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Cellular actors, Toll-like receptors, and local cytokine profile in acute coronary syndromes

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Aims Inflammation plays a key role in acute coronary syndromes (ACS). Toll-like receptors (TLR) on leucocytes mediate inflammation and immune responses. We characterized leucocytes and TLR expression within coronary thrombi and compared cytokine levels from the site of coronary occlusion with aortic blood (AB) in ACS patients.

Methods and results In 18 ACS patients, thrombi were collected by aspiration during primary percutaneous coronary intervention. Thrombi and AB from these patients as well as AB from 10 age-matched controls without coronary artery disease were assessed by FACS analysis for cellular distribution and TLR expression. For further discrimination of ACS specificity, seven non-coronary intravascular thrombi and eight thrombi generated *in vitro* were analysed. In 17 additional patients, cytokine levels were determined in blood samples from the site of coronary occlusion under distal occlusion and compared with AB. In coronary thrombi from ACS, the percentage of monocytes related to the total leucocyte count was greater than in AB (47 vs. 20%, $P = 0.0002$). In thrombi, TLR-4 and TLR-2 were overexpressed on CD14-labelled monocytes, and TLR-2 was increased on CD66b-labelled granulocytes, in comparison with leucocytes in AB. In contrast, *in vitro* and non-coronary thrombi exhibited no overexpression of TLR-4. Local blood samples taken under distal occlusion revealed elevated concentrations of chemokines (IL-8, MCP-1, eotaxin, MIP-1α, and IP-10) and cytokines (IL-1ra, IL-6, IL-7, IL-12, IL-17, IFN-α, and granulocyte-macrophage colony-stimulating factor) regulating both innate and adaptive immunity (all $P < 0.05$).

Conclusion In ACS patients, monocytes accumulate within thrombi and specifically overexpress TLR-4. Together with the local expression patterns of chemokines and cytokines, the increase of TLR-4 reflects a concerted activation of this inflammatory pathway at the site of coronary occlusion in ACS.

Keywords Acute coronary syndromes • Toll-like receptors • Inflammation • Thrombus

Background

Acute coronary syndromes (ACS) represent the major clinical events in the natural history of coronary artery disease (CAD).[1] Evidence is accumulating that inflammatory processes contribute to the development of ACS.[2–5] Activation of immune cells via different biological pathways leads to the production of inflammatory mediators,[6] thereby accelerating plaque vulnerability and eventually rupture. Toll-like receptors (TLR) are key regulators of both innate and adaptive immune responses.[7] Multiple endogenous

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ligands of TLR exist (e.g. heat shock proteins,[8] components of extracellular matrix,[9] fibrinogen,[10] and myeloid-related protein 8/14)[11] that activate the TLR signalling network;[12] TLR activation induces the production of proinflammatory cytokines [e.g. interleukin (IL) 6 (IL-6), IL-12, IL-1β, and tumour necrosis factor α (TNF-α)],[12,13] chemokines [e.g. IL-8,[14] monocyte chemoattractant protein 1 (MCP-1),[15] macrophage inflammatory protein (MIP)-1α,[16] MIP-1β, eotaxin,[17] and interferon-inducible protein-10 (IP-10)][18] as well as antimicrobial molecules such as nitric oxide.[19] These responses enable macrophages to eliminate invading microorganisms. Furthermore, after TLR-activation anti-inflammatory cytokines [e.g. IL-1 receptor antagonist (IL-1ra), IL-10, and IL-4][20] are released as well providing negative feedback regulation of the inflammatory response. Toll-like receptor-induced expression of co-stimulatory molecules sustains the activation of adaptive immunity.[19]

Multiple roles of TLR signalling in atherosclerosis have been postulated,[21] but the involvement of distinct members of the TLR family in the pathophysiology of ACS remains unknown. Thus, we investigated in patients with ACS undergoing primary percutaneous intervention (PCI) the composition of inflammatory cells as well as the expression of TLR within the occluding thrombus relative to the systemic circulation and compared cytokine levels from the site of coronary occlusion with aortic blood (AB).

Methods

Due to different sampling procedures, cellular analysis with TLR expression and cytokine levels were studied in two separate study populations (see Appendix 1). The institutional ethics committee approved the protocol. Written informed consent was obtained from all patients.

Cellular analysis and Toll-like receptors expression

Study population 1

Eighteen ACS patients with aspiration of macroscopically visible red thrombus during primary PCI and 10 patients with angiographically documented absence of CAD were consecutively studied.

Sample collection 1

Thrombus material was aspirated from the site of coronary occlusion during primary percutaneous coronary intervention (PCI) in patients with ACS using an Export® XT 6F Aspiration Catheter (Medtronic Inc., Minneapolis, MN, USA). The harvested thrombi were separated from blood with a sieve, placed in phosphate buffered saline (PBS), and immediately processed.

Analysis of cells within thrombi

The cellular content of thrombi and the TLR expression on the different cell types were assessed in patients with ACS. Thrombi were mechanically disrupted, cells resuspended in cell culture medium RPMI containing 10% foetal calf serum (FCS) and 0.001% tissue plasminogen activator (Actilyse®, Boehringer Ingelheim Pharma GmbH & Co., Germany) (to remove fibrin), and rotated for 24 h at 37°C before staining. Erythrolysis was performed by repetitive short mild osmotic shocks. The peripheral blood or thrombi mixture after incubation for 24 h at 37°C with Actilyse® were repeatedly incubated with distilled water for 5–10 s; then PBS was added (1:9 water:PBS) and the cells were centrifuged. This procedure has been repeated four to five times at 4°C to reduce the amount of red blood cells before the FACS analysis. The cells were labelled for 1 h at 4°C with the primary monoclonal antibodies (or the respective mixture of fluorochrome-labelled antibodies in the case of double-labelling) to differentiate leucocyte subpopulations, followed by 30 min at 4°C with the fluorochrome-labelled secondary antibodies (in the case of single staining) and finally were analysed using a FACScalibur (BD Bioscience, Heidelberg, Germany). The following monoclonal antibodies were used: anti-CD3, CD14, CD19, CD66b (unlabelled or fluorescein isothiocynate-conjugated, BD Bioscience) and anti-TLR-2, -3, -4, -9 (phycoerythrine-labelled, eBioscience, San Diego, CA, USA). Regarding the analysis of the different subpopulations, the first selection has been done on the basis of the SSC/FSC scatters, differentiating lymphocyte, monocyte, and granulocyte/blast gates (quadrant analysis). FL1 and FL2 were compensated, the FITC (FL1) and PE-labelled (FL2) antibodies (for example anti-CD14-FITC and anti-TLR-4-PE) were analysed separately or in combination. The given subpopulation was selected in the FL1 quadrant and the intensity of TLR-4 was analysed in a frequency histogram showing FL2 fluorescence. Mouse IgG1 was used as isotype control. The proportion of positive cells and median fluorescence intensity (mfi) were determined.

Analysis of aortic blood cells

Cell numbers were analysed using standard procedures (using a Casy1 counter, Schärfe System, Reutlingen, Germany). The same incubation conditions, labelling procedures, and flow cytometry analysis were applied for AB cells, as for the cells isolated from thrombi.

Control thrombi

To separate thrombus-specific rather than ACS-specific mechanisms and to determine potential effects of coagulation products on TLR expression, we analysed thrombi generated *in vitro*, and *in vivo* thrombi from other clinical settings than ACS.

In vitro thrombi

Peripheral blood samples from eight healthy subjects were separated into two parts: (i) either 500 μL of blood were incubated with 500 μL recombinant tissue factor (rTF, solution ready to use, Innovin, Dade-Behring, Marburg, Germany) at 37°C in cell culture media (RPMI-1240 including 10% FCS) for 1–6 h with continuous rotation to obtain an *in vitro* thrombus; or (ii) leucocytes were isolated by Ficoll-paque centrifugation and then incubated with the same concentration of tissue factor (to exclude any direct influence of rTF on the cells). The preparations were incubated for 6 and 12 h at 37°C. The control samples were treated in the same manner as cells of thrombi from *in vivo* settings. Cells were extracted using 0.001% Actilyse® stained for CD14 (FITC) and TLR-4 (PE), and analysed by flow cytometry. Measurements were performed over a time course from 1 to 12 h.

In vivo thrombi

We collected intravascular thrombi from other clinical settings and vascular beds than ACS (six peripheral arterial thrombi from patients with limb ischaemia and one pulmonary embolus) and analysed them according to the protocol for intracoronary thrombi and *in vitro* thrombi.

Immunohistochemistry

Immunohistochemistry of intracoronary thrombi was performed on paraffin-embedded parallel sections on separately collected specimens, using anti-TLR-4 and anti-TLR-2 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) as primary antibodies. Further, goat anti-mouse biotinylated antibodies and streptavidin conjugated to alkaline phosphatase (Jackson Laboratories, Bar Harbor, ME, USA) and Dako

Fast Red Substrate were used. Murine IgG was used as negative control for primary antibodies. Rheumatoid arthritis synovial membranes were used as positive controls.

Measurement of cytokine levels

Study population 2

This group consisted of 17 patients presenting with ACS and an identifiable culprit lesion at angiography during primary PCI plus at least two of the following three criteria: (i) coronary occlusion, (ii) thrombus formation, (iii) impairment of the wall motion in the supplied territory by left ventricular angiography. Exclusion criteria were a serum level of C-reactive protein $\geq$10 mg/L, myocarditis, pericarditis, or a left ventricular ejection fraction of $\leq$35%.

Sample collection 2

Serum levels of inflammatory markers were assessed in blood samples obtained simultaneously from the site of coronary occlusion and the aorta. In all patients, the culprit artery was intubated with a 7F guiding catheter and wired with the 0.014 inch PercuSurge™ GuardWire™ (Medtronic Inc., Minneapolis, MN, USA) as described.[6] An Export® XT aspiration catheter was used to obtain local blood samples. To minimize potential dilution effects with saline, the Export® catheter was immediately inserted without flushing over the PercuSurge GuardWire with the distal occlusion activated. The aspiration was started at the most distal point and stopped after one passage at the proximal point of the culprit coronary artery segment. Thus, only the filling volume of the Export® catheter might have caused a minor dilution with AB. This protocol resulted in relatively small volumes (0.5–2.0 mL) of intracoronary blood, however, sufficient for processing and analysis with the multiplex technology (see what follows). Only patients with poor flow ($\leq$ TIMI grade I) after wire passage were included to ensure preserved local conditions.

Measurement of cytokines/chemokines and apoptosis markers

Twenty-five cytokines and chemokines were analysed using bead-based multiplex technology: interleukin-1β (IL-1β), interleukin-1 receptor antagonist (IL-1ra), IL2, IL-2R, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12, IL-13, IL-15, IL-17, tumour necrosis factor (TNF)-α, interferon (IFN)-α, IFN-γ, granulocyte-macrophage colony-stimulating factor (GM-CSF), macrophage inflammatory protein (MIP)-1α, MIP-1β, interferon-γ-inducible protein (IP)-10, monokine induced by interferon-γ (MIG), eotaxin, RANTES (regulated on activation, normal T cell expressed and secreted), and monocyte chemoattractant protein (MCP)-1 (multiplex detection kit, Biosource International, Camarillo, CA, USA). The kit contains 96-well microtiter filter plates, coated with antibodies specific for certain cytokines or chemokines.

Serum concentrations of soluble vascular cell adhesion molecule (VCAM)-1, soluble intercellular adhesion molecule (sICAM)-1, sFas, soluble Fas ligand (sFasL); macrophage migration inhibitory factor (MIF) and tissue plasminogen activator inhibitor (tPAI)-1 were determined using a multiplex assay capable of simultaneously quantifying these six analytes (Linco Res. Inc., MO, USA). In addition, C-reactive protein was measured using a chemiluminescence immunoassay from Siemens diagnostics (formerly DPC) as previously reported.[6]

Statistical analysis

Continuous data are presented as median with the interquartile ranges (IQR). In the figures, data are displayed as box-and-whisker plot (the smallest observation, lower quartile, median, upper quartile, and largest observation). A sample size of 10 patients to detect a difference of 12 mfi units in TLR expression on monocytes (between thrombus vs. AB) given an alpha error of 0.05 and an error standard deviation of 10 mfi units would result in a power of 0.92. We used the Anderson–Darling, D'Agostino Skewness/Omnibus/Kurtosis, Shapiro–Wilk, Martinez–Iglewicz, and Kolmogorov–Smirnov test to check the normality. In samples from the same patient, variables were analysed using the Wilcoxon signed rank test (matched pairs), comparing samples from different patients we used the Mann–Whitney *U*-test (unmatched pairs). Spearman's rank correlation coefficient was used to measure the linear relationship between two variables. For regression-type problems (continuous dependent variable), we actually computed *F*-tests (with Bonferroni adjusted *P*-values). Furthermore, we used effect size (ES) measures (Cohen's *d*; Hedge's bias correction) to characterize the strength of the relationship between two variables. We defined ESs as 'small, $d \geq 0.2$,' 'medium, $d \geq 0.5$,' and 'large, $d \geq 0.8$'. Statistical analysis was performed with SPSS (version 15; SPSS Inc., Chicago, IL, USA), NCSS (Kaysville, Utah), and GraphPad Prism statistical software (GraphPad Software Inc., San Diego, CA, USA). A *P*-value < 0.05 was considered statistically significant.

Results

Cellular actors

Study population 1

Eighteen ACS patients with aspiration of macroscopically visible red thrombus during primary PCI were consecutively studied. TIMI-0 flow (corresponding to a total occluded artery) was present in 17 of 18 patients (95%, TIMI-1 flow in one patient). Mean age was 58 (SD $\pm$ 12) years, 94% were males. No diabetics were included, dyslipidaemia was present in 50% of the patients, arterial hypertension in 44%, 33% had a positive family history for premature cardiovascular events, and 50% were smokers (*Table 1*). Twenty-seven per cent of the patients were on aspirin and clopidogrel-pre-treatment was rare (6%). GP IIb/IIIa-inhibitors were initiated during PCI in 72% of the patients (33% abciximab and 39% tirofiban). Heparin was administered in 94%, one patient underwent the procedure with dalteparine. The AB samples of the control population were taken from 10 age-matched healthy controls with a similar risk factor profile and angiographically documented absence of CAD. Patients with ACS underwent cardiac catheterization on average 4 h after the onset of symptoms.

Cellular distribution of leucocytes in coronary thrombi vs. aortic blood

The cellular composition of the intracoronary thrombi from patients with ACS (study population 1) differed significantly from blood obtained in the aorta at the same point in time. Almost half of the cells, on median (IQR) 47% (37–54%) of the leucocytes within the thrombi were monocytes, whereas the proportion of these cells in the corresponding AB accounted for only 20% (9–28%) ($P = 0.0002$, ES $d = 2.3$). The second most common leucocytes within the thrombi were T-cells and granulocytes. Within the thrombi, the percentage of T-cells was significantly lower [24% (18–31%)] when compared with the AB [34% (30–41%), $P = 0.0035$, ES $d = 1.3$; *Figure 1*]. The lower percentage of T-cells within the thrombi was negatively correlated with the increased percentage of monocytes (Spearman's rank correlation coefficient -0.825). Similarly, the percentage of B-cells was

Table 1 Clinical characteristics of the study populations

	Population 1			Population 2
	ACS patients ($n = 18$)	Controls ($n = 10$)	*P*-value	ACS patients ($n = 17$)
Mean age (years)	59 (±12)	61(±11)	0.33	55 (±2)
Risk factors				
Dyslipidaemia	50%	44%	0.30	54%
Hypertension	44%	55%	0.29	42%
Diabetes	None	None	–	33%
Smoking	50%	44%	0.23	58%
Family history	33%	33%	0.28	13%
Previous statin medication	10%	33%	0.16	13%
Clinical presentation				
STEMI	77%	–		70%
Angiographic degree of CAD				
1-vessel disease	45%	–		58%
2-vessel disease	30%	–		25%
3-vessel disease	25%	–		17%
Culprit vessel				
LAD	50%	–		58%
CX	6%	–		29%
RCA	44%	–		13%
Lesion characteristics				
Total occlusion	94%	–		100%

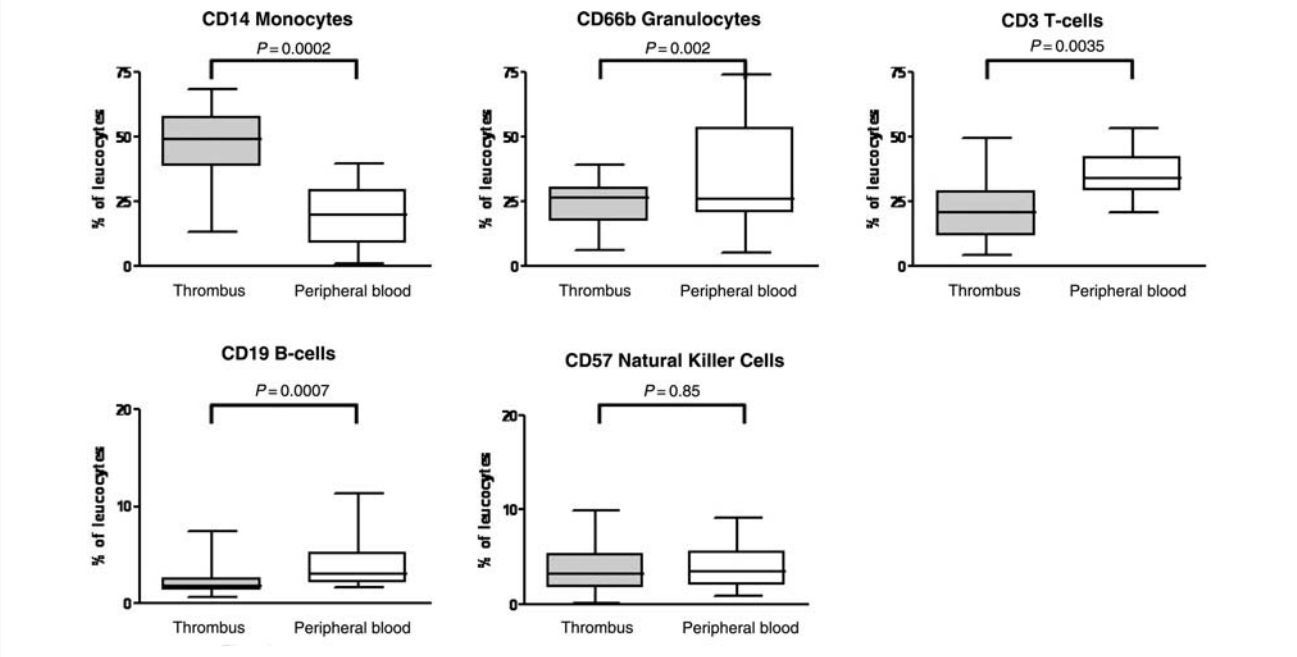

Figure 1 Distribution of leucocytes (%) in coronary thrombi compared with aortic blood samples in patients with acute coronary syndrome. Increased per cent of monocytes within coronary thrombi compared with monocytes from aortic blood was observed, whereas the per cent of granulocytes, T- and B-cells was decreased. Data are represented as box-and-whisker plot (the smallest observation, lower quartile, median, upper quartile, and largest observation).

lower in the thrombi [2% (1–3%)] compared with the AB [4% (2–5%), $P = 0.0007$, ES $d = 0.8$]. Finally, granulocytes were also less prevalent within thrombi [mean 23%, median 24% (15–30%)] than in the AB [mean 35%, median 26% (21–53%), $P = 0.002$, ES $d = 0.8$]. No such differences of local coronary and systemic levels could be documented for natural killer cells with 3.5% (2.3–5.4%) in the thrombi vs. 3.4% (2.0–5.5%) in the AB ($P = 0.85$, ES $d = 0.1$).

Cellular distribution of leucocytes in aortic blood of acute coronary syndrome patients vs. controls

Absolute total white cell blood count was significantly elevated in ACS patients [10.8 (8.5–12.7) $\times 10^3$ per μL compared with 7.4 (5.3–8.8) $\times 10^3$ per μL in controls; $P = 0.0068$]. Nevertheless, on comparison of the percental cellular composition of the AB of ACS patients with controls, no differences were found for the proportion either of monocytes ($P = 0.62$), B-cells ($P = 0.68$), natural killer cells ($P = 0.87$), or granulocytes ($P = 0.43$). There was only a trend to an increased percentage of T-cells ($P = 0.052$) in ACS patients (Appendix 2).

Toll-like receptors expression

Leucocytes within coronary thrombi

There was a significantly increased TLR-4 expression [43% (20–58)] on the leucocytes from the thrombus compared with those obtained from the AB [24% (19–29%), $P = 0.0013$, ES $d = 1.3$]. Similarly, TLR-2 were more markedly expressed [25% (16–52%)] in leucocytes within the coronary thrombi than in the corresponding AB [22% (10–24%), $P = 0.0046$, ES $d = 0.8$]. No such differences could be documented for the expression of TLR-3 ($P = 0.19$) and TLR-9 ($P = 1.0$) (Appendix 3).

Aortic leucocytes

The expression of TLR-4 was significantly more pronounced in leucocytes of ACS patients [24% (19–29%)] when compared with controls [12% (9–15%), $P = 0.0012$]. No such differences could be documented either for the expression of TLR-2 ($P = 0.23$), TLR-3 ($P = 0.11$), and TLR-9 ($P = 0.11$) (Appendix 4).

Toll-like receptors on leucocyte subtypes

FACS analysis revealed a markedly increased TLR-4 median fluorescence activity [mfi; 78 (64–85) mfi units] on local $CD14^+$ monocytes obtained from the thrombus compared with aortic monocytes [65 (57–70) mfi units, $P = 0.0061$]. Furthermore, TLR-4 mfi on monocytes of the controls was significantly lower compared with both thrombus and aortic monocytes of ACS patients [47 (40–49) mfi units, $P < 0.0001$]. TLR-2 median fluorescence activity on monocytes in the thrombus [153 (126–194) mfi units] was elevated compared with aortic monocytes of the same patients [144 (126–159) mfi units, $P = 0.041$] (*Figure 2*).

The median fluorescence activity of TLR-2 on $CD66b^+$ granulocytes was similar. Increased TLR-2 mfi was noted on local $CD66b^+$ granulocytes obtained from the thrombus compared with aortic granulocytes [76 (69–85) mfi units vs. 64 (54–81) mfi units, $P = 0.002$]. TLR-2 mfi on granulocytes of controls was even lower [48 (43–60) mfi units, $P = 0.019$]. However, TLR-4 median fluorescence activity on $CD66b^+$ granulocytes remained unchanged in the thrombus compared with aortic cells [47 (40–59) mfi units vs. 47 (41–53) mfi units, $P = 0.51$] but differed significantly from controls [30 (29–40) mfi units, $P = 0.0008$; *Figure 2*].

Control thrombi

In vitro thrombi

The control samples were treated in the same manner as cells of the thrombus, i.e. incubation with Actilyse® for 24 h at 37°C. The relative proportion of granulocytes is reduced by about 10% with this procedure, probably due to apoptosis. However, the yield of cells obtained from thrombi for FACS analysis is increased thereby. The procedure has no effect on the cell surface expression of TLR-4, as substantiated in cells before and after treatment (in three cases). Over a time-course of 12 h, the expression of TLR-4 on CD14+ monocytes remained in the same range as previously observed in AB cells from controls (median mfi: 0 h, 39; 1 h, 41; 2 h, 42; 4 h, 42; 6 h, 39; controls 45).

In vivo thrombi

We collected six thrombi from patients with peripheral arterial occlusions. Median age was 74 years (range 60–90 years). FACS analysis revealed a markedly lower TLR-4 median fluorescence activity [30 (24–39) mfi units] on local $CD14^+$ monocytes obtained from these arterial thrombi compared with monocytes from coronary thrombi [78 (64–85) mfi units, $P = 0.0004$, see Appendix 5]. Furthermore, we analysed an additional surgically extracted pulmonary embolus in a 60-year-old patient with deep venous thrombosis, which demonstrated a comparably lower TLR-4 expression (52 mfi).

Influence of thrombus age on Toll-like receptor-4 expression

We correlated the age of the thrombi, calculated according to the patients' history details, with TLR-4 expression on monocytes. Regarding the whole timeline of collected thrombi (event-to-balloon time range from 45 min to 5 days), no significant correlation between TLR-4 mfi on CD14+ monocytes could be detected (Spearman's rank correlation coefficient $r = 0.32$, $P = 0.18$). Even when including only thrombi of a probable age <12 h ($n = 14$), the correlation was very weak (Spearman's rank correlation coefficient $r = 0.45$, $P = 0.11$). Interestingly, *in vitro* thrombi (not exposed to their natural environment of cytokines, extracelluar matrix, necrotic debris, etc.) expressed TLR-4 on CD14+ monocytes at a still lower level and did not show any upregulation over the first 12 h (Spearman's rank correlation coefficient $r = -0.23$, $P = 0.66$) (see *Figure 3*).

Immunohistochemistry

TLR-4 expression was concentrated in distinct regions of the intracoronary thrombi (*Figure 4*). There were groups of mainly mononuclear leucocytes with high expression, whereas in regions with mainly polymorphonuclear leucocytes almost no TLR-4 activity was observed. Immunohistochemistry for TLR-2 demonstrated a reciprocal setting: regions with mainly polymorphonuclear leucocytes showed elevated TLR-2 activity, in contrast to mononuclear leucocyte clusters. In control thrombi, leucocytes showed generally a very weak TLR-4 expression. Furthermore, the nested

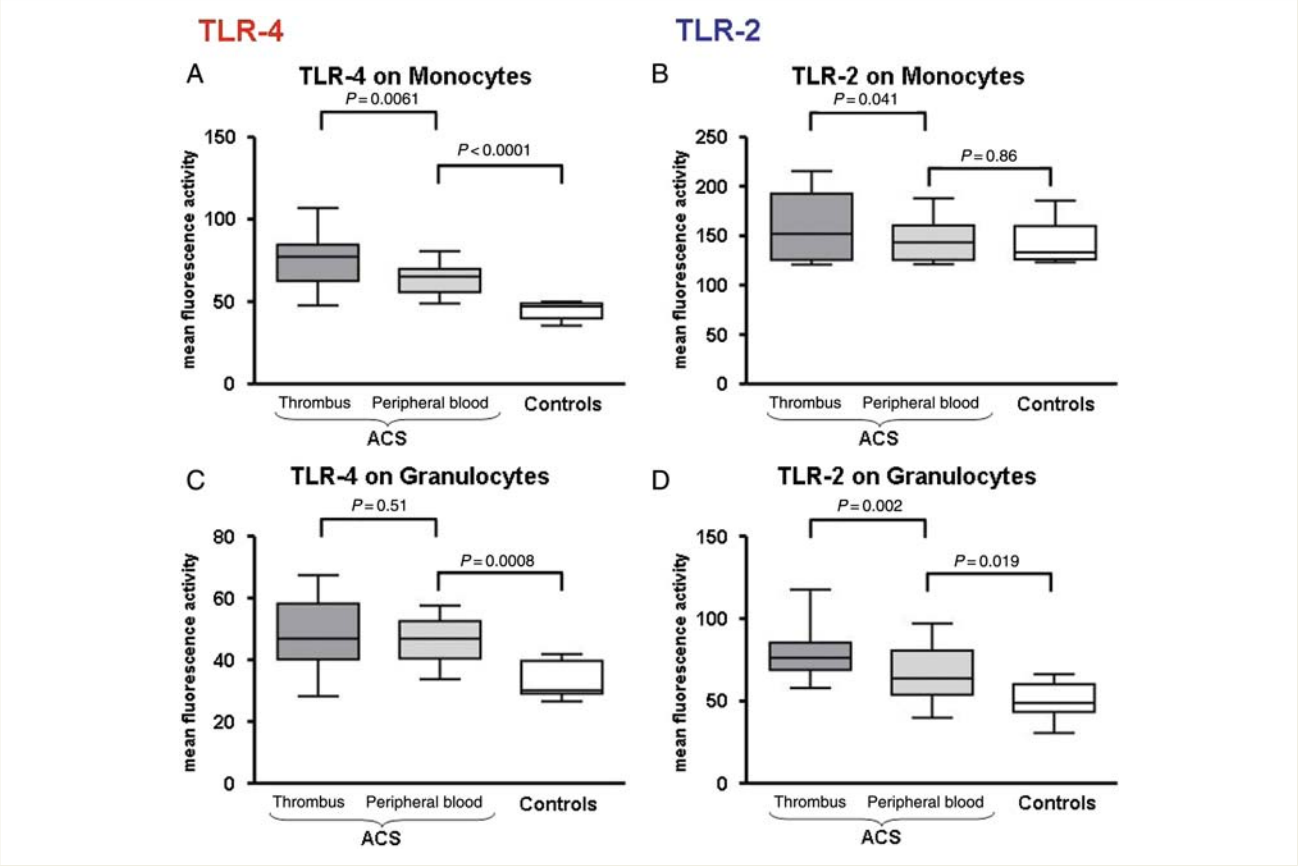

Figure 2 Expression of TLR-4 and TLR-2 on $CD14^+$ monocytes or $CD66b^+$ granulocytes within the thrombi, aortic blood samples of ACS patients and controls. Markedly increased expression of TLR-4 and TLR-2 was observed on $CD14^+$ monocytes obtained from the thrombus, when compared with aortic monocytes of the same ACS patients. Increased TLR-2 median fluorescence was noted on local $CD66b^+$ granulocytes from the thrombus, compared with aortic cells. The flow cytometric data from double-labelling (CD14-FITC/TLRs-PE or CD66b-FITC/TLRs-PE) are represented as box-and-whisker plot (the smallest observation, lower quartile, median, upper quartile, and largest observation).

appearance of leucocytes observed in intracoronary thrombi was missing, leucocytes in control thrombi appeared isolated within filamentous material and erythrocytes (Appendix 6).

Cytokine levels

Patient characteristics of study population 2 are shown in *Table 1*. All patients had totally occluded culprit arteries (TIMI-0 flow) at presentation. Only one patient had prior aspirin medication, no clopidogrel-pre-treatment was documented. Heparin was used as anticoagulant in all patients. GP IIb/IIIa-inhibitors were initiated during PCI in all patients (59% abciximab and 41% tirofiban).

Local concentrations of IL-17 ($P = 0.030$), GM-CSF ($P = 0.013$), IFN-α ($P = 0.007$), IL-6 ($P = 0.008$), IL-8 ($P = 0.013$), MIF ($P = 0.007$), eotaxin ($P = 0.019$), IL-1ra ($P = 0.013$), IL-12 ($P = 0.001$), MIP-1α ($P = 0.001$), MCP-1 ($P = 0.003$), tPAI ($P = 0.009$), and IP-10 ($P = 0.013$) were significantly elevated compared with aortic levels (*Figure 5*). The most prominent differences were identified for IL-12 as evidenced by the highest ES (0.81; power 0.94). No significant concentration gradients were detected for C-reactive protein ($P = 0.62$), TNF-α ($P = 0.06$), IL-1β ($P = 0.20$), IL-10 ($P = 0.23$), IL-4 ($P = 0.06$), IFN-γ ($P = 0.08$), MIG ($P = 0.80$), RANTES ($P = 0.09$), MIP-1β ($P = 0.14$), IL-2 ($P = 0.22$), IL-2R ($P = 0.25$), IL-7 ($P = 0.36$), IL-13 ($P = 0.06$), IL-15 ($P = 0.23$), sFas ($P = 0.83$), sFasL ($P = 0.06$), sICAM ($P = 0.55$), and sVCAM ($P = 0.18$).

Discussion

We have recently shown an increased local formation and secretion of cytokines such as interleukin-6, serum amyloid A,[6] and myeloid related protein 8/14[22] (MRP8/14) at the site of coronary occlusion in patients with ACS suggesting that local inflammatory responses are crucial in the development of this syndrome. However, many cellular sources and pathways of activation of these processes remain still unclear. In the present study, we characterize the cellular composition and TLR expression of occluding coronary thrombi and the local cytokine milieu in patients with ACS.

Compared with AB, coronary thrombi contained more CD14-positive monocytes, the most prominent cell type at the site of occlusion. Accordingly, B-cells and T-cells, as well as granulocytes, were less prevalent within thrombotic material.

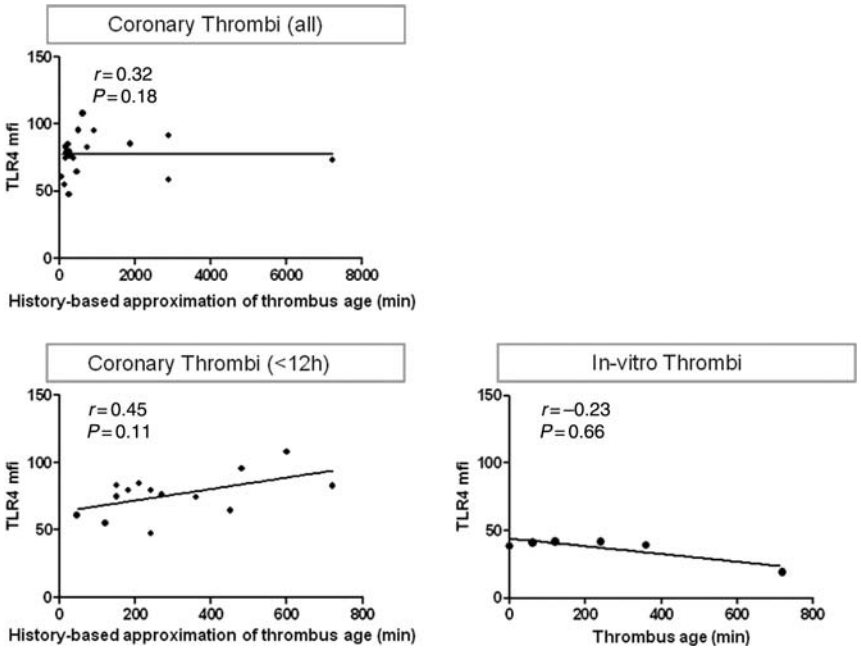

Figure 3 Influence of the age of the thrombi on TLR-4 expression. Regarding the whole timeline of collected thrombi (event-to-balloon time range from 45 min to 5 days), no significant correlation between TLR-4 mfi on CD14+ monocytes could be detected (Spearman's rank correlation coefficient $r = 0.32$, $P = 0.18$). By including only thrombi of suspected age <12 h ($n = 14$), there seemed to be a slight increase in TLR-4 expression over time, but correlation was very weak (Spearman's rank correlation coefficient $r = 0.45$, $P = 0.11$). In *in vitro* thrombi, no upregulation over the first 12 h could be documented (Spearman's rank correlation coefficient $r = -0.23$, $P = 0.66$).

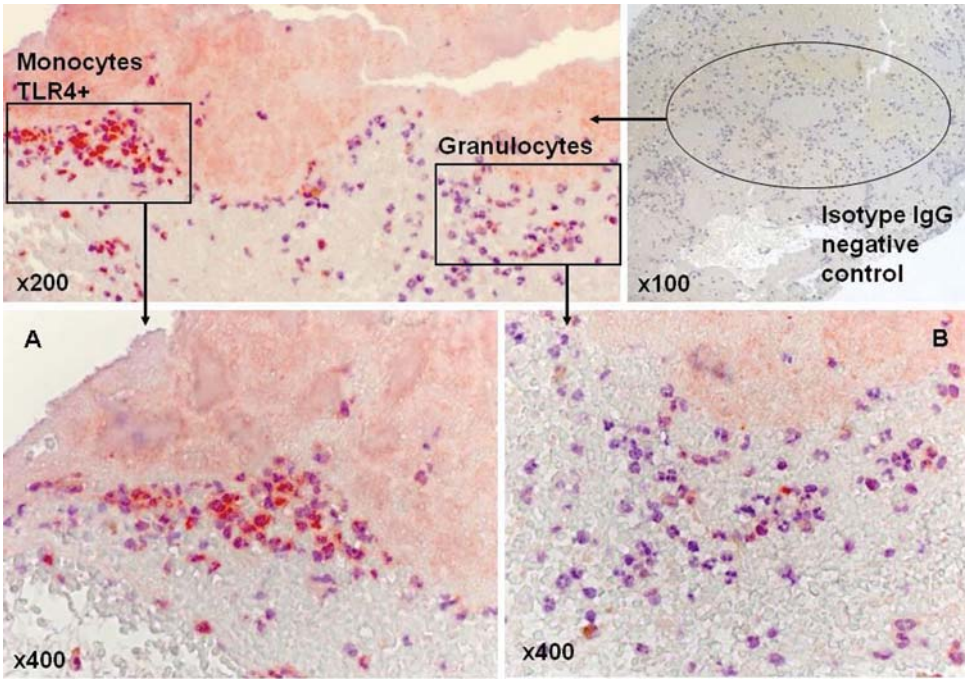

Figure 4 Expression of TLR-4 on leucocytes within the intracoronary thrombi. The expression of TLR-4 is concentrated in distinct regions of the intracoronary thrombi. There are groups of mainly mononuclear leucocytes with high expression (*A*), whereas in regions with mainly polymorphonuclear leucocytes almost no TLR-4 activity was observed (*B*).

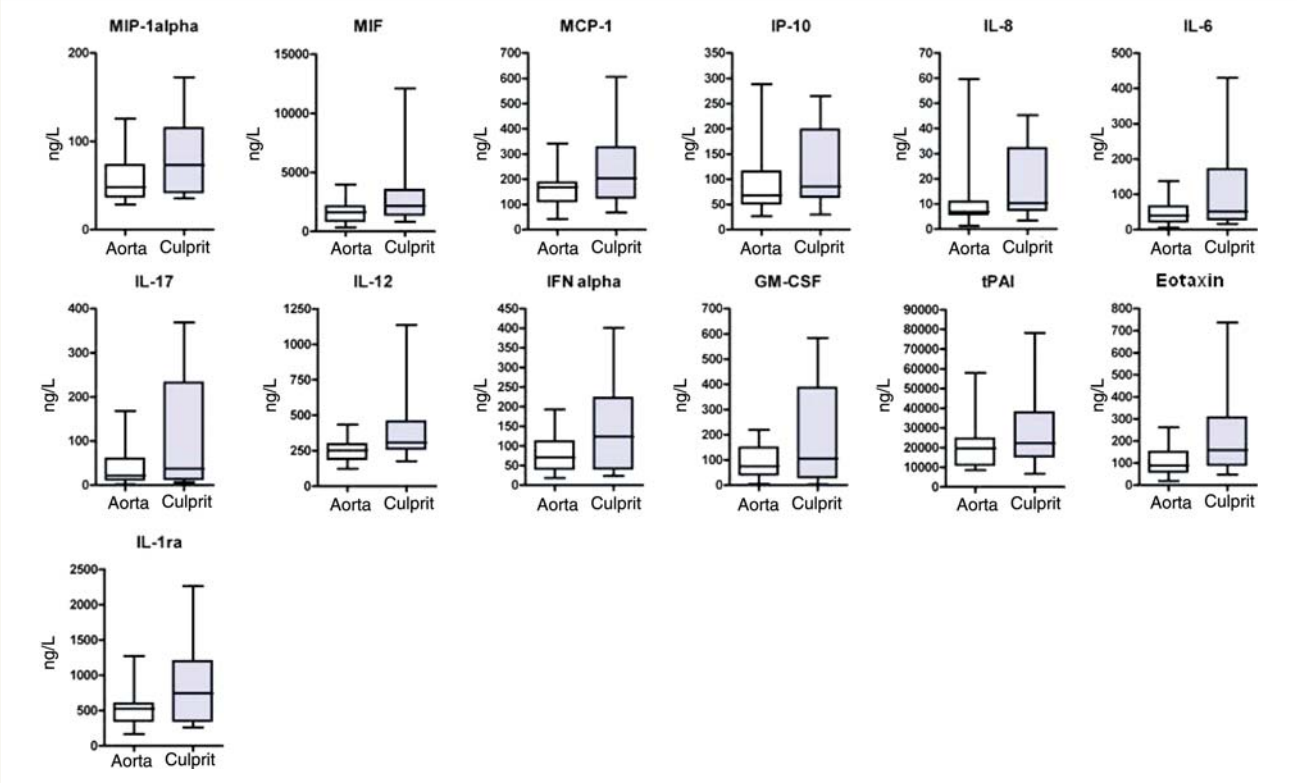

Figure 5 Locally elevated level of cytokines at the site of plaque rupture in ACS. In study population 2, local concentrations of IL-17, GM-CSF, IFN-α, IL-6, IL-8, MIF, eotaxin, IL-1ra, IL-12, MIP-1α, MCP-1, tPAI, and IP-10 were significantly elevated compared with aortic levels (all $P < 0.05$). Data are represented as box-and-whisker plot (the smallest observation, lower quartile, median, upper quartile, and largest observation).

Different levels of CD14-positive monocytes in the peripheral blood of various ACS subgroups have been described.[23] The fact that monocytes were more prevalent than other leucocytes at the site of coronary occlusion, however, suggests that they specifically accumulate in the thrombus on vulnerable plaques and contribute to the inflammatory reactions during coronary occlusion in ACS. Nevertheless, the origin and the time-course of appearance of monocytes in intracoronary thrombi remain unclear. Our methodological approach provides a snapshot of the intracoronary status at the time of primary PCI for ACS.

Toll-like receptors are expressed in atherosclerotic lesions and activated plaques.[24,25] In our study, also cells within the thrombus exhibited signs of activation, in particular overexpression of TLR. Compared with leucocytes in the AB, TLR-4 and TLR-2 were overexpressed on local CD14-labelled monocytes and TLR-2 on CD66b-labelled granulocytes. In contrast, no difference was observed in the expression of TLR-3 and TLR-9 between leucocytes obtained from the thrombus and AB. To clarify, whether this induction is ACS specific or whether this is an intrinsic feature of any *in vivo* thrombus, we correlated the suspected age of the thrombi with TLR-4 expression on monocytes according to the duration of clinical symptoms. We do have a considerable number of early presenters showing a significant activation of the TLR system in coronary thrombi. No correlation whatsoever can be shown over time. The fact that TLR were not activated in monocytes contained in thrombi produced *in vitro* demonstrates that the inflammatory milieu at the site of coronary occlusion specifically upregulates these receptors. The ACS specificity of TLR-4 overexpression on monocytes at the site of plaque rupture is further supported by the overexpression of TLR-4 in coronary thrombi compared with peripheral blood, and with clots in other settings (peripheral arterial thrombi/emboli and pulmonary embolus) as described earlier.

The association of CD14 and TLR-4 has been well documented. Indeed, CD14 is required to streamline LPS-induced signalling on TLR-4 by assembling with the MD2 protein[26] (*Figure 6*). Further, TLR-4 and TLR-2 activity is increased on CD14-positive monocytes in patients with CAD and ACS.[27–29] Intracellular signal transduction and cross-talk between TLR-2 and TLR-4[30] is complex, but eventually leads to the local release of cytokines and chemokines with proinflammatory, but also anti-inflammatory properties. Of note, particularly repetitive low-level activation of the TLR system could be protective to damage by promoting a pronounced anti-inflammatory cytokine release to dampen the inflammatory response during subsequent exposure,[20,31] while marked activation—such as must be the case in ACS—leads to a pronounced inflammatory response. In monocytes, the TLR-4 and the type I interleukin-1 receptor (IL-1R) have a unique intracellular TIR (Toll/IL-1 receptor) signalling domain. In response to activation by the corresponding ligands, TIR domains interact with the TIR

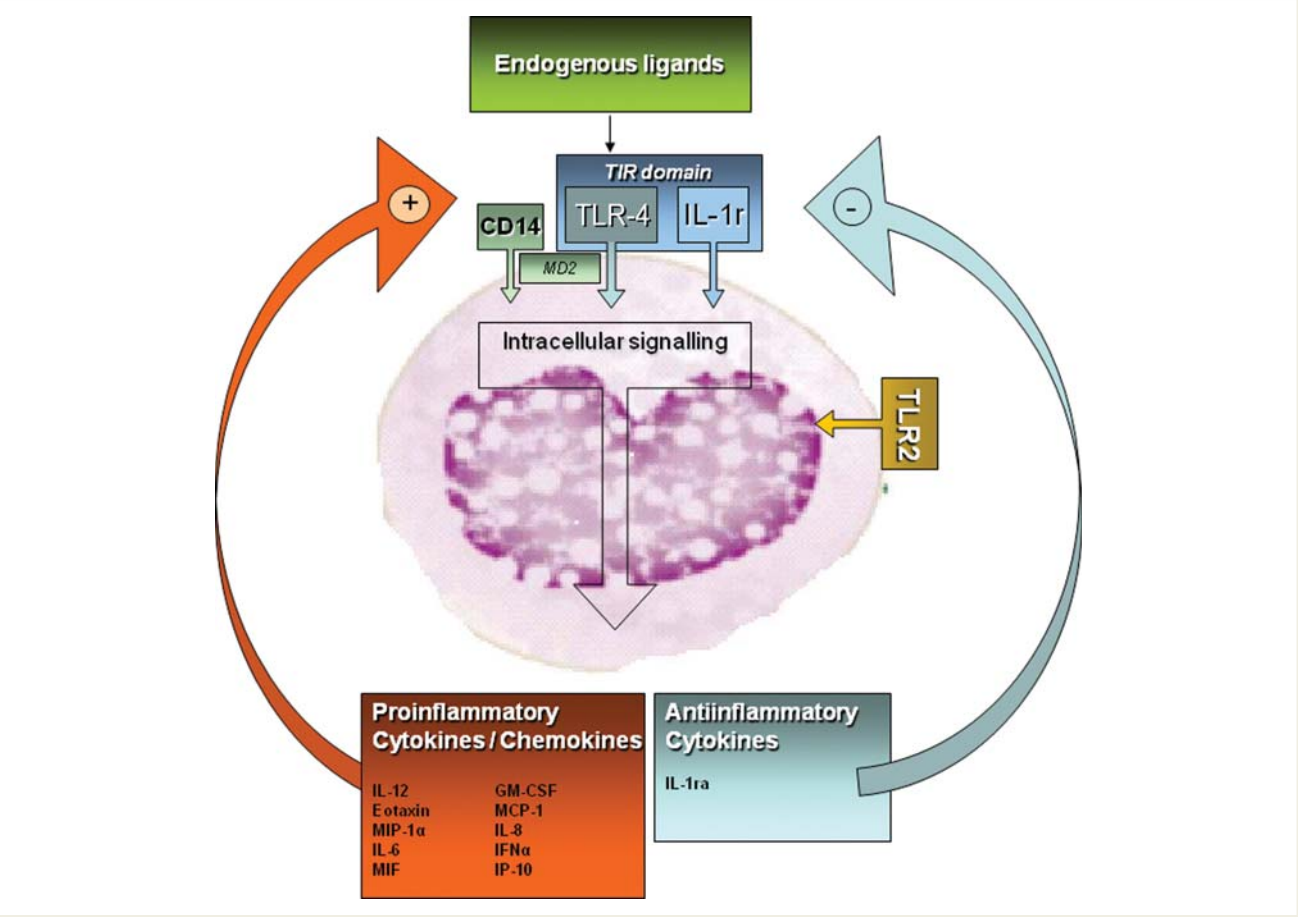

Figure 6 TLR system on monocytes and surrounding cytokine milieu in ACS. Several endogenous ligands to TLR-4 have been postulated. CD14 is required to streamline LPS-induced signalling on TLR-4 by the MD2-protein. In monocytes, the TLR-4 and the type I interleukin-1 receptor (IL-1R) have a unique intracellular TIR (Toll/IL-1 receptor) signalling domain. In response to the activation by the corresponding ligands, TIR domains interact with the TIR domain of the cytoplasmatic adaptor proteins. The subsequent intracellular signalling regulates the production and release of different cytokines. In our study, the cytokine milieu at the site of coronary occlusion was characterized by elevated concentrations of chemokines related to an innate immune response (e.g. IL-8, MCP-1, eotaxin, MIP-1α, and GM-CSF) and cytokines regulating adaptive immunity (e.g. IL-12, IL-7, IL-17)[33].

domain of the cytoplasmatic adaptor proteins. The subsequent intracellular signalling regulate the production and release of different cytokines[7,32] (*Figure 6*). In our study, the cytokine milieu at the site of coronary occlusion was characterized by elevated concentrations of chemokines related to an innate immune response (e.g. IL-8, MCP-1, eotaxin, MIP-1α, and GM-CSF) and cytokines regulating adaptive immunity (e.g. IL-12, IL-7, and IL-17).[33] The most prominent differences in local cytokine release were identified for IL-12, which underlines the important interplay of innate and adaptive immunity. The production of IL-12 but relative lack of IL-10 suggests that the local environment around the ruptured plaque is conducive for Th1 differentiation, which goes very well together with previously reported findings.[34] Interestingly, classic proinflammatory cytokines such as TNF-α or IL-1β usually released upon TLR-4 activation by LPS were locally not significantly elevated. Similarly, anti-inflammatory cytokines that directly and negatively regulate TLR-like IL-10 and IL-4 were not elevated at the site of plaque rupture. Nevertheless, we could observe a trend towards local elevation of TNF-α ($P = 0.06$) and IL-4 ($P = 0.06$), which might have become significant without the small potential dilution effects that cannot be excluded. The locally elevated levels of anti-inflammatory IL-1ra suggest a negative feedback regulation via the TLR-4-associated IL-1 receptor in the TIR domain. TLR-4–TLR-2 cross-talk induces a positive feedback signal leading to sustained and amplified activation[30] (*Figure 6*). Thus, these results suggest a rather specific pattern of expression of cytokines and chemokines after TLR activation possibly related to the endogenous ligands involved in coronary occlusion in ACS.

The present data suggest that the TLR-4 and TLR-2 signalling pathways on $CD14^+$ monocytes could represent a relevant signal transduction mechanism of innate immunity which mediates local vascular inflammation in ACS. In particular, control thrombi of healthy subjects expressed TLR-4 at lower levels, suggesting that the overexpression of these receptors on monocytes in ACS is indeed disease-specific. It has been suggested that the pleiotropic effects of statins involved in plaque stabilization may

be mediated via a reduced expression of TLR-4 on monocytes[35] leading to an attenuated immune response. Hypo-responsive TLR-4 genetic variants have been associated with a reduced risk of myocardial infarction,[36,37] albeit data on TLR-4 polymorphism are controversial.[38,39] Nevertheless, TLR-4 may provide a novel therapeutic target. Of note, TLR-ligand-based therapies have been considered recently for drug developement[40] and their therapeutic or preventive effects in infectious, malignant, autoimmune, and allergic diseases are under evaluation.[41] However, the complexity and redundancy of the TLR-system, as well as the growing number of endogenous ligands and a number of intracellular signalling molecules and transcription factors regulating pro- and anti-inflammatory cytokine production and release limit the predictability of medical interventions within this system. Thus, a better understanding of the role of TLR ligands and activation, signalling, and feedback regulation in ACS is necessary.

We acknowledge several limitations of this study. First, we did only investigate inflammatory pathways and mediators within thrombi and local blood samples. The inflammatory processes within the adjacent vessel wall are not addressed in the present study. The origin of the cells and cytokines accumulating in intracoronary thrombi may therefore be either derived from the circulating blood and/or the underlying vulnerable plaque.

Some dilution effect in particular due to the filling volume of the catheter is likely and cannot be excluded. This raises the question whether negative findings (such as the lack of significant increase in TNF-α or IL-1β, IL-10, and IL-4) might be confounded by dilution. Nevertheless, this limitation does certainly not negate the positive findings. Assumption of any minimal dilution effect, however, implicates reinforcement of significant elevations.

An ultimate proof for the ACS specifity of TLR upregulation is beyond the scope of this study. Notwithstanding, when correlating the calculated age of thrombi with TLR-4 expression, no influence of thrombus age was found. Although determination of the exact age of thrombi from other clinical settings than ACS, namely peripheral arterial/pulmonary emboli, is certainly more difficult (probably they are more often subacute and thus older than coronary thrombi), the difference in TLR-4 expression was highly significant and uniform. This finding further supports the ACS specificity of our findings.

It had been shown previously that antithrombotic therapy can influence gene expression in monocytes.[42] We cannot exclude a possible influence of an intensified antithrombotic therapy in ACS patients compared with control patients without antithrombotic regimen during catheterization. Nevertheless, in the analysis of local vs. systemic TLR upregulation on monocytes each patient is his own control, eliminating therefore a bias in periprocedural antithrombotic regimen.

Furthermore, there is a variety of pathophysiological variants in ACS (e.g. plaque morphology, heralded vs. unheralded infarctions, etc.), which may display different inflammatory reaction patterns that need further investigations.

In conclusion, monocytes accumulate in ACS patients within thrombi and markedly and specifically overexpress TLR-4. Together with the local expression patterns of chemokines and cytokines, the increase of TLR-4 reflects a concerted activation of this inflammatory pathway at the site of coronary occlusion in ACS. Through a better understanding of the role of TLR activation and signalling in ACS, specific TLR-targeted therapies could be beneficial in cardiovascular diseases in the future.

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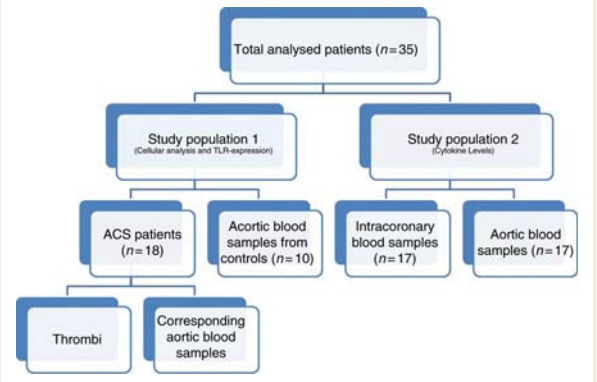
Conflict of interest: none declared.

Appendix 1

Due to different sampling procedures, cellular analysis with TLR-expression and cytokine levels were studied in two separate study populations.

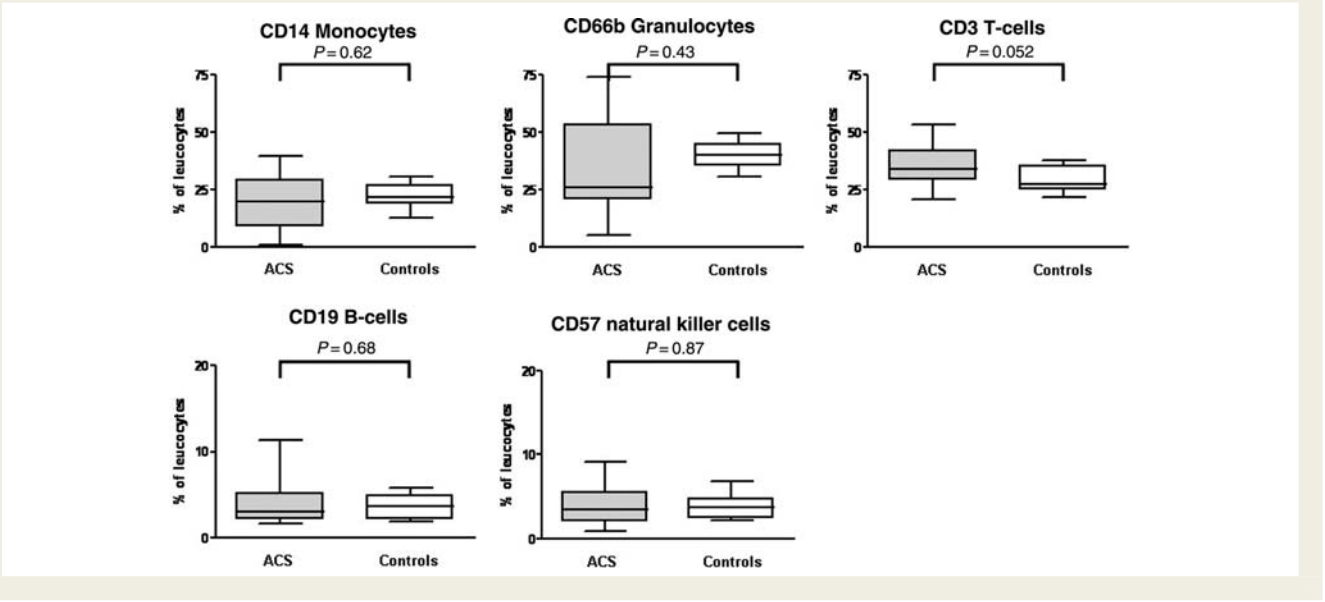


Appendix 2

Cellular distribution of leucocytes in aortic blood of ACS patients vs. controls

No differences were found for the proportion either of monocytes ($P = 0.62$), B-cells ($P = 0.68$), natural killer cells ($P = 0.87$), or granulocytes ($P = 0.43$) comparing the percental cellular composition of the AB of ACS patients with controls. There was only a trend to an increased percentage of T-cells ($P = 0.052$) in ACS patients.

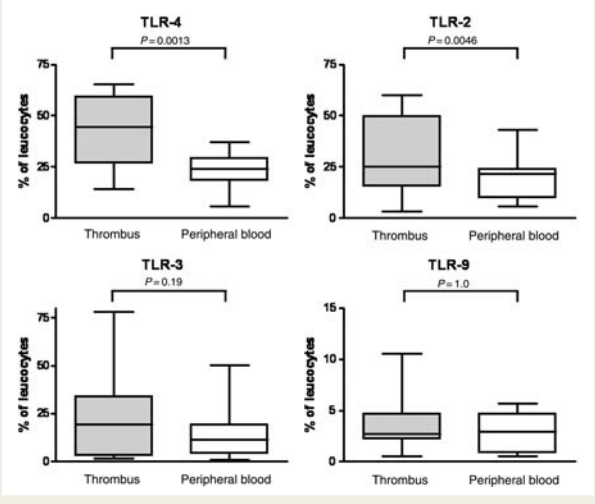
Data are expressed as per cent of specific leucocytes according to the total leucocyte count of the sample and represented as box-and-whisker plot (the smallest observation, lower quartile, median, upper quartile, and largest observation).



Appendix 3

Expression of Toll-like receptors (TLR) on leucocytes in thrombi, compared with AB

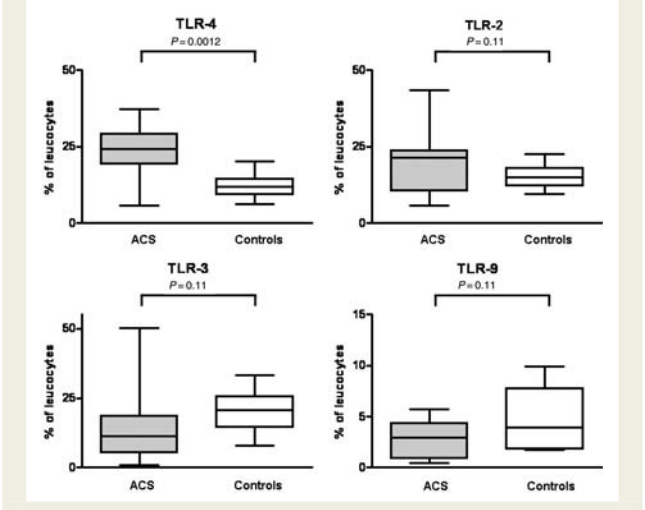
Significant increase of expression TLR-4 and TLR-2 on leucocytes enclosed within the thrombi compared with those obtained from the AB ($P < 0.01$ and $P < 0.01$, respectively). No such difference could be found for the expression of TLR-3 and TLR-9. Data are expressed as per cent of TLR positive leucocytes according to the total leucocyte count of the sample and represented as box-and-whisker plot (the smallest observation, lower quartile, median, upper quartile, and largest observation).



Appendix 4

Expression of Toll-like receptors (TLR) on aortic leucocytes of patients with acute coronary syndromes (ACS) compared with healthy controls

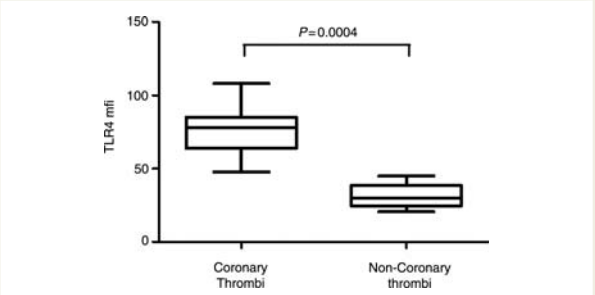
Significant increase of TLR-4 expression on aortic leucocytes in ACS patients compared with healthy controls. No such difference could be found for the expression of TLR-2, TLR-3, or TLR-9. Data are expressed as per cent of TLR positive leucocytes according to the total leucocyte count of the sample and represented as box-and-whisker plot (the smallest observation, lower quartile, median, upper quartile, and largest observation).



Appendix 5

Expression of TLR-4 on monocytes in coronary and non-coronary thrombi

We collected six thrombi from patients with arterial occlusions. FACS analysis revealed a markedly lower TLR-4 median fluorescence activity (30 mfi units; IQR 24–39) on local $CD14^+$ monocytes obtained from these arterial thrombi compared with monocytes from coronary thrombi (78 mfi units; IQR 64–85, $P = 0.0004$).

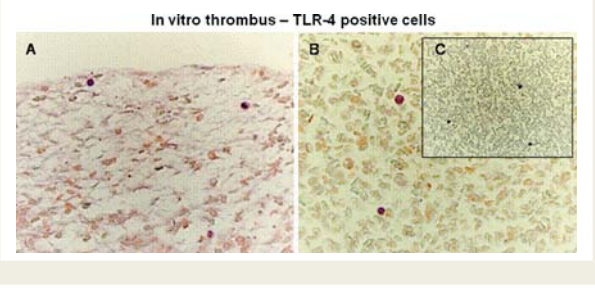


Appendix 6

Expression of TLR-4 on leucocytes within the *in vitro* thrombi

In artificial control thrombi, leucocytes showed generally a very weak expression of TLR-4. Furthermore, the nested appearance of leucocytes observed in intracoronary thrombi was missing, leucocytes in control thrombi appeared isolated within filamentous material and erythrocytes without any grouping of leucocytes.

Panel A, surface of the thrombus, with filamentous material; Panel B, inside of the thrombus, with isolated leucocytes and mostly erythrocytes; Panel C, isotype negative control (inset).



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CARDIOVASCULAR FLASHLIGHT

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Inter-ventricular septal thinning and high-degree atrio-ventricular block

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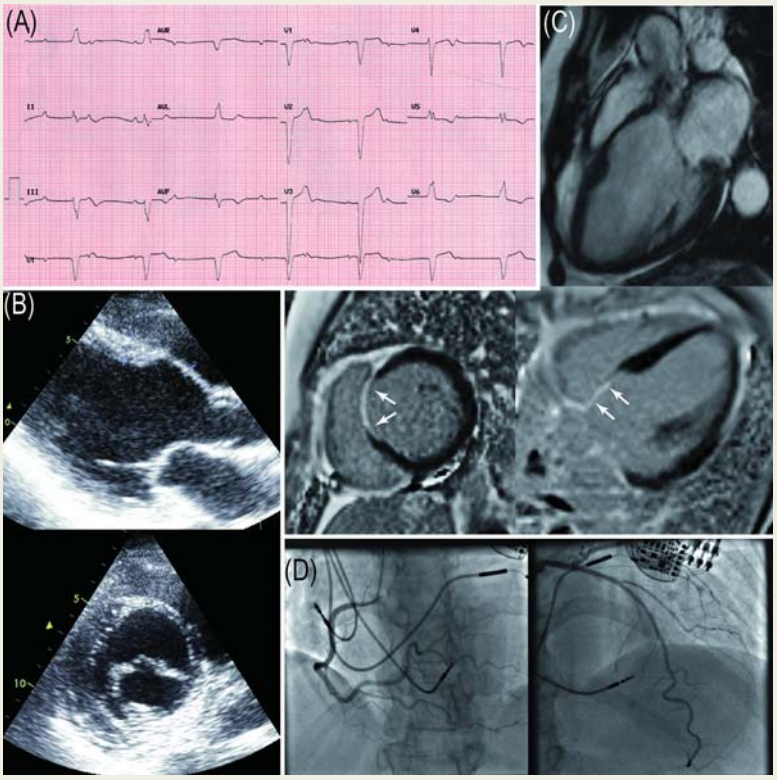
A 55-year-old woman was referred to our department for progressive dyspnoea. No personal nor familial history except a smoking habit was reported. Electrocardiogram showed a third-degree atrio-ventricular (A-V) block with ventricular rate at 35 b.p.m. (*Panel A*). Transthoracic echocardiography revealed a dilated myocardiopathy with ejection fraction estimated at 35%. Curiously, an abnormally thin aspect of the basal portion of the inter-ventricular septum (IVS) was noted. Maximum thickness was measured at 4 mm (*Panel B*). There was no septal defect. Cardiac magnetic resonance found a pathologically thin septum with transmural delayed enhancement in that area (arrows) in favour of a localized septobasal fibrosis (*Panel C*).



Confronted to a complete heart block with poor left ventricular function, a triple-chamber pacemaker was implanted. Coronary angiography revealed the absence of septal branch arising from the left descending artery before the origin of the first diagonal (*Panel D*). Blood tests were normal including troponin T and Lyme disease serology.

Three months later, the patient is found symptomless and NYHA class I. Echocardiography confirms the same aspect of the IVS with ejection fraction slightly improved at 40%.

To our knowledge, this observation is the first to describe a third-degree A-V block relevant of a missing septal branch with localized septal thinning. Aetiology remains imprecise, no patent argument was found for an acute coronary syndrome and isolated agenesis of a septal branch has never been noted. The origin of the myocardiopathy is also unknown and a prolonged A-V block could be incriminated.