Aging Cell (2013) **12**, pp682–689 Doi: 10.1111/acel.12095

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

Karel G. M. Beenakker,[1] Rudi G. J. Westendorp,[1,2] Anton J. M. de Craen,[1,2] Pieternella E. Slagboom,[2,3] Diana van Heemst[1,2] and Andrea B. Maier[1,4]

[1]Department of Gerontology and Geriatrics, Leiden University Medical Center, Leiden, the Netherlands
[2]Netherlands Consortium for Healthy Ageing, Leiden, the Netherlands
[3]Department of Medical Statistics, Molecular Epidemiology, Leiden University Medical Center, Leiden, the Netherlands
[4]Department of Internal Medicine, section of gerontology and geriatrics, VU University Medical Center, Amsterdam, the Netherlands

Summary

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. This study included 191 male and 195 female subjects (mean age 64.2 years (SD 6.4) and 61.9 ± 6.4, respectively) 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 (Pam_3Cys-SK_4), 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-1β) were measured. Analyses were adjusted for age, height, and body fat mass. Upon stimulation with LPS, the cytokine production capacity of INF-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 Pam_3Cys-SK_4, IL-6; TNF-α; and Il-1β were significantly positively associated with lean body mass and appendicular lean in women, but not in men. Taken together, this study shows that higher pro-inflammatory capacity of M1 monocytes upon stimulation is associated with muscle characteristics and sex dependent.

Key words: innate immunity; cytokines; monocyte; macrophage; inflammation; skeletal muscle; handgrip strength; gender differences.

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.*, 2011). Injection of ex vivo activated human macrophages in a rat model for myocardial infarction has been demonstrated to accelerate vascularization and tissue repair and improve cardiac remodeling 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.

In humans, a whole-blood stimulation assay has been developed for the assessment of the capacity of a subject's monocytes to produce inflammatory cytokines (van Furth *et al.*, 1994; Damsgaard *et al.*, 2009). 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 Fig. 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 (Pam_3Cys-SK_4) (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 *et al.*, 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 Pam_3Cys-SK_4 and assumed that the capacity to respond to LPS or Pam_3Cys-SK_4 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 was assessed, namely those that are known to stimulate M1 monocyte response including

Correspondence
Andrea B. Maier, MD, PhD, Department of Gerontology and Geriatrics, Leiden University Medical Center, 2300 RC Leiden, the Netherlands. Tel.: +31 71 526 6640; fax: +31 71 5248159; e-mail: a.maier@vumc.nl

Accepted for publication *14 April 2013*

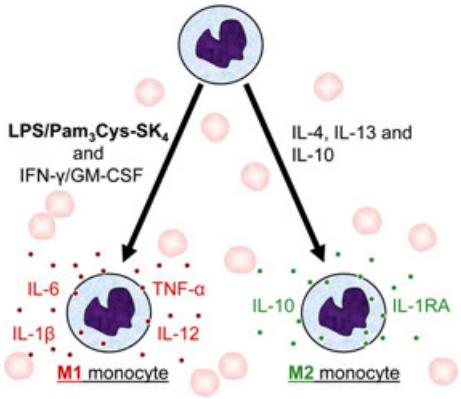

Fig. 1 Cytokines that stimulate M1 and M2 monocyte response and cytokines, which are secreted by M1 and M2 monocytes. Monocytes stimulated *ex vivo* with lipopolysaccharide (LPS) or tripalmitoyl-S-glycerylcysteine ($Pam_3Cys\text{-}SK_4$) differentiate into classically activated (M1) monocytes with a 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-alpha (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).

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-1β) (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 $Pam_3Cys\text{-}SK_4$ are related to muscle mass and strength as an indicator for repair and maintenance of muscle during lifetime history in middle-aged subjects.

Results

Table 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 analyzed 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-1β production capacity upon stimulation with LPS was all higher in men compared to women. Data on IFN-γ and GM-CSF production capacity upon stimulation with $Pam_3Cys\text{-}SK_4$ were not available because of values below the detection limit. Cytokine production capacity upon stimulation with $Pam_3Cys\text{-}SK_4$ was not different between men and women.

Table 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 per 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}	94 231 (78 474–121 669)	82 783 (67488–102 946)
TNF-α, pg mL^{-1}	9487 (6406–13 861)	8091 (5805–11 020)
IL-12, pg mL^{-1}	5003 (3899–6793)	4583 (3533–5941)
IL-1β, pg mL^{-1}	12 184 (9496–16 081)	10 080 (7428–13 145)
Cytokine production capacity upon stimulation with $Pam_3Cys\text{-}SK_4$		
Secretory capacity of M1 monocytes (median, IQR)		
IL- 6, pg mL^{-1}	5835 (1162–11 440)	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-1β, 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; $Pam_3Cys\text{-}SK_4$, tripalmitoyl-S-glycerylcysteine; M1, classically activated; IFN, interferon; GM-CSF, granulocyte macrophage colony-stimulating factor; IL, interleukin; TNF, tumor necrosis factor; IQR, interquartile range.

Cytokine production capacity upon stimulation with LPS

Table 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

Table 2 Production capacity of cytokines that stimulate M1 monocyte response upon stimulation with LPS 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-1β	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-1β	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

Values in bold are statistically significant ($P<0.05$).

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-1β values. Results were adjusted for age, height, and body fat mass.

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-1β) 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 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).

Cytokine production capacity upon stimulation with $Pam_3Cys\text{-}SK_4$

Table 3 shows the relation between production capacity of cytokines that are secreted by monocytes upon $Pam_3Cys\text{-}SK_4$ stimulation and lean body mass, appendicular lean mass, and handgrip

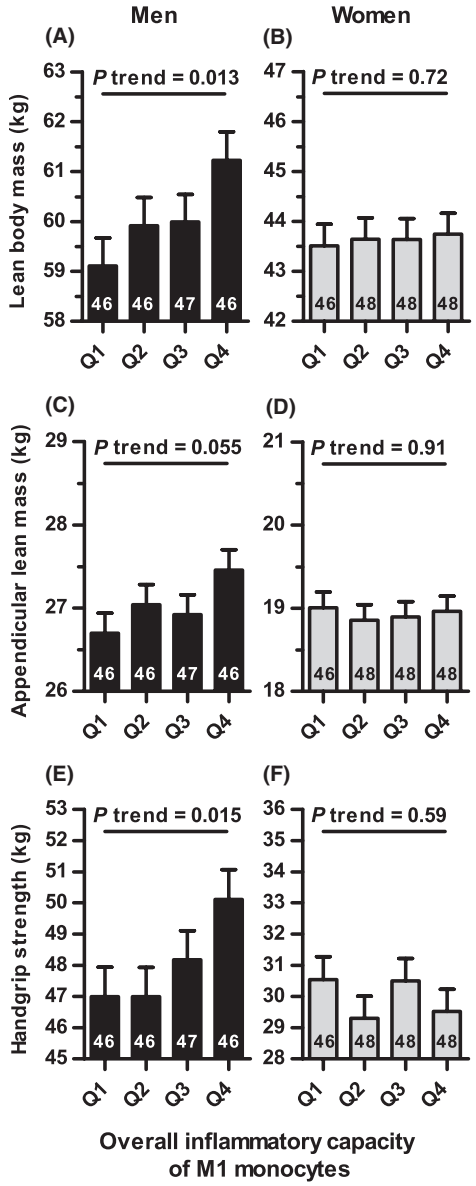

Fig. 2 Overall inflammatory capacity of M1 monocytes upon stimulation with LPS in men (A,C,E) and women (B,D,F) related to lean body mass (A,B), appendicular lean mass (C,D), and handgrip strength (E,F). Bars represent quartiles of mean overall inflammatory capacity of M1 monocytes upon stimulation with 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.

strength in men and women. In men, no significant associations were found. In women, a higher IL-6, TNF-α, and IL-1β 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-1β) 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).

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 associations between inflammatory capacity of M1 monocytes upon stimulation with LPS and muscle characteristics were found. Upon stimulation with $Pam_3Cys\text{-}SK_4$, IL-6; TNF-α; and IL-1β 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 $Pam_3Cys\text{-}SK_4$ was also positively associated with lean body mass.

Inflammatory capacity of M1 monocytes was measured by stimulating whole blood *ex vivo* with LPS or $Pam_3Cys\text{-}SK_4$, 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 intraindividual variation of cytokine production upon stimulation with LPS was over 50% of the interindividual day-to-day variation (van der Linden *et al.*, 1998; Damsgaard *et al.*, 2009). Furthermore, over 50% of this intraindividual 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.*, 2009). 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, which are involved in skeletal muscle repair and are stimulated by GM-CSF (Teixeira *et al.*, 2003). We assume that the capacity to produced 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

Table 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 (029)	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-1β	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-1β	**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

Values in bold are statistically significant (P<0.05).

Estimates are standardized per 1 SD increase in cytokine level. *P*, *P*-value; M1 monocyte, classically activated monocytes by $Pam_3Cys\text{-}SK_4$ 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-1β values. Results were adjusted for age, height, and body fat mass.

stimulating the muscle's progenitor cells (Arnold *et al.*, 2007). We assume that the capacity to respond to LPS or $Pam_3Cys\text{-}SK_4$ 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 (Murray *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 (Schroder *et al.*, 2006). IFN-γ, as well as GM-CSF, causes 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 $Pam_3Cys\text{-}SK_4$ 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 *et al.*, 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 *et al.*, 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 ($Pam_3Cys\text{-}SK_4$) 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 is 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-alpha 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 with TLR-2/1 stimulation (Dennison *et al.*, 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 $Pam_3Cys\text{-}SK_4$, 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, because we stimulated whole blood with LPS and $Pam_3Cys\text{-}SK_4$ and not with IL-4, IL-13, or IL-10 (see Fig. 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 $Pam_3Cys\text{-}SK_4$, is strongly positively associated with muscle mass.

Experimental procedures

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 center 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 leukemia ($n = 2$) 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.

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 $Pam_3Cys\text{-}SK_4$ as described 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 twofold with RPMI-1640 (Sigma, St. Louis, MO, USA) and incubated after addition of 10 or 50 $\mu g\ mL^{-1}$ *E. coli*-derived LPS (Difco Laboratories, Detroit, MI, USA) or 10 $\mu g\ mL^{-1}$N-Palmitoyl-S-[2,3-bis(palmitoyloxy)-(2RS)-propyl]-(R)-cysteinyl-(S)-seryl-(S)-lysyl-(S)-lysyl-(S)-lysyl-(S)-lysine, $Pam_3Cys\text{-}SK_4$, (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-1β using standard ELISA techniques (Central Laboratory of the Blood Transfusion Service, Amsterdam, the Netherlands). IFN-γ, IL-6, TNF-α, and IL-1β were measured in whole-blood samples stimulated with 10 $\mu g\ mL^{-1}$ LPS. GM-CSF and IL-12 were measured in whole-blood samples stimulated with 50 $\mu g\ mL^{-1}$ LPS. The interassay cubic variance (CV) for the LPS-stimulated cytokine assays, influenced also by dilutions for high cytokine values, was below 14% for IFN-γ; below 10% for GM-CSF and IL-6; below 18% for TNF-α and IL-1β; and below 21% for IL-12. The interassay CV for $Pam_3Cys\text{-}SK_4$-stimulated cytokine assays was below 5% for IL-6, below 15% for

TNF-α, below 10% for IL-12, and below 19% for IL-1β. The intraassay CV ranged up to 15%. Cytokine levels outside the range of three standard deviations were regarded as outliers and exclude from the analyses (0–4 values per cytokine).

Hematologic measurements

Leukocyte and monocyte concentrations were measured in venous blood samples using a Sysmex XE-2100 Hematology Analyzer (Sysmex, Etten-Leur, the Netherlands).

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 analyzer (GE healthcare, Madison, WI, USA). 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 estimated 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.

Handgrip strength

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

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 7 days. Only data on hours of vigorous activity per week were used because validation studies have shown that these data best correlate with objectively measured physical activity (Lee *et al.*, 2011).

Statistical analyses

The Statistical Package for the Social Sciences (SPSS) program version 20.0 (IBM, Armonk, New York, USA) 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 was 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-1β). 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 * 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.

Acknowledgments

This study was supported by the Netherlands Genomics/Netherlands Organization for scientific research (NGI/NWO; 05040202 and 050-060-810 NCHA) and the seventh framework program MYOAGE (HEALTH-2007-2.4.5-10). We thank the subjects of the Leiden Longevity Study for their cooperation. We thank Margo van Schie-Troost and Marja Kersbergen-van Oostrom for their excellent work on the whole-blood stimulation assays. None of the authors had conflict of interests.

Author contributions

Study concept and design: Beenakker and Maier. Analysis and interpretation of data: Beenakker, Maier, and Westendorp. Drafting of the manuscript: Beenakker and Maier. Statistical analysis: Beenakker and Maier. Critical revision of the manuscript for important intellectual content: Westendorp, de Craen, Slagboom, and van Heemst. Study supervision: Westendorp and Maier.

References

Arnold L, Henry A, Poron F, Baba-Amer Y, van Rooijen N, Plonquet A, Gherardi RK, Chazaud B (2007) Inflammatory monocytes recruited after skeletal muscle injury switch into anti-inflammatory macrophages to support myogenesis. *J. Exp. Med.* **204**, 1057–1069.

Aulock SV, Deininger S, Draing C, Gueinzius K, Dehus O, Hermann C (2006) Gender difference in cytokine secretion on immune stimulation with LPS and LTA. *J. Interferon Cytokine Res.* **26**, 887–892.

Bianchi ME, Manfredi AA (2009) Immunology. Dangers in and out. *Science* **323**, 1683–1684.

van den Biggelaar AH, Huizinga TW, de Craen AJ, Gussekloo J, Heijmans BT, Frolich M, Westendorp RG (2004) Impaired innate immunity predicts frailty in old age. The Leiden 85-plus study. *Exp. Gerontol.* **39**, 1407–1414.

Bundschuh DS, Barsig J, Hartung T, Randow F, Docke WD, Volk HD, Wendel A (1997) Granulocyte-macrophage colony-stimulating factor and IFN-gamma restore the systemic TNF-alpha response to endotoxin in lipopolysaccharide-desensitized mice. *J Immunol.* **158**, 2862–2871.

Cassatella MA (1995) The production of cytokines by polymorphonuclear neutrophils. *Immunol. Today* **16**, 21–26.

Chen SE, Jin B, Li YP (2007) TNF-alpha regulates myogenesis and muscle regeneration by activating p38 MAPK. *Am. J. Physiol. Cell Physiol.* **292**, C1660–1671.

Costelli P, Carbo N, Tessitore L, Bagby GJ, Lopez-Soriano FJ, Argiles JM, Baccino FM (1993) Tumor necrosis factor-alpha mediates changes in tissue protein turnover in a rat cancer cachexia model. *J Clin Invest.* **92**, 2783–2789.

de Craen AJ, Posthuma D, Remarque EJ, van den Biggelaar AH, Westendorp RG, Boomsma DI (2005) Heritability estimates of innate immunity: an extended twin study. *Genes Immun.* **6**, 167–170.

Damsgaard CT, Lauritzen L, Calder PC, Kjaer TM, Frokiaer H (2009) Whole-blood culture is a valid low-cost method to measure monocytic cytokines – a comparison of cytokine production in cultures of human whole-blood, mononuclear cells and monocytes. *J. Immunol. Methods* **340**, 95–101.

Dennison U, McKernan DP, Scully P, Clarke G, Cryan J, Dinan T (2012) Menstrual cycle influences Toll-like receptor responses. *NeuroImmunoModulation* **19**, 171–179.

van Furth AM, Steenwijk TM, Langermans JA, van Furth R (1994) In vitro effect of dexamethasone, pentoxifylline, and anti-endotoxin monoclonal antibody on the release of proinflammatory mediators by human leukocytes stimulated with *Haemophilus influenzae* type B. *Pediatr. Res.* **35**, 725–728.

Gabriel P, Cakman I, Rink L (2002) Overproduction of monokines by leukocytes after stimulation with lipopolysaccharide in the elderly. *Exp. Gerontol.* **37**, 235–247.

Gao Y, Li Y, Guo X, Wu Z, Zhang W (2012) Loss of STAT1 in bone marrow-derived cells accelerates skeletal muscle regeneration. *PLoS ONE* **7**, e37656.

Kamijo R, Le J, Shapiro D, Havell EA, Huang S, Aguet M, Bosland M, Vilcek J (1993) Mice that lack the interferon-gamma receptor have profoundly altered responses to infection with Bacillus Calmette-Guerin and subsequent challenge with lipopolysaccharide. *J. Exp. Med.* **178**, 1435–1440.

Lee PH, Macfarlane DJ, Lam TH, Stewart SM (2011) Validity of the International Physical Activity Questionnaire Short Form (IPAQ-SF): a systematic review. *Int. J. Behav. Nutr. Phys Act.* **8**, 115.

Leor J, Rozen L, Zuloff-Shani A, Feinberg MS, Amsalem Y, Barbash IM, Kachel E, Holbova R, Mardor Y, Daniels D, Ocherashvilli A, Orenstein A, Danon D (2006) Ex vivo activated human macrophages improve healing, remodeling, and function of the infarcted heart. *Circulation* **114**, I94–100.

van der Linden MW, Huizinga TW, Stoeken DJ, Sturk A, Westendorp RG (1998) Determination of tumour necrosis factor-alpha and interleukin-10 production in a whole blood stimulation system: assessment of laboratory error and individual variation. *J. Immunol. Methods* **218**, 63–71.

Ling CH, de Craen AJ, Slagboom PE, Gunn DA, Stokkel MP, Westendorp RG, Maier AB (2011) Accuracy of direct segmental multi-frequency bioimpedance analysis in the assessment of total body and segmental body composition in middle-aged adult population. *Clin Nutr.* **30**, 610–615.

Lu H, Huang D, Saederup N, Charo IF, Ransohoff RM, Zhou L (2011) Macrophages recruited via CCR2 produce insulin-like growth factor-1 to repair acute skeletal muscle injury. *FASEB J.* **25**, 358–369.

Mantovani A, Sozzani S, Locati M, Allavena P, Sica A (2002) Macrophage polarization: tumor-associated macrophages as a paradigm for polarized M2 mononuclear phagocytes. *Trends Immunol.* **23**, 549–555.

Mills CD, Kincaid K, Alt JM, Heilman MJ, Hill AM (2000) M-1/M-2 macrophages and the Th1/Th2 paradigm. *J Immunol.* **164**, 6166–6173.

Morley JE, Thomas DR, Wilson MM (2006) Cachexia: pathophysiology and clinical relevance. *Am. J. Clin. Nutr.* **83**, 735–743.

Murray HW, Scavuzzo D, Jacobs JL, Kaplan MH, Libby DM, Schindler J, Roberts RB (1987) In vitro and in vivo activation of human mononuclear phagocytes by interferon-gamma. Studies with normal and AIDS monocytes. *J Immunol.* **138**, 2457–2462.

Navarro-Xavier RA, Newson J, Silveira VL, Farrow SN, Gilroy DW, Bystrom J (2010) A new strategy for the identification of novel molecules with targeted proresolution of inflammation properties. *J Immunol.* **184**, 1516–1525.

Nyugen J, Agrawal S, Gollapudi S, Gupta S (2010) Impaired functions of peripheral blood monocyte subpopulations in aged humans. *J. Clin. Immunol.* **30**, 806–813.

Ono S, Tsujimoto H, Hiraki S, Takahata R, Kinoshita M, Mochizuki H (2005) Sex differences in cytokine production and surface antigen expression of peripheral blood mononuclear cells after surgery. *Am. J. Surg.* **190**, 439–444.

Ouyang Q, Cicek G, Westendorp RG, Cools HJ, van der Klis RJ, Remarque EJ (2000) Reduced IFN-gamma production in elderly people following in vitro stimulation with influenza vaccine and endotoxin. *Mech. Ageing Dev.* **121**, 131–137.

Przybyla B, Gurley C, Harvey JF, Bearden E, Kortebein P, Evans WJ, Sullivan DH, Peterson CA, Dennis RA (2006) Aging alters macrophage properties in human skeletal muscle both at rest and in response to acute resistance exercise. *Exp. Gerontol.* **41**, 320–327.

Roberts BJ, Dragon JA, Moussawi M, Huber SA (2012) Sex-specific signaling through Toll-Like Receptors 2 and 4 contributes to survival outcome of Coxsackievirus B3 infection in C57Bl/6 mice. *Biol Sex Differ.* **3**, 25.

Salomonsson S, Lundberg IE (2006) Cytokines in idiopathic inflammatory myopathies. *Autoimmunity* **39**, 177–190.

Schoenmaker M, de Craen AJ, de Meijer PH, Beekman M, Blauw GJ, Slagboom PE, Westendorp RG (2006) Evidence of genetic enrichment for exceptional survival using a family approach: the Leiden Longevity Study. *Eur. J. Hum. Genet.* **14**, 79–84.

Schroder K, Sweet MJ, Hume DA (2006) Signal integration between IFNgamma and TLR signalling pathways in macrophages. *Immunobiology* **211**, 511–524.

Solinas G, Germano G, Mantovani A, Allavena P (2009) Tumor-associated macrophages (TAM) as major players of the cancer-related inflammation. *J. Leukoc. Biol.* **86**, 1065–1073.

Summan M, Warren GL, Mercer RR, Chapman R, Hulderman T, Van Rooijen N, Simeonova PP (2006) Macrophages and skeletal muscle regeneration: a clodronate-containing liposome depletion study. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **290**, R1488–1495.

Taekema DG, Westendorp RG, Frolich M, Gussekloo J (2007) High innate production capacity of tumor necrosis factor-alpha and decline of handgrip strength in old age. *Mech. Ageing Dev.* **128**, 517–521.

Teixeira CF, Zamuner SR, Zuliani JP, Fernandes CM, Cruz-Hofling MA, Fernandes I, Chaves F, Gutierrez JM (2003) Neutrophils do not contribute to local tissue damage, but play a key role in skeletal muscle regeneration, in mice injected with Bothrops asper snake venom. *Muscle Nerve* **28**, 449–459.

Tidball JG, Wehling-Henricks M (2007) Macrophages promote muscle membrane repair and muscle fibre growth and regeneration during modified muscle loading in mice in vivo. *J. Physiol.* **578**, 327–336.

Trinchieri G (2003) Interleukin-12 and the regulation of innate resistance and adaptive immunity. *Nat. Rev. Immunol.* **3**, 133–146.

Van Bodegom D, May L, Meij HJ, Westendorp RG (2007) Regulation of human life histories: the role of the inflammatory host response. *Ann. N. Y. Acad. Sci.* **1100**, 84–97.

Wang Y, Wang YP, Zheng G, Lee VW, Ouyang L, Chang DH, Mahajan D, Coombs J, Wang YM, Alexander SI, Harris DC (2007) Ex vivo programmed macrophages ameliorate experimental chronic inflammatory renal disease. *Kidney Int.* **72**, 290–299.

Warren GL, Hulderman T, Jensen N, McKinstry M, Mishra M, Luster MI, Simeonova PP (2002) Physiological role of tumor necrosis factor alpha in traumatic muscle injury. *FASEB J.* **16**, 1630–1632.

Wijsman CA, Maier AB, de Craen AJ, van den Biggelaar AH, Westendorp RG (2011) An unopposed proinflammatory response is beneficial for survival in the oldest old. Results of the Leiden 85-plus Study. *J. Gerontol. A Biol. Sci. Med. Sci.* **66**, 393–399.

Williams GC (1957) Pleiotropy, natural-selection, and the evolution of senescence. *Evolution* **11**, 398–411.