Relationships between age, frailty, length of care home residence and biomarkers of immunity and inflammation in older care home residents in the UK

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Abstract

Ageing is associated with changes to the immune system, collectively termed immunosenescence and inflammageing. However, the relationships among age, frailty and immune parameters in older people resident in care homes are not well described. We assessed immune and inflammatory parameters in 184 UK care home residents aged over 65 yr and how they relate to age, frailty index and length of care home residence. Linear regression was used to identify the independent contribution of age, frailty and length of care home residence to the various immune parameters as dependent variables. Participants had a mean age (± SD) of 85.3 ± 7.5 yr, had been residing in the care home for a mean (± SD) of 1.9 ± 2.2 yr at the time of study commencement, and 33.2% were severely frail. Length of care home residence and frailty index were correlated but age and frailty index and age and length of care home residence were not significantly correlated. All components of the full blood count, apart from total lymphocytes, were within the reference range; 31% of participants had blood lymphocyte numbers below the lower value of the reference range. Among the

components of the full blood count, platelet numbers were positively associated with frailty index. Amongst plasma inflammatory markers, C-reactive protein (CRP), interleukin-1 receptor antagonist (IL-1ra), soluble E-selectin and interferon gamma-induced protein 10 (IP-10) were positively associated with frailty. Plasma soluble vascular cell adhesion molecule 1 (sVCAM-1), IP-10 and tumor necrosis factor receptor II (TNFRII) were positively associated with age. Plasma monocyte chemoattractant protein 1 was positively associated with length of care home residence. Frailty was an independent predictor of platelet numbers, plasma CRP, IL-1ra, IP-10 and sE-selectin. Age was an independent predictor of activated monocytes and plasma IP-10, TNFRII and sVCAM-1. Length of care home residence was an independent predictor of plasma MCP-1. This study concludes that there are independent links between increased frailty and inflammation and between increased age and inflammation amongst older people resident in care homes in the UK. Since, inflammation is known to contribute to morbidity and mortality in older people, the causes and consequences of inflammation in this population should be further explored.

Introduction

The number and proportion of older people is increasing in many societies (1, 2). Ageing increases the risk of morbidity, bringing with it loss of independence, increased health and social care costs, and for many older people, the need to move to a care home. Ageing is also associated with changes to the immune system, collectively termed immunosenescence (3-7) and inflammageing (8-11). Immunosenescence involves changes in the numbers of different immune cells in the bloodstream and reductions in their function (3-7). For example, there is reduced production and export of naïve T lymphocytes into the blood (and lymphoid tissues) during ageing with a loss in T cell receptor diversity and an accumulation of memory T lymphocytes (3-6). The overall result of these changes are lowered numbers of T lymphocytes in the blood and impaired T lymphocyte responsiveness (3-7). Immunosenescence also affects B lymphocyte numbers and function and the function of antigen presenting cells and some components of innate immunity (3-7). Inflammageing is seen as an increase in blood plasma or serum concentrations of the acute phase protein C-reactive protein (CRP) and of inflammatory cytokines like interleukin (IL)-6 (8-11). This may reflect sensitized pro-inflammatory signaling pathways in older people. Together these changes contribute to the increased prevalence and severity of infections (12, 13), the poorer responses to vaccinations (13-16) and the increased likelihood to suffer illness and disability (17) that occur with ageing. However, ageing is heterogeneous and occurs at different rates in different individuals; different settings may influence the ageing process, for example by providing different access to a good diet, physical activity, mental stimulation and social interactions. It is described that free-living older individuals have a significantly better quality of life when compared with older people in institutional care homes (18-20). This may relate to the different experiences offered outside and inside care homes which may themselves contribute to the ageing process.

Frailty is currently recognized as a “geriatric syndrome” (21, 22). Categorization of frailty has traditionally been according to physical mobility and strength (23), although there is also a cognitive component to frailty as recognized in some scales for evaluating the extent of frailty among older people (24). Frail older adults are at increased risk of adverse health outcomes, including falls, hospitalization, and mortality (25, 26). It has been suggested that one of the important pathways of frailty development is the immune/inflammatory pathway (27). Inflammation has also been linked to a wide range of chronic diseases of common prevalence within older populations (8, 9, 28). Age, frailty and length of care home residence might be linked to adverse outcomes (29). In order to better understand the relationships of age, frailty and length of care home residence with immunosenescence and inflammageing, we measured a range of immune and inflammatory markers

in 184 UK care home residents aged over 65 yr and investigated the relevant associations. We assessed static measures in blood (full blood count, immune phenotypes, plasma immune mediator concentrations, plasma CRP) as well as blood immune cell responses after ex vivo challenge (phagocytosis, blood culture responses to immune stimulation) and included components of both innate and acquired immunity. Many of these markers have not been well explored in the context of ageing or frailty or in older people in the care home setting.

Methods

Participants

This cross-sectional study is embedded within the "Probiotics to reduce infections in care home residents" (PRINCESS) trial which is a two-arm double-blind individually-randomized controlled trial, involving three academic centres in the UK (Universities of Cardiff, Oxford and Southampton). The full protocol (30) and the main outcomes (31) of the PRINCESS trial have been published. The PRINCESS trial was approved by the Wales REC 3 (15/WA/0306) and is registered at www.controlled-trials.com as ISRCTN16392920. Care home residents were eligible for participation if they were aged 65 yr or older and willing and able to give informed consent for participation; if they lacked capacity, a consultee could complete a consultee declaration for participation on their behalf. Exclusions were immunocompromise (ongoing immune-suppressants; long-term, high-dose, oral, intramuscular or intravenous steroids), lactose intolerance, taking ongoing regular probiotics, or temporary residence in the care home. Care homes were residential, nursing or mixed. Here we report frailty and immune parameters in a subset of participants whose data was available at study entry (n = 184, although not all immune parameters were available for all these participants). Data were not available for all participants in the main PRNCESS trial and in this sub-study because a) participants did not consent to take part in the immune sub-study of PRINCESS; or b) insufficient blood was collected to measure some or any of the immune parameters; or c) the blood arrived at the University of Southampton, where immune measurements were made, outside of a time window pre-determined based upon an earlier study (32).

Assessment of frailty

Frailty index was determined according to the scale described elsewhere (24). The scale has 9 categories defined as: 1 = Very fit for their age (active, energetic and motivated); 2 = Well (absent symptomatology of disease but less active); 3 = Managing well (medical problems under control but not regularly active); 4 = Vulnerable (symptoms that limit activities but not decedent on others); 5 = Mildly frail (impairment of daily activities); 6 = Moderately frail (progressive impairment and declined activities); 7 = Severely frail (completely dependent cognitively or physically but not terminally ill); 8 = Very severely frail (completely dependent and approaching the end of life); 9 = Terminally ill (life expectancy < 6 mo).

Measurement of immune parameters

Blood was collected into EDTA or heparin at the care homes and was posted to the University of Southampton. Whole blood was used to determine full blood count, for immune phenotyping, for assessment of neutrophil and monocyte phagocytosis, and for cultures to determine production of immune mediators after stimulation. Plasma was prepared for measurement of CRP and immune mediator concentrations. Immune parameters were measured as described in detail previously (32) . Briefly, full blood count was determined in blood collected into EDTA using an automated UniCel Beckman Coulter Dxl 800 (Beckman Coulter, High Wycombe, UK). Full blood collected into heparin was used for immune phenotyping using flow cytometry following staining with

fluorescently-labelled antibodies to immune cell surface structures. Blood (500 µl) was placed in BD Trucount™ tubes (BD Pharmingen Oxford, UK). Antibodies were purchased from BD Pharmingen (Oxford, UK). Table 1 lists the details of the immune phenotyping. Staining was performed at room temperature for 20 minutes and protected from light. BD-FACS lysing solution (1 ml; BD Pharmingen Oxford, UK) was added and tubes were incubated for 20 minutes. Tubes were vortexed and placed at room temperature in a dark place. Tubes were analysed on a BD FACS LSRF Fortessa TM X-20 Special order (BD Biosciences, San Jose, CA). Isotype controls were run at a medium flow rate. 10,000 events were collected for all samples in tubes containing Trucount beads. Beads were gated and 5,000 events were collected within the bead region. Data analyses were performed with BD FACSDiva 8.0.1 software. Instrument stability was checked daily using the cytometer setup and tracking to evaluate performance with Research Beads™ (BD Biosciences, Oxford, UK).

Phagocytic activity of blood neutrophils and monocytes towards *E. coli* was assessed in heparinsed whole blood (200 µl) using the commercially available Phagotest™ kit (Glycotope Biotechnology GmbH, Heidelberg Germany). Events (20,000) were collected using a BD FACSCalibur flow cytometer (BD Biosciences, San Jose, CA). Both the percentage of cells (neutrophils and monocytes) involved in phagocytosis and their geometric mean fluorescence intensity (reflecting the number of ingested bacteria per cell) were analysed.

For whole blood cultures, 500 µl heparinised whole blood was diluted 1:10 in Roswell Park Memorial Institute1640 culture medium supplemented with penicillin (50 U/ml), streptomycin (50 µg/ml) and L-glutamine (2 mM) (Sigma Aldrich, Gillingham, UK). Diluted blood (990 µl) was added to the wells of a 24-well flat-bottomed cell culture plate. Then, 10 µL of either medium, lipopolysaccharide (LPS; from *E. coli* K12 strain), peptidoglycan (PGN; from *Staphylococcus aureus*) or phytohaemagglutinin (PHA; from *Phaseolus vulgaris*) was added to the wells to obtain final concentrations of 10 µg/ml LPS, 5 µg/ml PGN or 5 µg/ml PHA respectively. Cultures were incubated for 24 hr at 37°C in an atmosphere of 95% air and 5% CO_2. Supernatants were collected by centrifuging the plate at 2000 rpm for 5 min and were then stored at -80°C for analysis. Once all supernatants were ready to be analysed, magnetic luminex assays (Bio-Techne, R&D Systems, Abingdon, UK) were used. Analytes were measured in negative controls and in the medium after stimulation with PGN or LPS and the assay limits of detection (pg/ml) were: tumour necrosis factor (TNF-α) (0.62), interleukin (IL)-1β (0.25), IL-6 (0.38), IL-10 (2.93) and IL-12p70 (2.39). Analytes measured following stimulation with PHA were TNF-α (limit of detection (pg/ml) (1.2) and interferon (IFN-γ) (0.4). Assays were performed according to manufacturer's instructions. Microparticles were resuspended in buffer and read using a Bio-Rad-plex Luminex Analyzer.

Plasma was prepared from 1 ml of heparinised whole blood by centrifugation at 1500 rpm for 5 min and stored at -20ºC prior to analysis. CRP, immune mediators and soluble receptors were measured by magnetic luminex assays (Bio-Techne, R&D Systems, Abingdon, UK). Analytes measured and the assay limits of detection (pg/ml) were CRP (116), TNF-α (0.54), IL-6 (0.31), IL-10 (0.24), IL-17 (1.8), IL-12p70 (2.96), IL-1ra (18), TNF receptor II (TNFRII; 0.5), monocyte chemoattract protein (MCP-1; 9.9), soluble vascular cell adhesion molecule (sVCAM-1; 238), soluble E-selectin (sE-selectin; 18.8), soluble intercellular adhesion molecule (sICAM-1; 87.9), and interferon gamma-induced protein 10 (IP-10; 1.18). Assays were performed according to manufacturer's instructions. Microparticles were resuspended in buffer and read using a Bio-Rad-plex Luminex Analyzer.

Statistical analysis

As this is an exploratory study no power calculation was done. Normality of data was assessed by visual inspection of histogram distributions and by using the Shapiro Wilk and Kolmogorov-Smirnov

tests. Data were not normally distributed. Thus, data are presented using median, interquartile range and percentiles. Comparisons of outcomes between sexes were made using the Mann-Whitney U test. Correlations amongst age (as a continuous variable), frailty index and length of care home residence (as a continuous variable) were investigated using Spearmans's test. Associations between age, frailty index, length of care home residence and each immune parameter were investigated using linear regression. Multivariate analysis using linear regression models was used to examine the independent influence of age, frailty and length of care home residence on each immune parameter. All data were log10 transformed prior to conducting these analyses. Data collation and analysis were performed in SPSS version 22, Microsoft Excel and PRISM software. In all cases a value for $p < 0.05$ was taken to indicate statistical significance; no correction for multiple testing was made.

Results

Participants characteristics

Table 2 shows the characteristics of the subset of participants studied here compared to those of the entire PRINCESS cohort; the characteristics are comparable. The age range of the included care home residents was 65 to 102 yr. They had a mean age (± SD) of 85.3 (± 7.5) yr and had been residing in the care home for a mean (± SD) of 1.89 (± 2.16) yr at the time of study commencement (Table 2), although it is not known if they had previously resided in another care home. There were more women than men (63.4% vs 36.6%). One-third (33.4%) of included participants were severely frail (category 7) and 34.3% were moderately or mildly frail (categories 6 and 5) (Table 2). Age, frailty and duration of care home residence did not differ between women and men (data not shown).

Association amongst age, frailty and length of care home residence

There was a significant positive correlation between length of care home residence and frailty index (Spearman's correlation coefficient = 0.185; $p = 0.023$) as shown in Figure 1a. Age and frailty index and age and length of care home residence were not significantly correlated (Figure 1b, 1c).

Full blood count and immune parameters

Data for the components of the full blood count were mainly within the reference range, apart from lymphocyte numbers (Table 3). Many participants had low blood lymphocyte numbers, with 31% (n = 49) having numbers below the lower value of the reference range. The percentage of women and men with lymphocyte numbers below the lower value of the reference range did not differ. Age, frailty and length of care home residence were not different between those with blood lymphocyte numbers below or within the reference range. A small proportion of participants (n =12; 7.6%) had platelet numbers above the upper value of the reference range. Platelet numbers were higher in women than men (median (10th and 90th centile) 293 (211, 389) vs 251 (168, 386) 10^9/l; $p = 0.039$). Data for immune phenotypes, neutrophil and monocyte phagocytosis, plasma CRP and immune mediator concentrations, and concentrations of immune mediators in stimulated whole blood cultures are shown in Tables 4, 5, 6 and 7, respectively. There are no reference values for these immune outcomes, but Table 4 lists a selection of previously reported vales for immune phenotypes in older individuals (33-35). Participants in the current study had lower numbers of T lymphocytes and natural killer cells and a lower ratio of $CD4^+$ to $CD8^+$ T lymphocytes than reported in these other studies of older adults. Ten percent of participants had a ratio of $CD4^+$ to $CD8^+$ T lymphocytes less than 1 (Table 4). The only immune outcome that differed between sexes was plasma IL-10 concentration, which was higher in men than women (median (10^{th} and 90^{th} centile) 0.66 (0.25, 3.59) vs 0.56 (0.12, 1.64) pg/ml; $p = 0.039$).

Relationship between immune markers and age, frailty and length of care home residence

Univariate analysis

Associations of each immune marker with age, frailty and length of care home residence were investigated. In most cases there was no statistically significant association (supplementary tables S1 to S7). Exceptions were:

- Platelet numbers were positively associated with frailty index ($p = 0.003$).
- Plasma CRP, IL-1ra, sE-selectin and IP-10 were positively associated with frailty index ($p = 0.014$, 0.023, 0.015 and 0.016, respectively) (Figure 2).
- PGN-stimulated IL-10 production was inversely associated with frailty index ($p = 0.031$).
- Plasma sVCAM-1, IP-10 and TNFRII were positively associated with age ($p = 0.023$, 0.002 and 0.002, respectively) (Figure 3).
- Plasma MCP-1 was positively associated with length of care home residence ($p = 0.012$).

Multivariate analysis

A linear regression model was used to identify the independent contribution of age, frailty and length of care home residence to the various immune parameters as dependent variables (supplementary tables S1 to S7). Among the parameters included as part of the full blood count, frailty was a significant predictive factor for platelet numbers (adjusted coefficient 0.23 (95% CI: 0.08, 0.37), $p = 0.002$; Table S1). Among the immune phenotypes, age was a significant predictive factor for activated monocytes as determined by CD86 expression (adjusted coefficient 2.78 (95% CI: 0.87, 4.70), $p = 0.005$; Table S2). Apart from these, none of the covariates was found to contribute significantly to the individual components of the full blood count (Table S1) or the immune cell phenotypes (Table S2). There were also no predictive associations between the covariates and neutrophil or monocyte phagocytosis (Table S3). For immune mediators after stimulation of whole blood cultures, the only predictive association was between frailty and PGN-stimulated IL-10 (adjusted coefficient -0.79 (95% CI: -1.54, -0.04), $p = 0.038$, Table S5). Frailty index, age and length of care home residence each independently predicted some plasma immune mediators (Table S4). Age was a significant predictor of plasma IP-10 (adjusted coefficient 1.77 (95% CI: 0.61, 2.93), $p = 0.003$), TNFRII (adjusted coefficient 1.76 (95% CI: 0.60, 2.92), $p = 0.003$) and sVCAM-1 (adjusted coefficient 1.19 (95% CI: 0.13, 2.26), $p = 0.029$). Frailty index was an independent predictor of CRP (adjusted coefficient 1.18 (95% CI: 0.34, 2.01), $p=0.006$), IL-1ra (adjusted coefficient 0.43 (95% CI: 0.00, 0.87), $p=0.050$), sE-selectin (adjusted coefficient 0.35 (95% CI: 0.05, 0.66), $p = 0.024$) and IP-10 (adjusted coefficient 0.32 (95% CI: 0.32, 0.64), $p = 0.042$). Lastly, length of care home residence was an independent predictor of MCP-1 (adjusted coefficient 0.10 (95% CI: 0.01, 0.19), $p = 0.026$).

Discussion

Few studies have described immune parameters in older people resident in care homes. Here we describe a selection of immune and inflammatory markers in blood and ex vivo immune cell functions in a sample of 184 older people resident in care homes aged 65 to 102 yr and their association with frailty, age and length of care home residence. Almost a third of the participants had low total lymphocyte numbers. Moreover, participants had lower numbers of T lymphocytes and natural killer cells and a lower ratio of $CD4^+$ to $CD8^+$ T lymphocytes than reported in other studies of older adults (33, 34). These findings are consistent with the hallmarks of immunosenescence (13, 36, 37) and would indicate an increased risk of infections and poor vaccination responses (12-16, 38). Lymphocyte numbers were not associated with age or frailty. This contrasts with the report of Collerton et al. (39) who found an inverse association of lymphocyte numbers with frailty, assessed

using two different models, in 845 85 yr olds in the UK. Furthermore, Bernabeu-Wittell et al. (40) identified that low lymphocyte numbers were associated with frailty in hospitalised older people with poly-pathologies; they also identified that frailty was a risk factor for mortality at 12 months. In another study, there was an inverse association between frailty score and lymphocyte count in institutionalised older people, but lymphocyte count did not predict hospitalisations or mortality, although frailty did predict mortality (41). Recently, low lymphocyte counts were shown to associate with frailty in patients with cardiovascular disease (42) .

Other associations identified in the current study indicate links between greater frailty and increased inflammation and between increasing age and increased inflammation. The association between frailty and inflammation is consistent with the proposal that frailty is an inflammatory condition (43, 44), while the associations between age and inflammatory markers or responses are consistent with the concept of inflammageing (45, 46).

A proportion of participants (7.6%) had a platelet count above the upper limit of the reference range. The exact threshold at which platelet numbers become a marker of chronic inflammation has not been clearly defined, but high platelet numbers are related to inflammatory conditions, cancer and infection as well as endothelial dysfunction (47, 48) and atherosclerotic plaque formation (49). Moreover, platelet numbers increased across categories of frailty, findings also confirmed through modelling, where frailty emerged as a significant independent predictor of platelet numbers. Recently, Bodolea et al. (42) found that platelet numbers associate with frailty in patients with cardiovascular disease. Fuentes et al. report that platelet oxidative stress is a novel marker of cardiovascular risk in frail older people (50) and Starr and Deary observed increased platelet numbers over a time-frame of 8 yr in individuals initially aged over 79 yr (51). The current study did not reveal a significant association of platelet numbers with age. Nevertheless, increased platelet numbers could be a marker of mortality risk through increased frailty. Platelets trigger leukocyte adhesion which favours their aggregation. The mechanism seems to be linked to platelet-induced production of adhesion molecules (52, 53).

CD80 and CD86 were used as markers of activated blood monocytes. The linear regression model showed that age was a significant independent predictor of $CD86^{+}$ monocytes over frailty and length of care home residence. Busse et al. demonstrated that monocytes expressing CD86 were increased in elderly individuals (54) and concluded this to be a consequence of immunosenescence/inflammageing, as this trait appeared in both a cohort of elderly individuals with dementia and in healthy age-matched controls (54).

Phagocytic function has been reported to decline with age leading to a failure to remove foreign antigenic particles and autologous senescent cells (55, 56). In the current study, phagocytic function of neutrophils and monocytes was not significantly associated with age, frailty or length of care home residence. These findings do not confirm what has been shown by others where phagocytic function, especially of neutrophils, declined with age (57, 58). However, this may be because the current study only investigated older participants. A previous comparison of neutrophil phagocytosis among three age groups (21-36, 38-56 and 62-83 yr) found a significant age-dependent reduction in the number of phagocytosed *E. coli* (59). Thus, that study investigated a much wider age range than in the current study. It is possible that beyond 65 yr of age, the alteration in phagocytic activity of neutrophils and monocytes becomes less dramatic than the change between young and older or middle-aged and older individuals.

Previous studies have associated markers of inflammation with different chronic and age-related conditions (e.g. cardiovascular disease and dementia (60, 61)). Others have reported that age and frailty are factors associated with inflammatory biomarkers (43, 44, 62). Indeed, researchers have reported that there is a characteristic "cytokinome" (63) in older people with physical frailty and sarcopenia (64), suggesting IP-10 to be a marker of frailty and sarcopenia. The current study identified that IP-10 was associated with frailty. In the current study frailty was also an independent predictor of CRP, IL-1ra and sE-selectin. Previous studies have shown that ageing is associated with increased concentrations of sICAM-1 and sVCAM-1 (8,61). The current study found that sVCAM-1 concentration had an association with age, as did IP-10 and TNFRII. These findings support the idea that inflammatory pathways are upregulated in ageing and in age-related diseases (65).

Beyer et al. suggest that inflammation is related to muscle wasting, facilitating progression of frailty: in a population of 33 geriatric individuals, those with higher MCP-1 showed a significantly lower grip strength and lower lean body mass (66). Animal research has suggested that MCP-1 is a potential biomarker of biological ageing (67). However, one study reported lower plasma MCP-1 in frail compared with non-frail older care home residents (64), while in the current study frailty was not a predictor of MCP-1 concentration.

Other inflammatory markers where frailty appeared as a significant contributory factor over age and length of stay at care home - identified through the regression model - were IL-1ra and the soluble adhesion molecule sE-selectin. IL-1ra opposes the action of pro-inflammatory IL-1 and may be released in an effort to mitigate inflammation. Nevertheless, IL-1ra has been linked as an independent risk factor of morbidity and mortality in the older people resident in care homes (44). Upregulation of the expression of adhesion molecules with frailty has been reported (68, 69).

Inflammageing, either low grade or chronic, is commonly linked to morbidity and mortality (70, 71). Our findings support an association of inflammation with frailty in older people resident in care homes. Inflammageing is a predictor of frailty in elderly (72). Edvardsson et al. have demonstrated that inflammatory markers are related to reduced survival in a follow-up study for one year with frail older people resident in care homes (73).

Experiments to assess cellular responses ex vivo were performed through whole blood cultures. These experiments allowed assessment of inflammatory and immune mediator production via stimulation of toll-like receptor (TLR)2 and TLR4 with PGN and LPS, respectively, as well as T cell stimulation with PHA. The activation of TLR2 and TLR4 leads to increased production of multiple cytokines (74, 75). Findings herein presented showed that IL-10, TNF-α and IL-1β were potently induced by LPS in comparison to PGN. LPS induced median production values 5-fold higher for IL-10, 3.9-fold higher for TNF-α and almost 12-fold higher for IL-1β when compared with PGN. Furthermore, a superior production of IL-12p70 was induced by LPS when compared with PGN, but the difference was less (two-fold). Lastly, IL-6 was similarly induced by both PGN and LPS. PHA stimulates T cells. The production of TNF-α following PHA stimulation was lower than with LPS and PGN. The potent effects exerted by LPS agree with what has been shown by others (76). The association of health and TLR responsiveness, particularly TLR4, in older people resident in care homes has not been widely explored. McFarlin et al. have suggested that TLR4 appears to have a role in regulating the linkage between cytokine production (IL-1β and TNF-α) and physically active lifestyle regardless of age. In that study, a group of older (60-80 yr) and young (18-30 yr) adults were categorised as "active" or "inactive". There were significantly higher production of IL-1β and TNF-α in the inactive group in both young and older people (77). McFarlin et al. also reported lower expression of TLR4 in the active group (77). Similar observations were reported in a group of older

342 women exposed to regular training (78). Current findings certainly suggest an active TLR4 pathway
343 in the older people resident in care homes according to the cytokine production detected in the
344 cultures following LPS stimulation. A predisposition to active responses of innate immune cells via
345 TLR4, and perhaps other pattern recognition receptors, may be one reason for higher circulating
346 concentrations of inflammatory cytokines in older people, one of the hallmarks of inflammageing.

347 IL-10 induced by PGN was significantly inversely associated with frailty. IL-10 is an anti-
348 inflammatory cytokine that counterbalances pro-inflammatory responses (79). The older people
349 resident in care homes appeared to show an imbalance in IL-10 and TNF-α.

350 Our findings may be compared with those of Collerton et al. (39) who measured a range of immune
351 and inflammatory parameters in 845 85 yr olds in the UK and related these to frailty assessed with
352 two different models. As mentioned earlier, that study reported an inverse association between frailty
353 and lymphocyte numbers which was not observed in the current study. This may represent
354 differences in the characteristics of the participants included in the two studies (all were resident in
355 care homes in the current study whereas this was not the case in Collerton et al.; age range was 66 to
356 102 yr in the current study but all participants were aged 85 yr in Collerton et al.) or the smaller
357 sample size of the current study. Collerton et al. also reported positive associations of frailty with
358 total leukocyte and neutrophil counts, which we did not observe. Collerton et al. (39) reported a
359 positive association between frailty and CRP concentrations, as observed in the current study. They
360 also identified a lack of association of frailty with monocyte, basophil and eosinophil counts, ratio of
361 $CD4^+$ to $CD8^+$ lymphocytes, and LPS-stimulated TNF-α and IL-6 production; our observations are
362 consistent with this. Collerton et al. (39) did not report platelet numbers or plasma concentrations of
363 inflammatory mediators, which were associated with frailty in the current study.

364 The current study has several strengths. There were few restrictions on participant inclusion. A broad
365 range of immune and inflammatory outcomes were measured, representing several different
366 components of the immune system; these included static measures in blood (full blood count,
367 immune phenotypes, plasma mediators, CRP) as well cell responses after challenge (phagocytosis,
368 blood culture responses to LPS, PGN and PHA) and components of innate (phagocytosis, blood
369 culture responses to LPS and PGN) and acquired immunity (blood culture responses to PHA).
370 Finally, linear regression modelling was used to identify independent effects of age, frailty and time
371 of care home residence on the outcomes reported. However, the study also has some limitations.
372 Firstly, not all immune outcomes were available for all 184 participants; this is mainly because some
373 blood samples did not arrive at the laboratory within a predetermined time to assure the viability of
374 the immune assay (32). Secondly, the samples were from participants in a randomised controlled trial
375 (30, 31) and this required exclusion of some of the care home residents; thus the findings are not
376 generalisable to all care home residents. Thirdly, we did not collect data on co-morbidities (other than
377 frailty index) or medication use, which might be relevant to immune and inflammatory biomarkers.
378 Finally, since the study was exploratory no power calculation was done, and so non-significant
379 findings must be interpreted with caution, and significant findings interpreted cautiously since we did
380 not correct for the multiple statistical comparisons performed.

381 **Conflict of Interest**

382 The authors declare that the research was conducted in the absence of any commercial or financial
383 relationships that could be construed as a potential conflict of interest.

384 **Author Contributions**

MLown, MLau, RL, KH, DG, FDRH, PL, CCB, and PCC conceptualized and designed the PRINCESS trial; EO-J and RL provided support for the PRINCESS trial; CCB oversaw the conduct of the PRINCESS trial; VMC-H conducted all laboratory research supported by HLF under the supervision of EAM and PCC; VMC-H, KH and DG conducted the statistical analysis; VMC-H and PCC drafted the manuscript; all authors commented on the manuscript and agreed the final version.

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600 **Figure captions**

601 Figure 1. Relationships between a) frailty index and length of care home residence, b) frailty index
602 and age, and c) age and length of care home residence. The relationship between frailty index and
603 length of care home residence was significant (p = 0,023)

604 Figure 2. Relationships between frailty index and plasma concentration of a) CRP, b) IL-1ra, c) sE-
605 selectin, and d) IP-10. All were significant.

606 Figure 3. Relationships between age and plasma concentration of a) IP-10,and b) TNF-RII. Both
607 were significant.

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Tables

Table 1. Details of immune phenotyping.

Immune cell population	CD or CD combination used to identify the population	Fluorochrome used	µl of antibody used/test
T cells	$CD45^+CD3^+$	PE-Cy5/AF647	20/5
Helper T cells	$CD45^+CD3^+CD4^+$	PE-Cy5/AF647/AF488	20/5/5
Cytotoxic T cells	$CD45^+CD3^+CD8^+$	PE-Cy5/AF647/BV605	20/5/5
Activated cytotoxic T cells	$CD45^+CD3^+CD8^+CD25^+$	PE-Cy5/AF647/BV605/PE	20/5/5/20
Regulatory T cells	$CD45^+CD3^+CD4^+CD8^-$ $CD25^{HI}CD127^{LO}$	PE-Cy5/AF647/AF488/ BV605/PE/BV421	20/5/5/5/20/5
Monocytes	$CD45^+CD14^+$	PE-Cy5/PE-Cy7	20/5
Activated monocytes	$CD45^+CD14^+CD80^+$	PE-Cy5/PE-Cy7/BV421	20/5/20
Activated monocytes	$CD45^+CD14^+CD86^+$	PE-Cy5/PE-Cy7/PE	20/5/20
B cells	$CD45^+CD3^-CD19^+$	PE-Cy5/AF647/AF488	20/5/5
Activated B cells	$CD45^+CD3^-$ $CD19^+CD80^+$	PE-Cy5/AF647/AF488/BV421	20/5/5/20
Activated B cells	$CD45^+CD3^-$ $CD19^+CD86^+$	PE-Cy5/AF647/AF488/PE	20/5/5/20
Natural killer cells	$CD45^+CD3^-CD16^+$	PE-Cy5/AF647/BV605	20/5/20

AF, Alexa Fluor; BV, Brilliant violet; Cy5, Cyanine 5; PE, phycoerythrin

Table 2. Characteristics of participants in this study and those of the full PRINCESS cohort at commencement of study and enrolment

Variable	Full PRINCESS cohort				Subset participating in this study			
	n	Mean (SD)	Median (IQR)	Min, Max	n	Mean (SD)	Median (IQR)	Min, Max
Age (yr)	310	85.3 (7.4)	86 (81 to 91)	65, 102	184	83.1 (15.7)	86 (80 to 91)	65, 102
Length of care home residence (yr)	307	1.7 (2.4)	1 (0 to 2)	0, 15	184	1.8 (2.2)	1 (0.4 to 2.4)	0, 15
		Frequency		%		Frequency		%
Sex:	310				183			
Male		103		33.2		67		36.6
Female		207		66.8		116		63.4
Frailty index:	310				140			
1 (Very fit)		4		1.3		1		0.7
2 (Well)		8		2.6		5		3.6
3 (Managing well)		19		6.1		13		9.3
4 (Vulnerable)		11		3.5		7		5.0
5 (Mildly frail)		20		6.5		13		9.3
6 (Moderately frail)		84		27.1		50		35.7
7 (Severely frail)		158		51.0		61		43.6
8 (Very severely frail)		6		1.9		0		0
9 (Terminally ill)		0		0		0		0

639 Table 3. Full blood count results for older people resident in care homes

Variable	Reference range (10^9/l)	n	Median	10^{th} percentile	90^{th} percentile
			Number of cells (10^9/l)		
Neutrophils	2.0 - 7.5	151	4.5	2.90	7.2
Lymphocytes	1.5 - 5.0	157	1.6	0.9	2.5
Monocytes	0.2 - 1.0	158	0.6	0.3	0.9
Eosinophils	0.0 - 0.5	153	0.1	0.1	0.3
Basophils	0.0 - 0.1	153	0.1	0	0.1
Total leukocytes	4 - 11	109	7.4	5.1	10.5
Platelets	140 - 400	158	268	191	390

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Table 4. Blood immunophenotypes in older people resident in care homes along with a comparison of values from the literature

Variable	n	Number of cells/µl			Tavares et al. (33) > 60 y (n=35)	Qin et al. (34) >65 y (n=41)	Seidler et al. (35) >50 y (n=60)
		Median	10th percentile	90th percentile	Mean (SD) cells/µl	Mean (SD) cells/µl	Median (range) cells/µl
T cells	148	1249	875	1726	1336 (630)	1946 (505)	-
Helper T cells	148	859	304	1391	780 (436)	699 (281)	-
Cytotoxic cells	148	648	402	1005	417 (313)	448 (235)	-
Activated cytotoxic T cells	142	224	126	367	-	191 (115)	-
Regulatory T cells	148	40	16	191	-	-	-
Ratio $CD4^+$:$CD8^+$	148	1.3	1.0	1.8	1.8 (1.3)	1.5 (1.2)	-
Monocytes	148	500	255	820	-	-	420 (165 – 903)
Activated monocytes ($CD80^+$)	148	152	36	379	-	-	-
Activated monocytes ($CD86^+$)	148	106	20	275	-	-	-
NK cells	98	81	49	116	-	448 (223)	-
B cells	148	221	102	342	191 (122)	198 (112)	-
Activated B cells ($CD80^+$)	148	119	68	213	-	-	-
Activated B cells ($CD86^+$)	148	118	72	220	-	-	-

Table 5. Phagocytosis of *E. coli* by blood neutrophils and monocytes from older people resident in care homes

Variable	n	Median	10th percentile	90th percentile
Neutrophils with phagocytic activity (%)	147	83.9	64.6	91.6
Geometric median fluorescence intensity (GMFI) of active neutrophils	142	256.8	158.6	378.5
Monocytes with phagocytic activity (%)	147	29.9	13.6	47.9
Geometric median fluorescence intensity (GMFI) of active monocytes	147	182.1	105.9	295.9

681 Table 6. Concentrations of CRP and immune mediators in plasma from older people resident in care
682 homes

Variable	n	Median	10th percentile	90th percentile
CRP (mg/l)	85	2.7	0.5	16.3
sICAM-1 (ng/ml)	95	386	208	764
IL-1ra (pg/ml)	95	1559	705	4644
sE-Selectin (ng/ml)	95	22.8	11.3	39.8
sVCAM-1 (ng/ml)	95	791	432	1391
MCP-1 (pg/ml)	95	356	165	691
IP-10 (pg/ml)	95	152	75	285
IL-17A (pg/ml)	95	0.9	0.6	6.9
TNFRII (pg/ml)	95	4072	2119	7963
IL-6 (pg/ml)	96	4.4	1.7	20.4
IL-10 (pg/ml)	96	0.6	0.1	1.8
TNF-α (pg/ml)	96	17.7	9.2	26.4

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690 Table 7. Immune mediator concentrations in stimulated cultures of whole blood from older people
691 resident in care homes

Variable	n	Median	10th percentile	90th percentile
Lipopolysaccharide-stimulated cultures				
IL-10 (pg/ml)	86	2428	473	10780
TNF-α (pg/ml)	86	13231	3358	32884
IL-6 (ng/ml)	86	47.6	15.7	87.2
IL-12p70 (pg/ml)	86	24.9	11.6	118.7
IL-1β (pg/ml)	86	4090	1476	14588
Peptidoglycan-stimulated cultures				
IL-10 (pg/ml)	86	468	90	2049
TNF-α (pg/ml)	86	3391	564	11334
IL-6 (ng/ml)	86	42.4	11.9	100.6
IL-12p70 (pg/ml)	86	14.3	5.3	64.0
IL-1β (pg/ml)	86	318	29	1448
Phytohaemagglutinin-stimulated cultures				
IFN-γ (pg/ml)	86	5.2	0.2	55.1
TNF-α (pg/ml)	86	1846	658	3472

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