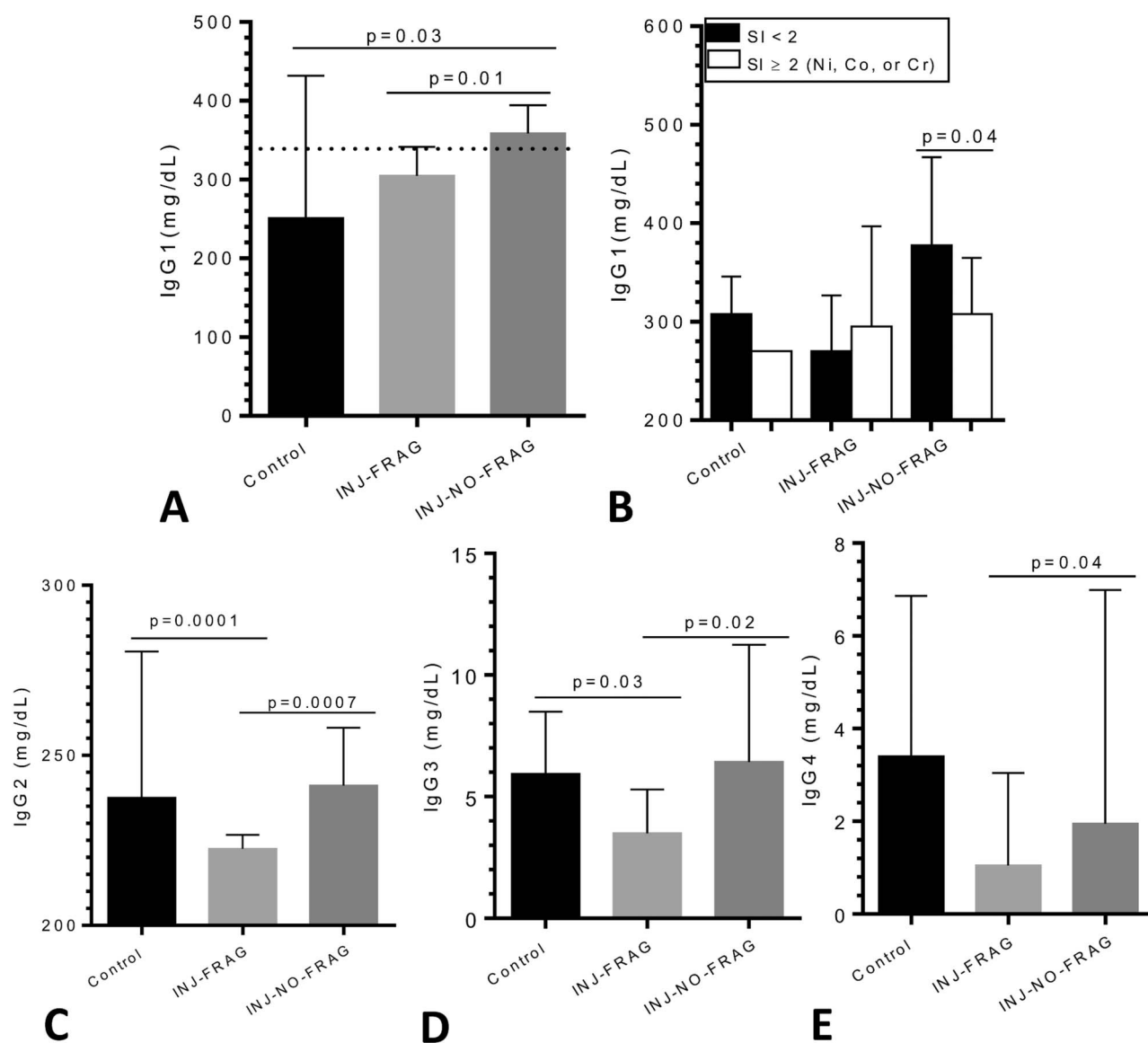


Fig. 6 A-E This figure shows our determination of serum immunoglobulin levels of (A) IgG1 in the control (n = 8), INJ-FRAG (n = 19), and INJ-NO-FRAG groups (n = 13), (B) IgG1 levels among patients with metal-reactivity who had a lymphocyte stimulation index of at least 2 for sensitivity to nickel, cobalt, or chromium; control stimulation index < 2 (n = 7), control stimulation index ≥ 2 (n = 1), INJ-FRAG stimulation index < 2 (n = 9), INJ-FRAG stimulation index ≥ 2 (n = 7), INJ-NO-FRAG stimulation index < 2 (n = 6), and INJ-NO-FRAG stimulation index ≥ 2 (n = 4), (C) IgG2, (D) IgG3, and (E) IgG4 in participants in the control (n = 8), INJ-FRAG (n = 18), and INJ-NO-FRAG groups (n = 14). Data are shown as the median with interquartile range, and statistical significance was determined with a nonparametric Mann-Whitney test.

reactivity of gunshot wound INJ-FRAG sub-group compared to blast/improvised explosive device (Fig. 2). Future work relating size to response may be able to determine this critical size. However, the inclusion of radiographic quantitation of fragment size or location may be problematic given the potential of numerous small fragments (< 1 mm in diameter) to release large quantities of metal by virtue of their surface area yet remain below the detection range of clinical radiographs; and, the inability to accurately size fragment volume due to radiograph scatter and

practical limitations of other modalities such as MRI with large magnetic fragments.

Lymphocyte Adaptive Immune Responses to Metal

Sustained metal fragments acquired during a battlefield-related injury may be considered severe (because of the initial tissue damage) biological analog of a nonarticulating orthopaedic implant. Adaptive immune hypersensitivity

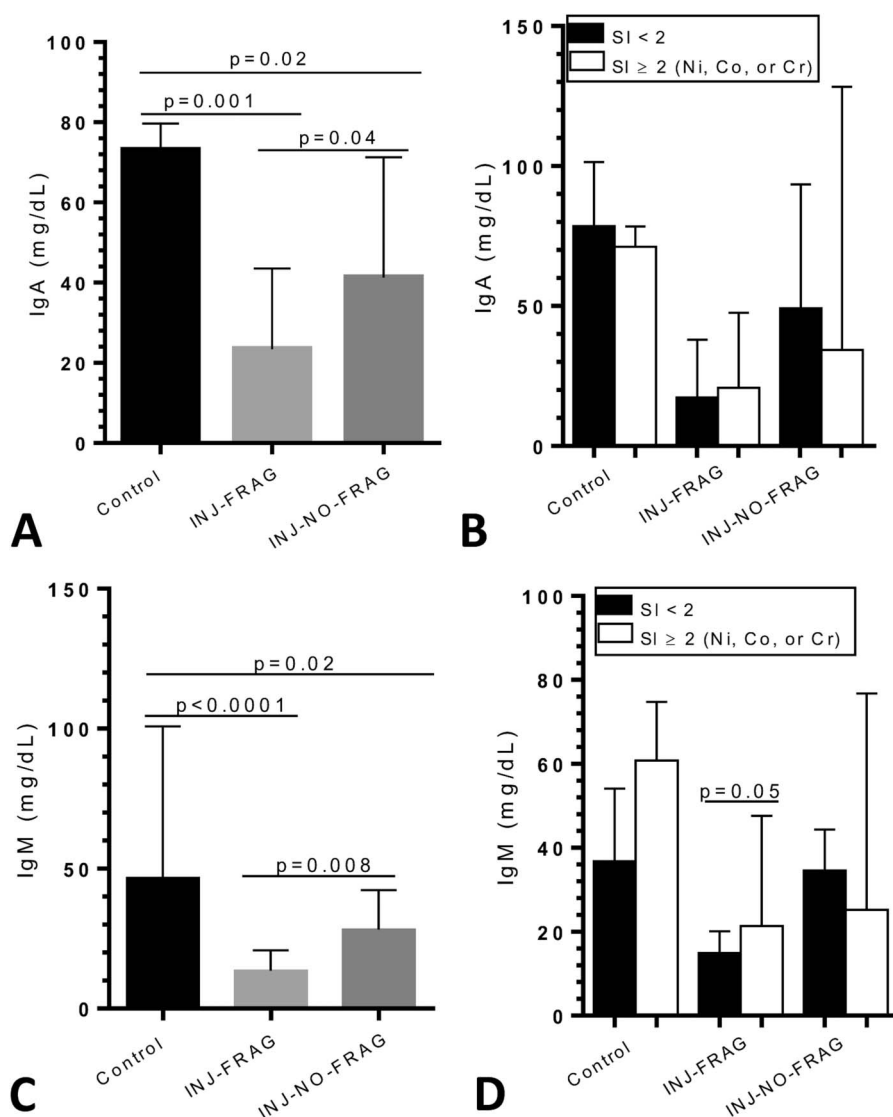


Fig. 7 A-D This figure shows our determination of serum immunoglobulin levels of (A) IgA from the control (n = 8), INJ-FRAG (n = 19), and INJ-NO-FRAG groups (n = 13), (B) IgA levels among participants with metal-reactivity who had a lymphocyte stimulation index of at least 2 for reactivity to nickel, cobalt, or chromium; control stimulation index < 2 (n = 7), control stimulation index ≥ 2 (n = 1), INJ-FRAG stimulation index < 2 (n = 9), INJ-FRAG stimulation index ≥ 2 (n = 7), INJ-NO-FRAG stimulation index < 2 (n = 6), and INJ-NO-FRAG stimulation index ≥ 2 (n = 4), (C) IgM in participants in the control (n = 8), INJ-FRAG (n = 19), and INJ-NO-FRAG groups (n = 13), and (D) IgM levels among participants with metal-reactivity who had a lymphocyte stimulation index of at least 2 for reactivity to nickel, cobalt, or chromium; control stimulation index < 2 (n = 7), control stimulation index ≥ 2 (n = 1), INJ-FRAG stimulation index < 2 (n = 9), INJ-FRAG stimulation index ≥ 2 (n = 7), INJ-NO-FRAG stimulation index < 2 (n = 6), and INJ-NO-FRAG stimulation index ≥ 2 (n = 4). Data are shown as the median with interquartile range, and statistical significance was determined with a nonparametric Mann-Whitney test.

responses to implant metals are well-documented in case and group studies in which there was a link between in vivo metal exposure and lymphocyte reactivity [25, 29, 35]. Similarly, our results indicate that in vivo exposure to a

retained metal fragment more than 11 years after a battlefield-related injury increases lymphocyte reactivity. Specifically, in our study, people with an indwelling metal fragment sustained during a battlefield-related injury had a

higher proportion and severity of lymphocyte reactivity to nickel and cobalt than did noninjured male controls (Fig. 1A-C). In contrast, people with injury and no metal fragment did not have a similarly increased metal-related adaptive immune profile. These results indicate a contributing relationship between in vivo exposure to metal fragments and altered adaptive lymphocyte metal reactivity responses over the long term. The need for further study of these interactions in military personnel with battlefield injuries that eventually need a metal orthopaedic implant is supported by this pilot study. Further study may be particularly important given that osteoarthritis has been reported as the main disability in military participants with battlefield injuries [48]. This was reflected in our study groups: Six of 20 people with injury and metal fragments had osteoarthritis, and of those with injury and no fragment only two of 14 people had osteoarthritis and one of 14 had rheumatoid arthritis. Thus, while the group sizes are too small to isolate and test for differences between these subsets of individuals, further study with larger participant groups are needed to isolate the effect of these participants who may have altered immunoprofiles. However, there was no difference in the lymphocyte metal-reactivity indices between participants with osteoarthritis in the INJ-FRAG group compared with those in the INJ-NO-FRAG group. Additional immunoassays are required to determine the profiles of patients with activated lymphocytes; for example, identifying CD4 + T-cells or B-cells or using flow cytometry to measure metal antigens to delineate specific pathomechanisms associated with indwelling metal fragments. Overall, diagnostic testing such as metal-reactivity LTT testing, may be useful for risk assessment of those with battlefield-acquired indwelling metal fragments and osteoarthritis to help anticipate the likelihood of future orthopaedic implant surgery like a total hip replacement. This may be particularly important for the INJ-FRAG group where metal lymphocyte reactivity was shown elevated and the level of osteoarthritis was found to be 30% (six of 20) at a relatively young average age of 35 years (compared with < 1% of the general population for hip, knee or hand osteoarthritis at younger than 45 years) [33]. Thus, people with battlefield-acquired metal fragment injury may have a greater than a 30-fold increase in the likelihood of progression to total joint replacement than the general population.

Serum Metal Ion Levels

Despite having increased lymphocyte reactivity to metal, injured individuals with indwelling metal fragments did not have increased serum metal ion levels. Metal ion levels in serum were within normal ranges. The current consensus statement of the American Association of Hip and Knee

Surgeons [34] states that serum metal ion levels for cobalt and chromium below 3 parts per billion (< 3 µg/L), compared with orthopaedic implants, are considered low risk for metal-on-metal articulating joint implants, where the risk corresponds to implant performance and not general toxicity [34, 59]. Our data indicated that retained metal fragments in military participants, unlike some types of orthopaedic implants, do not release readily detectable systemically elevated levels of metal ions in serum, and thus did not correlate with metal reactivity levels. This may be due to the initial removal of readily identifiable larger metal fragments.

Immunoglobulin (IgE, IgG) Serum Levels

Immunoglobulins (antibodies) are produced by lymphocytes and are critically involved in immune responses by attaching to antigens or pathogens targeted for destruction. The least-abundant antibody in human serum is IgE, but it plays an important role in allergic diseases, specifically Type I immediate hypersensitivity reactions mediated by mast cells and basophils (Table 3) [39]. Additionally, cancer, infections, and autoimmune and inflammatory diseases may also result in elevated concentrations of IgE [37, 42, 55]. Thus, observed increases in lymphocyte metal reactivity and IgE serum levels in military participants with an indwelling metal fragment may also reflect an increased state of inflammation. Individuals who were metal-reactive in the INJ-FRAG group (stimulation index of at least 2 for sensitivity to nickel, cobalt, or chromium) had increased median serum IgE levels (Fig. 5B) than individuals who were metal reactive in the INJ-NO-FRAG group who had nonmetal retained fragment injuries.

An additional examination showed that within the INJ-FRAG group, IgE was increased in those with osteoarthritis compared with those without, suggesting that immunoglobulin levels may reflect inflammation induced by osteoarthritis (Fig 7C). A direct comparison between stimulation index and IgE levels in these groups (retained

Table 3. This table shows normal, accepted levels of antibodies and their respective total concentrations in human [20].

Immunological parameter	Normal levels	Total concentration in serum
IgE IU/mL	< 214	Trace Ig
IgG1 mg/dL	341 to 894	65%
IgG2 mg/dL	171 to 632	25%
IgG3 mg/dL	18.4 to 106	6%
IgG4 mg/dL	2.4 to 121.0	4%
IgA mg/dL	61 to 356	15%
IgM mg/dL	37 to 286	5%

versus unretained) and subgroups (osteoarthritis versus no osteoarthritis) demonstrated no direct relationship (Spearman rank r ; $p > 0.1$), largely owing to the relatively few participants with osteoarthritis. Relatedly, reduced serum IgA and IgM concentrations may be a result of complex compensatory reactions to increased IgE and/or Type IV delayed type hypersensitivity immune reactivity in military participants with retained metal fragments.

Overall, these findings suggest that retained metal fragments may have long-term immunomodulatory effects. Specifically, our data indicate that chronic exposure to metal in vivo may contribute to increased activation of T-cells and B-cells, which translates to lymphocyte metal reactivity/proliferation. There are no comparison studies we are aware of relating immune function to indwelling metal fragments. However, studies of orthopaedic patients with lymphocyte reactivity to metal implant debris have shown utility for using LTT both clinically and in cohort studies. For more than 30 years, LTT has been validated and included within Medicare guidelines. A stimulation index > 2 has been used clinically to determine reactivity (hypersensitivity) to metals, latex, drugs [14, 54, 57, 58, 61, 62] with sensitivity and specificities as high as 80% to 90% for specific metals [8, 9, 16, 41, 45, 60]. It is important to note metal-LTT diagnostic test specificity and sensitivity data do not define comorbidity risk, such as implant outcome. Using this testing in orthopaedic research has shown T-cell reactivity [27] associated with metal-debris-induced adaptive immune reactivity [28]. Also, innate immune activation (activated macrophages) through danger signaling (inflammasome activation) has been shown to be central to metal implant debris adaptive immunity and characterized as pleotropic for T-cell and B-cell activation, where IL-17 based cytokines indicate a Th17 type of T-cell connected to innate immune inflammasome activation (macrophage) associated with metal implant debris [1, 2, 22, 28, 52]. The results of this pilot study indicate the clinical utility to further investigate if these same responses (for example if orthopaedic implant debris-induced inflammasome-to-Th17-cell activation) are also present in people with battlefield injury and retained metal fragments.

Conclusions

We found adaptive immune profile alterations in injured military participants with an indwelling metal fragment compared with injured military participants without a retained metal fragment and noninjured controls. This suggests that a battlefield trauma combined with metal exposure can lead to systemic long-term adaptive immune reactivity as evidenced by lymphocyte reactivity to metals such as nickel and increased serum antibody levels. Thus,

retained metal fragments are likely not biologically inert. Given that battle-injured personnel with retained metal fragments have increased levels of osteoarthritis, metal-induced lymphocyte responses, and general immunoglobulin levels, there is a continuing need for more comprehensive long-term study on how this affects general health and (when applicable) subsequent orthopaedic implant outcome.

Acknowledgments None.

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