Depressive symptoms in hip fracture patients are associated with reduced monocyte superoxide production

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ABSTRACT

Ageing is accompanied by reduced functioning of the immune system, termed immunesenescence which is associated with increased risk of infection and mortality. However the immune system does not operate in isolation and can be modified by many environmental factors, including stress. In this study we determined whether physical stress (hip fracture) and psychological distress (depressive symptoms) had additive effects upon the aged immune system, specifically on monocyte numbers and function. We assessed immune function in 101 hip fracture patients (81 female) 6 weeks and 6 months after injury and 43 healthy age matched controls (28 females). Thirty-eight of the hip fracture group were found to be depressed at the 6 week sampling. No differences in peripheral monocyte count, distribution of monocyte subsets or TNFα secretion were observed between hip fracture patients and healthy controls. However we observed significantly reduced superoxide production in response to *Escherichia coli* in the monocytes of hip fracture patients who developed depressive symptoms compared with non-depressed hip fracture patients (p = 0.002) or healthy controls (p = 0.008) 6 weeks after the fracture which remained decreased 6 months following injury. In previous studies we have shown an effect of depression on neutrophil superoxide generation in hip fracture patients, suggesting a particular susceptibility of this aspect of immune cell function to psychological stress.

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1. Introduction

Stress is a frequent precipitant of major depression (Leonard and Myint, 2009) and chronic age-related diseases such as cardiovascular disease, rheumatoid arthritis, cancer, and lung disease have long been known to have a negative impact on the psychological wellbeing of older people, resulting in development of depressive symptomatology (Bisschop et al., 2004). Hip fracture is a devastating condition and a major health issue in old age (Abrahamsen et al., 2009) and a high prevalence of depression ranging from 9% to 47% has been reported post-hip fracture in older adults (Holmes and House, 2000). Importantly, depression in hip fracture patients has been associated with increased risk of infections and poor survival (Nightingale et al., 2001) and an impaired ability to regain pre-fracture levels of physical functioning (Mossey et al., 1990). A 1–3% increase in the frequency of hip fractures per year has been reported worldwide (Cummings and Melton, 2002).

Thus, understanding the health implications of this common injury for older people and the additive effect of development of depressive symptoms is now imperative.

Ageing results in altered immunological dynamics and reduced immunity (Dorshkind et al., 2009; Panda et al., 2009), termed immunesenescence, contributing towards an increased risk of morbidity and mortality in older adults most notably an increase in susceptibility to infections (Bonomo, 2002; Gavazzi and Krause, 2002). Monocytes and their tissue equivalent macrophages, play a key role in innate immunity acting as early sensors of infections by detecting pathogens via their Toll-like receptors (TLRs) for pathogen associated molecular patterns (PAMPs) and releasing pro-inflammatory cytokines to recruit additional leukocytes. The differential expression of CD14 and CD16 have been used to define three peripheral subsets of monocytes: classical (CD14$^+$ CD16$^-$) represent approximately 90% of monocytes in a healthy individual; intermediate (CD14$^+$ CD16$^+$) and non-classical monocytes (CD14$^+$ CD16^{++}) make up the remaining fraction (Passlick et al., 1989). There is also considerable complexity in the functionality of monocyte lineages, with these cells having the potential for differentiation into not only macrophages but also specific classes of inflammatory dendritic cells (Auffray et al., 2009). In this way

monocytes and their differentiated end cells help to integrate the innate and adaptive immune system in their role as antigen presenting cells.

Monocyte numbers do not change significantly with ageing (Takahashi et al., 1985; Gomez et al., 2008) but there are changes to the distribution of monocyte subsets with age: non-classical $CD14^+CD16^+$ monocytes increase significantly with age resulting in a shift from classical to non-classical monocytes (Seidler et al., 2010; Nyugen et al., 2010). Early studies reported impaired phagocytic function (Hearps et al., 2012) and a decline in reactive oxygen and nitrogen species release in monocytes from old donors (McLachlan et al., 1995; Alvarez and Santa Maria, 1996). Monocytes are also significant producers of pro-inflammatory cytokines and a study of monocytes from 154 young and old individuals found an age-associated reduction in TNF-α and IL-6 secretion after stimulation through the TLR1/2 heterodimer and a decrease in TLR7-mediated IL-6 production though TNF-α and IL-6 production in response to ligation of TLR2/6, TLR4, and TLR5 was unaffected by ageing (van Duin et al., 2007a). In addition, TLR-induced upregulation of the costimulatory molecule CD80 on monocytes after stimulation through TLR1/2, TLR2/6, TLR4, TLR5, and TLR7/8 all showed a substantial age-associated decline (van Duin et al., 2007a).

It is well accepted that stress is a potent suppressor of immune function (Segerstrom and Miller, 2004; Zorrilla et al., 2001). However, data regarding the effect of stress on monocytes are limited; there are only a few studies done in depressed patients (Schlatter et al., 2004; Lisi et al., 2013) and there is one study exploring the effect of surgical stress which has reported dysregulated monocyte functioning (Ono et al., 2001). Importantly, normal ageing is associated with increased incidence of psychological distress (Bauer et al., 2009), which may therefore further compromise or be a key component of the normal age-related decline in immunity seen in older adults. The physiological response to stress requires activation of the hypothalamus–pituitary–adrenal (HPA) axis involving increased secretion of glucocorticoids (Charmandari et al., 2005), which have potent immunosuppressive properties including altered cytokine production (Tsianakas et al., 2012) and suppression of superoxide generation (Tsianakas et al., 2012; Butcher et al., 2005). Dehydroepiandrosterone (DHEA) is also secreted by the adrenals and is present in the circulation in its sulphated form DHEAS at micromolar concentrations. In contrast to glucocorticoids DHEA and DHEAS have been reported to have immune-enhancing properties, including enhanced monocyte function (McLachlan et al., 1996) and increased human neutrophil function (Radford et al., 2010). Normal ageing is accompanied by an altered HPA axis due to the sharp decline in the production of DHEA/S, termed adrenopause (Orentreich et al., 1984; Hazeldine et al., 2010) and an exaggerated HPA axis after stress (Wilkinson et al., 1997; Pedersen et al., 2001). The result is increased cortisol levels as compared to young individuals in stressful conditions which may compromise further the age-associated immune decline.

We set out to test the hypothesis that ageing, physical stress and psychological stress are key interacting factors that could have an additive detrimental effect on immune functioning in older adults. We examined the combined effect of the physical stress of hip fracture and psychological stress, specifically depression, on monocyte subset distribution, bactericidal properties, cytokine production and antigen presentation and co-stimulatory capacity in older adults.

2. Materials and methods

2.1. Participants

101 older hip fracture patients were recruited from five hospitals in Birmingham, UK between 2010 and 2012. Inclusion criteria were that participants had to be aged 60 years and over with a hip fracture sustained 4–6 weeks previously but with no chronic immune-related disorders e.g., cancer, diabetes, or taking any regular medications that might modify immunity, e.g., immunosuppressants. Additionally patients must not have had any diagnosis of depression by a physician prior to age 50 years or be taking or have previously taken anti-depressant medication, in order to pick up patients with depressive symptoms emerging post-hip fracture rather than those with a prior history of and thus propensity to depression. 43 healthy older adults were also recruited from the community as controls. These controls also had to meet the inclusion criteria above but not have a current hip fracture. The study was approved by South Birmingham Local Research Ethics Committee and all participants provided written informed consent (study ref: 09/H1203/80).

2.2. Study design and procedure

The study was a prospective case–control design with three groups of older adults: hip fracture patients with or without depressive symptoms and healthy older adults. Consent was gained whilst patients were still in hospital. All patients completed questionnaires, structured interviews and provided a blood sample 4–6 weeks and six months after hip fracture. Control participants completed a depression and anxiety symptoms scale and basic demographic information when attending the university for a single blood sampling. Blood samples were taken between 09.00 and 11.00 to minimise any effect of diurnal variations in steroid levels. None of the participants had an acute infection at the time of blood sampling. Interviews were performed either in the hospital or in the patient's home for hip fracture patients and at the university for control participants.

The psychological status of the participant was assessed by means of standardised psychometric questionnaires. Depression was evaluated by a Geriatric Depression Scale (Yesavage et al., 1982). Depression was defined as a GDS score greater than or equal to 6 (Sheikh and Yesavage, 1986). The Hospital Anxiety and Depression Scale was also used to measure depression and anxiety (Zigmond and Snaith, 1983). The scale contains 14 items, scored from 0 (not present) to 3 (considerable), with seven assessing aspects of depression and seven assessing anxiety.

2.3. Monocyte function assays

Monocyte phagocytic ability was measured in whole blood using a commercially available kit (Phagotest kit, Orpegen Pharma, Germany) and the assays were performed according to manufacturer's instructions. Briefly, FITC-labelled opsonised Escherichia coli were added to whole blood and incubated at 37 °C for 10 min and the control tube was kept on ice for 10 min. Following incubation, a quenching solution was added to each tube to stop the reaction and red blood cells were lysed. Following the lysis step the cell suspension was resuspended in DNA staining solution and fluorescence was analysed immediately using a Cyan™ ADP flow cytometer (Dako Ltd., Cambridge, UK). Data were analysed using Summit V 4.3. The phagocytic index was used as a measurement of the phagocytic capabilities of monocytes and was calculated as the percentage of cells that had ingested bacteria, multiplied by the mean fluorescence intensity (MFI), divided by 100.

Monocyte superoxide burst was measured in whole blood using a commercially available kit (Phagoburst kit, Orpegen Pharma). The assays were performed according to manufacturer's instructions. Whole blood was treated with 100 nM fMLP (Sigma-Aldrich, Dorset, UK), 20 nM PMA (Sigma-Aldrich) or opsonised E. coli (Orpegen Pharma) at 37 °C for 10 min. Following incubation, fluorogenic substrate dihydrorhodamine (DHR) 123 (Dako) was added to the blood sample at 37 °C for 10 min. Post-incubation, cells were lysed after which DNA staining solution was added. Oxidation of substrate DHR123 was analysed by flow Cytometry using a Cyan™ ADP flow cytometer. The oxidative burst production is indicated by the MFI of the monocyte population.

2.4. Isolation of PBMCs and immunostaining for phenotypic analysis of monocytes

PBMCs were isolated from peripheral blood by density centrifugation using Ficoll-Paque™ PLUS (GE Healthcare, Uppsala, Sweden). The blood was layered on top of 6 ml of Ficoll-Paque™ PLUS in a 25 ml Universal tube and the tube was centrifuged at 650 $\times g$ for 30 min with no break. Post-centrifugation, the mononuclear cell layer containing PBMC was removed and added to a new universal tube and the cells were washed twice with PBS and were counted on a haemocytometer. Isolated PBMCs were frozen down by re-suspending cells in freezing medium consisting of 10% DMSO (Sigma-Aldrich, UK) in heat inactived foetal calf serum (Biosera, UK) and transferring them in small aliquots into cryovials. The cryovials were transferred into a freezing container (Mr. Frosty, Sigma-Aldrich, UK) containing isopropanol (VWR International, UK). Cells were then stored at −80 °C.

Frozen cells were thawed in a water bath at 37 °C and were washed in 10 ml of RPMI 1640 (Sigma-Aldrich, UK) to remove DMSO. Post-washing, PBMCs were resuspended in PBS at a concentration of 1×10^6/ml. For phenotypic characterisation of monocytes, isolated PBMCs were stained with a combination of fluorochrome conjugated antibodies including; CD14-Pac blue (Bio Legend, UK; clone M5E2), CD16-FITC (eBiosciences, UK; clone CB16), HLADR-PE (Serotec, clone: MCA1879), CD80-APC (Bio legend, UK; clone 2D10) and CD86-APC (Bio legend, UK; clone IT2.2). Appropriate isotype controls were used for setting gates. Following incubation, cells were washed and resuspended in PBS for flow cytometric analysis using a Cyan™ ADP flow cytometer (Dako).

2.5. Monocyte stimulation — lipopolysaccharide (LPS)

Frozen PBMCs were resuspended in RPMI medium containing 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin (Sigma-Aldrich) supplemented with 10% heat-inactivated foetal calf serum (Sera Laboratories International, Sussex, UK) at a concentration of 1×10^6/ml for functional analysis. Time course experiments were performed to examine the TNFα production by monocytes at 1 h, 2 h, 4 h, 8 h, 24 h and 48 h stimulations (data not shown). From these data, monocytes were stimulated with LPS isolated from *E. coli* serotype 0111:B4 (1 μg/ml; Sigma-Aldrich) for 4 h in the presence of Brefeldin A (10 μg/ml; Sigma-Aldrich) to inhibit cytokine secretion. Cells were then washed and stained for CD14 to identify the monocytes and cells were then fixed (Reagent A; Fix and Perm kit, Invitrogen Ltd., Paisley, Scotland) and permeabilised (Reagent B; Fix and Perm kit, Invitrogen). Permeabilised cells were stained with anti-human TNFα-PE antibody (Bio legend, clone: MAb11) for intracellular staining. Appropriate isotype controls were used for gate setting for measuring cytokine expression. Cells were analysed using a Cyan™ ADP (Dako). Data analysis was carried out using Summit v4.3x.

Additionally, monocytes were also stimulated with LPS isolated from *E. coli* serotype 0111:B4 (1 μg/ml; Sigma-Aldrich) for 4 h in the absence of Brefeldin A. These culture supernatants were snap frozen in liquid nitrogen and stored at −80 °C for subsequent analysis of TNFα secretion by monocytes. TNFα levels in supernatants were measured by enzyme-linked immunosorbent assays (ELISA) using a commercial kit (Abcam, Cambridge, UK), according to manufacturer's instructions.

2.6. Serum cortisol and DHEAS assays

Serum cortisol and DHEAS levels were measured by ELISA using a commercial kit (IBL International, Hamburg, Germany) according to manufacturer's instructions.

2.7. Statistical analysis

Univariate ANOVA with least significant difference post-hoc tests were used to assess differences between the three groups (hip fracture with depressive symptoms, hip fracture without depressive symptoms, and healthy controls). Where demographic variables differed significantly between the groups, analyses were rerun adjusting for these variables using ANCOVA. Pearson's correlations were used to examine associations between depression score and monocyte function and hormone levels. Repeated measures ANOVA models were used to examine changes in monocyte functioning between the 6 week and 6 month sampling time points.

3. Results

3.1. Participant demographics

The full demographic statistics for the study participants have been reported previously (Phillips et al., 2013). There were no differences between the patients who developed depression and those who did not for major demographic features, though the healthy control group were younger (74.9 ± 5.64 years, p < 0.001) than the hip fracture only (83.8 ± 7.48 years) and hip fracture with depression groups (84.0 ± 8.62 years) and also had a higher BMI (27.5 ± 5.02, p < 0.001) than either of the patient groups (23.5 ± 3.81 hip fracture alone; 22.7 ± 4.03 hip fracture with depression). All statistical comparisons were therefore adjusted for these two measures. Patients were classified into two groups on the basis of their GDS scores: hip fracture patients with a GDS score of 5 or less were classified as non-depressed (HF; hip fracture only), those with a score of greater than 5 were categorised as depressed (HF + D; Hip fracture patients with depression). None of the recruited patients had a prior history of depression and in this study we observed that 38 (37%) of the hip fracture patients had developed depression after their injury. This is in line with previous studies that have reported a high prevalence of depression in hip fracture patients (Nightingale et al., 2001).

3.2. Distribution of monocyte subsets

The contribution of the three subsets of monocytes: classical (CD14$^+$ CD16$^−$), intermediate (CD14$^+$ CD16$^+$) and non-classical (CD14$^+$ CD16^{++}), to the monocyte pool was determined in the peripheral blood of hip fracture patients with and without depression with healthy older adults. There was no significant difference in total monocyte count between the three groups, F (2, 65) = .37, p = .69, η^2 = .01 [Fig. 1A]. We also found no difference in the percentage of classical (CD14$^+$ CD16$^−$) monocytes between the two groups of hip fracture patients and healthy controls F (2, 67) = .23, p = .79, η^2 = .007 [Fig. 1B], or in the percentage of 'intermediate' monocytes F (2, 67) = .30, p = .73, η^2 = .009 or 'non-classical' monocytes F (2, 67) = .43, p = .64, η^2 = .01 between the three groups [Fig. 1C and D].

3.3. Monocyte function in hip fracture patients

On comparing monocyte phagocytic ability between the three groups although we observed a trend towards an increase in phagocytic ability of monocytes in the hip fracture patients, though this failed to reach statistical significance F (2, 81) = 2.15, p = .12, η^2 = .05 (Fig. 2A). Phagocytosis of pathogens by monocytes is followed by intracellular killing via superoxide production. Upon examining the ability of monocytes to generate superoxide in response to opsonised *E. coli*, we observed significant differences between the three groups F (2, 82) = 7.41, p = .001, η^2 = .15, but the significant impairment in superoxide generation was restricted to the hip fracture patients who developed depression, when compared either to healthy controls (p = .008) or to hip fracture patients without depression
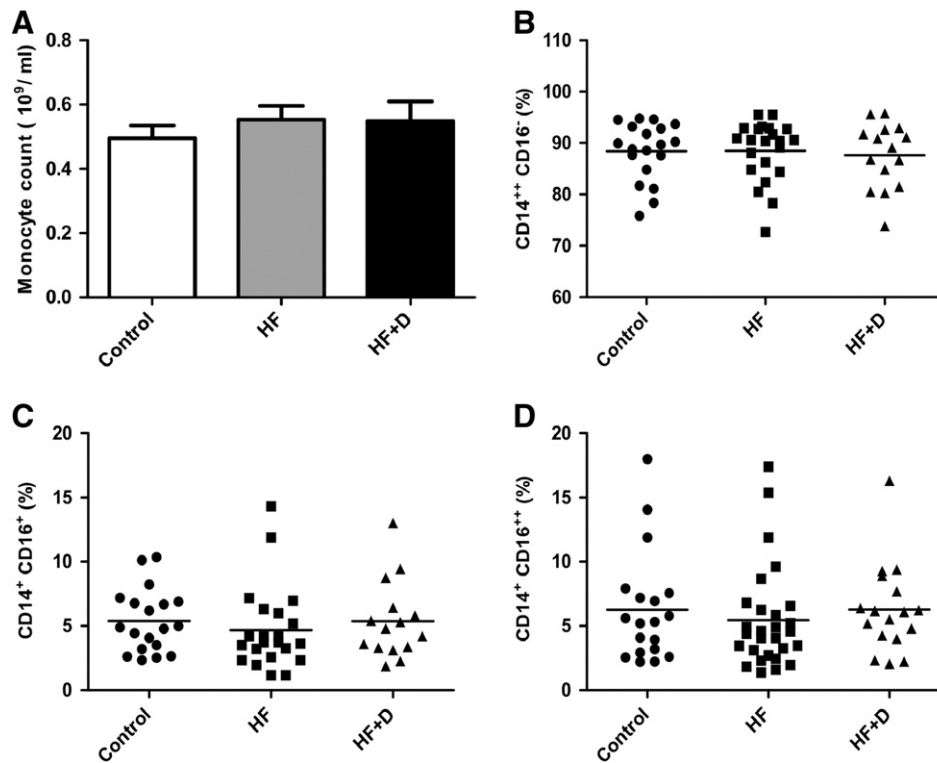
Fig. 1. Monocyte counts and monocyte subsets in hip fracture patients six weeks post-surgery. (A) The absolute numbers of monocytes in peripheral blood of hip fracture patients and controls; the percentage of (B) CD14$^+$ CD16$^-$, (C) CD14$^+$ CD16$^+$ and (D) CD14$^+$ CD16^{++} monocytes in peripheral blood of hip fracture patients and controls. Data are mean ± SEM.

(p = .002) (Fig. 2B). When the above analyses were repeated with adjustment for age, sex and BMI, the superoxide production in response to *E. coli* F (2, 70) = 6.72, p = .002, η^2 = .16 remained significant for the hip fracture plus depression group (data not shown).

We also evaluated the long term effect of hip fracture and depressive symptoms on immune functioning of older adults. On examining monocyte superoxide production in hip fracture patients six months after surgery, the main effect of time on both groups of hip fracture patients was not significant F (1, 38) = 2.31, p = .13, η^2 = .05, and no significant improvement in superoxide production by monocytes was seen at 6 months compared with 6 weeks in the hip fracture group with depression (Fig. 2C).

3.4. Cytokine secretion by monocytes

In addition to anti-microbial activity monocytes are capable of participating in the inflammatory response by producing pro-inflammatory cytokines such as TNFα and IL1-β (Orentreich et al., 1984). On assessing cytokine production capacity of monocytes by intracellular cytokine detection using immunostaining and flow cytometry, we failed to find any significant differences in the percentage of monocytes producing TNFα between the three groups in untreated cells F (2, 33) = 1.24, p = .30, η^2 = .07 (data not shown) or after 4 h LPS stimulation F (2, 32) = .08, p = .92, η^2 = .05 (Fig. 3A). To examine TNFα secretion by monocytes we performed an ELISA on cell culture supernatants. There was no difference in TNFα secretion between the three groups in the untreated condition F (2, 26) = .04, p = .95, η^2 = .003 (data not shown) or after 4 h LPS stimulation F (2, 22) = .20, p = .75, η^2 = .02 (Fig. 3B).

3.5. Expression of co-stimulatory molecules and MHC by monocytes

Next, we evaluated the expression of co-stimulatory molecules CD80 and CD86 on monocytes of hip fracture patients and healthy controls. The percentage of monocytes expressing CD80 F (2, 41) = 1.67,

p = .20, η^2 = .07 and its expression levels F (2, 40) = 1.86, p = .16, η^2 = .08 did not differ between the three groups [Fig. 4A and B respectively] and neither did the percentage of monocytes expressing CD86, F (2, 49) = 0.13, p = .87, η^2 = .005 or CD86 expression levels F (2, 49) = 1.41, p = .25, η^2 = .05 [Fig. 4C and D respectively]. Additionally, we also examined the expression of co-stimulatory molecules CD80 and CD86 on the three monocyte subsets and failed to find any significant differences between the hip fracture groups and healthy controls (data not shown).

A reduction in human leukocyte antigen class II (HLA-DR) expression in monocytes has been reported during stressful situations, including after surgery (Handy et al., 2010), during systemic inflammation (Kim et al., 2010), or sepsis (Fingerle et al., 1993) and has been associated with poor outcome. However, the effect of physical distress or depression on HLADR expression in monocytes has not been reported. In this study, we did not observe any differences in the percentage of monocytes expressing HLADR, F (2, 50) = 1.58, p = .21, η^2 = .06 [Fig. 4E] or in the surface expression of this molecule, F (2, 50) = 0.62, p = .53, η^2 = .02 [Fig. 4F] between the three groups. It is possible that the HLADR expression on monocytes was reduced immediately after hip fracture surgery, but we have only examined HLADR expression on monocytes six weeks after injury and surgery.

3.5.1. Cortisol:DHEAS ratio and monocyte superoxide production

To try and determine if depression might be driving the reduced monocyte superoxide response we correlated GDS score with superoxide generation. The data show an association between monocyte superoxide production and depressive symptoms in hip fracture patients, GDS score predicted monocyte superoxide production in hip fracture patients β = −.32, p = .01, ΔR^2 = .10 [Fig. 5A]. We have recently reported an increase in the cortisol and the cortisol:DHEAS ratio in the patients with hip fracture and depression compared to hip fracture alone or to healthy controls (Duggal et al., 2013). However, we did not find a significant association between the serum cortisol:DHEAS ratio, β = −.12, p = .37, ΔR^2 = .01 (data not shown), or serum cortisol,
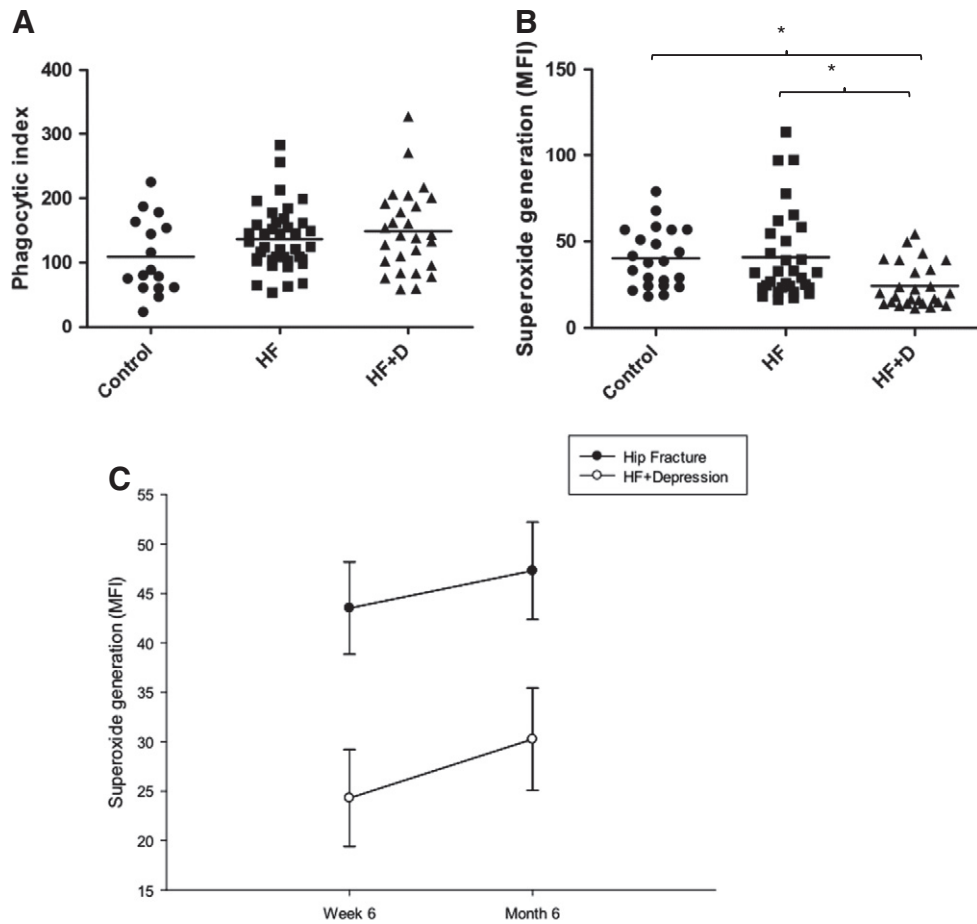
Fig. 2. Monocyte phagocytosis and superoxide production on stimulation with *E. coli* in hip fracture patients. (A) Monocyte phagocytic index for hip fracture patients with depression (n = 26), hip fracture patients without depression (n = 31) and healthy controls (n = 18). The solid bar represents the mean value. (B) Monocyte superoxide production (MFI) for hip fracture patients with depression (n = 27), hip fracture patients without depression (n = 40) and healthy controls (n = 22). The solid bar represents the mean value. (C) Mean monocyte superoxide generation for hip fracture patients with depression (n = 21) at 6 weeks and 6 months post-injury. Data are mean ± SEM and * indicates p < 0.05 and ** indicates p < 0.005.

$\beta = -.129$ p = .13, $\Delta R^2 = .03$, and monocyte superoxide production in the hip fracture patients [Fig. 5B].

4. Discussion

The high incidence of depression after hip fracture, seen in approximately one-third of our hip fracture patients at six weeks post-surgery, is consistent with previous studies of depression in hip fracture patients (Holmes and House, 2000; Lenze et al., 2007). In this study, for the first time we report that the psychological stress of depression rather than the physical stress of a hip fracture had a negative effect on monocyte function. Interestingly, the effect of depression on monocyte function was not universal and was restricted to impairment in bactericidal properties, specifically superoxide generation, with no effect on phagocytic ability, TNFα secretion or expression of co-stimulatory and antigen presenting molecules. Further, this suppressed monocyte functioning observed in hip fracture patients with depression persisted up to six months after injury, suggesting that depression in hip fracture patients results in a long term suppression of monocyte bactericidal properties.
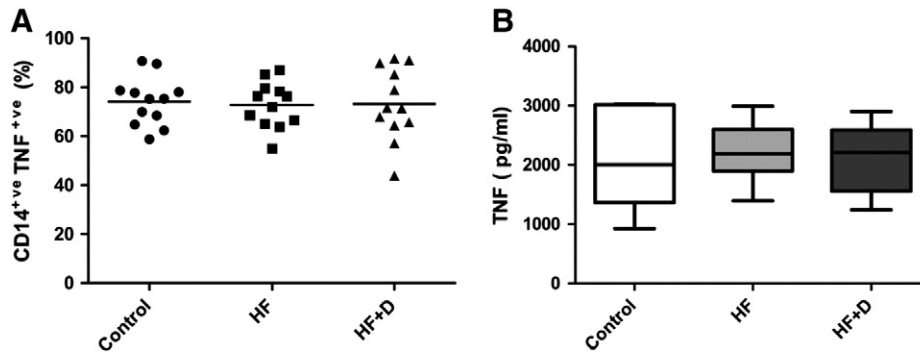

Fig. 3. Monocyte cytokine production capacity upon LPS stimulation in hip fracture patients six weeks post-surgery. (A) The percentage of monocytes producing TNFα for hip fracture patients with depression (n = 12), hip fracture patients without depression (n = 12) and healthy controls (n = 12). The solid bar represents the mean value. (B) TNFα secretion by monocytes upon LPS stimulation in hip fracture patients with depression (n = 12), hip fracture patients without depression (n = 12) and healthy controls (n = 12). Data are mean ± SEM.
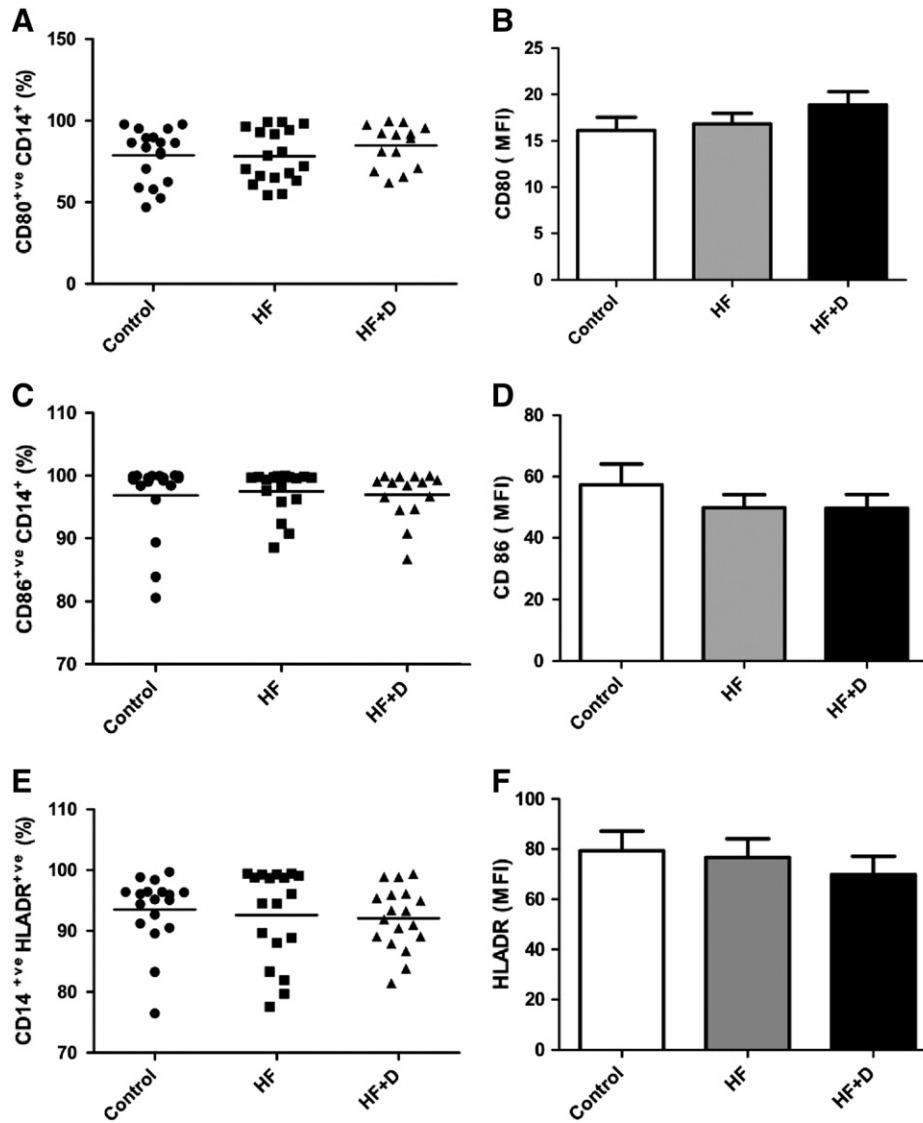
Fig. 4. Monocyte expression of co-stimulatory molecules and HLA-DR in hip fracture patients six weeks post-surgery. (A) The percentage of CD80$^+$ monocytes in hip fracture patients with depression (n = 15), hip fracture patients without depression (n = 16) and healthy controls (n = 18). (B) CD80 expression by monocytes from hip fracture patients with depression (n = 15), hip fracture patients without depression (n = 16) and healthy controls (n = 18). (C) The percentage of CD86$^+$ monocytes in hip fracture patients with depression (n = 15), hip fracture patients without depression (n = 18) and healthy controls (n = 18). (D) CD86 expression by monocytes from hip fracture patients with depression (n = 15), hip fracture patients without depression (n = 18) and healthy controls (n = 18). (E) The percentage of HLADR$^+$ monocytes in hip fracture patients with depression (n = 18), hip fracture patients without depression (n = 18) and healthy controls (n = 18). (F) HLADR expression by monocytes from hip fracture patients with depression (n = 18), hip fracture patients without depression (n = 18) and healthy controls (n = 18).
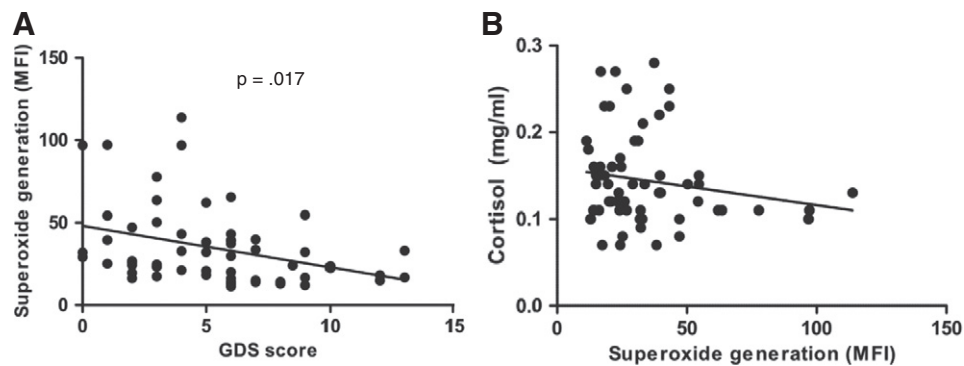

Fig. 5. Relationship of cortisol and cortisol:DHEAS ratio with monocyte superoxide production in hip fracture patients with depression. Linear regression plots for the relationship between (A) GDS score and superoxide generation by monocytes; (B) serum cortisol and superoxide generation by monocytes in hip fracture patients (n = 62).

On examining the effect of physical trauma (hip fracture) and psychological stress (depressive symptoms) on peripheral monocyte counts we did not observe any significant differences in peripheral monocyte count between our three groups, which is in line with previous findings of unaltered circulating monocyte counts in depressed individuals (Schlatter et al., 2004). Further, the presence of depressive symptoms in hip fracture patients does not have an effect on the peripheral frequency of $CD14^{+ve}CD16^{-ve}$ or $CD14^{+ve}CD16^{+ve}$ monocytes, which has also been previously reported in depressed patients (Schlatter et al., 2004).

Previous studies evaluating the impact of ageing on innate immunity have reported impairments in monocyte reactive oxygen species generation in older adults (Panda et al., 2009; Alvarez and Santa Maria, 1996). The question addressed here was whether stress would further worsen the age associated impairments in monocyte bactericidal properties. Our previous work has reported reduced superoxide generation by neutrophils in older hip fracture patients (Butcher et al., 2005), and our recent analysis of neutrophil function in this patient cohort showed that hip fracture patients with depression had reduced superoxide generation compared to hip fracture patients without depression and healthy controls (Duggal et al., 2013). Previous studies have also associated psychological stressors, such as bereavement in older adults with impaired neutrophil superoxide generation (Khanfer et al., 2011), but to our knowledge our study is the first to examine the effect of stress on superoxide production by monocytes in older adults. However, one study reported an increase in superoxide generation by monocytes in depressed individuals, but in this study the monocyte parameters were measured in only ten individuals with major depression and these were all young subjects (Schlatter et al., 2004). Taken together, our data suggest that superoxide generation by innate immune cells is most susceptible to psychological stress. This impairment in neutrophil and monocyte defences may be of clinical significance and might underlie the increased susceptibility to infections previously reported in hip fracture patients (Butcher et al., 2005), though this study was not adequately powered to detect a difference in infection rates.

Neuro-hormonal dysregulation is a prominent finding in depression (Gold and Chrousos, 2002). Previous studies have reported an association between psychological wellbeing and HPA axis activity, specifically an increased cortisol level has been reported in depressed patients (Gold et al., 1988; Burke et al., 2005). We have also previously reported an increased serum cortisol:DHEAS ratio in hip fracture patients (Butcher et al., 2005) and the same was found here for both cortisol and the cortisol:DHEAS ratio, but only for the patients who developed depression. In vitro studies have reported a suppressive effect of glucocorticoids on monocyte bactericidal properties, especially superoxide generation (Tsianakas et al., 2012; Ehrchen et al., 2007). The activation of the NADPH oxidase complex involving association of cytosolic components $p47^{phox}$, $p67^{phox}$, and $p40^{phox}$ with membrane bound $gp91^{phox}$ and $p22^{phox}$, catalyses electron transfer from NADPH to oxygen resulting in superoxide generation (Babior et al., 2002). A study examining the effect of dexamethasone on the human monocytic THP-1 cell line reported a down regulation of NADPH oxidase system due to inhibition of expression of $p47^{phox}$ genes (Condino-Neto et al., 1998). However we found no association between either cortisol or the cortisol:DHEAS and superoxide generation, but we did find an association for depression (GDS) and the cortisol:DHEAS ratio.

An age-associated impairment in cytokine production by monocytes (IL6, TNFα) has been reported on stimulation with TLR1/2 agonists (van Duin et al., 2007b; Delpedro et al., 1998). However, the additional effect of physical stress of hip fracture and psychological distress of depression does not appear to further exaggerate the age-associated decline in cytokine production by monocytes. Our findings are consistent with other studies that have reported intact monocyte cytokine production upon stimulation with bacterial components in depressed younger patients (Landmann et al., 1997), suggesting that the ability of monocytes to respond to mitogen stimuli remains unaffected by exposure to chronic stress in both young and older adults.

Monocytes express a spectrum of cell surface molecules including co-stimulatory molecules CD80 and CD86 that play an essential role in for mediating T cell response to infectious agents (van Duin et al., 2007a; Fleischer et al., 1996). Ageing is accompanied by a diminished monocyte co-stimulatory activity, due to reduced CD80 expression (Strohacker et al., 2012). However, the additional physical stress of hip fracture and psychological distress of depression did not affect expression of B7 molecules (CD80 and CD86) on monocytes in older adults. In addition to providing co-stimulatory signals, monocytes are also capable of antigen presentation to T cells via MHC-II molecule human leukocyte antigen DR (HLA-DR), which has been reported to remain unaltered with major depression (Landmann et al., 1997). Our findings are consistent with this study as we found no significant differences in HLADR expression between the two groups of hip fracture patients and healthy controls.

Recovery after hip fracture is a long process and only one-third of patients have been reported to return to their pre-fracture functional status one year after the fracture (Magaziner et al., 2000). Previous findings have suggested that stress induced immune dysregulation may persist even after chronic stress has ablated (Kiecolt-Glaser et al., 1996). This lasting, detrimental effect of stress might reflect a premature ageing of immune system associated with chronic stress (Gouin et al., 2008). In an attempt to examine the long term effect of depression on immune suppression we examined the monocyte function on hip fracture patients with post-fracture depression at six months after surgery and reported a significant suppression in monocyte superoxide generation in hip fracture patients with depression even 6 months post-surgery. The effects of stress on monocyte superoxide function in hip fracture patients are therefore long-lasting.

It should be acknowledged that the present study is not without limitations. With an observational design, direction of causality cannot be determined, thus it is possible that the monocyte function changes and depressive symptoms observed could both result from a confounding variable such as variations in clinical outcomes, e.g., pain, or surgical complications. Our study did not include self-reported measures of pain, however, pain medication and other medications that patients were taking were not associated with either monocyte function or depressive symptoms. Further, neither were there any associations with surgery type or anaesthetic type, which might be expected to influence clinical outcomes. Nonetheless, it should be noted that some unmeasured third factor such as pain might underlie our observed associations.

4.1. Conclusions

In this study, we have proposed that the development of depression in hip fracture patients post-surgery results in immune dysregulation, due to reduced bactericidal functioning of monocytes. The persistence of impairment in monocyte functioning in depressed hip fracture patients six months after the surgery raises the possibility of a long term and detrimental effect of depression and hip fracture on immune function. Indeed these data supplement our previous reports of this patient cohort showing reduced long term physical recovery in those who developed depressive symptoms (Phillips et al., 2013). Despite numerous reports of high prevalence of depression in hip fracture patients, the treatment and prevention of depression in these patients have received limited attention. Our results suggest that management of depression in these patients may have an impact on the recovery of these patients.

Conflict of interest

The authors have no conflicts of interest to declare.

Acknowledgments

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