



Aging is associated with chronic innate immune activation and dysregulation of monocyte phenotype and function

Anna C. Hearps,^{1,2} Genevieve E. Martin,^{1,2} Thomas A. Angelovich,^{1,3} Wan-Jung Cheng,¹ Anna Maisa,¹ Alan L. Landay,⁴ Anthony Jaworowski^{1,2,5†} and Suzanne M. Crowe^{1,2,6†}

¹Centre for Virology, Burnet Institute, GPO Box 2284, Melbourne, Vic. 3004, Australia

²Department of Medicine, Monash University, 99 Commercial Road, Melbourne, Vic. 3004, Australia

³School of Applied Sciences, Royal Melbourne Institute of Technology, Building 223, Plenty Rd, Bundoora, Vic. 3083, Australia

⁴Rush University Medical Center, 1735 W. Harrison St, Chicago, IL, 60612, USA

⁵Department of Immunology, Monash University, Commercial Road, Melbourne, Vic. 3004, Australia

⁶Infectious Diseases Unit, Alfred Hospital, PO Box 315, Melbourne, Vic. 3181, Australia

Summary

Chronic inflammation in older individuals is thought to contribute to inflammatory, age-related diseases. Human monocytes are comprised of three subsets (classical, intermediate and nonclassical subsets), and despite being critical regulators of inflammation, the effect of age on the functionality of monocyte subsets remains to be fully defined. In a cross-sectional study involving 91 healthy male (aged 20–84 years, median 52.4) and 55 female (aged 20–82 years, median 48.3) individuals, we found age was associated with an increased proportion of intermediate and nonclassical monocytes ($P = 0.002$ and 0.04 , respectively) and altered phenotype of specific monocyte subsets (e.g. increased expression of CD11b and decreased expression of CD38, CD62L and CD115). Plasma levels of the innate immune activation markers CXCL10, neopterin ($P < 0.001$ for both) and sCD163 ($P = 0.003$) were significantly increased with age. Whilst similar age-related changes were observed in both sexes, monocytes from women were phenotypically different to men [e.g. lower proportion of nonclassical monocytes ($P = 0.002$) and higher CD115 and CD62L but lower CD38 expression] and women exhibited higher levels of CXCL10 ($P = 0.012$) and sCD163 ($P < 0.001$) but lower sCD14 levels ($P < 0.001$). Monocytes from older individuals exhibit impaired phagocytosis ($P < 0.05$) but contain shortened telomeres ($P < 0.001$) and significantly higher intracellular levels of TNF both at baseline and following TLR4 stimulation ($P < 0.05$ for both), suggesting a dysregulation of monocyte function in the aged. These data show that aging is associated with chronic innate immune activation and significant changes in monocyte function, which may have implications for the development of age-related diseases.

Key words: aging; innate immunity; monocytes; phagocytosis; phenotype; TLR4.

Introduction

Aging is associated with a decline in immune function and increased susceptibility to infections, which is juxtaposed with inflammation and an increased risk of chronic disease. Chronic inflammation and immune activation are pathogenically linked with immune senescence, and the development of inflammatory age-related diseases and pro-inflammatory cytokine levels are sensitive predictors of disease and mortality in older adults. Increased levels of TNF and/or IL-6 are associated with mortality (Bruunsgaard *et al.*, 2003) and increased risk of age-related diseases including cardiovascular disease (Ridker *et al.*, 2000), Alzheimer's disease (Licastro *et al.*, 2003), disability (Ferrucci *et al.*, 1999) and frailty (Leng *et al.*, 2011). The mechanisms responsible for impaired immunity and inflammation in older adults are unclear, but cells of the innate immune system including monocytes and macrophages are the first line of defence against infections and are critical mediators and regulators of the inflammatory response. Despite the fact that these cells play an important role in the pathogenesis of many inflammatory diseases associated with aging such as atherosclerosis (Gerrity & Naito, 1980), the impact of aging on monocyte phenotype and function remains to be fully defined.

Human monocyte subsets are defined on the basis of CD14 and CD16 expression. The major population of monocytes are CD14⁺⁺ CD16[−] (classical monocytes) whilst CD16⁺ monocytes are segregated into intermediate (CD14⁺⁺ CD16⁺) and nonclassical (CD14⁺ CD16⁺⁺) subsets [Ziegler-Heitbrock *et al.*, 2010] and Fig. 1]. Transcriptional profiling suggests that monocytes expressing CD16 (nonclassical and intermediate subsets) exhibit a phenotype similar to specific dendritic cell subsets (DCs) and macrophages and may represent an alternative state of differentiation (Ancuta *et al.*, 2009). Consistent with this, nonclassical monocytes reportedly patrol the endothelium and sense and respond to viral antigens, showing similarities to monocyte-derived DCs (Cros *et al.*, 2010). CD16⁺ monocytes produce more pro-inflammatory cytokines upon stimulation, whilst CD14⁺⁺ monocytes (classical and intermediate) are more efficient phagocytes and classical monocytes are the major producers of reactive oxygen species (Cros *et al.*, 2010). The three monocyte subsets exhibit different cytokine production profiles following Toll-like receptor (TLR) ligation (Cros *et al.*, 2010; Nyugen *et al.*, 2010), reinforcing their discreet functions; however, the functional roles of these subsets and how these properties change with age remain to be fully characterized.

Increasing age is associated with altered monocyte phenotype including an expansion of the CD16⁺ monocyte population (Sadeghi *et al.*, 1999; Nyugen *et al.*, 2010), which also occurs during bacterial (Nockher & Scherberich, 1998) and untreated HIV infection (Hearps *et al.*, 2012). Surface expression of certain proteins such as HLA-DR, CX₃CR1 and CD62L on monocytes is also reduced in older adults, which may affect monocyte survival, adherence and migration to sites of inflammation (De Martinis *et al.*, 2004; Seidler *et al.*, 2010). Whether there are age-related changes in human monocyte function, such as phagocytosis and pro-inflammatory cytokine production, is unclear, due in part to conflicting

Correspondence

Dr Anna C. Hearps, Centre for Virology, Burnet Institute, GPO Box 2284, Melbourne, Vic. 3001, Australia. Tel.: +61 3 9282 2150; fax: +61 3 9282 2142; e-mail: annah@burnet.edu.au

[†]These authors contributed equally to this work.

Accepted for publication 13 June 2012

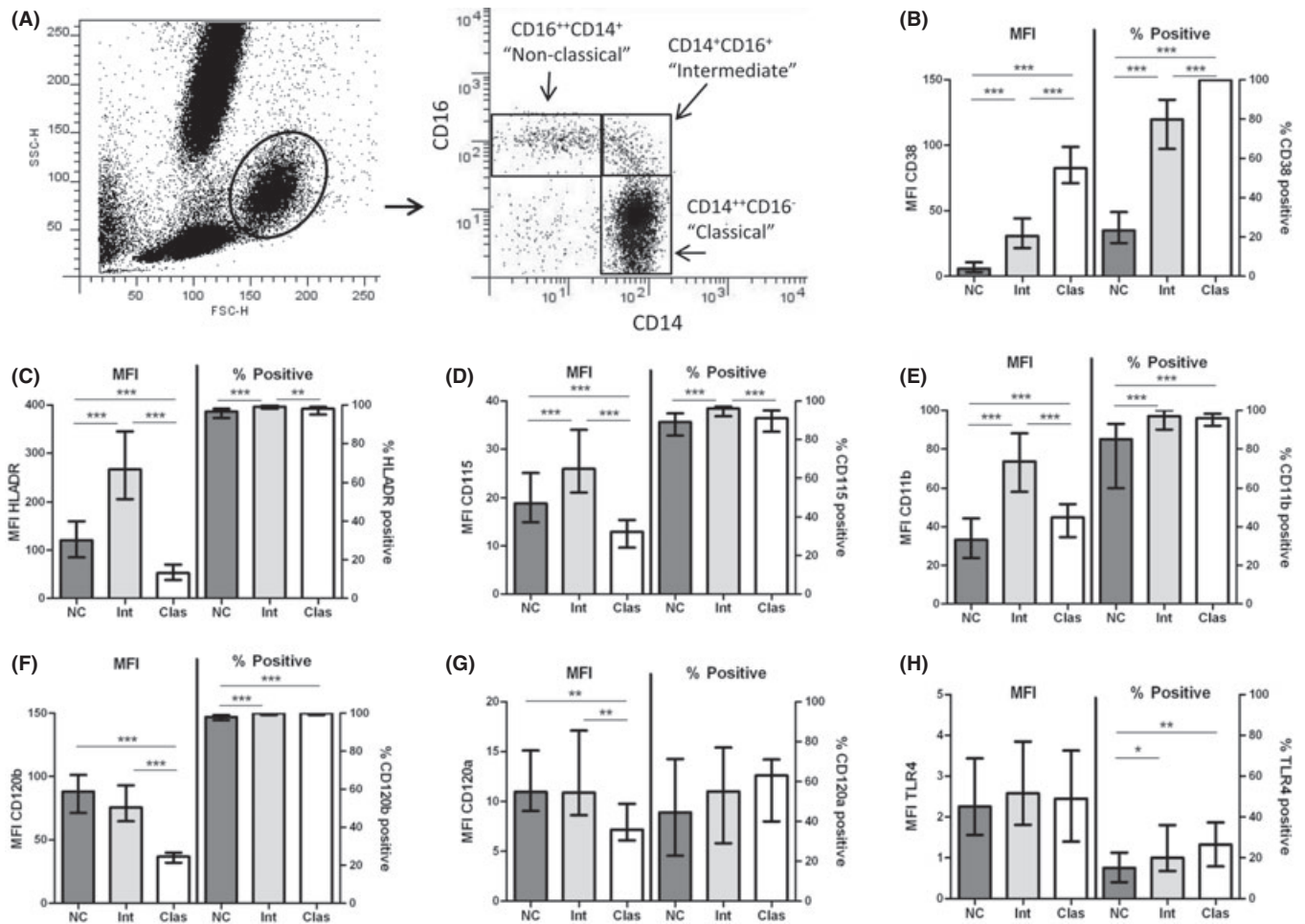


Fig. 1 Monocyte phenotyping. (A) Total peripheral blood monocytes were gated via forward and side scatter and monocyte subsets identified based on expression of CD14 and CD16 as indicated. Expression of CD38 (B), HLA-DR (C), CD115 (D), CD11b (E), CD120b (F), CD120a (G) and TLR4 (H) was determined on monocyte subsets using data from all study participants. Overall expression level (indicated by geometric mean fluorescence intensity (MFI) of stain minus isotype control; left y-axis) and proportion of cells positive (%; right y-axis) for each marker are indicated. Median values and interquartile ranges are shown. *P* values were determined by Mann–Whitney analysis; ****P* < 0.001, ***P* < 0.01, **P* < 0.05.

data arising from the use of different experimental models and recruitment criteria of 'aged' individuals (Panda *et al.*, 2009). An increased basal level of pro-inflammatory cytokines in monocyte-derived macrophages from aged individuals has been reported (Sadeghi *et al.*, 1999), but is disputed elsewhere (Seidler *et al.*, 2010). Production of pro-inflammatory cytokines by monocytes in response to TLR1/2 ligands *ex vivo* is impaired in older adults (van Duin *et al.*, 2007; Nyugen *et al.*, 2010) although whether age affects response to other TLR agonists such as the bacterial endotoxin lipopolysaccharide (LPS, a TLR4-agonist) remains controversial [reviewed in (van Duin & Shaw, 2007)]. Thus, older adults appear to exhibit a dysregulated inflammatory response and an increase in markers of inflammation, although the mechanism of these changes remains unclear.

Sex-related differences in monocyte and innate immune function have received little attention to date; however, a lower incidence and severity of sepsis in women as compared with men (Wichmann *et al.*, 2000) suggests that innate immunity may vary with sex. Some studies (Bouman *et al.*, 2004), but not all (Hodkinson *et al.*, 2006), report decreased numbers of monocytes in women, whilst women exhibit a reduced proportion of CD16⁺ monocytes (Heimbeck *et al.*,

2010) with a similar phagocytic capacity (Hodkinson *et al.*, 2006) as men. A study of plasma pro-inflammatory cytokine levels in aged men and women found no significant sex-related differences (Forsey *et al.*, 2003); however, elevated TNF levels are linked with all-cause mortality in men, but not women, whilst IL-6 levels are a predictor in both sexes (Bruunsgaard *et al.*, 2003). Although limited, these data indicate that significant differences in innate immune function exist between men and women and justify the consideration of sex in gerontological immune studies.

In this study, we sought to determine the impact of age on the phenotype, function and activation of monocyte subsets and further explore whether these effects differ between men and women. We found alterations in monocyte phenotype, increased inflammatory function and elevated levels of plasma innate immune activation markers in aged individuals that could have implications for the development of inflammatory diseases such as cardiovascular disease. Whilst most of these changes were consistent in men and women, some sex-related differences in monocyte and innate immune activation markers were identified, indicating a differential impact of age on certain aspects of monocyte function in men and women.

Results

Phenotype analysis of monocyte subsets

We measured surface expression of phenotypic markers of monocyte function and activation on classical, intermediate and nonclassical monocyte subsets (defined as in Fig. 1a) in all study participants. The median percentage of intermediate and nonclassical monocytes was 5.1% and 5.0%, respectively. As previously reported (Ziegler-Heitbrock, 2007), CD62L was expressed exclusively on classical monocytes [median mean fluorescence intensity (MFI) 55.9 (IQR: 41.2–69.9); median per cent positive 98.1% (IQR: 93.4–99.4%)], which also exhibited the highest expression of CD38 ($P < 0.0001$ for both MFI and per cent positive as compared with intermediate or nonclassical subsets, Fig. 1b). HLA-DR, CD115, CD11b and CD120b were expressed ubiquitously on monocyte subsets (Fig. 1c–f), whilst a similar proportion of all subsets were positive for CD120a (Fig. 1g). With respect to overall expression levels (as indicated by MFI), the intermediate monocyte subset exhibited the highest expression of HLA-DR, CD11b and CD115 ($P < 0.0001$ for all) and equal highest expression of CD120a and CD120b (with the nonclassical subset, Fig. 1a–g). Classical monocytes showed the lowest expression of HLA-DR, CD115, CD11b and CD120a and b. Expression of TLR4 was similar on all three subsets, although fewer nonclassical monocytes were positive for TLR4 compared with the intermediate and classical subsets (Fig. 1h).

These data confirm that the three monocyte subsets are phenotypically distinct and indicate that CD14⁺⁺ CD16⁺ intermediate monocytes have the highest expression of a number of key functional and activation markers.

Age- and sex-related changes in monocyte phenotype

We used linear regression analysis to identify associations between age and the expression of monocyte phenotypic markers (i.e. HLA-DR, CD38, CD62L, CD11b, CD115, CD120a/b and TLR4) as well as the proportion of monocyte subsets. The parameters that were found to be significantly associated with age are listed in Table 1. The relative proportion of the three monocyte subsets was significantly altered by age with the percentage of nonclassical and intermediate monocytes increasing ($P = 0.004$ and 0.002 , respectively) and the percentage of classical monocytes decreasing ($P = 0.029$) with age (Table 1). Overall expression (as indicated by MFI) of CD38 (on all three monocyte subsets), CD62L and CD115 (both on classical monocytes) and TLR4 (on nonclassical and intermediate monocytes) was significantly decreased with age, whilst expression of CD11b (classical and nonclassical monocytes) was significantly increased with age (Table 1). Confirming previous reports (Wikby *et al.*, 2002), phenotypic markers indicative of T-cell senescence were also found to be significantly altered with age; the percentage of CD3⁺ CD8⁺ lymphocytes positive for CD28 was decreased ($P = 0.037$) whilst the

Table 1 Monocyte/T-cell phenotypic and soluble innate immune activation markers significantly associated with age and the influence of sex on this association

			Association with age (adjusted for sex)†				Association with sex (adjusted for age)‡			
	<i>n</i>	Subset*	<i>P</i> value	Coefficient	95% CI		<i>P</i> value	Coefficient§	95% CI	
Monocyte phenotypic markers										
CD38 (MFI)¶	58	NC	0.030	−0.0125	−0.0238	−0.00126	0.416			
CD38 (MFI)**	70	Int	0.033	−0.00513	−0.00983	−0.000417	0.001	0.346	0.142	0.550
CD38 (MFI)**	71	Class	0.036	−0.00275	−0.00530	−0.000190	0.318			
CD62L (MFI)	67	Class	0.001	−0.322	−0.508	−0.136	0.016	−9.90	−17.93	−1.88
CD11b (MFI)	65	NC	0.015	0.186	0.0370	0.335	0.054			
CD11b (MFI)	74	Class	0.025	0.160	0.0205	0.300	0.958			
CD115 (MFI)	75	Class	0.019	−0.0519	−0.0951	−0.00871	0.047	−1.912	−3.80	−0.0244
TLR4 (MFI)††	31	NC	0.033	−0.0258	−0.00230	−0.0494	ND			
TLR4 (MFI)††	31	Int	0.026	−0.0345	−0.00457	−0.0644	ND			
Proportion (%)‡‡	76	NC	0.004	0.000588	0.000193	0.000983	0.002	0.0279	0.0106	0.0452
Proportion (%)**	76	Int	0.002	0.00910	0.00336	0.0148	0.765			
Proportion (%)‡‡	76	Class	0.029	−0.000825	−0.00156	−8.83e−5	0.164			
T-cell senescence markers										
CD57 (%)	64	CD3+ CD8+	0.008	0.00319	0.000856	0.00551	0.126			
CD28 (%)¶	64	CD3+ CD8+	0.037	−0.00147	−0.00285	−9.37e−5	0.849			
CD57+ CD28− (%)¶	64	CD3+ CD8+	0.014	0.00276	0.000580	0.00493	0.427			
Soluble innate immune activation markers										
sCD14	129		0.984	−0.0318	−3.11	3.05	<0.001	220	106	333
sCD163	111		0.003	3.91	1.39	6.43	<0.001	−173	−250	−96.6
Neopterin**	138		<0.001	0.00464	0.00216	0.00713	0.434			
CXCL10**	87		<0.001	0.0123	0.00806	0.0167	0.012	−0.222	−0.395	−0.0492

*Cell subset on which the parameter was analysed. NC, nonclassical monocytes; Int, intermediate monocytes; Class, classical monocytes.

†Determined by linear regression analysis (unless otherwise indicated) adjusting for sex.

‡Determined by linear regression analysis (unless otherwise indicated) adjusting for age.

§Indicates the difference in the regression analysis owing to sex. A value >0 indicates a higher level of the parameter in men, a value <0 indicates a lower level of the parameter in men.

Data were transformed as required by either square root (¶) or log (**).

††Analysis performed using data from men only.

‡‡Analysis was performed using median regression.

percentage of CD57⁺ ($P = 0.008$) and CD28⁺CD57⁺ ($P = 0.014$) CD8⁺ T cells increased significantly with age. These data confirm that in addition to well-documented changes in T-cell phenotype, aging is also associated with significant changes in monocyte phenotype.

Regression analysis was also used to identify potential sex-related differences in the relationship between age and monocyte phenotype. Of the innate parameters found to be significantly associated with aging, differences between men and women were observed in the expression levels of certain parameters, with men showing a higher expression of CD38 (MFI on classical monocytes, $P = 0.001$) but lower expression of CD62L and CD115 (MFI on classical monocytes for both, $P = 0.016$ and 0.047 respectively) as compared with women of a similar age (Table 1). There were no sex-related differences in the slope of the regression curves (data not shown), suggesting that monocyte phenotype changes at the same rate with age in both men and women. Men and women had similar proportions of classical [median and interquartile range 87.5% (81.8–90.5) and 89.0% (85.1–93.2), respectively] and intermediate [5.3% (4.1–8.7) and 7.0% (4.7–9.0), respectively] but men had significantly more nonclassical monocytes than women [6.1% (4.6–10.2) and 3.5% (2.0–6.1), respectively, P value=0.002 by regression analysis, Table 1].

Age-related changes in plasma markers of innate immune activation

To further investigate the impact of age on monocyte and innate immune activation, we measured levels of soluble markers of innate immune activation (neopterin, CXCL10, sCD163 and sCD14) in plasma. Using linear regression analysis, plasma levels of sCD163 ($P = 0.003$), neopterin and CXCL10 ($P < 0.001$ for both) were found to be significantly increased with age (Table 1 and Fig. 2a–c). As observed with monocyte phenotype, there were no significant sex-related differences in the slope of the

association between plasma markers and age (Fig. 2), but men showed significantly higher overall levels of sCD14 ($P < 0.001$) but lower levels of sCD163 ($P < 0.001$) and CXCL10 ($P = 0.012$) as compared with women of an equivalent age (Table 1 and Fig. 2a, c and d). These data show that aging is associated with an increase in plasma markers of innate immune activation irrespective of sex, but that differences exist in the levels of CXCL10 and sCD14 between men and women.

Correlation of plasma activation markers with phenotypic cellular markers of immune aging revealed significant associations between CXCL10 and neopterin levels and both monocyte and T-cell aging biomarkers. Significant associations were found between CXCL10 and neopterin and the proportion of the three monocyte subsets and the proportion of CD57⁺ and CD28⁺ T cells (Table 2). Significant correlations were also found between CXCL10 levels and CD11b expression (classical and nonclassical monocytes) and between neopterin levels and expression of CD38 (intermediate monocytes, Table 2). These data suggest soluble activation markers such as CXCL10 and neopterin may be useful indicators of age-related phenotypic changes in both monocytes and T cells.

The impact of age on monocyte function

Monocytes from aged individuals have been reported to exhibit an impaired pro-inflammatory response to TLR1/2 agonists (van Duin *et al.*, 2007; Nyugen *et al.*, 2010), but the effect of age on monocyte TLR4 response remains unclear. To investigate the effect of age on the pro-inflammatory response of monocyte subsets to TLR4 stimulation, we measured intracellular levels of TNF in monocyte subsets at baseline and following LPS stimulation. Similar basal levels of TNF were present in all three monocyte subsets; however, monocytes from aged individuals showed significantly higher levels of TNF in unstimulated classical ($P = 0.0005$), intermediate ($P < 0.0001$) and nonclassical ($P = 0.016$)

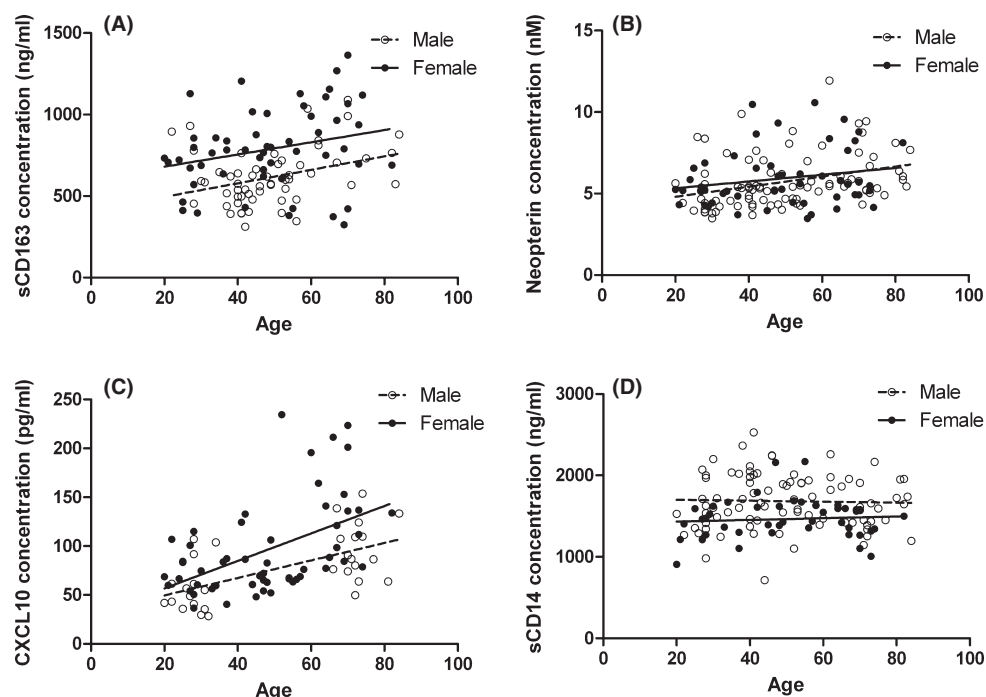


Fig. 2 Plasma markers of innate immune activation. Plasma levels of (A) sCD163, (B) neopterin, (C) CXCL10 and (D) sCD14 were determined via ELISA. Values and individual linear regression curves are shown for female (closed circles, solid line) and male (open circles, broken line) participants.

Table 2 Significant correlations between plasma and cellular aging biomarkers*

Cellular marker	CXCL10		Neopterin	
	ρ	P value	ρ	P value
% Classical monocytes	-0.338	0.002	-0.239	0.024
% Intermediate monocytes	-0.405	0.001	0.214	0.045
% Nonclassical monocytes	-0.230	0.033	0.229	0.031
CD11b expression – classical monocytes	0.348	0.007		NS
CD11b expression – nonclassical monocytes	0.405	0.003		NS
CD38 expression – intermediate monocytes		NS	0.293	0.020
% CD57 ⁺ CD8 ⁺ T cells	0.224	0.042	0.248	0.029
CD28-CD8 ⁺ T cells	-0.245	0.026	0.019	-0.267

*Determined by Spearman's rank correlation.

NS, not statistically significant.

monocyte subsets (Fig. 3a). Substantial amounts of TNF were produced by all monocytes subsets following LPS stimulation, with intermediate monocytes displaying the highest level of TNF production and classical monocytes the lowest. Intermediate and nonclassical monocytes from aged individuals produced significantly higher levels of TNF compared with young individuals ($P = 0.034$ and 0.006 , respectively, Fig. 3b), suggesting age is associated with a heightened inflammatory response of monocytes to TLR4 stimulation.

To further explore the function of monocytes in aged individuals, we assessed the ability of monocytes to phagocytose a bacterial target (fluorescently labelled *Escherichia coli*) in a whole blood assay. Monocytes with low CD14 expression are not efficient phagocytes (Cros et al., 2010) and were thus not analysed in these experiments. CD14⁺ monocytes (classical and intermediate) from older individuals exhibited significantly impaired phagocytosis of a bacterial target as compared with young individuals (Fig. 3c, $P = 0.04$).

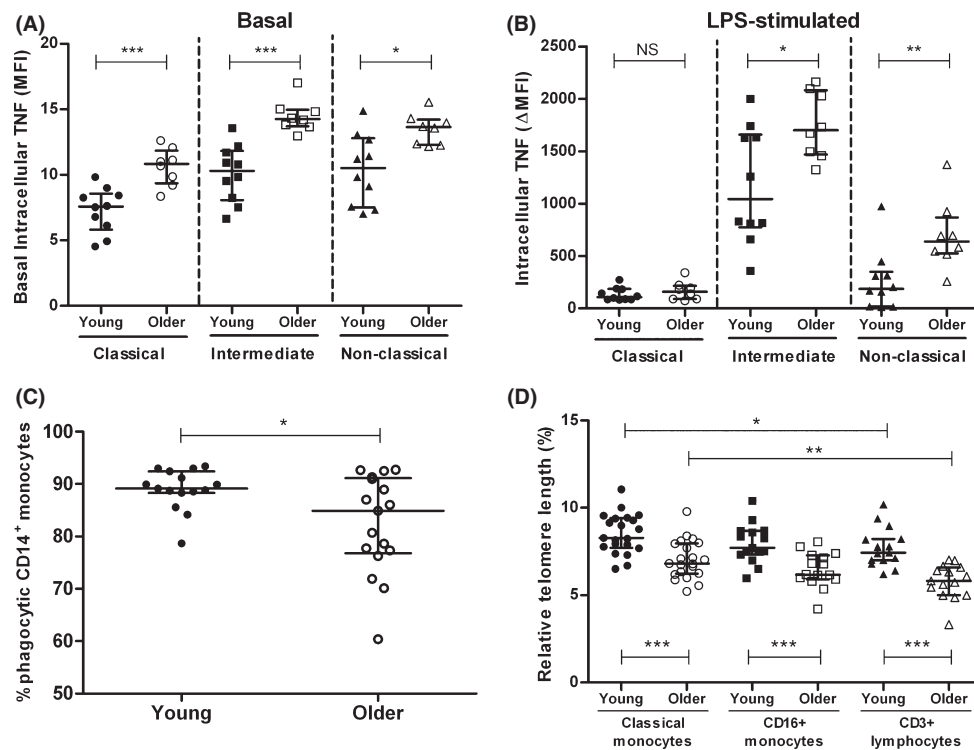


Fig. 3 Monocyte LPS response, phagocytosis and telomere length. Intracellular TNF in classical, intermediate and nonclassical monocyte subsets (circles, squares and triangles, respectively) was determined in whole blood from young (closed symbols, median age 31 years, range 21–45) and older (open symbols, median age 68 years, range 66–72) individuals. Geometric mean fluorescence intensity (MFI of stain minus isotype control) of TNF is shown for samples at baseline (A), and delta (Δ) MFI (MFI of treated sample minus untreated sample incubated in parallel) shown following stimulation with 10 ng mL^{-1} LPS for 4 h (B). (C) The percentage of CD14⁺ monocytes that phagocytosed a pHRODO-labelled *Escherichia coli* target are shown for young (closed circles, median age 28 years, range 20–34) and older men (open circles, median age 72 years, range 67–84). (D) Telomere length of classical (CD14⁺ CD16⁻, circles) and CD16⁺ (CD16⁺ CD14^{variable}, squares) monocyte subsets and CD3⁺ lymphocytes (triangles) was determined via FISH-Flow and immunophenotyping. Values represent relative telomere length (RTL) expressed as a percentage of the internal control cell line 1301 for young (closed symbols; median age 28, range 20–34 years) and older individuals (open symbol; median age 73, range 62–84 years). (a–c) Median values and interquartile ranges are shown. P values were determined by Mann–Whitney analysis; *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$.

Age- and sex-associated changes in telomere length in monocyte subsets

Aging is associated with telomere shortening in a number of leucocyte populations; however, the telomere length of monocyte subsets from young and aged individuals has not been directly compared. Simultaneous measurement of relative telomere length in multiple cell populations was performed using a FISH-Flow protocol. Nonclassical and intermediate monocyte subsets were pooled for this analysis (and termed CD16⁺ monocytes) owing to low cell yields following FISH-Flow analysis. No significant sex-related differences in classical or CD16⁺ monocytes or CD3⁺ T cells were found (data not shown); thus, data from men and women were pooled for further analysis. We found both classical and CD16⁺ monocytes from aged individuals contained significantly shorter telomeres than those from young individuals (Fig. 3d, $P = 0.0005$ and 0.001 respectively). Consistent with previous reports (Rufer *et al.*, 1999), telomere length in CD3⁺ lymphocytes was also significantly shorter in aged as compared with young participants ($P < 0.0001$). Telomere length in classical, but not CD16⁺ monocytes, was significantly shorter than CD3⁺ lymphocytes ($P = 0.04$ and 0.002 for young and aged individuals, respectively). There was no statistically significant difference in telomere length between classical and CD16⁺ monocytes in either young or aged individuals.

Discussion

Significant morbidity in older adults arises from impaired immunity to pathogens and age-related diseases driven by chronic inflammation. Monocytes are important cells for both immune defence and inflammation, and we therefore investigated the impact of age on their phenotype and function and determined whether these changes differed between sexes.

Phenotype and function of monocyte subsets

Phenotypic analysis of monocyte subsets revealed that the CD14⁺⁺ CD16⁺ intermediate subset has the highest expression of HLA-DR, CD11b and CD115 and equal highest expression of CD120a, CD120b and TLR4, suggesting that phenotypically this subset is not intermediate between the classical and nonclassical subsets. Higher level of expression of HLA-DR on the intermediate as compared with other subsets suggests they may be more efficient at antigen presentation, whilst increased CD11b indicates a more activated phenotype. The high expression of both TLR4 and CD14 (required for LPS signal transduction) on intermediate monocytes corresponds with their considerably higher production of the pro-inflammatory cytokine TNF in response to the TLR4-agonist LPS shown here and by others (Cros *et al.*, 2010). Nonclassical monocytes show the greatest level of TNF production following TLR1/2 (Nyugen *et al.*, 2010) and TLR7/8 ligation (Cros *et al.*, 2010), indicating that monocyte subsets may have specialized responses to different TLR agonists. These findings suggest that the intermediate subset represents a specific and highly active monocyte population with heightened responsiveness to certain stimuli rather than an intermediate stage of monocyte differentiation.

Age- and sex-related changes in monocyte phenotype

We identified a number of age-related changes in the phenotype of all three monocyte subsets, including increased expression of CD11b

on nonclassical and classical subsets. CD11b is involved in mediating transendothelial migration of monocytes into atherosclerotic vessels (Sotiriou *et al.*, 2006), and increased CD11b expression has been associated with increased atherosclerotic plaque formation in mice (van Royen *et al.*, 2003). Reduced monocyte expression of CD62L in aged individuals shown here and previously (De Martinis *et al.*, 2004) is consistent with a generalized downregulation of CD62L expression on lymphocytes in older adults (De Martinis *et al.*, 2000). CD62L is responsible for monocyte rolling and adhesion to endothelial cells; downregulation of CD62L is hypothesized to impair rolling and potentially increase firm attachment of cells to vessels, which is a precursor to endothelial migration (De Martinis *et al.*, 2004). Altered expression of CD11b and CD62L on monocytes from older individuals may therefore affect monocyte migration and promote atherosclerotic plaque formation. Our finding that CD11b expression is an independent predictor of arterial wall thickening (an indicator of atherosclerosis, unpublished data) supports this hypothesis. The phenotypic changes in CD11b and CD62L expression we observed on monocytes from the aged are similar to those observed following treatment of monocytes with LPS or TNF *in vitro* (Griffin *et al.*, 1990), suggesting the inflammatory milieu may contribute to phenotypic changes in monocytes in older individuals.

Age was also associated with a significant reduction in CD115 and TLR4 expression on classical monocytes. CD115 internalization can be induced by LPS (Chen *et al.*, 1993) and IFN γ (Delneste *et al.*, 2003). We have previously shown LPS levels are elevated in aged individuals (Hearps *et al.*, 2012), and the association between age and increased CXCL10 levels shown here suggests elevated plasma IFN γ levels in older adults. This is consistent with findings of heightened IFN γ production by CD8⁺ CD28⁻ T cells in older individuals (Eylar *et al.*, 2001) and expansion of the CD8⁺ CD28⁻ T-cell population in the aged. The effect of reduced CD115 expression on monocyte function is not known, but it may skew differentiation away from M-CSF-induced anti-inflammatory macrophages with a repair phenotype (M2), towards production of inflammatory M1 macrophages.

We show that increased proportion of CD16⁺ monocytes in older adults seen here and elsewhere (Sadeghi *et al.*, 1999; Seidler *et al.*, 2010) is attributed to significant expansion of both the intermediate and nonclassical subsets, although we also observed a significantly decreased proportion of classical monocytes not seen in other cohorts (Nyugen *et al.*, 2010). The absolute number of CD16⁺ monocytes is reportedly increased in aged individuals, whilst the number of classical monocytes is not significantly altered (Nyugen *et al.*, 2010; Seidler *et al.*, 2010). Intermediate and nonclassical monocytes are significant producers of pro-inflammatory cytokines and expansion of these populations may contribute to chronic inflammation in the aged. Our finding of a significantly lower proportion of nonclassical monocytes in women as compared with men is consistent with a previous report of decreased CD16⁺ monocytes in young/middle-aged women (Heimbeck *et al.*, 2010). These data suggest that there are sex-related differences in the distribution of monocyte subsets which may translate into an altered functional profile.

Age- and sex-related changes in soluble markers of innate immune activation

To the best of our knowledge, we are the first to show that sCD163 levels increase with age, even though sCD163 levels are a predictor of numerous age-associated diseases including rheumatoid arthritis (Greisen *et al.*, 2011) and coronary atherosclerosis (Aristoteli *et al.*, 2006). sCD163 is shed from the surface of monocytes and serves as a marker of

monocyte activation. Increased levels of the monocyte/macrophage product, neopterin, shown here and elsewhere (Spencer *et al.*, 2010), provide further evidence of persistent monocyte activation in older adults irrespective of sex. Neopterin is also a sensitive indicator of reactive oxygen species levels (Murr *et al.*, 2002), thus elevated neopterin is consistent with the known role of oxidative stress in immune aging. Our finding of an age-related increase in the monocyte/macrophage activation marker CXCL10 confirms a previous report (Miles *et al.*, 2008), but the finding of higher levels of CXCL10 in women as compared with men is novel and warrants further investigation into the functional and clinical implications of this. CXCL10 and sCD163 production is stimulated by IFN γ , and T cells from women produce significantly more IFN γ following T-cell receptor stimulation (Goetzl *et al.*, 2010), which is consistent with the findings shown here. CXCL10 and neopterin levels correlated significantly with a number of age-related changes in both monocyte and T cell phenotype, suggesting these proteins may be useful biomarkers of cellular aging of both adaptive and innate immune cells in future gerontological studies.

The impact of age on monocyte function and telomere length

Our data indicating impaired phagocytosis but heightened pro-inflammatory response to LPS suggest monocyte function is dysregulated in the aged. Published data regarding monocyte function in aged individuals are conflicting, likely due to different experimental systems including the use of purified or cultured monocytes, which is known to alter monocyte phenotype (Lundahl *et al.*, 1995) and TLR response (Gabriel *et al.*, 2002; Qian *et al.*, 2011). To avoid these confounders, we used *ex vivo* whole blood models, which have the additional benefit of maintaining the presence of soluble blood factors (e.g. LPS-binding proteins, complement) to gain a more accurate picture of *in vivo* monocyte function. Our finding of impaired phagocytosis of *E. coli* by monocytes from older adults may at least partially explain their increased susceptibility to bacterial infection. Consistent with our results, others report increased pro-inflammatory cytokine levels in LPS-stimulated whole blood from older adults (Gabriel *et al.*, 2002), whilst data from studies using PBMC or plate-adhered monocytes are inconsistent (Pietschmann *et al.*, 2003; van Duin *et al.*, 2007; Qian *et al.*, 2011). Thus, extrinsic soluble factors may play an important role in modulating monocyte LPS response *in vivo*. We also found monocytes from older individuals contained significantly increased basal levels of TNF, suggesting persistently higher pro-inflammatory activity of these cells *in vivo*. Whilst these increases were slight, chronically elevated production of pro-inflammatory cytokines such as TNF over a period of years is likely to have a significant impact on immune activation and function. In contrast to our findings of heightened TLR4 response, pro-inflammatory response to TLR1/2 ligation by monocytes is impaired in the aged (van Duin *et al.*, 2007; Nyugen *et al.*, 2010), suggesting aging has a differential effect on individual TLR responses in monocytes.

Reduced TLR4 expression on CD16⁺ monocyte subsets in older adults shown here is consistent with previous reports of reduced TLR4 expression on total monocytes (van Duin *et al.*, 2007; Qian *et al.*, 2011). Taken together with our previous finding of increased LPS levels in the aged and the heightened pro-inflammatory response to LPS shown here, reduced expression and/or internalization of TLR4 may be a mechanism to reduce the inflammatory response of monocytes to endotoxaemia. Interestingly, levels of sCD14 (considered a marker of TLR4-mediated activation of monocytes) were not significantly altered with age in this study, although elucidating the complex relationship between TLR4 expression,

CD14 shedding and endotoxaemia requires more thorough investigation in larger, directed studies.

Cellular division of monocytes in the periphery is uncommon; thus, our finding of telomere shortening in peripheral monocytes may reflect shortening in haematopoietic bone marrow precursor cells. Indeed, monocyte and peripheral CD34⁺ progenitor cell telomere lengths correlate (Spyridopoulos *et al.*, 2009), and shortened telomeres in granulocytes from older individuals (Rufer *et al.*, 1999) suggest telomere shortening of a common myeloid precursor. It is not known whether telomere attrition affects monocyte function, although telomere shortening in leucocytes is associated with a range of diseases including chronic heart disease (Spyridopoulos *et al.*, 2009).

Summary

This study has shown that aging is associated with significant changes in monocyte phenotype and dysregulated monocyte function, which may contribute to impaired immunity to infections in older adults. Furthermore, significant differences exist between men and women in the levels of certain monocyte phenotypic and soluble activation markers and the functional implications of these differences warrants further exploration. Future investigations of the impact of altered expression of molecules such as CD11b and CD62L on monocyte function may help elucidate the mechanism of age-related diseases such as atherosclerosis. Older individuals exhibit a state of chronic innate immune activation, and this, coupled with a hyper-inflammatory response to TLR4-ligation and increased endotoxaemia, may contribute to the pathogenesis of inflammatory diseases in the aging population. Future longitudinal studies will determine the value of monocyte and innate immune aging markers in predicting age-related disease and may justify their inclusion in disease risk screening algorithms.

Experimental procedures

Subject recruitment and blood processing

Healthy male [$n = 91$, mean age 52.4 (range 20–84) years; mean body mass index 25 (range 16–29); non-Caucasian race, 8.1%; current smokers, 3.3%] and female [$n = 55$, mean age 48.3 (range 20–82) years; mean body mass index 25 (range 18–42); non-Caucasian race, 14.5%; current smokers, 7.3%] participants were recruited from the community with informed consent, and blood was collected into EDTA and heparin anticoagulant tubes. Whole blood was processed and analysed within 2 h of blood draw. Peripheral blood mononuclear cells (PBMCs) were isolated via Ficoll density gradient centrifugation. Plasma and PBMCs were stored at -80°C and -140°C , respectively, for later analysis. Information regarding medical history, use of medications and demographic details was collected from each participant by interview. Exclusion criteria included regular use of anti-inflammatory medication, recent (within 3 weeks) self-reported mild illness, vaccination or injury and major illness (e.g. cancer, cardiovascular event, surgery) within the preceding 6 months. This study received ethical approval from The Alfred Research and Ethics Committee, Rush University Medical Centre Institutional Review Board and Monash University Human Research Ethics Committee.

Analysis of monocyte and T cell phenotype

Owing to previous findings by ourselves and others that PBMC preparation and storage alters the expression of some monocyte phenotypic

markers (Lundahl *et al.*, 1995), we analysed monocyte phenotype via whole blood staining. Blood collected into EDTA anticoagulant was mixed with a 20 × volume of 1 × FACS lysing solution (BD Biosciences, Franklin Lakes, NJ, USA) to lyse erythrocytes (a process that was verified to not significantly alter expression of the surface markers measured in this study), incubated on ice for 10 min and then washed twice with FACS wash [1% heat-inactivated cosmic calf serum, 2 mM EDTA in calcium- and magnesium-free phosphate-buffered saline (Invitrogen, Carlsbad, CA, USA)]. Cells were stained on ice for 30 min using pretitrated volumes of the following antibodies: CD14-APC, CD16-PE-Cy7, CD38-PE, HLA-DR-FITC, CD11b-PE, CD62L-FITC, CD28-APC, CD8-PE, CD57-FITC, CD4-PCP-Cy5 (BD Biosciences, San Diego, CA, USA), TLR4-FITC (R&D Systems, Minneapolis, MN, USA) and CD115-PE (eBiosciences, San Diego, CA, USA), or appropriate isotype control antibodies. Cells were washed in FACS wash, fixed in 1% formaldehyde and analysed on a dual-laser BD FACSCalibur flow cytometer.

Assessment of monocyte phagocytosis

The ability of monocytes to phagocytose a bacterial target was assessed using heat-killed *E. coli* labelled with the pH-dependent dye pHRODO (Invitrogen) as per the manufacturer's instructions. 2×10^8 labelled *E. coli* were added to 100 µL of whole blood collected into heparin anticoagulant and incubated either at 37 °C or on ice for 10 min. Blood cells were washed with FACS wash and monocytes labelled with anti-CD14-APC and analysed as described above.

Intracellular cytokine staining

Basal and LPS-stimulated levels of TNF were determined in monocyte subsets via intracellular staining. One hundred microlitre of whole blood (collected into heparin anticoagulant) was mixed 1:1 with Iscove's modified Dulbecco's medium (Invitrogen) supplemented with L-glutamine, penicillin, streptomycin and a final concentration of $3 \mu\text{g mL}^{-1}$ Brefeldin A (eBioscience) and $1 \mu\text{g mL}^{-1}$ GolgiStop (BD Biosciences). Cells were stimulated with a final concentration of 10 ng mL^{-1} LPS (Sigma-Aldrich, St Louis, MO, USA) for 4 h in a humidified 37 °C incubator. Following stimulation (or at baseline for determination of basal TNF levels), cells were washed with FACS wash, surface CD14 and CD16 stained as described above and red cells lysed using FACS lysis buffer (BD Biosciences). Cells were then permeabilized with Perm/Wash Buffer I (BD Biosciences) and intracellular cytokines labelled with anti-TNF- α -PE (BD Biosciences) for 30 min on ice in the dark. Stained cells were washed, fixed in a final concentration of 1% formaldehyde and stored at 4 °C until analysis on a BD FACSCalibur.

Analysis of telomere length

Telomere length in monocyte subsets and CD3⁺ lymphocytes was determined via immunophenotyping and FISH-Flow using modification of a previously published protocol (Schmid & Jamieson, 2004). PBMCs were labelled with anti-CD14-Qdot 800, anti-CD3-AlexaFluor 405 (both from Invitrogen) and anti-CD16-AlexaFluor 647 (Biolegend, San Diego, CA, USA) and cross-linked with 4 mM bis(sulfosuccinimidyl)suberate. Telomere length in labelled cells was subsequently determined using the FITC FISH-Flow telomere labelling kit (Dako, Glostrup, Denmark) as per the manufacturer's instructions, except the kitsupplied DNA stain was substituted with a 7-AAD stain ($0.1 \mu\text{g mL}^{-1}$). Samples were analysed on a LSRII flow cytometer and relative telomere length determined as a percentage of the internal control cell line 1301.

Measurement of soluble markers of innate immune activation

Plasma protein levels were determined using frozen EDTA plasma (subjected to only one freeze-thaw) clarified via centrifugation at 10 000 g for 10 min prior to analysis. Commercial ELISA kits were used to determine levels of sCD163 (IQ products, Cat. # IQP-383), neopterin (Screening EIA, Brahms, Cat. # 99R.096), sCD14 and CXCL10/IP-10 (Cat. # DC140 and DIP100, respectively, all from Quantikine, R&D Systems) as per the manufacturer's instructions.

Statistical analysis

Significant differences between grouped data were identified via Mann-Whitney U test for nonparametric data using GraphPad Prism 5 software. Associations between immune parameters and age were identified via linear regression (or median regression where appropriate) using Stata software and adjusting for sex and/or age as indicated in the results tables. Data were transformed as required.

Acknowledgments

The authors wish to thank Dr Clare Westhorpe for input into study design and Maelenn Gouillou for assistance with statistical analysis. The authors gratefully acknowledge the contribution to this work of the Victorian Operational Infrastructure Support Program. AM is supported by the Postdoctoral Programme of the German Academic Exchange Service (DAAD), and SC is supported by a Principal Research Fellowship from the Australian National Health and Medical Research Council (NHMRC). The work was funded via NHMRC project grant 543137 to AJ and SC.

Author contributions

AH, AJ and SC designed the study, and AH, GM, WC, TA and AM produced experimental data in the laboratories of AJ/SC and AL. AH prepared the manuscript with critical review from all authors.

References

- Ancuta P, Liu KY, Misra V, Wacliche VS, Gosselin A, Zhou X, Gabuzda D (2009) Transcriptional profiling reveals developmental relationship and distinct biological functions of CD16⁺ and CD16⁻ monocyte subsets. *BMC Genomics* **10**, 403.
- Aristoteli LP, Moller HJ, Bailey B, Moestrup SK, Kritharides L (2006) The monocytic lineage specific soluble CD163 is a plasma marker of coronary atherosclerosis. *Atherosclerosis* **184**, 342–347.
- Bouman A, Schipper M, Heineman MJ, Faas MM (2004) Gender difference in the non-specific and specific immune response in humans. *Am. J. Reprod. Immunol.* **52**, 19–26.
- Bruunsgaard H, Ladelund S, Pedersen AN, Schroll M, Jorgensen T, Pedersen BK (2003) Predicting death from tumour necrosis factor- α and interleukin-6 in 80-year-old people. *Clin. Exp. Immunol.* **132**, 24–31.
- Chen BD, Chou TH, Sensenbrenner L (1993) Downregulation of M-CSF receptors by lipopolysaccharide in murine peritoneal exudate macrophages is mediated through a phospholipase C dependent pathway. *Exp. Hematol.* **21**, 623–628.
- Cros J, Cagnard N, Woollard K, Patey N, Zhang SY, Senechal B, Puel A, Biswas SK, Moshous D, Picard C, Jais JP, D'Cruz D, Casanova JL, Trouillet C, Geissmann F (2010) Human CD14^{dim} monocytes patrol and sense nucleic acids and viruses via TLR7 and TLR8 receptors. *Immunity* **33**, 375–386.
- De Martinis M, Modesti M, Loreto MF, Quaglini D, Ginaldi L (2000) Adhesion molecules on peripheral blood lymphocyte subpopulations in the elderly. *Life Sci.* **68**, 139–151.

- De Martinis M, Modesti M, Ginaldi L (2004) Phenotypic and functional changes of circulating monocytes and polymorphonuclear leucocytes from elderly persons. *Immunol. Cell Biol.* **82**, 415–420.
- Delneste Y, Charbonnier P, Herbault N, Magistrelli G, Caron G, Bonnefoy JY, Jeannin P (2003) Interferon-gamma switches monocyte differentiation from dendritic cells to macrophages. *Blood* **101**, 143–150.
- van Duin D, Shaw AC (2007) Toll-like receptors in older adults. *J. Am. Geriatr. Soc.* **55**, 1438–1444.
- van Duin D, Mohanty S, Thomas V, Ginter S, Montgomery RR, Fikrig E, Allore HG, Medzhitov R, Shaw AC (2007) Age-associated defect in human TLR-1/2 function. *J. Immunol.* **178**, 970–975.
- Eylar EH, Lefranc CE, Yamamura Y, Baez I, Colon-Martinez SL, Rodriguez N, Breithaupt TB (2003) HIV infection and aging: enhanced interferon- and tumor necrosis factor- α production by the CD8+ CD28- T subset. *BMC Immunol.* **2**, 10.
- Ferrucci L, Harris TB, Guralnik JM, Tracy RP, Corti MC, Cohen HJ, Penninx B, Pahor M, Wallace R, Havlik RJ (1999) Serum IL-6 level and the development of disability in older persons. *J. Am. Geriatr. Soc.* **47**, 639–646.
- Forsey RJ, Thompson JM, Ernerudh J, Hurst TL, Strindhall J, Johansson B, Nilsson BO, Wikby A (2003) Plasma cytokine profiles in elderly humans. *Mech. Ageing Dev.* **124**, 487–493.
- Gabriel P, Cakman I, Rink L (2002) Overproduction of monokines by leukocytes after stimulation with lipopolysaccharide in the elderly. *Exp. Gerontol.* **37**, 235–247.
- Gerrity RG, Naito HK (1980) Ultrastructural identification of monocyte-derived foam cells in fatty streak lesions. *Artery* **8**, 208–214.
- Goetz EJ, Huang MC, Kon J, Patel K, Schwartz JB, Fast K, Ferrucci L, Madara K, Taub DD, Longo DL (2010) Gender specificity of altered human immune cytokine profiles in aging. *FASEB J.* **24**, 3580–3589.
- Greisen SR, Moller HJ, Stengaard-Pedersen K, Hetland ML, Horslev-Petersen K, Jorgensen A, Hvid M, Deleuran B (2011) Soluble macrophage-derived CD163 is a marker of disease activity and progression in early rheumatoid arthritis. *Clin. Exp. Rheumatol.* **29**, 689–692.
- Griffin JD, Spertini O, Ernst TJ, Belvin MP, Levine HB, Kanakura Y, Tedder TF (1990) Granulocyte-macrophage colony-stimulating factor and other cytokines regulate surface expression of the leukocyte adhesion molecule-1 on human neutrophils, monocytes, and their precursors. *J. Immunol.* **145**, 576–584.
- Hearps AC, Maisa A, Cheng WJ, Angelovich TA, Lichtfuss GF, Palmer CS, Landay AL, Jaworowski A, Crowe SM (2012) HIV infection induces age-related changes to monocytes and innate immune activation in young males which persist despite cART. *AIDS (London, England)* **26**, 843–53.
- Heimbeck I, Hofer TP, Eder C, Wright AK, Frankenberger M, Marei A, Boghdadi G, Scherberich J, Ziegler-Heitbrock L (2010) Standardized single-platform assay for human monocyte subpopulations: lower CD14+CD16++ monocytes in females. *Cytometry A* **77**, 823–830.
- Hodkinson CF, O'Connor JM, Alexander HD, Bradbury I, Bonham MP, Hannigan BM, Gilmore WS, Strain JJ, Wallace JM (2006) Whole blood analysis of phagocytosis, apoptosis, cytokine production, and leukocyte subsets in healthy older men and women: the ZENITH study. *J. Gerontol. A Biol. Sci. Med. Sci.* **61**, 907–917.
- Leng SX, Tian X, Matteini A, Li H, Hughes J, Jain A, Walston JD, Fedarko NS (2011) IL-6-independent association of elevated serum neopterin levels with prevalent frailty in community-dwelling older adults. *Age Ageing* **40**, 475–481.
- Licastro F, Grimaldi LM, Bonafe M, Martina C, Olivieri F, Cavallone L, Giovannetti S, Masliah E, Franceschi C (2003) Interleukin-6 gene alleles affect the risk of Alzheimer's disease and levels of the cytokine in blood and brain. *Neurobiol. Aging* **24**, 921–926.
- Lundahl J, Hallden G, Hallgren M, Skold CM, Hed J (1995) Altered expression of CD11b/CD18 and CD62L on human monocytes after cell preparation procedures. *J. Immunol. Methods* **180**, 93–100.
- Miles EA, Rees D, Banerjee T, Cazzola R, Lewis S, Wood R, Oates R, Tallant A, Cestaro B, Yaqoob P, Wahle KW, Calder PC (2008) Age-related increases in circulating inflammatory markers in men are independent of BMI, blood pressure and blood lipid concentrations. *Atherosclerosis* **196**, 298–305.
- Murr C, Widner B, Wirleitner B, Fuchs D (2002) Neopterin as a marker for immune system activation. *Curr. Drug Metab.* **3**, 175–187.
- Nockher WA, Scherberich JE (1998) Expanded CD14+ CD16+ monocyte subpopulation in patients with acute and chronic infections undergoing hemodialysis. *Infect. Immun.* **66**, 2782–2790.
- 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.
- Panda A, Arjona A, Sapey E, Bai F, Fikrig E, Montgomery RR, Lord JM, Shaw AC (2009) Human innate immunosenescence: causes and consequences for immunity in old age. *Trends Immunol.* **30**, 325–333.
- Pietschmann P, Gollob E, Brosch S, Hahn P, Kudlacek S, Willheim M, Woloszczuk W, Peterlik M, Tragl KH (2003) The effect of age and gender on cytokine production by human peripheral blood mononuclear cells and markers of bone metabolism. *Exp. Gerontol.* **38**, 1119–1127.
- Qian F, Wang X, Zhang L, Chen S, Piecychna M, Allore H, Bockenstedt L, Malawista S, Bucala R, Shaw AC, Fikrig E, Montgomery RR (2012) Age-associated elevation in TLR5 leads to increased inflammatory responses in the elderly. *Aging Cell* **11**, 104–110.
- Ridker PM, Rifai N, Stampfer MJ, Hennekens CH (2000) Plasma concentration of interleukin-6 and the risk of future myocardial infarction among apparently healthy men. *Circulation* **101**, 1767–1772.
- van Royen N, Hoefler I, Bottinger M, Hua J, Grundmann S, Voskuil M, Bode C, Schaper W, Buschmann I, Piek JJ (2003) Local monocyte chemoattractant protein-1 therapy increases collateral artery formation in apolipoprotein E-deficient mice but induces systemic monocytic CD11b expression, neointimal formation, and plaque progression. *Circ. Res.* **92**, 218–225.
- Rufer N, Brummendorf TH, Kolvrass S, Bischoff C, Christensen K, Wadsworth L, Schulzer M, Lansdorp PM (1999) Telomere fluorescence measurements in granulocytes and T lymphocyte subsets point to a high turnover of hematopoietic stem cells and memory T cells in early childhood. *J. Exp. Med.* **190**, 157–167.
- Sadeghi HM, Schnelle JF, Thoma JK, Nishanian P, Fahey JL (1999) Phenotypic and functional characteristics of circulating monocytes of elderly persons. *Exp. Gerontol.* **34**, 959–970.
- Schmid I, Jamieson BD (2004). Assessment of telomere length, phenotype, and DNA content. *Curr. Protoc. Cytom.* Chapter 7, Unit 7.26.
- Seidler S, Zimmermann HW, Bartneck M, Trautwein C, Tacke F (2010) Age-dependent alterations of monocyte subsets and monocyte-related chemokine pathways in healthy adults. *BMC Immunol.* **11**, 30.
- Sotiriou SN, Orlova VV, Al-Fakhri N, Ihanus E, Economopoulou M, Isermann B, Bdeir K, Nawroth PP, Preissner KT, Gahmberg CG, Koschinsky ML, Chavakis T (2006) Lipoprotein(a) in atherosclerotic plaques recruits inflammatory cells through interaction with Mac-1 integrin. *FASEB J.* **20**, 559–561.
- Spencer ME, Jain A, Matteini A, Beamer BA, Wang NY, Leng SX, Punjabi NM, Walston JD, Fedarko NS (2010) Serum levels of the immune activation marker neopterin change with age and gender and are modified by race, BMI, and percentage of body fat. *J. Gerontol. A Biol. Sci. Med. Sci.* **65**, 858–865.
- Spyridopoulos I, Hoffmann J, Aicher A, Brummendorf TH, Doerr HW, Zeiher AM, Dimmeler S (2009) Accelerated telomere shortening in leukocyte subpopulations of patients with coronary heart disease: role of cytomegalovirus seropositivity. *Circulation* **120**, 1364–1372.
- Wichmann MW, Inthorn D, Andress HJ, Schildberg FW (2000) Incidence and mortality of severe sepsis in surgical intensive care patients: the influence of patient gender on disease process and outcome. *Intensive Care Med.* **26**, 167–172.
- Wikby A, Johansson B, Olsson J, Lofgren S, Nilsson BO, Ferguson F (2002) Expansions of peripheral blood CD8 T-lymphocyte subpopulations and an association with cytomegalovirus seropositivity in the elderly: the Swedish NONA immune study. *Exp. Gerontol.* **37**, 445–453.
- Ziegler-Heitbrock L (2007) The CD14+ CD16+ blood monocytes: their role in infection and inflammation. *J. Leukoc. Biol.* **81**, 584–592.
- Ziegler-Heitbrock L, Ancuta P, Crowe S, Dalod M, Grau V, Hart DN, Leenen PJ, Liu YJ, MacPherson G, Randolph GJ, Scherberich J, Schmitz J, Shortman K, Sozzani S, Strobl H, Zembala M, Austyn JM, Lutz MB (2010) Nomenclature of monocytes and dendritic cells in blood. *Blood* **116**, e74–e80.