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The role of inflammation in muscle aging

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Chapter 6

Pro-inflammatory capacity of classically activated monocytes relates positively to muscle mass and strength

Abstract

Background: In mice, monocytes that exhibit a pro-inflammatory profile enter muscle tissue after muscle injury and are crucial for clearance of necrotic tissue and stimulation of muscle progenitor cell proliferation and differentiation. The aim of this study was to test if pro-inflammatory capacity of classically activated (M1) monocytes relates to muscle mass and strength in humans.

Methods: This study included 191 male and 195 female subjects (mean age 64.2 years and 61.9 years, respectively, standard deviation 6.4) of the Leiden Longevity Study. Pro-inflammatory capacity of M1 monocytes was assessed by ex vivo stimulation of whole blood with Toll-like receptor (TLR) 4 agonist lipopolysaccharide (LPS) and TLR-2/1 agonist tripalmitoyl-S-glycerylcysteine (Pam3Cys-SK4), both M1 phenotype activators. Cytokines that stimulate M1 monocyte response (IFN- γ and GM-CSF) as well as cytokines that are secreted by M1 monocytes (IL-6, TNF- α , IL-12 and IL-1 β) were measured. Analyses were adjusted for age, height and body fat mass.

Results: Upon stimulation with LPS, the cytokine production capacity of IFN- γ , GM-CSF, and TNF- α was significantly positively associated with lean body mass, appendicular lean mass and handgrip strength in men, but not in women. Upon stimulation with Pam3Cys-SK4, IL-6, TNF- α and IL-1 β was significantly positively associated with lean body mass and appendicular lean in women, but not in men.

Conclusions: Taken together, this study shows that higher pro-inflammatory capacity of M1 monocytes upon stimulation is associated with muscle characteristics and sex dependent.

6.1 Introduction

In mice, monocytes and macrophages are crucial for muscle regeneration. After skeletal muscle injury, monocytes with a pro-inflammatory profile migrate from the blood into the muscle tissue and differentiate into pro-inflammatory cytokine producing macrophages, which clear necrotic tissue and stimulate muscle progenitor proliferation (Arnold *et al.*, 2007; Tidball & Wehling-Henricks, 2007). Two days after injury, pro-inflammatory macrophages in mice change into macrophages with an anti-inflammatory phenotype to stimulate further differentiation of muscle progenitor cells and fusion of myotubes (Arnold *et al.*, 2007; Lu *et al.*, 2011a). Injection of ex vivo activated human

macrophages in a rat model for myocardial infarction has been demonstrated to accelerate vascularisation and tissue repair and improve cardiac remodelling and function (Leor *et al.*, 2006). Others showed in mice that loss of signal transducer and activator of transcription 1 (STAT-1) in bone marrow-derived cells increases the levels of pro-inflammatory cytokines in the skeletal muscle after injury and also accelerates muscle regeneration (Gao *et al.*, 2012). The pro-inflammatory role of monocytes and macrophages in human skeletal muscle has not been investigated yet.

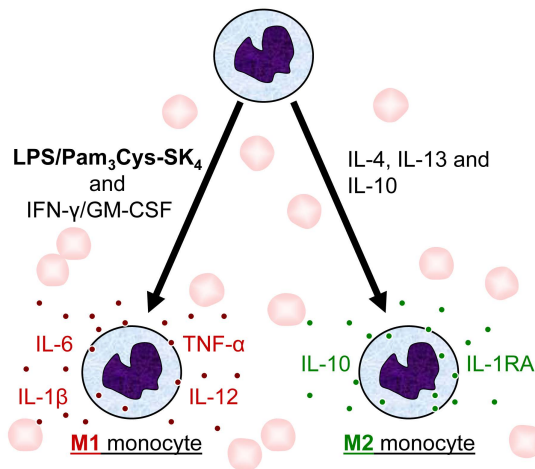


Figure 6.1. Cytokines that stimulate M1 and M2 monocyte response and cytokines, which are secreted by M1 and M2 monocytes. Monocytes stimulated ex-vivo with lipopolysaccharide (LPS) or tripalmitoyl-S-glycerylcysteine (Pam3Cys-SK4) differentiate into classically activated (M1) monocytes with a pro-inflammatory phenotype (Mills *et al.*, 2000; Wang *et al.*, 2007; Navarro-Xavier *et al.*, 2010). Exposure to interferon gamma (IFN-γ) or granulocyte/macrophage colony-stimulating factor (GM-CSF) has a priming and stimulatory effect on M1 monocytes (Kamijo *et al.*, 1993; Bundschuh *et al.*, 1997). Characteristic for M1 monocytes is the production of the pro-inflammatory cytokines interleukin (IL)-6 (IL-6), tumor necrosis factor-α (TNF-α), IL-12, and IL-1β (Mantovani *et al.*, 2002). Monocytes differentiate into alternative activated monocytes (M2) with an anti-inflammatory phenotype upon stimulation with IL-13 and IL-4 alone, or together with IL-10 (Mantovani *et al.*, 2002). Characteristic for M2 monocytes is the production of the anti-inflammatory cytokines IL-1 receptor antagonist (IL-1RA) and IL-10 (Mantovani *et al.*, 2002).

In humans, a whole blood stimulation assay has been developed for the assessment of the capacity of a subject's monocytes to produce inflamma-

tory cytokines (Van Furth *et al.*, 1994; Damsgaard *et al.*, 2009a). Earlier studies have shown that the capacity to produce cytokines measured by this assay is highly reproducible (Van der Linden *et al.*, 1998) and under genetic control (De Craen *et al.*, 2005). Depending on the stimulant, monocytes and macrophages express a classically activated (M1) pro-inflammatory phenotype or an alternatively activated (M2) anti-inflammatory phenotype (as schematically depicted in Figure 6.1). In elderly humans, the capacity of monocytes to respond to M1 phenotype activators like toll-like receptor 4 (TLR-4) agonist lipopolysaccharide (LPS) and TLR-2/1 agonist tripalmitoyl-S-glycerylcysteine (Pam3Cys-SK4) (Mills *et al.*, 2000; Wang *et al.*, 2007; Navarro-Xavier *et al.*, 2010), has been reported to be lower compared to younger subjects (Ouyang *et al.*, 2000; Van den Biggelaar *et al.*, 2004; Nyugen *et al.*, 2010), although some found other results (Gabriel, Cakman & Rink, 2002). Whether cytokine production capacity of monocytes plays a role in age related loss of muscle mass and strength is unknown. Earlier we have shown that a pro-inflammatory cytokine production capacity is beneficial for survival in oldest old subjects (Wijsman *et al.*, 2011). We also found that tumor necrosis factor- α (TNF- α) production capacity relates to decline in handgrip strength in a selected group of subjects aged 85 years (Taekema *et al.*, 2007).

In the present study we further explored the relation between pro-inflammatory cytokine production capacity and skeletal muscle in a cohort of middle aged subjects. We stimulated whole blood ex-vivo with LPS or Pam3Cys-SK4 and assumed that the capacity to respond to LPS or Pam3Cys-SK4 is a proxy for the capacity to respond to stimuli from the skeletal muscle like muscle injury. The production capacity of two types of cytokines were assessed, namely those that are known to stimulate M1 monocyte response including interferon- γ (IFN- γ) and granulocyte macrophage colony-stimulating factor (GM-CSF) and those that are known to be secreted by M1 monocytes including interleukin-6 (IL-6), tumor necrosis factor- α (TNF- α), interleukin-12p40 (IL-12) and interleukin 1- β (IL-1 β) (Mantovani *et al.*, 2002; Solinas *et al.*, 2009). We investigated whether the capacity to stimulate M1 monocyte response and the secretory capacity of M1 monocytes upon stimulation with LPS or Pam3Cys-SK4 are related to muscle mass and strength as an indicator for repair and maintenance of muscle during lifetime history in middle aged subjects.

6.2 Experimental Procedures

6.2.1 Study characteristics

The Leiden Longevity Study consists of offspring from long-lived Caucasian siblings and the partners thereof (Schoenmaker *et al.*, 2006). There were no selection criteria for health or demographic characteristics. Between November 2006 and May 2008, 392 subjects visited the study centre where measurements of innate immune capacity and muscle characteristics were performed. All measurements were carried out by trained research nurses. Information on medical history was obtained from the subjects' general practitioner (response 88.3%). Seven chronic diseases were documented including myocardial infarct, stroke, hypertension, diabetes mellitus, neoplasm, chronic obstructive disease and rheumatoid arthritis. Subjects diagnosed with leukaemia ($n = 2$) or use of oral corticosteroids ($n = 4$) were excluded from the analysis. The Medical Ethical Committee of the Leiden University Medical Centre approved the study and informed consent was obtained from all subjects.

6.2.2 Pro-inflammatory capacity of M1 monocytes

The pro-inflammatory capacity of M1 monocytes was assessed by measuring the cytokine production capacity of whole blood samples upon *ex vivo* stimulation with LPS or Pam3Cys-SK4 as describe elsewhere (Van Furth *et al.*, 1994; Van der Linden *et al.*, 1998). In short, cytokine production capacity was assessed by stimulating *ex vivo* using 2 ml of whole-blood. All venous blood samples were drawn in the morning before 11:00 am to exclude circadian variation. The blood was collected in heparinized tubes and samples were diluted two-fold with RPMI-1640 (Sigma, St. Louis, MO) and incubated after addition of $10 \mu\text{g mL}^{-1}$ or $50 \mu\text{g mL}^{-1}$ *E. coli*-derived LPS (Difco Laboratories, Detroit, MI) or $10 \mu\text{g mL}^{-1}$ N-Palmitoyl-S-[2,3-bis(palmitoyloxy)-(2RS)-propyl]-(R)-cysteinyl-(S)-seryl-(S)-lysyl-(S)-lysyl-(S)-lysyl-(S)-lysine, Pam3Cys-SK4, (EMC Microcollections, Tübingen, Germany) at 37°C and 5% CO_2 for 24 hours. After centrifugation, the supernatants were stored at -80°C until assayed for IFN- γ , GM-CSF, IL-6, TNF- α , IL-12 and IL-1 β using standard ELISA techniques (Central Laboratory of the Blood Transfusion Service, Amsterdam, The Netherlands). IFN- γ , IL-6, TNF- α and IL-1 β were measured in whole blood samples stimulated with $10 \mu\text{g mL}^{-1}$ LPS. GM-CSF and IL-12 were measured in whole blood samples stimulated with $50 \mu\text{g mL}^{-1}$ LPS. The

inter-assay cubic variance (CV) for the LPS stimulated cytokine assays, influenced also by dilutions for high cytokine values, were below 14% for IFN- γ ; below 10% for GM-CSF and IL-6; below 18% for TNF- α and IL-1 β and below 21% for IL-12. The inter-assay CV for Pam3Cys-SK4 stimulated cytokine assays were below 5% for IL-6, below 15% for TNF- α ; below 10% for IL-12 and below 19% for IL-1 β . The intra-assay CV ranged up to 15%. Cytokine levels outside the range of three standard deviations were regarded as outliers and exclude from the analyses (0 to 4 values per cytokine).

6.2.3 Hematologic measurements

Leukocyte and monocyte concentration was measured in venous blood samples using a Sysmex XE-2100 Hematology Analyzer (Sysmex, Etten-Leur, The Netherlands).

6.2.4 Body composition

Next to measurements of body weight and height, a Direct Segmental Multi-frequency Bioelectrical Impedance Analysis (DSM-BIA) was performed using the In-Body (720) body composition analyser (GE healthcare, Madison, WI). Previously we have shown this technique to be a valid tool for the assessment of whole body composition and segmental lean mass measurements in our study population (Ling *et al.*, 2011). A tetrapolar eight-point tactile electrode system was used, which separately measures impedance of the subject's trunk, arms and legs at six different frequencies (1, 5, 50, 250, 500, 1000 kHz) for each of the body segment. Total body water (TBW) was estimate from area, volume, length, impedance and a constant proportion (specific resistivity). Lean body mass was estimated as $TBW/0.73$. Body fat mass was calculated as the difference between TBW and lean body mass. Appendicular lean mass calculation was based on lean mass of both the right and left arms and legs. Subjects wore normal indoor clothing and were asked to stand barefoot on the machine platform with their arms abducted and hands gripping on to the handles. Data on body composition measurement were unavailable in two subjects.

6.2.5 Handgrip strength

Handgrip strength of the dominant side was measured (to the nearest kilogram) using a JAMAR hand dynamometer (Sammons Preston Inc., Boling-

brook, IL) with the subject in an upright position. Subjects were advised to exert maximal force and one test trial was allowed, followed by three test measurements. The best measure recorded was taken for the final analysis.

6.2.6 Physical activity

Physical activity was self-reported using the Dutch version of the International Physical Activity Questionnaire (IPAQ) Short Version. IPAQ includes questions about hours per week spent in vigorous, moderate, slow activity, walking and sitting during the last seven days. Only data on hours of vigorous activity per week were used because validation studies have shown that these data best correlates with objectively measured physical activity (Lee *et al.*, 2011).

6.2.7 Statistical analyses

The Statistical Package for the Social Sciences (SPSS) program version 20.0 (IBM, Armonk, New York) was used for data analysis. Cytokine production capacity values were natural log transformed due to skewness. The production capacity of cytokines that stimulate M1 monocyte response and that are secreted by M1 monocytes were calculated based on sex specific Z-scores of each cytokine. This was performed to be able to compare effect-sizes of the different cytokine production capacities and to combine them in composite scores. A composite score was defined as the mean of the Z-scores of production capacity of cytokines that stimulate M1 monocytes response (IFN- γ and GM-CSF) or that are secreted by M1 monocytes (IL-6, TNF- α , IL-12, IL-1 β). Linear regression analyses included lean body mass, appendicular lean mass and handgrip strength as dependent variables and the production capacity of individual cytokines or their composite scores as independent variables. All analyses were adjusted for age, height and body fat mass. Normalization cytokine production capacity values for monocyte count was performed by dividing the amount of produced cytokine in pg/L by the number of monocytes $\times 10^9/L$. Then the cytokine production levels normalized for monocyte count were naturally log transformed due to skewness. To evaluate the influence of the capacity to stimulate M1 monocyte response upon stimulation with LPS and the secretory capacity of M1 monocytes upon stimulation with LPS together, for each subject the overall mean of the composite scores of the capacity to stimulate M1 monocyte response upon stimulation with LPS and the secretory capacity of M1 monocytes upon stimulation with

LPS were calculated. All analyses were performed for males and females separately and were adjusted for age, height and body fat mass. P -values < 0.05 were regarded as statistically significant.

6.3 Results

Table 6.1 lists the characteristics of the included 386 participants of The Leiden Longevity Study, a study consisting of offspring from long-lived Caucasian siblings and the partners thereof. As some innate immune characteristics have been shown to be sex specific, data were analysed stratified for men and women. Mean age was 64.2 years (SD 6.5) for men and 61.9 years (SD 6.4) for women. In men, mean lean body mass, mean appendicular lean mass and mean handgrip strength were higher compared to women. Median IFN- γ production capacity upon stimulation with LPS was higher in men, and median GM-CSF production capacity upon stimulation with LPS was higher in women. Median IL-6, TNF- α , IL-12 and IL-1 β production capacity upon stimulation with LPS were all higher in men compared to women. Data on IFN- γ and GM-CSF production capacity upon stimulation with Pam3Cys-SK4 were not available because of values below the detection limit. Cytokine production capacity upon stimulation with Pam3Cys-SK4 was not different between men and women.

6.3.1 Cytokine production capacity upon stimulation with LPS

Table 6.2 shows the association of production capacity levels of cytokines that stimulate M1 monocyte response upon stimulation with LPS and production capacity levels of cytokines that are secreted by M1 monocytes upon stimulation with LPS with lean body mass, appendicular lean mass and handgrip strength. In men, a higher IFN- γ and GM-CSF production capacity was significantly associated with higher lean body mass, higher appendicular lean mass and higher handgrip strength. Furthermore, a composite score was calculated from the standardized values of the production capacity levels of cytokines that stimulate M1 monocyte response (IFN- γ and GM-CSF). This composite score was significantly positively associated with lean body mass ($p = 0.014$), appendicular lean mass ($p = 0.018$) and handgrip strength ($p = 0.018$) in men. From the cytokines known to be secreted by M1 monocytes, a higher TNF- α production capacity was significantly positively associated with higher lean body mass ($p = 0.006$), higher appendicular lean mass

($p = 0.027$) and higher handgrip strength ($p = 0.020$) in men. A higher IL-12 production capacity was also significantly associated with higher handgrip strength ($p = 0.007$), but not with lean body mass ($p = 0.21$) and appendicular lean mass ($p = 0.47$). The composite score made up from the production capacity levels of cytokines that are secreted by M1 monocytes (IL-6, TNF- α , IL-12 and IL-1 β) was borderline significantly associated with lean body mass ($p = 0.059$) and handgrip strength ($p = 0.09$) in men. In women, no significant associations were found. Additional adjustment for comorbidities, family trait for longevity and physical activity did not materially change the results (data not shown). Also normalizing cytokine production levels for monocyte count and adjustment for monocyte concentration did not materially change the results (data not shown).

Next, subjects were categorized into quartiles of overall inflammatory capacity of M1 monocytes depending on the overall mean of the composite scores of the production capacity levels of cytokines that stimulate M1 monocyte response and the production capacity levels of cytokines that are secreted by M1 monocytes. Figure 6.2 shows that in men, higher overall inflammatory capacity of M1 monocytes upon stimulation with LPS was associated with higher lean body mass (p for trend = 0.013), higher appendicular lean mass (p for trend = 0.055) and higher handgrip strength (p for trend = 0.015). Men in the lowest overall inflammatory capacity of M1 monocytes quartile ($n = 46$) had on average 59.1 kg (SE 0.6) lean body mass, 26.7 kg (SE 0.2) appendicular lean mass and 47.0 kg (SE 1.0) handgrip strength. Men in the highest overall inflammatory capacity of M1 monocytes quartile ($n = 46$) had on average 61.2 kg (SE 0.6) lean body mass, 27.5 kg (SE 0.2) appendicular lean mass and 50.1 kg (SE 1.0) handgrip strength. In women, no significant associations were found. Additional adjustment for comorbidities, family trait for longevity and physical activity did not materially change the results (data not shown). Also normalizing cytokine production levels for monocyte count and adjustment for monocyte concentration did not materially change the results (data not shown).

6.3.2 Cytokine production capacity upon stimulation with Pam3Cys-SK4

Table 6.3 shows the relation between production capacity of cytokines that are secreted by monocytes upon Pam3Cys-SK4 stimulation and lean body mass, appendicular lean mass and handgrip strength in men and women. In men, no significant associations were found. In women, a higher IL-

6, TNF- α and IL-1 β production capacity was significantly associated with a higher lean body mass and higher appendicular lean mass, but not with handgrip strength. A higher IL-12 production capacity was in women significantly associated with a higher lean body mass. The composite score calculated from the production capacity levels of cytokines that are secreted by M1 monocytes (IL-6, TNF- α , IL-12 and IL-1 β) was significantly associated with lean body mass ($p = 0.022$) and appendicular lean mass ($p = 0.028$) in women, but not in men. Additional adjustment for comorbidities, family trait for longevity and physical activity did not change the results significantly (data not shown). Normalizing the cytokine production levels for monocyte count together with an adjustment for monocyte concentration did also not materially change the results (data not shown).

6.4 Discussion

The aim of this study was to investigate whether the pro-inflammatory capacity of classically activated (M1) monocytes relates to muscle mass and strength in humans. We have shown that in men IFN- γ and GM-CSF production capacity as well as TNF- α production capacity upon stimulation with LPS were positively associated with lean body mass, appendicular lean mass and handgrip strength. IL-12 production capacity upon stimulation with LPS was also positively associated with handgrip strength. In addition, male subjects with a higher overall inflammatory capacity of M1 monocytes upon stimulation with LPS had higher lean body mass and higher handgrip strength. In women no association between inflammatory capacity of M1 monocytes upon stimulation with LPS and muscle characteristics were found. Upon stimulation with Pam3Cys-SK4, IL-6, TNF- α and IL-1 β were positively associated with lean body mass and appendicular lean mass in women, but not in men. In women, IL-12 cytokine production capacity upon stimulation with Pam3Cys-SK4 was also positively associated with lean body mass.

Inflammatory capacity of M1 monocytes was measured by stimulating whole blood *ex vivo* with LPS or Pam3Cys-SK4, both M1 phenotype activators in a highly standardized way (Mills *et al.*, 2000; Wang *et al.*, 2007; Navarro-Xavier *et al.*, 2010). Earlier studies showed that the intra-individual variation of cytokine production upon stimulation with LPS was over 50% of the inter-individual day-to-day variation (Van der Linden *et al.*, 1998; Damsgaard *et al.*, 2009a). Furthermore, over 50% of this intra-individual variation is known to be genetically determined (De Craen *et al.*, 2005). Whole blood

stimulated cytokine production is known to be well correlated with isolated monocytic cytokine production (Damsgaard *et al.*, 2009a). However, also other immune cells than monocytes produce cytokines upon stimulation and could therefore have had a contribution to the total amount of produced cytokines (Cassatella, 1995). In fact, cytokine production upon stimulation in whole blood reflects the complex interaction between different cell types *in vivo*. Neutrophil granulocytes migrate into the tissue after injury before the migration of monocytes, are involved in skeletal muscle repair and are stimulated by GM-CSF (Teixeira *et al.*, 2003). We assume that the capacity to produce cytokines in general is most clinically relevant for the skeletal muscle. We found that normalizing and adjusting the pro-inflammatory capacity of M1 monocytes for monocyte count did not change the results. This suggests that a high cytokine production capacity is not the result of a high white monocyte count *per se*, but a functional characteristic of monocytes themselves.

In mice, monocytes with a pro-inflammatory phenotype like M1 monocytes are known to respond to muscle injury by migrating from the blood into the muscle tissue, clearing necrotic tissue and stimulating the muscle's progenitor cells (Arnold *et al.*, 2007). We assume that the capacity to respond to LPS or Pam3Cys-SK4 is a proxy for the capacity to respond to stimuli from the skeletal muscle like muscle injury. In the present study, we measured the capacity to stimulate M1 monocyte response as well as the secretory capacity of M1 monocytes. The cytokines that stimulate M1 monocyte response are mainly produced by immune cells in the whole blood other than monocytes and induce not only cytokine production response (Kamijo *et al.*, 1993; Bundschuh *et al.*, 1997), but also the phagocytic capacity of M1 monocytes (Mur *et al.*, 1987). The stimulatory capacity of M1 monocytes upon stimulation with LPS was positively associated with lean body mass, appendicular lean mass and handgrip strength in men. This suggests that stimulation of M1 monocytes is important for the function of M1 macrophages within the muscle. It is known that IFN- γ has a "priming" function on monocytes by shifting the dose response curve of monocytes such that the threshold for LPS to affect gene regulation in monocytes is lower (Schoenmaker *et al.*, 2006). IFN- γ , as well as GM-CSF, cause a more sensitive and heightened response to LPS (Kamijo *et al.*, 1993; Bundschuh *et al.*, 1997) and assumingly also to stimuli from the skeletal muscle like muscle injury. In line with this explanation, we report a positive association between handgrip strength and the capacity of M1 monocytes to secrete IL-12 upon LPS stimulation, a cytokine which is known to induce T- cells and NK cells to produce IFN- γ , GM-CSF and TNF- α

(Trinchieri, 2003).

We observed in men a positive relation between the secretory production capacity of TNF- α by M1 monocytes upon stimulation LPS and lean body mass, appendicular lean mass and handgrip strength. In women, we observed a positive relation between the secretory production capacity of TNF- α by M1 monocytes upon stimulation Pam3Cys-SK4 and lean body mass and appendicular lean mass. TNF- α is an extensively studied cytokine with pleiotropic effects. The physiological effect of TNF- α is to regulate myogenesis and muscle regeneration through its activation of p38 MAPK (Warren *et al.*, 2002; Chen, Jin & Li, 2007). This effect is known to be independent of IL-6 (Warren *et al.*, 2002), which could explain the finding that IL-6 production capacity by M1 monocytes upon stimulation with LPS was not significantly associated with muscle mass and strength in our study. In pathologic conditions, like inflammatory myopathies, cachexia and age-related chronic systemic inflammation, TNF- α is related to muscle wasting (Morley, Thomas & Wilson, 2006; Salomonsson & Lundberg, 2006) and is capable to induce muscle proteolysis (Costelli *et al.*, 1993). However, in these pathological conditions TNF- α production is a chronic reaction cellular damage. During chronological aging, the level of systemically circulating cytokines increase, whereas the acutely produced cytokines by leukocytes in response to TLR stimulation or resistance exercise decrease (Van den Biggelaar *et al.*, 2004; Przybyla *et al.*, 2006). Furthermore, a high pro-inflammatory cytokine production capacity upon stimulation with LPS has been associated with low C-reactive proteins levels in subjects aged 85 years (Wijsman *et al.*, 2011). This suggests that cytokine production capacity upon stimulation with LPS should therefore be interpreted differently from levels of systemically circulating cytokines. Earlier we found in a selected group of subjects aged 85 years that the decline over time in handgrip strength, is higher in subjects with a higher TNF- α production capacity upon stimulation with LPS (Taekema *et al.*, 2007). A possible explanation is that TNF- α production capacity is protective for the skeletal muscle earlier in life, but has detrimental effects on age-related loss of muscle mass and strength later in life, as proposed by the theory of antagonistic pleiotropy (Williams, 1957; Van Bodegom *et al.*, 2007). Longitudinal studies in young subjects are needed to prove this explanation.

In contrast to men, no relation between pro-inflammatory capacity of M1 monocytes upon stimulation with TLR-4 agonist (LPS) and muscle mass and muscle strength was found in women. In women however, we did find significant relations between pro-inflammatory capacity of M1 monocytes upon

stimulation with TLR-2/1 agonist (Pam3Cys-SK4) and muscle mass. These findings indicate that within the investigated relation the TLR-4 pathway is predominant in men and the TLR-2/1 pathway in predominant in women. A recent study in mice showed that coxsackievirus B3 infection causes in leukocytes and hearth muscle tissue an up-regulation of TLR-4 in males and up-regulation of TLR-2/1 in females (Roberts *et al.*, 2012). In humans it has been shown that monocytic TLR-2/1 expression, but not monocytic TLR-4 expression, was significantly higher in women compared to men (Ono *et al.*, 2005). We found that the cytokine production capacity in men compared to women was more prominent upon TLR-4 stimulation, than upon TLR-2/1 stimulation. Others found that TNF- α cytokine production capacity per monocyte was higher in men compared to women (Aulock *et al.*, 2006). However, they also found that this difference was similar when a TLR-2/1 instead of a TLR-4 stimulator was used. Nevertheless, changes in sex hormones during the menstrual cycle are known to influence the cytokine production upon TLR-4 stimulation differently in comparison to TLR-2/1 stimulation (Dennison, 2012). Future studies are needed to investigate sex differences in the TLR-4 and TLR-2/1 pathways.

Our findings are consistent with studies showing that inflammatory cells have a beneficial effect on skeletal muscle. Studies in mice have shown that depletion of monocytes is associated with a prolonged clearance of necrotic myofibers, a tendency for increased muscle fat accumulation, an impaired membrane lesion repair and satellite cell differentiation and prevention of muscle fiber growth after muscle injury (Summan *et al.*, 2006; Arnold *et al.*, 2007; Tidball & Wehling-Henricks, 2007). We are the first to report that a higher overall inflammatory capacity of M1 monocytes is associated with a higher lean body mass and higher handgrip strength in men. These results suggest that future anti-inflammatory therapies targeting the effects of inflammation mediated by the innate immune system on age-related diseases like sarcopenia and dynapenia may be potentially harmful for human skeletal muscle.

The strengths of this study include the relatively large number of subjects in which innate immune capacity was measured and the availability of measurements for muscle mass as well as muscle strength. A first limitation is the cross-sectional study design, which makes causal inference difficult. Another limitation is that the inflammatory capacity of M1 monocytes was measured using a ligand secreted by bacteria and not by injured human muscle. However, trauma and pathogens elicit the same inflammatory response and

TLR-4 and TLR-2/1, the pathogen recognition receptors (PRR) for LPS and Pam3Cys-SK4, respectively, are also known to recognize endogenous danger signals (Bianchi & Manfredi, 2009). Furthermore, in the present study we did not investigate the inflammatory capacity of M2 monocytes, since we stimulated whole blood with LPS and Pam3Cys-SK4 and not with IL-4, IL-13 or IL-10 (see Figure 6.1). Future studies are needed to investigate the relation between inflammatory capacity of M1 and M2 monocytes and the relation between the inflammatory capacity of monocytes with respect to that of macrophages.

In conclusion, within a middle aged human population we showed that the inflammatory capacity of M1 monocytes, measured by stimulating whole blood ex-vivo with LPS, is strongly positively associated with muscle mass and strength in men. In women, the inflammatory capacity of M1 monocytes, measured by stimulating whole blood ex-vivo with Pam3Cys-SK4, is strongly positively associated with muscle mass.

Table 6.1. Characteristics of Leiden Longevity Study including middle aged men and women.

Characteristic	Men N=191	Women N=195
Clinical characteristics		
Age, years	64.2 (6.5)	61.9 (6.4)
Comorbidity n, (%)		
Diabetes mellitus	12 (7.1)	8 (4.7)
Hypertension	38 (22.5)	50 (29.1)
Myocardial infarction	7 (4.1)	1 (0.6)
Stroke	7 (4.1)	4 (2.3)
History of malignancy	9 (5.3)	13 (7.6)
Rheumatoid arthritis	0	2 (1.2)
Family longevity trait n, (%)	111 (58.1)	83 (42.6)
Body composition		
Height, cm	177.8 (7.0)	165.4 (6.0)
Weight, kg	84.9 (11.4)	71.5 (12.3)
Body fat mass, kg	21.3 (7.4)	25.2 (9.1)
Lean body mass, kg	60.0 (6.9)	43.6 (4.8)
Appendicular lean mass, kg	27.0 (3.4)	18.9 (2.5)
Handgrip strength, kg	48.0 (8.1)	30.0 (5.5)
IPAQ vigorous activity, h/week (median, IQR)	0.3 (0.0-5.0)	0.0 (0.0-3.5)
Hematologic measurements		
Leukocyte concentration *10 ⁹ /L	6.43 (1.44)	6.34 (1.42)
Monocytes concentration *10 ⁹ /L	0.55 (0.20)	0.47 (0.14)
Cytokine production capacity upon stimulation with LPS		
Capacity to stimulate M1 monocyte response (median, IQR)		
IFN- γ , pg mL ⁻¹	1877 (652-4757)	1766 (856-4097)
GM-CSF, pg mL ⁻¹	118 (71-208)	154 (91-289)
Secretory capacity of M1 monocytes (median, IQR)		
IL-6, pg mL ⁻¹	94231 (78474-121669)	82783 (67488-102946)
TNF- α , pg mL ⁻¹	9487 (6406-13861)	8091 (5805-11020)
IL-12, pg mL ⁻¹	5003 (3899-6793)	4583 (3533-5941)
IL-1 β , pg mL ⁻¹	12184 (9496-16081)	10080 (7428-13145)
Cytokine production capacity upon stimulation with Pam ₃ Cys-SK ₄		
Secretory capacity of M1 monocytes (median, IQR)		
IL-6, pg mL ⁻¹	5835 (1162-11440)	4605 (971-9959)
TNF- α , pg mL ⁻¹	135 (37-365)	122 (33-314)
IL-12, pg mL ⁻¹	463 (125-959)	367 (125-833)
IL-1 β , pg mL ⁻¹	66 (32-160)	55 (24-115)

Data are presented as mean (SD) unless stated otherwise. Family longevity trait: offspring of nonagenarian siblings. LPS: lipopolysaccharide. Pam₃Cys-SK₄: tripalmitoyl-S-glycerylcysteine, M1: classically activated. IFN: interferon. GM-CSF: granulocyte macrophage colony-stimulating factor. IL: interleukin. TNF: tumor necrosis factor. IQR: interquartile range.

Table 6.2. Production capacity of cytokines that stimulate M1 monocyte response upon stimulation with LPS or are secreted by M1 monocytes upon stimulation with LPS related to lean body mass, appendicular lean mass and handgrip strength in men and women.

	Lean body mass		Appendicular lean mass		Handgrip strength	
	kg (SE)	p	kg (SE)	p	kg (SE)	p
Men						
Capacity to stimulate M1 monocyte response						
IFN- γ	0.78 (0.31)	0.013	0.30 (0.13)	0.025	1.35 (0.52)	0.018
GM-CSF	0.60 (0.29)	0.038	0.27 (0.12)	0.032	0.97 (0.48)	0.044
Composite score	0.79 (0.32)	0.014	0.33 (0.14)	0.018	1.28 (0.54)	0.018
Secretory capacity of M1 monocytes						
IL-6	0.46 (0.30)	0.14	0.12 (0.13)	0.37	0.23 (0.50)	0.64
TNF- α	0.78 (0.28)	0.006	0.27 (0.12)	0.027	1.10 (0.47)	0.020
IL-12	0.35 (0.35)	0.21	0.09 (0.12)	0.47	1.26 (0.46)	0.007
IL-1 β	0.19 (0.31)	0.53	0.04 (0.13)	0.76	0.22 (0.51)	0.66
Composite score	0.69 (0.36)	0.059	0.20 (0.16)	0.20	1.03 (0.60)	0.09
Women						
Capacity to stimulate M1 monocyte response						
IFN- γ	0.01 (0.23)	0.96	-0.07 (0.10)	0.53	-0.13 (0.38)	0.74
GM-CSF	0.34 (0.22)	0.12	0.14 (0.10)	0.15	-0.10 (0.36)	0.78
Composite score	0.21 (0.24)	0.39	0.04 (0.11)	0.70	-0.13 (0.40)	0.74
Secretory capacity of M1 monocytes						
IL-6	0.05 (0.25)	0.83	-0.01 (0.11)	0.90	0.20 (0.42)	0.64
TNF- α	-0.10 (0.23)	0.65	-0.10 (0.10)	0.34	-0.07 (0.38)	0.86
IL-12	-0.18 (0.24)	0.46	-0.11 (0.11)	0.32	0.34 (0.40)	0.40
IL-1 β	0.18 (0.22)	0.41	0.06 (0.10)	0.53	0.27 (0.36)	0.46
Composite score	0.05 (0.28)	0.87	-0.03 (0.12)	0.84	0.30 (0.48)	0.53

Estimates are standardized per 1 SD increase in cytokine level. p: p-value. M1 monocyte: classically activated monocytes by LPS stimulation. IFN: interferon. GM-CSF: granulocyte macrophage colony-stimulating factor. IL: interleukin. TNF: tumor necrosis factor. SE= standard error. Cytokine values were natural log transformed. Capacity to stimulate M1 monocyte response composite score: mean Z-score of IFN- γ and GM-CSF values. Secretory capacity of M1 monocytes composite score: mean Z-score of IL-6, TNF- α , IL-12 and IL-1 β values. Results were adjusted for age, height and body fat mass. Values in bold are statistically significant ($P < 0.05$).

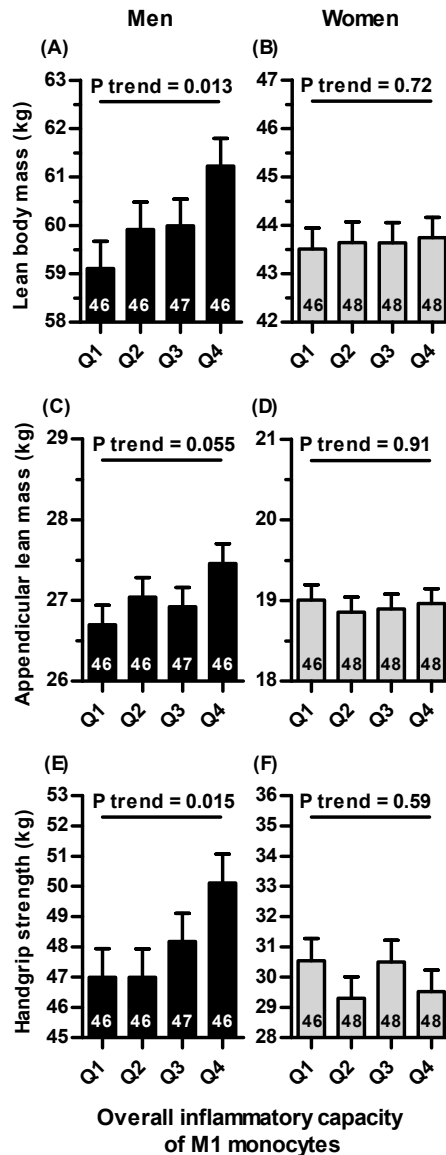


Figure 6.2. Overall inflammatory capacity of M1 monocytes upon stimulation with LPS in men (A,C,E) and women (B,D,F) related to lean body mass (A,B), appendicular lean mass (C,D) and handgrip strength (E,F). Bars represent quartiles of mean overall inflammatory capacity of M1 monocytes upon stimulation with LPS (SE) adjusted for age, height and body fat mass. Overall inflammatory capacity of M1 monocytes was defined as overall mean of the composite scores of the capacity to stimulate M1 response and the secretory capacity of M1 monocytes upon stimulation with LPS. M1: classically activated. Kg: kilogram. Numbers in bars indicate the number of subjects. Data and *p*-for trends are calculated using linear regression with adjustment for age, height and body fat mass. Values in bold are statistically significant ($P < 0.05$).

Table 6.3. Production capacity of cytokines that are secreted by M1 monocytes upon stimulation with Pam3Cys-SK4 related to lean body mass, appendicular lean mass and handgrip strength in men and women.

	Lean body mass		Appendicular lean mass		Handgrip strength	
	kg (SE)	p	kg (SE)	p	kg (SE)	p
Men						
Secretory capacity of M1 monocytes						
IL-6	0.26 (0.29)	0.36	0.10 (0.12)	0.40	-0.10 (0.47)	0.83
TNF- α	0.40 (0.29)	0.17	0.14 (0.13)	0.26	0.04 (0.49)	0.93
IL-12	0.35 (0.29)	0.23	0.11 (0.13)	0.37	0.16 (0.49)	0.75
IL-1 β	0.10 (0.32)	0.75	0.03 (0.14)	0.85	-0.03 (0.52)	0.53
Composite score	0.36 (0.32)	0.26	0.14 (0.14)	0.97	-0.02 (0.54)	0.97
Women						
Secretory capacity of M1 monocytes						
IL-6	0.53 (0.22)	0.016	0.20 (0.10)	0.038	0.41 (0.36)	0.25
TNF- α	0.49 (0.22)	0.027	0.21 (0.10)	0.038	0.21 (0.37)	0.57
IL-12	0.43 (0.21)	0.044	0.18 (0.10)	0.065	0.29 (0.35)	0.41
IL-1 β	0.68 (0.26)	0.009	0.31 (0.11)	0.008	0.29 (0.43)	0.49
Composite score	0.59 (0.25)	0.022	0.25 (0.11)	0.028	0.25 (0.42)	0.54

Estimates are standardized per 1 SD increase in cytokine level. p: p-value. M1 monocyte: classically activated monocytes by Pam₃Cys-SK₄ stimulation. IL: interleukin. TNF: tumor necrosis factor. SE= standard error. Cytokine values were natural log transformed. Secretory capacity of M1 monocytes composite score: mean Z-score of IL-6, TNF- α , IL-12 and IL-1 β values. Results were adjusted for age, height and body fat mass. Values in bold are statistically significant (P<0.05).