The role of inflammation in muscle aging

Beenakker, K.G.M.

Chapter 4

Immune responsiveness associates with cardiovascular mortality independent of circulating markers of inflammation

Submitted for publication by: K.G.M. Beenakker, S. Chen, M.P. Rozing, members of the PROSPER-group, R.G.J. Westendorp, A.B. Maier

Abstract

Background: Recent studies showed that the risk of a cardiovascular event is transiently increased after infection. This suggests a possible role for acute elevation of cytokines during an immune response in the development of cardiovascular disease. The aim of this study is to investigate whether immune responsiveness associates with cardiovascular, non-cardiovascular and all-cause mortality and whether this association is dependent on circulating markers of inflammation.
Methods: In 403 subjects from the PROspective Study of Pravastatin in the Elderly at Risk (PROSPER) trail, with a mean age of 75.1 years, we determined immune responsiveness at baseline by ex-vivo stimulating whole-blood samples with lipopolysaccharide (LPS) and measuring the interleukin (IL)-6, tumor necrosis factor (TNF)-α, IL-12, IL-1β, IL-10, and IL-1RA production within 24 hours. An immune responsiveness composite score was obtained by averaging the individual cytokines' sex-specific standardized production responsiveness values. Serum IL-6 and high-sensitivity C-reactive protein (Hs-CRP) were measured as circulating markers of inflammation. Subjects were followed for 12.8 years and cardiovascular, non-cardiovascular and all-cause mortality was recorded.
Results: A higher IL-6, TNF-α, and IL-1β production responsiveness was associated with a significantly higher cardiovascular and all-cause mortality. The hazard ratio (95% confidence interval; P-value) per standard deviation increment in immune responsiveness composite score was 1.89 (1.26–2.85; $P = 0.002$) for cardiovascular mortality and 1.39 (1.12–1.74; $P = 0.003$) for all-cause mortality. Adjusting these relations for circulating markers of inflammation did not change the results.
Conclusion: Immune responsiveness associates positively with the risk of cardiovascular and all-cause mortality independent of circulating markers of inflammation.

4.1 Introduction

Inflammatory processes are considered major contributors to the development of cardiovascular disease. These processes are regulated by cytokines that interact with endothelial cells, vascular smooth muscle cells, and extracellular matrix and that are associated with vascular dysfunction and atherosclerosis (Sprague & Khalil, 2009). Recent genetic studies showed that the

receptor of interleukin-6 (IL-6) plays a causal role in coronary heart disease (Collaboration IRGCERF, 2012; IL6RMR Consortium, 2012). Chronically elevated levels of circulating cytokines like serum IL-6 and other circulating markers of inflammation such as high-sensitivity C-reactive protein (Hs-CRP) are well-known risk factors for cardiovascular disease (Singh & Newman, 2011). Moreover, it has also been shown that there is a transient increase in the risk of a vascular event after infection (Warren-Gash, Smeeth & Hayward, 2009). It has therefore been suggested that acute elevations of cytokines levels are also involved in the development of cardiovascular disease (Smeeth *et al.*, 2004).

An *ex-vivo* whole-blood stimulation assay has been developed to investigate immune responsiveness (Desch *et al.*, 1989). This stimulation assay is well reproducible and assesses primarily subject's monocytic cytokine production response upon stimulation with lipopolysaccharide (LPS) (Damsgaard *et al.*, 2009a), which is under thigh genetic control (De Craen *et al.*, 2005). Immune responsiveness measured by this method has been associated with cardiovascular mortality in women aged 85 year old (Van den Biggelaar *et al.*, 2004). However, it is currently unknown whether immune responsiveness also relates to cardiovascular mortality in men and younger individuals and whether elevated levels of circulating markers of inflammation play a role in this association.

The aim of the present study was to investigate whether immune responsiveness, determined by measuring whole-blood's cytokine production responsiveness upon stimulation with LPS, relates to cardiovascular, non-cardiovascular and all-cause mortality. Furthermore, we assessed whether this relation is dependent on circulating markers of inflammation.

4.2 Material and Methods

4.2.1 Study design and subjects

Within the PROspective Study of Pravastatin in the Elderly at Risk (PROSPER), a large multicenter randomized placebo-controlled trial including 5804 subjects, it was assessed whether treatment with pravastatin decreases the risk of major vascular events in elderly. A detailed description of the protocol has been published elsewhere (Shepherd *et al.*, 1999, 2002). Between December 1997 and May 1999 subjects were screened and enrolled in Scotland (Glasgow), Ireland (Cork), and the Netherlands (Leiden). Subjects were

men or women aged 70-82 years with either preexisting vascular disease or increased risk of vascular disease because of smoking, hypertension, or diabetes mellitus. Subjects with congestive heart failure, arrhythmia or a history of malignancy within 5 years prior to the trial were not eligible to participate. In a substudy, including a random sample of 403 subjects (30%) of the subjects in the Netherlands, immune responsiveness was measured at baseline.

4.2.2 Laboratory measurements

Immune responsiveness was assessed by measuring the level of IL-6, tumor necrosis factor (TNF)-α, IL-12, IL-1β, IL-10, and IL-1RA produced by whole-blood samples upon ex-vivo stimulation with 10 ng/ml LPS during 24 hours at standard culture condition (37˚C, 5% CO_2), as described earlier (Van der Linden *et al.*, 1998). Cytokine levels outside the range of three standard deviations were regarded as outliers and excluded from the analyses (0.7% of the cytokine values). Hs-CRP was measured on stored (at −80˚C) and previously unthawed samples by automated particle-enhanced immunoturbidimetric assay (Roche UK, Welwyn Garden City, UK). Serum IL-6 was assayed using a high-sensitivity ELISA (R & D Systems, Oxford, UK).

4.2.3 Other baseline measurements

A research nurse interviewed all subjects to obtain data on demographic characteristics. Body mass index was calculated using standard protocols. Each participant's general practitioner provided information about history of vascular diseases (coronary, cerebral, or peripheral). Diabetes mellitus was defined by self-reported history, a fasting glucose concentration of 7.0 mmol/L, or self-reported use of anti-diabetic medication.

4.2.4 Follow-up measurements

Subjects were followed for mortality until January 1, 2012 in an average follow-up period of 12.8 years. Dates of deaths were obtained from the Dutch civil registry and specific data on causes of death from the Dutch Central Bureau of Statistics. Death due to cardiovascular mortality was classified as ICD-10 codes 100–199 and death due to other reasons was classified as non-cardiovascular mortality.

4.2.5 Statistical analyses

Immune responsiveness values as well as circulating markers of inflammation values were natural log-transformed due to skewness. For each of the cytokines a sex-specific Z-score was calculated to be able to compare effect sizes and to combine them in composite scores. The immune responsiveness composite score was obtained by averaging the IL-6, TNF-α, IL-12, IL-1β, IL-10, and IL-1RA sex-specific Z-scores. Data on one (n = 21), two (n = 1) or three (n = 3) of these cytokines were not available. In case of missing data, the composite score was obtained by averaging the sex-specific Z-scores of the remaining cytokines. The circulating markers of inflammation composite score was obtained by averaging the sex-specific Z-scores for serum IL-6 and Hs-CRP. If data on one of these circulating markers was not available (n = 1), then the sex-specific Z-scores of the remaining marker was used. A partial correlation between immune responsiveness values and circulating markers of inflammation values was calculated adjusted for age, smoking status and diabetes. A Cox-proportional hazard model was used to analyze whether immune responsiveness and circulating markers of inflammation are associated with mortality. The associations were adjusted for age, smoking status, diabetes and pravastatin allocation. In an additional analysis, we adjusted the association between markers of inflammation and mortality for the immune responsiveness composite score and we adjusted the association between immune responsiveness and mortality for the circulating markers of inflammation composite score. In order to account for a possible modulatory effect of pravastatin (Methe *et al.*, 2005; Niessner *et al.*, 2006; Shepherd *et al.*, 2002) the analyses were repeated stratified for subjects who received placebo (n = 205) and pravastatin (n = 198). Kaplan-Meier survival curves were used for visualization. For this visualisation we dichotomized the study population into a “High” and a “Low” immune responsiveness category based on the groups mean immune responsiveness composite score. The P-values for the Kaplan-Meier survival curves were calculated using log-rank tests. All analyses were performed using SPSS software version 20.0 (IBM, Armonk, New York, USA) and StataCorp Stata/SE version 12.0. P-values < 0.05 were regarded as statistically significant.

4.3 Results

Table 4.1. Baseline characteristics.

Characteristic	Study population n=403
Age, years	75.1 (3.3)
Sex, % men	53.6
BMI, kg/m²	26.9 (3.6)
Current smokers, %	23.8
Comorbidities	
Hypertension, %	65.5
Diabetes, %	19.6
History of myocardial infarction, %	13.2
History of stroke or TIA, %	15.1
History of vascular disease, %	44.9
Laboratory measurements	
Total cholesterol, mmol/L	5.8 (0.8)
HDL cholesterol, mmol/L	1.3 (0.3)
Circulating markers of chronic inflammation, median (IQR)	
Hs-CRP, mg/L,	2.9 (1.7-5.2)
Serum IL-6, pg/mL	2.7 (2.0-4.1)
Immune responsiveness[#], median (IQR)	
IL-6, pg/ml	71351 (54884-95568)
TNF-α, pg/ml	14334 (9199-22279)
IL-12, pg/ml	12601 (8102-19720)
IL-1β, pg/ml	8105 (5208-13208)
IL-10, pg/ml	1573 (1115-2238)
IL-1RA, pg/ml	35918 (27423-50975)

Data are presented as mean (SD) unless stated otherwise. BMI: body mass index; TIA: transient ischemic attack; HDL: high-density lipoprotein; Hs-CRP: high-sensitivity C-reactive protein; IQR: Interquartile range. [#] cytokine production response upon whole-blood stimulation with lipopolysaccharide.

Table 4.1 shows the baseline characteristics in the study population including 403 subjects. The mean age was 75.1 years (SD 3.3) and 53.6% of the population was men. The median follow-up time was 12.8 years (interquartile range: 12.8–12.9). In this period, 217 subjects (53.8%) died. Sixty-six subjects (16.3%) died due to a cardiovascular cause and 148 sub-

jects (36.7%) died due to a non-cardiovascular cause. For three subjects (1.4%) the cause of death was not recorded.

Table 4.2. Correlation between circulating markers of inflammation and immune responsiveness at baseline.

	Circulating markers of inflammation		
Immune responsiveness[#]	Hs-CRP *r*	Serum IL-6 *r*	Composite score *r*
IL-6	0.08	-0.01	0.04
TNF-α	-0.01	-0.04	-0.02
IL-12	-0.02	-0.09	-0.06
IL-1β	-0.02	-0.02	-0.02
IL-10	0.07	0.08	0.09
IL-1RA	0.24**	0.19**	0.25**
Composite score	0.08	0.01	0.05

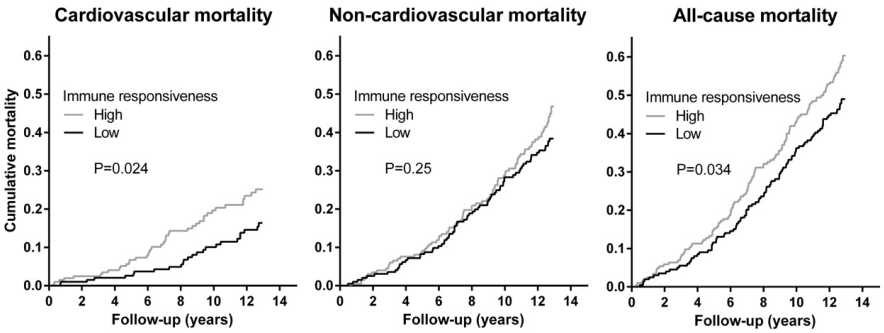
r: Correlation coefficient corrected for sex, age, smoking and diabetes; Immune responsiveness was determined by measuring cytokine production response upon whole-blood stimulation with lipopolysaccharide. Immune responsiveness composite score: the average sex-specific Z-score of IL-6, TNF-α, IL-12, IL-1β, IL-10, and IL-1RA production responsiveness; Circulating markers of inflammation composite score: the average sex-specific Z-score of Hs-CRP and serum IL-6; **P<0.001.

Table 4.2 shows the correlation between circulating markers of inflammation and IL-6, TNF-α, IL-12, IL-1β, IL-10, and IL-1RA production responsiveness. None of the correlations were significant, except that IL-1RA production responsiveness was significantly positively correlated with Hs-CRP, serum IL-6 and the circulating markers of inflammation composite score.

Table 4.3. Mortality risks dependent on circulating markers of inflammation or immune responsiveness.

	Cardiovascular mortality		Non-cardiovascular mortality		All-cause mortality	
	HR (95% CI)	P-value	HR (95% CI)	P-value	HR (95% CI)	P-value
Circulating markers of inflammation						
Hs-CRP, per SD increment	1.21 (0.95-1.53)	0.13	1.11 (0.94-1.31)	0.22	1.13 (0.99-1.29)	0.067
Serum IL-6, per SD increment	1.18 (0.95-1.47)	0.14	**1.22 (1.05-1.42)**	**0.008**	**1.21 (1.07-1.37)**	**0.002**
Composite score, per SD increment	1.25 (0.97-1.62)	0.086	**1.21 (1.02-1.44)**	**0.030**	**1.22 (1.06-1.41)**	**0.006**
Immune responsiveness[#]						
IL-6, per SD increment	**1.50 (1.12-2.00)**	**0.003**	1.16 (0.97-1.40)	0.11	**1.26 (1.08-1.47)**	**0.003**
TNF-α, per SD increment	**1.32 (1.03-1.69)**	**0.031**	1.11 (0.94-1.32)	0.22	**1.16 (1.01-1.33)**	**0.036**
IL-12, per SD increment	1.25 (0.94-1.67)	0.12	1.06 (0.89-1.28)	0.51	1.11 (0.95-1.29)	0.19
IL-1β, per SD increment	**1.41 (1.07-1.87)**	**0.015**	1.14 (0.95-1.37)	0.17	**1.20 (1.03-1.40)**	**0.018**
IL-10, per SD increment	1.09 (0.78-1.54)	0.61	1.15 (0.92-1.44)	0.23	1.13 (0.94-1.36)	0.20
IL-1RA, per SD increment	**1.41 (1.09-1.83)**	**0.010**	1.08 (0.90-1.30)	0.38	**1.17 (1.01-1.36)**	**0.036**
Composite score, per SD increment	**1.89 (1.26-2.85)**	**0.002**	1.24 (0.95-1.63)	0.11	**1.39 (1.12-1.74)**	**0.003**

Values in bold are statistically significant (P<0.05). Results are presented as hazard rates (HR) with corresponding confidence intervals (95% CI) and were adjusted for age, diabetes, smoking and pravastatin allocation. Circulating markers of inflammation composite score: the average sex-specific Z-score of Hs-CRP and serum IL-6. [#] cytokine production response upon whole-blood stimulation with lipopolysaccharide. Immune responsiveness composite score: the average sex-specific Z-score of IL-6, TNF-α, IL-12, IL-1β, IL-10, and IL-1RA production responsiveness.



Immune responsiveness: The average sex-specific Z-score of IL-6, TNF-α, IL-12, IL-1β, IL-10, and IL-1RA production response upon whole-blood stimulation with lipopolysaccharide. High: above the groups mean, Low: below the groups mean; P-values were calculated using log-rank tests.

Figure 4.1. Kaplan-Meier curves.

Table 4.3 shows the Cox-proportional hazard analyses of circulating markers of inflammation and immune responsiveness with mortality. Serum IL-6 and the circulating markers of inflammation composite score were significantly positively associated with non-cardiovascular and all-cause mortality, but not with cardiovascular mortality. IL-6, TNF-α, IL-1β, and IL-1RA production responsiveness and the immune responsiveness composite score were significantly positively associated with cardiovascular and all-cause mortality, but not with non-cardiovascular mortality. Figure 4.1 depicts Kaplan-Meier curves of subjects with a high or a low immune responsiveness composite score in relation to cardiovascular, non-cardiovascular and all-cause mortality.

Table 4.4 shows the reciprocal adjustment of circulating markers of inflammation and immune responsiveness on the relation with mortality. Results did not change significantly. Supplementary table 4.5 and 4.6 show that the relation between immune responsiveness and cardiovascular mortality was more pronounced in subjects with placebo allocation compared to subjects with pravastatin allocation. However this difference was not statistically significant (p for interaction = 0.22).

Table 4.4. Reciprocally adjusted mortality risks dependent on markers of inflammation and immune responsiveness.

	Cardiovascular mortality		Non-cardiovascular mortality		All-cause mortality	
	HR (95% CI)	P-value	HR (95% CI)	P-value	HR (95% CI)	P-value
Circulating markers of inflammation*						
Hs-CRP, per SD increment	1.18 (0.93-1.51)	0.18	1.10 (0.93-1.30)	0.25	1.12 (0.98-1.28)	0.097
Serum IL-6, per SD increment	1.20 (0.96-1.50)	0.10	**1.23 (1.06-1.42)**	**0.006**	**1.22 (1.08-1.39)**	**0.001**
Composite score, per SD increment	1.25 (0.97-1.62)	0.089	**1.21 (1.02-1.44)**	**0.031**	**1.22 (1.06-1.41)**	**0.007**
Immune responsiveness°#						
IL-6, per SD increment	**1.50 (1.13-2.00)**	**0.005**	1.17 (0.97-1.40)	0.099	**1.26 (1.08-1.47)**	**0.003**
TNF-α, per SD increment	**1.34 (1.05-1.72)**	**0.021**	1.13 (0.96-1.34)	0.15	**1.18 (1.03-1.36)**	**0.018**
IL-12, per SD increment	1.28 (0.97-1.70)	0.86	1.08 (0.90-1.30)	0.41	1.13 (0.97-1.31)	0.12
IL-1β, per SD increment	**1.41 (1.07-1.87)**	**0.014**	1.15 (0.96-1.38)	0.13	**1.21 (1.04-1.41)**	**0.012**
IL-10, per SD increment	1.08 (0.78-1.55)	0.60	1.17 (0.93-1.47)	0.19	1.15 (0.95-1.38)	0.16
IL-1RA, per SD increment	**1.37 (1.05-1.80)**	**0.022**	1.04 (0.86-1.25)	0.72	1.13 (0.96-1.31)	0.13
Composite score, per SD increment	**1.90 (1.26-2.86)**	**0.002**	1.26 (0.97-1.65)	0.085	**1.41 (1.13-1.76)**	**0.002**

Values in bold are statistically significant (P<0.05). Results are presented as hazard rates (HR) with corresponding 95% confidence intervals (CI) and were adjusted for age, diabetes, smoking and pravastatin allocation. * additionally adjusted for immune responsiveness composite score. ° additionally adjusted for circulating markers of inflammation composite score. # cytokine production response upon whole-blood stimulation with lipopolysaccharide. Circulating markers of inflammation composite score: the average sex-specific Z-score of Hs-CRP and serum IL-6. Immune responsiveness composite score: the average sex-specific Z-score of IL-6, TNF-α, IL-12, IL-1β, IL-10, and IL-1RA production responsiveness;

4.4 Discussion

Higher IL-6, TNF-α, and IL-1β production responsiveness and a higher immune responsiveness composite score were associated with a significantly higher risk of cardiovascular and all-cause mortality. Adjusting these relations for the circulating markers of inflammation did not change these results.

In the present study we measured immune responsiveness in whole blood samples using a highly standardized stimulation assay, known to be a well-suited low-cost proxy-measure of monocytic cytokine production responsiveness (Damsgaard *et al.*, 2009a; Van der Linden *et al.*, 1998). Therefore, our results suggest that monocytes are an important source of the cytokines that are involved in cardiovascular mortality. Interestingly, monocytes are known to be recruited into atherosclerotic plaques and to be involved in the remodelling of cardiac tissue after a myocardial infarction (Nahrendorf, Pittet & Swirski, 2010; Randolph, 2013). Immune responsiveness differs from circulating markers of inflammation, since these circulating markers have as their source a wide variety of cell types including lymphoid cells as well as non-lymphoid cells like endothelial cells, fibroblasts and adipocytes (Naka, Nishimoto & Kishimoto, 2002). Circulating markers of inflammation are therefore a reflection of the systemical inflammatory status. Immune responsiveness reflects the ability of blood cells to produce, when challenged by infectious agents, high amounts of cytokines within 24 hours. We used the stimulus LPS which is relevant in relation to the observation that there is a transient increase in risk for a vascular event after an infection (Smeeth *et al.*, 2004).

Table 4.5. Circulating markers of chronic inflammation and cytokine production response in relation to mortality in the placebo group.

	Cardiovascular mortality		Non-cardiovascular mortality		All-cause mortality	
	HR (95% CI)	P-value	HR (95% CI)	P-value	HR (95% CI)	P-value
Circulating markers of inflammation						
Hs-CRP, per SD increment	1.18 (0.86-1.63)	0.30	1.24 (0.98-1.55)	0.070	**1.22 (1.01-1.47)**	**0.037**
Serum IL-6, per SD increment	1.17 (0.89-1.55)	0.26	**1.22 (1.00-1.49)**	**0.049**	**1.21 (1.02-1.42)**	**0.024**
Composite score, per SD increment	1.24 (0.88-1.75)	0.21	**1.29 (1.02-1.65)**	**0.036**	**1.28 (1.05-1.55)**	**0.015**
Immune responsiveness[#]						
IL-6, per SD increment	**1.90 (1.25-2.89)**	**0.003**	1.08 (0.81-1.43)	0.60	**1.30 (1.03-1.64)**	**0.028**
TNF-α, per SD increment	**1.43 (1.02-2.00)**	**0.036**	1.03 (0.81-1.30)	0.83	1.15 (0.95-1.39)	0.15
IL-12, per SD increment	1.40 (0.95-2.06)	0.085	0.91 (0.71-1.18)	0.49	1.05 (0.85-1.29)	0.66
IL-1β, per SD increment	1.42 (0.98-2.04)	0.060	1.04 (0.82-1.34)	0.73	1.15 (0.94-1.41)	0.17
IL-10, per SD increment	1.39 (0.87-2.21)	0.16	1.12 (0.80-1.57)	0.52	1.21 (0.92-1.58)	0.18
IL-1RA, per SD increment	1.37 (0.96-1.97)	0.086	1.21 (0.93-1.57)	0.17	**1.26 (1.02-1.56)**	**0.033**
Composite score, per SD increment	**2.46 (1.36-4.44)**	**0.003**	1.12 (0.75-1.66)	0.58	**1.45 (1.04-2.00)**	**0.026**

Values in bold are statistically significant (P<0.05). Results are presented as hazard rates (HR) with corresponding 95% confidence intervals (CI) and were adjusted for age, diabetes and smoking. [#] cytokine production response upon whole-blood stimulation with lipopolysaccharide. Immune responsiveness composite score: the average sex-specific Z-score of IL-6, TNF-α, IL-12, IL-1β, IL-10, and IL-1RA production responsiveness. Circulating markers of inflammation composite score: the average sex-specific Z-score of Hs-CRP and serum IL-6.

Our data suggests that the transiently increased risk for vascular events may be related to immune responsiveness of blood cells, although this needs to be further investigated. The stimulus LPS is an agonist of toll-like receptor 4 (TLR-4), which is known to recognize also endogenous danger signals like high-mobility group box-1 (HMGB-1) (Bianchi & Manfredi, 2009). HMGB-1 is released by necrotic cells and acts as an early mediator of inflammation and organ damage in ischemia/reperfusion damage of the heart (Andrassy *et al.*, 2008). Therefore, the immune response upon stimulation with LPS could also be seen as a proxy for the immune response upon a myocardial infarction. This could be an explanation for the finding that immune responsiveness was associated with cardiovascular mortality and this association was independent of circulating markers of inflammation which are associated with cardiovascular mortality via different pathophysiolical mechanisms like the formation of atherosclerosis (Sprague & Khalil, 2009).

Our study was performed in a clinical trial population at risk for cardiovascular disease with a mean age of 75 years. The findings from this study are different from a population based study in elderly aged 85 years showing an higher risk for all-cause mortality in subjects with a low pro- and low anti-inflammatory immune responsiveness (Wijsman *et al.*, 2011). Probably, a low immune responsiveness is in this population of oldest old more a reflection of an age related impaired immune function and that this increased the risk for fatal infections (Bruunsgaard *et al.*, 1999; Van den Biggelaar *et al.*, 2004). In line with our findings, a higher TNF-α production responsiveness has in this population been related with a higher risk of cardiovascular

Table 4.6. Mortality risks dependent on circulating markers of inflammation and immune responsiveness in subjects with pravastatin allocation.

	Cardiovascular mortality		Non-cardiovascular mortality		All-cause mortality	
	HR (95% CI)	P-value	HR (95% CI)	P-value	HR (95% CI)	P-value
Circulating markers of inflammation						
Hs-CRP, per SD increment	1.23 (0.85-1.78)	0.27	1.35 (0.99-1.83)	0.056	1.06 (0.87-1.29)	0.54
Serum IL-6, per SD increment	1.19 (0.83-1.71)	0.34	1.00 (0.79-1.27)	0.99	**1.21 (1.01-1.46)**	**0.041**
Composite score, per SD increment	1.26 (0.85-1.86)	0.25	1.21 (0.97-1.50)	0.091	1.17 (0.95-1.44)	0.14
Immune responsiveness[#]						
IL-6, per SD increment	1.17 (0.78-1.77)	0.45	1.13 (0.88-1.46)	0.34	1.23 (1.00-1.51)	0.053
TNF-α, per SD increment	1.17 (0.79-1.75)	0.43	1.21 (0.95-1.58)	0.092	1.17 (0.95-1.43)	0.14
IL-12, per SD increment	1.08 (0.70-1.67)	0.72	1.26 (0.96-1.65)	0.094	1.18 (0.94-1.48)	0.15
IL-1β, per SD increment	1.40 (0.91-2.16)	0.13	1.28 (0.97-1.67)	0.075	**1.28 (1.02-1.61)**	**0.033**
IL-10, per SD increment	0.81 (0.48-1.35)	0.41	1.25 (0.91-1.73)	0.17	1.07 (0.82-1.38)	0.63
IL-1RA, per SD increment	1.43 (0.98-2.09)	0.067	0.78 (0.74-1.25)	0.78	1.10 (0.89-1.35)	0.39
Composite score, per SD increment	1.41 (0.77-2.56)	0.26	1.40 (0.97-2.01)	0.073	1.35 (0.99-1.83)	0.056

Values in bold are statistically significant (P<0.05). Results are presented as hazard rates (HR) with corresponding 95% confidence intervals (CI) and were adjusted for age, diabetes and smoking. Circulating markers of inflammation composite score: the average sex-specific Z-score of Hs-CRP and serum IL-6. [#]cytokine production response upon whole-blood stimulation with lipopolysaccharide. Immune responsiveness composite score: the average sex-specific Z-score of IL-6, TNF-α, IL-12, IL-1β, IL-10, and IL-1RA production responsiveness.

mortality (Van den Biggelaar *et al.*, 2004). However, in the present study no significant relation between IL-10 production responsiveness and cardiovascular mortality was found, while in these elderly aged 85 years it has been shown that a higher IL-10 production responsiveness associates with a lower risk for fatal stroke and cardiovascular mortality (Van Exel *et al.*, 2002; Van den Biggelaar *et al.*, 2004). An explanation could be that in the latter population the heterogeneity among subjects was higher due to their high age and due to the absence of selection criteria for health characteristics in the study.

Although immune responsiveness, determined by measuring cytokine production response upon whole-blood stimulation with LPS, is over 50% genetically determined, recent studies showed that infections and vaccinations not only challenge cytokine production responsiveness acutely, but also impacts the epigenetic reprogramming of monocytes (Kleinnijenhuis *et al.*, 2012; Quintin *et al.*, 2012). Bacille Calmette-Guerin (BCG), the vaccine against tuberculosis, induces in men an increased immune response upon *ex-vivo* stimulation with unrelated microbial products for at least three months (Kleinnijenhuis *et al.*, 2012). A similar prolonged changed immune responsiveness was observed by *Candida albicans* infection in mice (Quintin *et al.*, 2012). These findings suggest that immune responsiveness can be a novel target for the prevention of cardiovascular diseases (Bekkering *et al.*, 2013).

In conclusion, within elderly at risk for cardiovascular disease, we showed that immune responsiveness is associated with cardiovascular mortality and all-cause mortality independent of circulating markers of inflammation. Immune responsiveness may be a target for future therapy and screening pa-

tients for immune responsiveness may contribute to a better cardiovascular mortality risk prediction.