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Age Differences in Cytokine Expression Under Conditions of Health Using Experimental Pain Models

Yenisel Cruz-Almeida^{1,2,3,*}, Maria Aguirre^{1,3}, Heather L. Sorenson⁴, Patrick Tighe^{1,5}, Shannon M. Wallet⁴, and Joseph L. Riley III^{1,3}

¹Pain Research & Intervention Center of Excellence (PRICE)

²Institute of Aging, College of Medicine

³Department of Community Dentistry and Behavioral Science, College of Dentistry

⁴Department of Oral Biology, College of Dentistry

⁵Department of Anesthesiology, College of Medicine

Abstract

Older adults are at an increased risk to develop frequent and prolonged pain. Emerging evidence proposes a link between immune changes and pain, which is consistent with the inflammation theory of aging and the increased incidence of age-related diseases. This study tested the hypothesis that older adults show greater immune responses to experimental pain compared to younger individuals. Study subjects (8 younger and 9 older healthy adults) underwent 3 experimental sessions using well-validated human experimental pain models: the cold pressor task (CPT), focal heat pain (FHP), and a non-painful thermal control. Blood was collected through an indwelling catheter at baseline and 3, 15, 30, 45, 60, and 90 minutes post-stimuli administration. Pro-inflammatory cytokines (TNF- α , IL-6 and IL-8) peaked at the same time points for both groups, with greater elevations among older subjects for TNF- α and IL-8 in both pain models and elevations in IL-6 only for CPT. Antiinflammatory cytokines (IL-4, IL-5, and IL-10) generally peaked later for the older subjects, with increased elevations for FHP but not the CPT. These data is consistent with the assertion that age-related immune system dysregulation may account for the increased prevalence of pain in older adults.

Keywords

aging; pain; cytokine; biomarkers; pro-inflammatory; anti-inflammatory; experimental pain; acute pain

*Corresponding Author: Yenisel Cruz-Almeida, MSPH, PhD, Pain Research & Intervention Center of Excellence, University of Florida, Clinical and Translational Research Building (CTRB), Room 2142, 2004 Mowry Road, Gainesville, FL 32610, Phone: (352) 294-5845, Fax: (352).

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1. Introduction

Aging is a biological process characterized by progressive physiological decline. The inflammation theory of aging, also called “inflamm-aging” (inflammation + aging), proposes a link between immune changes and the increased incidence of age-related diseases (Franceschi et al., 2000; Pizza et al., 2011). This theory is supported by the observation that serum concentrations of inflammatory mediators are elevated in old age and these elevations are associated with the pathogenesis of many age-related diseases (Krabbe et al., 2004; Singh and Newman, 2011). Older adults are also at an increased risk for frequent and prolonged pain and experience greater pain-related loss of function compared to younger cohorts, whereby, up to 60% of older adults have reported chronic pain (Molton & Terrill, 2014 for review).

Recent evidence suggests that an overactive inflammatory response to an injury or pathogen (Kwok et al., 2012) may lead to peripheral and central sensitization of pain (Kawasaki et al., 2008), which could confer increased pain sensitivity and exacerbation of clinical symptoms. Prior data suggest that peripheral pro-inflammatory cytokines (IL-1 β , IL-6, TNF- α) sensitize peripheral nociceptors and induce spinal release of cytokines (Sommer and Kress, 2004; Stürmer et al., 2005). Evidence animal models suggest that central pro-inflammatory cytokines induce sensitization of spinal dorsal horn neurons by enhancing excitatory neurotransmission (IL-1 β , TNF- α) and/or suppressing inhibitory neurotransmission (IL-1 β , IL-6) (Kawasaki et al., 2008). Furthermore, systemic release of pro-inflammatory cytokines can activate glial cells in the central nervous system (Ledeboer et al., 2005; McMahon et al., 2005). This inflammation-linked glial activation may be central to the initiation and progression of chronic pain (Gwak et al., 2012; Grace et al., 2011; Milligan and Watkins, 2009).

To this end, there is strong evidence for the association between immune biomarkers and clinical pain sensitivity. Most studies have reported systemic elevations of pro-inflammatory cytokines when chronic pain patients were compared to healthy controls with higher levels of these pro-inflammatory cytokines correlating with greater clinical pain (DeVon et al., 2014). However, this evidence is correlational and does not provide evidence of causality. To date, there are no studies that have tested for age differences in basal and pain-evoked plasma levels of inflammatory mediators under conditions of systemic health.

The overall goal of the present investigation was to characterize the time course and magnitude of changes of pro- and anti-inflammatory cytokines in plasma across three separate experimental sessions using well-validated experimental pain models: (a) the cold pressor task, (b) focal heat pain and (c) a non-painful thermal control, in healthy younger and older individuals. We hypothesized that: 1) that the cold pressor task and the focal heat pain application, but not a thermal non-painful control would induce an immune response; and 2) older adults would have a greater inflammatory response to painful cold and heat stimulation. Our findings offer insights on the type of immune response associated with acute pain in healthy individuals without chronic pain, which may be used to compare to the immune responses of those individuals who experience chronic pain across the age range.

2. Materials and Methods

2.1. Participants

The University of Florida Institutional Review Board approved the study. The study subjects were 8 younger healthy adults (4 males, 4 females) with a mean age of 21.4 (SD = 5.8) and 9 older healthy adults (5 males, 4 females) with a mean age of 68.1 (SD = 6.0). The older adults reported a greater level of academic achievement ($p = 0.04$), however five of the younger subjects were college students and had not yet reached their terminal level of education. There were no differences in body mass index between the younger sample (24.6, SD = 3.0) and older sample (24.8, SD = 2.5). All four older females reported post-menopausal status and the four younger females reported having regular menstrual cycles and were not taking birth control pills. Seventy-five percent ($n = 6$) of the younger sample and 67% of the older sample ($n = 6$) reported they exercised regularly. Potential participants were screened over the phone and again in person during the first visit. Exclusion criteria included were: current use of narcotics or tobacco products, chronic or current use of analgesics, uncontrolled hypertension, other serious systemic disease (e.g. diabetes, thyroid problems, etc.), neurological problems with significant changes in somatosensory and pain perception, serious psychiatric conditions (e.g., schizophrenia, bipolar disorder), currently receiving treatment for chronic pain (e.g., low-back pain, postherpetic neuralgia) or any ongoing pain problem (headaches, arthritis, injury-related pain etc), pregnant or trying to get pregnant (for females). Once enrolled, participants were instructed a day prior to each scheduled session to avoid any heavy, vigorous exercise past 12 AM, to have breakfast no later than 1 hour before the scheduled experimental session and to avoid dairy products, foods with high sugar, acidity, or fat, drinks with caffeine content, or any alcoholic beverage. In addition, participants were asked to avoid staying up late the night before the experiments and to try to go to bed at their usual bedtime. All experimental sessions were scheduled at the same time for all participants and were always after 11 AM.

2.2. Experimental Sessions

Sessions were all conducted in standardized, quiet rooms at the University of Florida Clinical and Translational Science Institute Clinical Research Clinic (CRC). Staff nurses supervised urine collection for pregnancy testing (women only). Sessions began with subjects sitting in a reclining chair and resting for 5 minutes. Two blood pressure readings separated by 5 minutes were taken and if there was a change in blood pressure greater than 5%, subjects were instructed to rest for an additional 5 minutes and a third blood pressure measurement was taken. Staff nurses inserted an indwelling catheter in the left forearm and subjects rested for an additional 15 minutes. Testing began with collection of a baseline blood sample followed by exposure to the painful stimuli for that session (i.e., cold pressor task, focal heat, warm control). Additional samples of blood were collected at 3, 15, 30, 45, 60, and 90 minutes. The order of experimental sessions was counterbalanced across subjects.

2.2.1. Cold Pressor Task (CPT)—Subjects immersed their foot in 8°C (males) or 10°C (females) water, which was constantly recirculated to prevent local warming or cooling around the submerged foot. Our earlier work has shown that males and females report equivalent levels of pain at these temperatures (Riley et al., 2010). The cold stimulus was

administered in four 60-second trials with a 60-second rest/withdrawal period between trials. The water level was set at a height of 7 cm in order to keep the stimulated area consistent. Subjects provided ratings of cold pain during each of four 60-second trials at 15-sec, 30-sec, and 45-sec for a total of 12 pain ratings. Testing temperatures were adjusted between trials if needed to obtain targeted pain ratings of between 40 and 50 (VAS: 0=no pain and 100=intolerable pain). For example, if subjects provided a peak VAS pain rating above 50 during a CPT trial, the temperature was adjusted upwards for the next trial.

2.2.2. Focal Heat Pain (FHP)—Similar to the CPT, painful heat stimuli were administered in four 60-second trials with a 60-second rest/withdrawal period between trials. Starting temperatures of 48°C (males) and 47°C (females) were used and adjusted to reach the targeted pain ratings of 40–50 VAS as needed. The Pathway system (Medoc, USA) was used for the thermal stimulation using a 16×16mm ATS thermode.

2.2.3. Non-painful Control—Application of a non-painful warm stimulus (32–34°C) was used as the control session, which was identical to the CPT and FHP sessions except for the stimulation temperature used. The warm stimulus was similarly administered in four trials of about 60-second each with a 60-second rest/withdrawal period between trials. Subjects were asked to rate the warm stimulus using a VAS (0=No pain sensation and 100=Maximal pain sensation). No subjects rated any of the control trials as painful. The Pathway system (Medoc, USA) was used for the thermal stimulation using a 16×16mm ATS thermode.

2.3. Plasma Collection and Soluble Mediator Analysis

Established protocols were followed for blood collection using heparin-coated tubes. All samples were immediately placed on ice and transported to the CRC laboratory where they were centrifuged at 12,000 rpm for 15 min. All plasma was stored at –80°C until assays could be performed. Plasma soluble mediators were qualitatively and quantitatively evaluated, utilizing the MILLIPLEX XMAP human cytokine/chemokine-premixed 13-Plex assay (Millipore, Billerica, MA). The soluble mediators GM-CSF, IFN γ , IL-1 β , IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12 (p70), IL-13, and TNF α were probed for according to the manufacturer protocols. Briefly, plasma and antibody-coated beads were added to 96-well primed filter plate and allowed to incubate overnight at 4°C. Following three washes, biotinylated detection antibodies were allowed to incubate for 1 h at room temperature, after which streptavidin (SAV)-phycoerythrin was allowed to incubate for 30 min. Following three washes, beads were resuspended in sheath fluid and reactivity acquired using a Luminex 200 IS system (Luminex, Austin, Tx). Concentrations in pg/ml were determined using a standard curve, 5-parameter logistics and Milliplex analyst (Viagene).

2.4. Statistical methods

The dependent variables for this study were circulating pro-inflammatory (TNF- α , IL-6-, IL-8) and anti-inflammatory (IL-10, IL-4, IL-5) cytokine concentrations expressed in pg/ml, adjusted for baseline levels calculated as *post-stimulation – baseline*. This study tested the hypotheses that older adults will show greater pro-inflammatory responses and lower anti-inflammatory responses to painful hot and cold stimuli compared with younger adults. We also tested whether greater pro-inflammatory response and anti-inflammatory response will

be observed for CPT immersion compared to FHP. To test Hypothesis 1, differences in plasma concentrations at baseline, 3, 15, 30, 45, 60, and 90 minutes for the warm session were tested using repeated-measures ANOVA. The primary independent variable of interest for this hypothesis was “time”, which tested the magnitude of the overall biomarker response. Sex and age were used as covariates in the second set of analyses. For Hypothesis 2, age differences in each biomarker profile for both CPT and FHP sessions were tested using repeated-measures ANOVA. Comparisons were made using 4 time-points, 30-min, 45-min, 60-min, and 90-min post stimulation, unless the peak plasma concentration was observed at 30-min, in which time-points, 15-min, 30-min, 45-min, and 60-min were used. The primary independent variable of interest for hypothesis 2 was “age”, which tested for age differences in the magnitude of the overall response. The “age $\times$ time” interaction effect was also examined and tested for age differences in the time course of biomarker expression. Sex and body mass index was used as a covariate in this set of analyses. When interaction effects were significant, pair-wise comparisons were made only across each time-point and not between time-points to reduce the number of statistical tests performed. Mauchly’s Test of Sphericity assessed whether the assumption of sphericity had been violated (Mauchly, 1940); and if so, we applied the Greenhouse-Geisser correction (Greenhouse and Geisser, 1959). Baseline differences across age groups using ANOVAs were also tested. In addition, Pearson Product Moment Correlations coefficients were used to document the associations between the peak change in circulating biomarkers within and across CPT and FHP sessions and between baseline levels and peak changes. Data analysis was performed using IBM SPSS version 22.

3. Results

The mean pain rating for the experimental sessions, which were calculated by averaging across the 12 pain ratings taken during each of the four administrations of CPT or FHP using a 0–100 numerical rating scale, was 43.6 (SD = 19.7) for CPT and 50.2 (SD = 19.4) for FHP. A repeated measures ANOVA (session $\times$ age) indicated that the pain ratings were not significantly different between the sessions ($p = 0.174$) or between the age groups ($p = 0.799$). The physiological response to both cold and heat pain testing was initially evaluated by systolic (SBP) and diastolic (DBP) blood pressure at baseline, stimuli induction and at discharge (Table 1). The mean changes for the CPT session were 7.7 (SD = 11.8) for SBP and 4.4 (SD = 13.4) for DBP, while for the FHP session, the mean change was 5.6 (SD = 7.8) for SBP and 3.1 (SD = 7.2) for DBP. Repeated measures ANOVA indicated that there were no differences between sessions ($p = 0.203$). Additionally, the effect for age ($p = 0.507$) and the age $\times$ stimuli interaction ($p = 0.514$) were not significant.

The physiological response to both cold and heat pain testing was also investigated by evaluating the soluble mediator profile within the plasma of healthy individuals at intervals indicated for up to 90 minutes following the completion of each testing paradigm. As a control, the plasma levels of these mediators in the absence of pain testing were also evaluated for the same time period. Importantly, none of these mediators were significantly elevated compared to baseline for any of the cytokines tested in the absence of pain application supporting Hypothesis 1. The pro-inflammatory cytokines, IL-6, IL-8 and TNF α

and the anti-inflammatory cytokines IL-4, IL-5 and IL-10 were affected by both pain paradigms and are thus the focus of this analysis (Fig. 1–4).

3.1. Baseline levels

For baseline levels of TNF- α , IL-6 and IL-8, the main effect of “age” was significant [$F(1, 13) = 222.956, p < 0.0001$; $F(1, 13) = 85.847, p < 0.0001$; $F(1, 13) = 33.9633, p < 0.0001$] indicating that older adults had higher levels of circulating pro-inflammatory cytokines than younger adults. Importantly, the “session” variable (CPT vs. FHP) was not significant for any of the pro-inflammatory biomarkers. Similarly, for baseline levels of the anti-inflammatory markers IL-10, IL-4 and IL-5, the main effect of “age” was significant [$F(1, 13) = 5.352, p = 0.038$; $F(1, 13) = 5.495, p = 0.036$; $F(1, 13) = 6.190, p = 0.027$] indicating that older adults also had higher levels of circulating anti-inflammatory markers than younger adults. Again, the “session” effect (CPT vs. FHP) was not significant indicating that the baseline levels did not differ between cold and heat pain application. Baseline data collapsed across trials for each biomarker marker are presented in Figure 1.

3.2. Cold Pressor Task

After CPT, plasma TNF- α levels peaked at 30 minutes post-testing while IL-8 levels peaked at 45 minutes (Fig. 2A,C). IL-6 had an initial induction occurring with TNF- α at 15–30 minutes post-testing followed by a more robust induction at 60 minutes post-testing (Fig. 2B). While the TNF- α response was resolved by 60 minutes post-testing, the IL-8 and the IL-6 responses were not resolved until 90 minutes post-testing (Fig. 2A–C). Here, the main effect of “age” was significant [$F(1, 13) = 5.634, p = 0.034$] for TNF- α with greater overall plasma levels of TNF- α for the older adults compared to the younger sample. In addition, the “time $\times$ age” interaction for TNF- α did not reach statistical significance [$F(1.148, 14.922) = 3.703, p = 0.069$]. For IL-6, both the main effect of “age” [$F(1, 13) = 14.223, p = 0.002$] and the “time $\times$ age” interaction [$F(1.274, 16.562) = 17.333, p < 0.001$] were significant. Pairwise comparisons indicated that significant “age” differences occurred at 60-min with older sample of adults exhibiting greater plasma levels of IL-6 than the younger sample (Fig. 2B). For IL-8, the main effect of “age” was not significant [$F(1, 13) = 0.155, p = 0.701$], whereas the “time $\times$ age” interaction was significant [$F(1.566, 20.355) = 5.431, p = 0.018$]. Pairwise comparisons indicated that significant age differences occurred at 45 minutes with the older sample of adults exhibiting greater plasma levels of IL-8 than the younger sample (Fig. 2C).

Several anti-inflammatory cytokines were also upregulated following the CPT, yet their peak induction was somewhat later than that observed with the pro-inflammatory cytokines (Fig. 2D–F). Specifically, while IL-4, IL-5 and IL-10 were detected as early as 30 minutes post-testing, peak expression for IL-4 and IL-10 occurred at 45 minutes post-testing while IL-5 peak expression was around 60 minutes (Fig. 2D–F). In addition, all anti-inflammatory responses were completely resolved by 90 minutes post-testing (Fig. 2D–F). For IL-10, the main effect of “age” was not significant [$F(1, 13) = 1.901, p = 0.191$], whereas the “time $\times$ age” interaction was significant [$F(2.012, 26.159) = 5.841, p = 0.008$]. Pairwise comparisons indicated that significant age differences occurred at 45-min with older adults exhibiting lower plasma levels of IL-10 than the younger sample (Fig. 2D). For IL-4, the

main effect of “age” was not significant [$F(1, 13) = 1.818, p = 0.201$], whereas the “time $\times$ age” interaction was significant [$F(1.775, 23.074) = 5.104, p = 0.017$]. Pairwise comparisons indicated that significant age differences occurred at 45-min with the older sample of adults exhibiting lower plasma levels of IL-4 than the younger sample (Fig. 2E). For IL-5, neither the main effect of “age” [$F(1, 13) = 0.896, p = 0.361$] and the “time $\times$ age” interaction were significant [$F(1.481, 19.259) = 0.566, p = 0.566$] (Fig. F).

Interestingly, within a session, associations for pro-inflammatory markers following administration of CPT stimuli were weak to moderate and failed to reach significance, while associations between changes in plasma levels for anti-inflammatory markers following the CPT were strong and statistically significant (Table 2). In addition, changes from baseline to peak plasma concentration were significant for IL-6, $r = 0.84, p < 0.001$; IL-8, $r = 0.49, p = 0.044$; IL-4, $r = -0.49, p = 0.046$; and IL-5, $r = -0.50, p = 0.040$, but not TNF α or IL-10 (Table 3).

3.3. Focal Heat Pain

Following FHP, the same soluble mediators were elicited as CPT. Here, TNF- α and IL-8 expression peaked at 45 minutes post-testing (Fig. 3A, C) and IL-6 peaked at 60 minutes post-testing (Fig. 3B). While IL-8 expression resolved by 90 minutes post-testing, both IL-6 and TNF- α remained elevated at 90 minutes (Fig. 3A–C). For TNF- α and IL-6, the main effect of “age” was not significant [$F(1, 13) = 2.639, p = 0.128$; $F(1, 13) = .781, p = 0.393$], whereas the “time $\times$ age” interaction [$F(1.072, 13.934) = 5.410, p = 0.034$; $F(2.046, 26.602) = 8.909, p = 0.001$] was significant. Pairwise comparisons indicated that significant age differences occurred with older adults exhibiting greater plasma levels of TNF- α at 45-min than the younger sample and greater plasma levels of IL-6 response at 30-min, 45-min, and 90-min than the younger adults (Fig. 3A, B). For IL-8, the main effect of age was significant [$F(1, 13) = 8.156, p = 0.014$], along with the “time $\times$ age” interaction [$F(1.024, 13.276) = 13.276, p = 0.003$]. Pairwise comparisons indicated that significant age differences occurred with older adults exhibiting greater levels at 45-min than the younger sample (Fig. 3C).

Similar to CPT, the peak induction of the anti-inflammatory mediators by FHP was later than that observed with the pro-inflammatory cytokines. Specifically, IL-4, IL-5 and IL-10 were not observed at any appreciable level until 45 minutes post-testing, whereby all peaked at 60-min for the younger sample and 90-min for the older sample (Fig. 3D–F). Here, for IL-10, IL-4, and IL-5 the main effect of “age” [$F(1, 13) = 14.248, p = 0.002$; $F(1, 13) = 9.916, p = 0.008$; $F(1, 13) = 12.961, p = 0.003$] and the “time $\times$ age” interaction effect were both significant [$F(1.338, 17.397) = 11.128, p = 0.002$; $F(1.398, 18.172) = 10.586, p = 0.002$; $F(1.146, 14.893) = 12.881, p = 0.002$]. Pairwise comparisons indicated that for IL-10 significant age differences occurred at 45-min and 90-min, while for IL-4 significant age differences occurred at 45-min and 90-min, and for IL-5 significant age differences occurred at 45-min, 60-min, and 90-min with older sample of adults always exhibiting greater plasma levels than the younger sample (Fig. 3D–F).

Associations following FHP between pro-inflammatory markers were small to strong with 2 of 3 reaching statistical significance (Table 2), while similar to that observed in CPT,

associations between changes in plasma levels for anti-inflammatory markers following FHP were strong and statistically significant (Table 2). In addition, changes from baseline to peak plasma concentration were significant for TNF- α , $r = 0.55$, $p = 0.022$ and IL-6, $r = 0.84$, $p < 0.001$, but not IL-8 or any of the anti-inflammatory mediators evaluated (Table 3).

4. Discussion

While previous research has supported associations between pain and inflammation using samples of chronic pain patients, to our knowledge, this is the first experimental study demonstrating that application of a standardized painful stimulus results in an immune response that differs in magnitude and time course between healthy older and younger individuals. The importance of interactions between immune mediators and nervous systems in inflammatory pain and neuropathic pain is emerging, and both occur most frequently in older adults (Dieleman et al., 2008; Watkins et al., 2005). In inflammatory pain, peripheral cytokines released by immune and glial cells influence the sensation of pain by increasing the sensitization of nociceptors (Guillot, 2012). This peripheral inflammation, if persistent, induces central sensitization with mechanical allodynia and thermal hyperalgesia (Kawasaki et al., 2008; Ren and Dubner, 2010). Neuropathic pain following injury to peripheral nerves results in amplification of responses to painful stimuli at the first synapse in the nociceptive pathway, leading to excessive nociceptive signaling in the central nervous system (Clark et al., 2013; Marchand et al., 2005).

The three major findings were: 1) baseline levels of both pro- and anti-inflammatory cytokines were greater in older compared to younger participants; 2) pro-inflammatory cytokines peaked at the same time points for both younger and older groups, with greater elevations among older subjects for TNF- α and IL-8 with both pain models and IL-6 only for CPT; and 3) anti-inflammatory cytokines generally peaked later for the older subjects compared younger subjects but not the CPT.

4.1. Baseline levels

Even though our study inclusion criteria ensured the healthy status of our participants, older adults had increased circulating levels of both pro- and anti-inflammatory cytokines at baseline. This is consistent with the literature supporting increased presence of systemic immune biomarkers such as IL-6 and TNF- α at rest in older adults, which appears to be a hallmark of aging (Mishto et al 2003). A review by DeVon (2014) reported most studies of persons with painful conditions showed elevated baseline levels of various inflammatory cytokines that were positively associated with more severe pain, suggesting similar vulnerability to chronic pain for older adults.

The mechanisms underlying baseline immune elevation differences are not completely understood. Emerging evidence suggests that aged immune cells can promote the secretion of pro-inflammatory cytokines (i.e., IL-6, IL-8 among others) in a process initiated by IL-1 α , regulated by TGF- β and mediated by the p38MAP kinase and NF κ B systems (Campisi et al., 2011). In turn, these pro-inflammatory cytokines can induce anti-inflammatory cytokine production, which will further regulate pro-inflammatory cytokine expression and function. For example, IL-6 can inhibit TNF- α , while IL-10 can suppress the

ability of macrophages to release TNF- α and IL-6 (Austin 2010; de Oliveira, 2011). Evidence also suggests that age-related changes in systemic levels of catecholamines and sex hormones can either inhibit or upregulate inflammatory responses through complex effects on innate/adaptive immune cells (Ricci et al., 1999; Rohleder et al., 2002; Pfeilschifter et al., 2002).

4.2. Expression of pro-inflammatory cytokines

We found that pro-inflammatory cytokines peaked at the same time points for both younger and older groups, with greater elevations among older subjects for TNF- α and IL-8 in both pain models and only IL-6 using CPT. Although there are no previous studies specifically examining age differences in immune function in response to pain, Ono et al., (2001) found that elderly subjects had an intrinsically increased capacity to produce IL-6 in response to surgical stress. Along those lines, animal studies have examined the contribution of several of the immune mediators measured in the current study, whereby they have been described as direct and indirect mediators of nociceptor activation. For instance, it has been demonstrated that pro-inflammatory cytokines can directly sensitize nociceptors (Ren and Dubner, 2011 for review). This was confirmed upon the knockout of TNF- α receptors, which resulted in reduced heat hyperalgesia after intraplantar injection of an adjuvant inducing inflammation in mice (Kawasaki, 2008).

In the current human study TNF- α was one of the first plasma cytokines induced at 30 and 45 minutes post-testing (Figs. 2, 3) and this upregulation was more robust in older individuals (Table 2, 3). Given the increased circulating baseline levels of TNF- α in our older participants, this may actually indicate that TNF- α could have enhanced pain responses in older adults and subsequent induction of factors like IL-6 and IL-8. Indeed, a significant induction of plasma IL-6 and IL-8 was observed following both CPT and FHP (Figs. 2, 3). Furthermore, both IL-6 and TNF- α -mediated signaling are known to play key gateway function for inflammatory signal amplification (Tsirpanlis et al., 2009). Hence, the baseline chronic low-grade inflammation measured in the older participants would be expected to propagate pro-inflammatory signals and further facilitate nociceptive processing leading to increased risk for pain.

4.3. Expression of anti-inflammatory cytokines

Healthy older adults had a significantly greater induction of circulation anti-inflammatory cytokines compared to younger participants in response only to the FHP. These data suggest that the anti-inflammatory response differs and/or is more effective in certain pain paradigms. Production of IL-10 and IL-4 has been shown to be greater in stimulated lymphocytes from older compared to younger individuals and may suggest dysregulation of the anti-inflammatory response and contribute to the reduced cellular immunity in older individual (Rink et al., 1998). Similar observations have been reported in individuals with chronic pain with serum levels of IL-10 correlating with clinical pain intensity (Miller et al., 2002; Imamura et al., 2015).

Anti-inflammatory cytokines such as IL-4 and IL-10 have been demonstrated to produce antinociceptive effects. For instance, injection of IL-4 following mechanical hyperalgesia

evoked by carrageenan, bradykinin, and TNF- α in rats resulted in decreased hyperalgesia and a reduced production of TNF- α , IL-1 β and release of prostaglandins (Cunha et al., 1999). Similarly, healthy IL-4 knockout mice displayed tactile allodynia, while responses to heat, cold and pressure stimuli were unchanged (Uceyler et al., 2011) consistent with our own findings where the heat pain paradigm produced stronger induction. However, gene expression analysis demonstrated overexpression of IL-10 and IL-13 in the spinal cord, which may have had a compensatory action and could explain the lack of differences in these nociceptive responses (Wang et al., 2012).

Unlike the pro-inflammatory cytokines, the anti-inflammatory biomarkers were delayed in both pain models for the older adults (Figs. 2 > Figs. 2, 3). The importance of a homeostatic balance between pro-inflammatory and anti-inflammatory mechanisms is well established for health as anti-inflammatory cytokines serve as negative-feedback regulators (Elenkov, 2005). The time lag we observed in the release of endogenous anti-inflammatory cytokines in older adults would appear to delay this protective, self-limited inflammatory response. There is already evidence of an age-related shift from a Th1 (pro-inflammatory) to a Th2 (anti-inflammatory) response in immune cells from elderly individuals (Rink et al., 1998).

A weakness of many studies in older adults is that they examine a limited number of pro-inflammatory cytokines (i.e., IL-6, and TNF- α), and fail to report Th2 cytokines. Specifically, IL-6, has been the most studied cytokine in aging, and so called the “cytokine for gerontologists” (Maggio et al., 2006). However, IL-6 is pleiotropic, so it has both pro- and anti-inflammatory actions including the induction of IL-10 (Opal and DePalo, 2000). Nevertheless, a recent review has concluded that there are no reports of IL-6 showing direct anti-nociceptive properties (Busch-Dienstfertig and González-Rodríguez, 2013). Based on our data, this Th1/Th2 dysregulation is plausible since the magnitude of IL-10 induction was greater than IL-6 and remained significantly elevated in older, but not younger adults. It is likely that the mechanisms above are not mutually exclusive, and that there are inter-individual differences in the predominant mechanism within each person. Thus, mechanism-based studies including larger sample sizes are needed to characterize and measure these possible mechanistic variations that will likely provide insights into inflammatory phenotypes associated with successful aging.

4.4. Strengths and limitations

Strengths of this study included the use of multiple painful stimuli (CPT and FHP) tested on separate days. Our participants were carefully screened for health and instructed not to perform activities that are known to impact immune responses and/or pain sensitivity including exercise, alcohol, caffeine and changes in sleep-wake cycles. In addition, our study measured multiple cytokines simultaneously representative of Th1 and Th2 immune responses. This study also has limitations. First, its generalizability is reduced by inclusion of only healthy participants. However, this allowed a more valid age comparison without differing levels of disease across age cohorts. Second, we did not assess the heterogeneity in genotype variants and hormones that are known to impact immune function and pain sensitivity given our small sample size. Third, our small sample size limited our statistical analyses and fourth, we did not obtain *in-vitro* data to ascertain the precise source of the

cytokines measured. Finally, because of ethical issues in human experimental pain paradigms, participants understand that the experimental painful stimulation may be terminated soon or upon request, lacking the unpredictability that makes chronic pain a strong stressor. Nevertheless, these findings also provide information within the context of acute pain. Larger samples of community-dwelling older adults whose health better reflects the general population would allow greater external validity but at the cost of internal validity in comparisons across age cohorts. Studies need to make comparison between individuals with and without chronic pain, and examine the contribution of inflammatory versus non-inflammatory pain. Future work should include hormone/genetic markers. In addition, it may be useful to take an integrated systems approach to characterize the function of other neurobiological mechanisms (i.e., oxidative stress, apoptotic pathways, mitochondrial function) that either alone or in combination with cytokines may be significant contributors to chronic pain development.

5. Conclusions

In summary, these data indicate that acute experimental pain can induce an immune response, which in older adults, is more robust and remains elevated longer than in younger individuals. These findings coupled with the higher baseline levels of these cytokines in older individuals corroborate animal studies implicating inflammation as a plausible underlying mechanism in the increased sensitization of pain observed in older individuals (Riley et al., 2010, 2014). Our results suggest that each biomarker may have a unique trajectory and thus, one time point of analysis is not sufficient to provide insight into the underlying mechanisms associated with the immune-pain axis, thus highlighting the need for optimization of sample collection protocols based on both the biomarker of choice as well as the pain paradigm being utilized.

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Highlights

- To our knowledge, this is the first experimental study demonstrating that application of a standardized painful stimuli result in an immune response that differs in magnitude and time course between healthy older and younger individuals.
- Pro-inflammatory cytokines peaked at the same time points for both younger and older groups, with greater elevations among older subjects for TNF- α and IL-8 with both pain models and IL-6 only for Cold Pressor Task;
- Anti-inflammatory cytokines generally peaked later for the older subjects compared to younger subjects only in the Focal heat Pain model, but not the Cold Pressor Task.

Figure 1.
Baseline expression of cytokines prior to experimental pain testing collapsed across sessions.

Figure 2.

Cytokine plasma levels following Cold Pressor Task. Dotted lines are change from baseline in older adults while solid lines are change from baseline in younger participants.

Figure 3.

Cytokine plasma levels following Focal Heat Pain. Dotted lines are change from baseline in older adults while solid lines are change from baseline in younger participants.

Table 1

Mean SBP and DBP across the testing sessions for the total sample and by age group.

	Total sample Mean (SD)	Younger Mean (SD)	Older Mean (SD)
CPT session			
SBP - baseline	113.8 (10.0)	111.5 (5.4)	115.8 (12.9)
SBP – stimuli administration	121.4 (14.1)	121.4 (14.6)	121.6 (14.6)
SBP - discharge	119.5 (17.1)	109.8 (7.7)	128.1 (18.8)
DBP - baseline	68.8 (9.2)	68.0 (8.9)	69.7 (10.0)
DBP – stimuli administration	73.3 (12.7)	74.1 (13.6)	72.6 (12.6)
DBP - discharge	72.8 (11.1)	67.1 (6.9)	77.9 (12.0)
FHP session			
SBP - baseline	117.9 (14.5)	111.4 (12.0)	123.8 (14.5)
SBP – stimuli administration	123.4 (14.5)	116.5 (11.7)	129.7 (14.4)
SBP - discharge	119.5 (14.5)	114.9 (10.6)	123.1 (16.6)
DBP - baseline	67.2 (9.5)	62.0 (3.5)	71.8 (10.8)
DBP – stimuli administration	70.2 (13.8)	61.3 (9.1)	78.2 (12.4)
DBP - discharge	69.4 (11.2)	64.8 (8.8)	73.4 (12.0)

SBP= Systolic Blood Pressure

DBP= Diastolic Blood Pressure

CPT= Cold Pressor Task

FHP= Focal Heat Pain

Table 2
Correlations between peak plasma concentrations for pairs of biomarkers for both the CPT and FHP sessions.

	TNF- α	IL-6	IL-8	IL-10	IL-4	IL-5
TNF- α	-----	-0.21	0.36	-0.11	-0.09	0.08
IL-6	-0.12	-----	0.47	0.13	0.02	0.06
IL-8	0.88**	-0.21	-----	0.28	0.21	0.28
IL-10	0.90**	-0.20	0.81**	-----	0.91**	0.77**
IL-4	0.89**	-0.14	0.84**	0.93**	-----	0.80**
IL-5	0.92**	-0.14	0.83**	0.92**	0.91**	-----

* p < 0.05,

** p < 0.01

Values above the diagonal in bold are for the CPT paradigm and below the diagonal in italics are for the FHP.
(CPT= Cold Pressor Pain; FHP= Focal Heat Pain)

Table 3

Correlations between baseline and peak plasma concentrations for pairs of biomarkers for both the CPT and FHP paradigms and for peak concentrations across stimuli sessions.

	TNF- α	IL-6	IL-8	IL-10	IL-4	IL-5
CPT (baseline vs peak)	0.17	0.84**	0.49*	-0.36	-0.49*	-0.50*
FHP (baseline vs peak)	0.55*	0.69**	0.13	-0.12	-0.21	-0.21
CPT peak vs. peak FHP	0.09	0.52*	0.67**	0.25	0.22	0.30

*
p < 0.05,
**
p < 0.01

Values above the diagonal are for the CPT paradigm and below the diagonal for the FHP paradigm.

(CPT= Cold Pressor Pain; FHP= Focal Heat Pain)