Circulating Monocytes in Patients with Acute Coronary Syndromes Lack Sufficient Interleukin-10 Production after LPS stimulation


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Acute coronary syndromes (ACS) are associated with inflammation resulting from monocyte activation. We sought for differences in the production of pro- and anti-inflammatory cytokines by monocytes from patients with ACS. C-reactive protein (CRP) and neopterin were measured in 22 patients with acute coronary syndromes, 50 patients with stable vascular disease and 22 healthy controls. Production of TNF-α and IL-10 was determined after respectively 6 and 24 h of incubation of full blood with LPS. Levels of CRP (median, inter-quartile range (IQR): 1.5 mg/L (0.8-4.5) vs. 2.1 (0.9-3.6) vs. 0.4 (0.3-1.2); P < 0.001) and neopterin (7.4 nmol/L (6.0-8.7) vs. 7.1 (6.0-8.9) vs. 6.4 (5.6-7.3); P = 0.07) were higher in patients. IL-10 production after LPS stimulation was greatly reduced in patients with acute coronary syndromes (16,175 pg/ml, 7,559-28,470 pg/ml) as opposed to patients with stable disease (28,379 pg/ml, 12,601-73,968 pg/ml) and healthy controls (63,830 pg/ml, 22,040-168,000 pg/ml; P = 0.003). TNF-α production was not different between groups (7,313 pg/ml (4,740-12,615) vs. 11,002 (5,913-14,190) vs. 8,229 (5,225-11,364); P = 0.24). We conclude that circulating monocytes in unstable coronary syndromes produce equal amounts of TNF-α but less IL-10 after stimulation with LPS in vitro. We hypothesize that, in acute coronary syndromes, the production pro-inflammatory cytokines is not counter-balanced by anti-inflammatory cytokines such as IL-10.

INTRODUCTION

Atherosclerosis is an inflammatory disease of multi-factorial origin, resulting in vascular occlusion. Monocytes/macrophages are culprit cells in atherosclerotic plaque formation [1-3]. Recently, it was shown that patients with coronary artery disease have higher levels of neopterin, a marker of monocyte/macrophage activation [4-6]. This implicates that monocyte/macrophage activation is an important determinant of the inflammatory response in coronary artery disease (CAD). Also, levels of IL-6, a pro-inflammatory cytokine that is mainly produced by activated monocytes/macrophages, are elevated in patients with CAD [7;8]. Finally, levels of C-reactive protein (CRP), a systemic marker of inflammation, are prognostic for the development and outcome of acute coronary syndromes.[9;10].

Thus, a pro-inflammatory immune response, caused by monocyte/macrophage activation, contributes to the development of acute coronary syndromes.

Less is known about the role of anti-inflammatory cytokines in human atherosclerosis. In mice, however, recent reports suggest an important role for IL-10 in preventing atherogenesis [11;12]. Since an adverse outcome in ACS is associated with an elevated level of several pro-inflammatory cytokines [10], and the anti-inflammatory cytokine IL-10 is supposed to be protective in atherosclerosis, we hypothesized that a disturbed cytokine balance by monocytes might be present in patients with ACS.

MATERIALS AND METHODS

Patients and controls
Three groups of subjects were studied. Group 1 consisted of 22 consecutive patients admitted to our hospital with an acute coronary syndrome. Seventeen patients had an acute myocardial infarction defined as: typical chest pain, ST-elevation > 0.1 mV in at least
Decreased IL-10 production in acute coronary syndromes

2 contiguous leads and creatin kinase (CK) levels of more than twice the upper limit or elevated troponin levels. Unstable angina pectoris was diagnosed in the other 7 patients and was defined as: 2 episodes of chest pain at rest or an episode of chest pain lasting over 20 min in combination with ST-elevations > 0.1 mV during pain, without elevations of CK.

Group 2 comprised 50 consecutive patients from our outpatient clinic with stable vascular disease. This group had either coronary artery disease (CAD) (n =31), peripheral vascular disease (PVD) (n =17) or both (n =2). CAD was defined as a positive exercise test result, significant stenosis (> 70%) at coronary angiography, previous admission for an ACS, previous percutaneous trans-luminal coronary angioplasty or previous coronary artery bypass grafting. Patients had to be stable for at least 6 months. Patients with PVD were recruited from the outpatient clinic for vascular surgery. PVD was defined as the presence of intermittent claudication, and was confirmed by a decreased ankle/brachial index (<0.8), Doppler ultrasonography, digital subtraction angiography or prior vascular surgery. Twenty-two healthy volunteers were recruited from the hospital staff and served as controls. Persons with evidence of recent infectious disease, immunologic disorders, fever, use of anti-inflammatory drugs, major surgery or neoplastic disease were excluded from the study. All subjects gave written informed consent. The study was approved by the local medical ethical committee.

Blood samples

From each patient 1 tube of 10 ml containing NH 143 I.U. (Vacutainer system, Becton, Dickinson and Company, Plymouth, UK) and 1 tube of 10 ml containing 0.12 ml EDTA-K3 15% (Vacutainer system, Becton, Dickinson and Company) was drawn by venapuncture. In case of an ACS this was done immediately at admission, before any intervention had occurred. The tubes were transported on ice. A white blood cell count was performed immediately. Blood was then centrifuged at 1255 xg for 10 min. The plasma supernatant was removed and stored at -20°C until further analysis. The heparin tubes were immediately used for lipopolysaccharide stimulation tests.

In vitro whole blood lipopolysaccharide (LPS) stimulation

Cytokine production was measured using a whole blood culture system as described elsewhere [13]. Briefly, under sterile conditions, aliquots of 1 ml of heparinized whole blood were drawn into blank 5ml tubes (Vacutainer system, Becton Dickinson and Company). The LPS stimulated samples were treated with 20 µL LPS (Escherichia Coli, serotype 0127-B8, 2.5 mg/ml, Sigma Chemical Co, St. Louis, MO), to a final concentration of 2.5 µg/mL blood. All tubes were filled up to 2 mL with RPMI 1640 (with Heps and L-glutamine, cat Nº BE12-115F, Bio Whittaker Europe, Verviers, Belgium). LPS-treated samples, together with an untreated control sample, were then incubated at 37°C in an atmosphere containing 5% CO2. After 6 h of incubation the samples for TNF-α measurement were centrifuged at 1076 xg for 5 min. The IL-10 samples were centrifuged after 24 h of incubation. The plasma supernatant was removed and stored at -20°C for further analysis. The first 14 whole blood cultures were co-incubated with Polymixin B. Polymixin B occupies the CD14 molecule, the major LPS-receptor that is mainly present on monocytes [14]. These samples showed no enhanced production of cytokines after stimulation. This shows
that enhanced cytokine production after LPS stimulation is brought off exclusively by the CD14 molecule and therefore by monocytes.

**CRP, TNF-α, IL-10 and neopterin measurement**

CRP, TNF-α and IL-10 levels were determined by using a validated sandwich enzyme-linked immunosorbent assays (ELISA) [15-17]. In brief, Costar plates number 9018 (Costar, Badhoeve-dorp, The Netherlands) were respectively coated with antibodies to CRP (A-073, Dakopatts, Glostrup, Danmark), TNF-α (MAB610, R&D systems inc., Minneapolis, USA) and IL-10 (Moab 18551D, BD Pharmigen, Bedford, USA) overnight. Subsequently, for CRP measurements plates were incubated with samples in duplo in a 1:125 dilution and samples were diluted with incubation buffer containing: 0.05 M Tris-HCl, 0.30 M NaCl and 0.05% Tween-20. For TNF-α and IL-10 measurements plates were incubated with TNF-α and IL-10 samples in 1:2, 8, 32 and 128 dilutions, whereas these samples were diluted with incubation buffer containing: 0.01 M PBS, 0.05% Tween-20 and 0.2% gelatin. CRP, TNF-α and IL-10 standards were made respectively with: 4 µg/ml ORCE 02/03 (Boehringer, Mannheim, Ger-many), 20 µg/ml r-h TNF-α (Boehringer-ger) and 500ng/ml r-h IL-10 (BD Pharmigen). CRP, TNF-α and IL-10 detection was respectively done with 1:2000 rat anti-human CRP bound to horseradish peroxidase (HRP) (P-227, Dakopatts), 1:2000 goat anti-human TNF-α-Biotin (Nr. 21335, Pierce, Rock-ford, USA) and 1:500 monoclonal antibody 18562D (BD Pharmigen). The TNF-α and IL-10 plates were then treated with 1:8000 Streptavidin labeled with poly-HRP (CLB, Amsterdam, The Netherlands).

Between each step the plates were washed 5 times with washing buffer containing: 0.025 M Tris-HCl, 0.15 M NaCl and 0.05% Tween-20. In all 3 ELISA’s tetramethylbenzidin (TMB; Brunschwig Chemie, Amsterdam, The Netherlands) was used as chromogen. The chromogen reaction was stopped by adding 2N sulphuric acid. Extinctions were measured with an Emax scanner (Molecular devices, Sunnyvale, USA) at 450-575 nm and calculated with Softmax Pro software (Molecular devices, Sunnyvale, USA). Neopterin levels were measured by commercially available ELISA (Brahms Diagnostica GmbH, Germany). All ELISA’s had a C.V. of less than 10%.

Cytokine production in whole blood after LPS-stimulation was calculated by subtracting the cytokine concentration in the unstimulated sample from that in the LPS-incubated sample. Cytokine production per 10E6 monocytes was calculated by dividing the total cytokine production by the number of monocytes in the peripheral blood smear.

**Statistical analysis**

For the comparison of the baseline characteristics between groups, continuous variables were analysed by Kruskall-Wallis or Wilcoxon's 2-sample test. In case of categorical variables, a Chi-square test or a Fisher's exact test was used, when appropriate. Descriptive statistics are presented as median values and interquartile range or as percentages. Univariate ANOVA was performed to identify factors influencing the inflammatory parameters. Variables having a univariate P-value <0.15 were included.
RESULTS

Patient characteristics are presented in table 1. The patients with ACS were predominantly male as compared to the patients with stable vascular disease and the healthy controls (95 vs. 66 vs. 68 % male, $P = 0.03$). However, multivariate analysis showed that gender was not a major determinant influencing outcome. Risk factors for cardiovascular disease were not different between the patients groups.

With respect to cardiovascular medication patients with stable vascular disease used more lipid-lowering medication, beta-blockers, ACE-inhibitors and nitrates, whereas the use of aspirin, diuretics and calcium antagonists did not differ between both groups.

**Levels of CRP, neopterin and white blood cell counts**

As expected CRP levels were higher in patients suffering from vascular disease compared with the healthy controls ($P <0.001$). However, no difference was found between CRP values of both patient groups ($P = 0.90$; table 2, figure 3). CRP levels correlated with neopterin levels ($r = 0.401$, $P <0.001$) and white blood cell count ($r = 0.272$, $P = 0.004$).

White blood cell counts were highest in patients with ACS as compared to patients with stable atherosclerotic disease and healthy control groups respectively ($P <0.001$; table 2, figure 2).

**LPS-induced IL-10 production**

IL-10 levels are provided in table 2 and graphically displayed in figure 1. IL-10 production per 10E6 monocytes ($P = 0.002$) as well as total IL-10 production ($P = 0.003$) after LPS stimulation was markedly and significantly lower in the ACS group as compared to patients with

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**Table 1. Baseline characteristics.**

<table>
<thead>
<tr>
<th></th>
<th>Acute coronary syndromes (n=22)</th>
<th>Stable atherosclerosis (n=50)</th>
<th>Healthy controls (n=22)</th>
<th>P-value*</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age, y</strong></td>
<td>52(41-65)</td>
<td>54(46-60)</td>
<td>48(44-53)</td>
<td>0.09</td>
</tr>
<tr>
<td><strong>Sex (M/F,(%M))</strong></td>
<td>21/1(95)</td>
<td>33/17(66)</td>
<td>15/7(68)</td>
<td>0.03</td>
</tr>
<tr>
<td><strong>Systolic blood pressure (mmHg)</strong></td>
<td>135(115-153)</td>
<td>140(130-158)</td>
<td></td>
<td>0.24</td>
</tr>
<tr>
<td><strong>Total cholesterol (mmol/L)</strong></td>
<td>5.8(5.3-6.9)</td>
<td>5.6(4.7-6.2)</td>
<td></td>
<td>0.11</td>
</tr>
<tr>
<td><strong>Medication</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aspirin, n(%)</td>
<td>14(64)</td>
<td>33(66)</td>
<td></td>
<td>1.00</td>
</tr>
<tr>
<td>Nitrates, n(%)</td>
<td>10(45)</td>
<td>8(16)</td>
<td></td>
<td>0.02</td>
</tr>
<tr>
<td>Statins, n(%)</td>
<td>4(18)</td>
<td>36(72)</td>
<td></td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Beta-blockers, n(%)</td>
<td>6(27)</td>
<td>33(66)</td>
<td></td>
<td>0.04</td>
</tr>
<tr>
<td>ACE-inhibitors, n(%)</td>
<td>2(9)</td>
<td>16(32)</td>
<td></td>
<td>0.04</td>
</tr>
<tr>
<td>Calcium antagonists, n(%)</td>
<td>4(18)</td>
<td>9(18)</td>
<td></td>
<td>1.00</td>
</tr>
<tr>
<td>Diuretics, n(%)</td>
<td>1(5)</td>
<td>6(12)</td>
<td></td>
<td>0.43</td>
</tr>
<tr>
<td><strong>Risk factors</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Smoking, n(%)</td>
<td>14(64)</td>
<td>38(76)</td>
<td></td>
<td>0.39</td>
</tr>
<tr>
<td>Family history, n(%)</td>
<td>7(32)</td>
<td>25(50)</td>
<td></td>
<td>0.20</td>
</tr>
</tbody>
</table>

* The $P$-values for three groups were calculated with the Kruskall-Wallis or Chi-square test, where appropriate. The $P$-values for two groups were calculated using either Wilcoxon's two sample test or Fisher's exact test.
Table 2. Biological and experimental parameters.

<table>
<thead>
<tr>
<th></th>
<th>Acute coronary syndromes (n=22)</th>
<th>Stable atherosclerosis (n=50)</th>
<th>Healthy controls (n=22)</th>
<th>P-value*</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>CRP, mg/L</strong></td>
<td>1.51(0.84-4.50)</td>
<td>2.09(0.91-3.57)</td>
<td>0.43(0.30-1.20)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td><strong>IL-10 after LPS challenge (pg/mL)</strong></td>
<td>16,175(7,559-28,470)</td>
<td>28,379(12,601-73,968)</td>
<td>63,830(22,040-168,000)</td>
<td>0.003</td>
</tr>
<tr>
<td><strong>IL-10 (pg per 10E6 monocytes)</strong></td>
<td>30,339(15,150-44,049)</td>
<td>45,730(22,876-189,000)†</td>
<td>110,000(38,259-357,000)</td>
<td>0.002</td>
</tr>
<tr>
<td><strong>TNF-α after LPS challenge (pg/mL)</strong></td>
<td>7,313(4,740-12,615)</td>
<td>11,002(5,913-14,190)†</td>
<td>8,229(5,227-11,364)</td>
<td>0.29</td>
</tr>
<tr>
<td><strong>TNF-α (pg per 10E6 monocytes)</strong></td>
<td>15,234(9,263-20,356)</td>
<td>17,950(11,585-25,670)†</td>
<td>18,980(11,616-25,827)</td>
<td>0.35</td>
</tr>
<tr>
<td><strong>White blood cell count, (×10E6 cells)</strong></td>
<td>9.2(7.5-12.4)</td>
<td>7.1(6.3-8.4)</td>
<td>5.7(5.2-6.7)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td><strong>Neopterin (nmol/L)</strong></td>
<td>7.4(6.0-8.7)</td>
<td>7.1(6.0-8.9)</td>
<td>6.4(5.6-7.3)</td>
<td>0.07</td>
</tr>
</tbody>
</table>

* The P-values for three groups were calculated with the Kruskall-Wallis or Chi-square test, where appropriate. † n=49

stable atherosclerosis and healthy controls. In the unstimulated samples, an insignificant spontaneous production of cytokines was found (data not shown). IL-10 production in stable atherosclerosis patients was not significantly lower as compared to healthy controls (P = 0.163). In the overall population, IL-10 production after monocyte activation was negatively correlated with neopterin levels (r = -0.192, P = 0.042) and white blood cell count (r = -0.276, P = 0.003).

**LPS-induced TNF-α production**
LPS-induced TNF-α per 10E6 monocytes as well as total TNF-α levels for the ACS-, stable vascular disease- and healthy control group were not significantly different between the groups (P = 0.29; table 2, figure 4) Linear correlations for the whole population were found between TNF-α and monocyte count (r = 0.347, P<0.001).

**DISCUSSION**
The pathogenesis of plaque rupture is characterised by an inflammatory process in which activated monocytes play an important role [2]. Monocyte activation in unstable coronary syndromes is thought to be a result of enhanced cytokine production by T-lymphocytes [18;19]. Pro- and anti-inflammatory cytokines can either activate or deactivate this inflammatory reaction.

We demonstrate, for the first time, that in patients with acute coronary syndromes, activated monocytes produce lower amounts of IL-10 after LPS-stimulation, but that the LPS-induced production of TNF-α, a pro-inflammatory cytokine, by monocytes did not differ between stable and unstable atherosclerotic disease. Patients with stable and unstable atherosclerotic disease displayed a comparable inflammatory state, reflected by elevated levels of CRP and white blood cell counts. Furthermore, levels of neopterin tended to be higher in our patients as well, indicating monocyte/macrophage activation as was reported previously [4-6]. Our findings suggest that a lack of anti-inflammatory counterbalance might be the culprit for enhanced inflammation as found in patients with unstable atherosclerotic disease.
Interleukin-10 is a major immuno-modulatory cytokine that is produced by monocytes/macrophages, T helper type 2 cells, regulatory T-cells and B-cells. It inhibits a broad array of inflammatory processes such as T helper type 1 cell (pro-inflammatory) cytokine production, antigen presentation and antigen-specific T-cell proliferation [20]. It is demonstrated that IL-10 decreases inflammatory activity in vivo, as shown in T helper type 1 mediated diseases such as psoriasis [22].

In recent years a lot of attention has been given to the role of IL-10 in the pathogenesis of atherosclerosis and the acute coronary syndromes. A protective role for IL-10 in atherosclerosis has been emphasised by a number of studies. Uyemura et al. [20] demonstrated that IL-10 was virtually absent in atherosclerotic plaques and that it had deactivating properties on the T helper 1 immune response. Recently, a protective role for IL-10 in atherosclerosis was suggested by demonstrating that IL-10 transgenic mice had reduced lesion size after an atherogenic diet [11], and Mallat et al. [12] demonstrated abundant atherosclerosis in IL-10 -/- mice, a process that could partly be prevented by the transfer of murine IL-10. In a clinical study, Smith et al. [23] noticed that levels of IL-10 were decreased in patients with acute coronary syndromes. In line with their findings, we demonstrate that in particular monocytes of these patients have a deficient production of this apparently important cytokine.

**Figures 1-4.** Levels of IL-10 per 10E6 monocytes; White blood cell counts; Levels of CRP; Levels of TNF-α per 10E6 monocytes.
The study as presented here is cross-sectional and samples are small, therefore we cannot provide insight in whether the lack of IL-10 production by monocytes is transient or persistent in our patients. It may, therefore, have been that the monocytes of the patients with ACS were 'exhausted' in their IL-10 production, this could explain the negative overall correlation that was found between IL-10 levels and levels of neopterin and white blood cell counts. However, one would have expected a lower TNF-α production as well. The fact that patients with stable atherosclerotic disease have a decreased IL-10 production as compared to healthy controls, but higher than in patients with ACS, suggests that it is a chronic feature of atherosclerotic cardiovascular disease that may be enhanced by an acute exacerbation. It can therefore be speculated that restoration of the inflammatory cytokine balance, e.g. by supplying recombinant IL-10, may prove beneficial in patients with ACS. However, this issue remains speculative, but it provides a challenge for future research.

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REFERENCES

15. Hazenberg BP, Limburg PC, Bijzet J, van Rijswijk MH. SAA versus CRP serum levels in different inflammatory conditions, studied by ELISA using polyclonal anti-AA and monoclonal anti-SAA antibodies. In: Isobe T,
Decreased IL-10 production in acute coronary syndromes


