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Chemokines in atherosclerotic lesion development and stability : from mice to man

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CCL3 (MIP-1 α) Levels are Elevated During Acute Coronary Syndromes and Show Strong Prognostic Power for Future Ischemic Events



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Abstract

Objectives: As chemokines are considered instrumental in thrombotic plaque rupture and erosion as well as in ischemia-reperfusion injury processes, we aimed to identify previously unknown chemokines associated with acute coronary syndromes.

Methods: Plasma of 44 patients with acute myocardial infarction (AMI) and 22 controls were profiled for a panel of chemokines by multiplex analysis. Levels of CCL3 were prospectively verified in 54 patients with unstable angina pectoris (UAP). An AMI mouse model was used to assess the relationship between differentially expressed chemokines and myocardial ischemia.

Results: CCL3 levels were significantly elevated in AMI vs. controls ($p=0.02$) albeit, that adjustment for confounding factors attenuated this association. In support of a direct association with cardiac ischemia CCL3 levels were also seen to be elevated in patients with UAP at baseline and significantly down-regulated after 180 days ($p<0.001$). Importantly, baseline upper quartile levels were strongly correlated with future acute coronary syndromes (Likelihood Ratio 11.5; $p<0.01$). Furthermore circulating levels of CCL3 were significantly enhanced after AMI in mice ($p=0.02$), while CCR5⁺ T-cell numbers were increased as well, suggestive of CCL3 driven T-cell homing towards the ischemic area.

Conclusions: CCL3 levels are elevated during ACS and released upon ischemia. Since CCL3 specifically predicts future cardiovascular events, it may serve as a predictive biomarker.

Introduction

Given the vital role of leukocyte recruitment at all stages of cardiovascular disease progression from early plaque development, via plaque rupture and erosion to ischemia-reperfusion injury following acute myocardial infarction (AMI) the chemokine family is thought to contribute significantly to cardiovascular disease. Illustratively, it has been shown that the chemokine CCL5 is secreted from activated platelets and several CCL5 related polymorphisms have been implicated in atherosclerosis and cardiac mortality¹⁻³. Various chemokines were suggested to play a direct role in post-ischemic injury repair after AMI not only by mediating the recruitment of neutrophils, mast cells and stem cells to the lesion site but also indirectly, by modulating necrosis and angiogenesis⁴⁻⁹. CXCL10 for instance was shown to be up-regulated in ischemic tissue very early after injury to inhibit premature angiogenesis, allowing the infarct to be cleared of debris and thus the formation of scar tissue¹⁰. These considerations point to a crucial role of chemokines in acute coronary syndromes (ACS). However, while individual chemokines have been reported to impart an increased risk for AMI, a broader study comparing plasma chemokine profiles from AMI patients versus controls is lacking.

In this study, we aimed to identify myocardial ischemia related chemokines in two independent patient cohorts of AMI and unstable angina pectoris (UAP), respectively using a multiplex and ELISA verification strategy. We show that CCL3 is highly up-regulated while CXCL10 is down-regulated respectively in patients with AMI. CCL3 levels were also seen to be transiently increased in patients with UAP, indicating that elevated CCL3 levels are related to the ischemic process itself rather than to the underlying atherosclerosis and more importantly correlated with new episodes of ACS during follow-up. To more specifically define the origin of CCL3 and CXCL10 in ischemia we performed coronary artery ligations in mice and monitored chemokine levels. Similar to the obtained data in patient cohorts, murine plasma levels of CCL3 were significantly elevated after ligation. Flow cytometric analysis of PBMCs in ligated mice revealed a CCR5 dependent migratory T-cell response. Altogether our data point to a prominent role of CCL3 in ischemic events, not only as a biomarker for, but also as mediator in the ischemic process itself.

Methods

Patient Cohorts

Study populations for chemokine profiling were compiled from the previously defined MISSION! intervention study¹¹. In brief, 44 patients who were admitted to the emergency department of Leiden University Medical Center diagnosed with acute myocardial infarction, as defined by the presence of typical ECG changes (STEMI) combined with the presence of enzymatic myocardial damage, defined as an increase in cardiac biomarkers (Creatine Kinase and/or Troponin T), were included. Twenty-two non-symptomatic age and sex matched subjects not suffering from manifest coronary artery disease (CAD) were included as controls to the MISSION! patient cohort (Table 1). Percutaneous coronary intervention (PCI) was used as primary reperfusion strategy, within 60 minutes upon presentation of symptoms. Eligible PCI patients received abciximab (0.25 mg/kg bolus followed by an infusion of 0.125 µg/kg/minute during 12 hours) in the absence of contraindications, where after PCI was performed. Baseline venous blood samples of AMI patients were obtained within 2 hours after hospitalization, before primary PCI and maximally within 6 hours upon onset of AMI. Samples were centrifuged and aliquots were stored at -80°C until further analysis.

Plasma samples of patients with unstable angina, derived from the well defined Acute Phase ReAction and Ischemic Syndromes (APRAIS) study (Table 2), were used to determine circulating CCL3 levels¹². In brief, 54 patients who were admitted to the emergency department of Leiden University Medical Center between March and September 1995 with UAP Braunwald class IIIB were included and followed for up to 18

months at the out-patient clinic. Follow-up primary end points were a new ACS, coronary re-vascularisation (PTCA or CABG) or cardiac death. Venous blood samples were obtained on admission at the emergency department (t=0), after 2 (t=2) and 180 days after admission (t=180), centrifuged and plasma aliquots were stored at -80°C until further analysis. All patients received standard medical therapy unless contraindicated, i.e. aspirin 300 mg orally, nitro-glycerine intravenously and heparin infusion based titrated to the activated partial thromboplastin time. Patients received other standard treatments at the physicians' discretion. In general, patients suffering from autoimmune disease, malignancies or chronic inflammatory diseases were excluded from the studies. All subjects gave written informed consent and both study protocols were approved by the Ethics Committee of the Leiden University Medical Center.

Multiplex Chemokine Assay

Circulating chemokine levels of CCL2, CCL3, CCL5, CCL11, CCL17, CCL18, CCL22, CXCL8, CXCL9 and CXCL10 as well as four reference cytokines (Interleukin-2 (IL-2), Interleukin-6 (IL-6), Tumor Necrosis Factor- α (TNF α) and soluble Intra Cellular Adhesion Molecule-1 (sICAM-1)) were determined in the MISSION! cohort, and CCL3 levels in the APRAIS cohort, by using a highly sensitive fluorescent microsphere based readout as described earlier^{13, 14}. Briefly, plasma samples were filtered and subsequently diluted with 10% normal rat and mouse serum (1:2) (Rockland, Gilbertsville, PA) to block residual non-specific antibody binding. 1000 microspheres were added per chemokine (10 μ l/well) in a total volume of 60 μ l, together with standard and blank samples, and the suspension incubated for 1 hour in a 96 well filter plate at room temperature (RT). Then, 10 μ l of biotinylated antibody mix (16.5 μ g/ml) was added and incubated for 1 hour at RT. After washing, beads were incubated with 50 ng/well streptavidin R-phycoerythrin (BD Biosciences, San Diego, CA) for 10 minutes. Finally, beads were washed again and the fluorescence intensity was measured in a final volume of 100 μ l high-performance ELISA buffer (Sanquin, Amsterdam, the Netherlands). Measurements and data analysis were performed with the Bio-Plex Suspension Array system in combination with the Bio-Plex Manager software version 3.0 (Bio-Rad laboratories, Hercules, CA). The multiplex assay has been well validated and intra-assay variation ranged from 6.5% (CCL5) to 16.2% (CXCL8) with an average variability of 11.4%^{13, 14}. Spiking of the assay with a fixed amount of recombinant protein revealed recoveries of 81 to 121%. Sensitivity varied between cytokines and ranged from 1.2 pg/ml for TNF α to 26.4 pg/ml for sICAM-1.

Murine Myocardial Infarction

Mice were anaesthetized and artificially ventilated (rate 200 breaths/min, stroke volume of 200 μ l) with a mixture of oxygen/N₂O [1:2 (vol/vol)]/ 2-2.5% isoflurane (Abbott Laboratories, Hoofddorp, the Netherlands) using a rodent ventilator (Harvard Apparatus, Holliston, MA). Myocardial infarction was induced by permanent ligation of the proximal left anterior descending coronary artery with a sterile Ethicon 7/0 silk suture (Johnson & Johnson, Amersfoort, the Netherlands). Sham operated animals were prepared in a similar manner but without tightening the suture around the LAD. After three hours, ligated and sham operated mice were sacrificed, PBMCs and spleens were isolated for flow cytometric analysis and plasma was harvested for chemokine detection. All animal procedures were approved by the Animal Ethics Committee of Leiden University, and were conducted in compliance with the Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85-23, revised 1996).

Plasma analysis

Human as well as murine CCL3 (Biosource, Carlsbad, CA), murine CXCL10 (R&D systems, Minneapolis, MN), murine IL-6 (eBioscience, San Diego, CA) and murine KC (CXCL8, Biosource) levels were determined by sandwich Elisa according to the manufacturers protocol. Baseline inflammatory parameters in the APRAIS cohort, such as C-reactive protein, fibrinogen and erythrocyte sedimentation rate (ESR), were determined as

described previously¹². Soluble CD40 ligand (sCD40L) was determined via a Quantakine highly sensitive immunoassay (R&D Systems, Minneapolis, MN).

Flow Cytometry

Peripheral Blood Mononuclear Cells (PBMCs) were isolated from whole blood after selective ablation of erythrocytes. Splenocytes were isolated by squeezing spleens gently through a 70µm cell strainer (BD Falcon, BD Biosciences, San Jose, CA). Crude total blood cell and splenocyte preps were incubated with erythrocyte lysis buffer (155mM NH₄CL in 10mM Tris/HCL, pH 7.2) for 5 minutes on ice. Cells were centrifuged for 5 minutes, resuspended in lysis buffer and residual erythrocytes were lysed by 5 minute incubation on ice. Cells were washed twice with PBS and counted. Subsequently cells were stained for CD4-PerCP, CCR3-A647, CCR5-biotin (BD Biosciences), CD8-FITC, F4/80-APC (eBioscience) and CXCR3 (US biological, Swampscott, MA) surface markers by adding 0.25 µg antibody per sample (2x10⁵ cells, 100µl). CCR5 biotinylated antibody was pre-incubated with 0.125 µg streptavidin PE (eBioscience), while CXCR3 was pre-incubated with 0.25 µg anti-rabbit-biotin (BD Biosciences) and 0.125µg streptavidin PE for 15 minutes before adding to the cells. After 45 minutes incubation on ice, cells were washed with PBS and analyzed by flow cytometry (FACScalibur, BD Biosciences).

Statistical Methods

Statistical analysis was performed using SPSS version 13.0 (SPSS, Chicago, IL). Chemokine data were tested for normal distribution by use of a Kolmogorov-Smirnov analysis. All values are expressed as mean ± SD and median ± Inter Quartile Range (IQR) in case of skewed distribution. Non-Gaussian distributed data were analyzed by a Mann-Whitney U test, whereas normally distributed variables were analyzed by Student's t-test. Multiple groups were analyzed by one-way ANOVA followed by Bonferroni multi-comparison test for Gaussian data, or by Kruskal-Wallis followed by Dunnet comparison testing for non-Gaussian data. Differences in risk factor distribution between the control and the AMI group were analyzed with a Fishers Exact probability test. Correlation analysis of inflammatory parameters was performed by Spearman's rank correlation test. Co-variate adjustment for risk factors was performed by multivariate linear regression test. Non-Gaussian distributed data were log transformed prior to analysis. Quartile distribution of CCL3 was used for Chi-Square testing to associate elevated levels of CCL3 with future cardiovascular events. A *p*-value < 0.05 was considered significant.

Results

MISSION! Patient Statistics

Pilot studies revealed that the standard deviation in cytokine levels in the AMI population was on average 1.5 fold higher than that of the control subjects. Therefore the patient and control sub-cohorts were compiled at a 2:1 ratio, and matched for gender, sex, age, type 2 diabetes mellitus, hypertension and hyperlipidemia. The AMI cohort encompassed a higher fraction of smokers and ex-smokers than the control cohort (56.8% in AMI compared to 22.7% in controls; *p*=0.01; Table 1). All chemokine values were therefore adjusted for smoking by univariate analysis. Cytokines were well within detection range of the multiplex immuno-assay.

Reference Panel

A panel of reference cytokines and cell adhesion markers was included in the analysis as a control for the validity of the multiplex assay. In compliance with previous findings plasma levels of IL-2 (0.07 ± 0.26 pg/ml in controls vs. 0.65 ± 1.83 in AMI; *p*=0.003), TNF-α (0.55 pg/ml (0.00-1.55 IQR) in controls vs. 1.40 (0.51-2.35 IQR) in AMI; *p*=0.03), sICAM-1 (476.1 ± 369 ng/ml in controls vs. 713.0 ± 327 in AMI; *p*=0.04) and IL-6 (9.8 ± 18.85 in controls compared to 23.7 ± 52.26 pg/ml in AMI; *p*=0.04) were significantly elevated in AMI patients (Table 3). Levels of other general inflammation markers, such

as IL-1 α , IFN- γ and soluble VCAM-1, remained unchanged (data not shown), showing that at this early time point AMI patients were not displaying a systemic hyperinflammatory response.

	Controls (N=22)	Acute Myocardial Infarction (N=44)	p-value
Age (years)	61.7 $\pm$ 12.1	61.8 $\pm$ 11.6	0.96
Male/Female	12/10	24/20	1.00
Diabetes Mellitus	3 (13.6%)	6 (13.6%)	1.00
Hypertension	8 (36.3%)	11 (25 %)	0.39
Total Cholesterol	5.6 $\pm$ 1.32 mmol/L	6.0 $\pm$ 0.83 mmol/L	0.14
Smoking	5 (22.7%)	25 (56.8%)	0.01
Number Vessel Disease	-	50 % 1-vessel disease 45.5 % 2-vessel disease 4.5 % 3-vessel disease	
Troponin-T max	-	6.80 $\pm$ 6.67	
Medication	-		
β -blocker	-	11.4%	
Statin	-	4.5%	
ACE inhibitor	-	2.3%	
Anti-coagulants	-	4.5%	
Anti-platelet	-	0%	

Table 1: MISSION! patient characteristics. Values are mean $\pm$ SD when appropriate

Chemokines

Plasma levels of the CC chemokines CCL3 (39.8 pg/ml, IQR 21.3-50 in controls compared to 47.8 pg/ml, IQR 39.6-67.2 in AMI; $p=0.01$) and CCL5 (13.4 ng/ml, IQR 6.4-29.2 in controls compared to 33.3 ng/ml, 19.1-45.3 in AMI; $p=0.001$) were significantly up-regulated in AMI compared to control patients (Table 3). After correction for smoking behavior CCL3 and CCL5 remained significantly elevated during AMI ($p=0.025$ and $p=0.006$ respectively). Of the CXC chemokines only CXCL8 (4.2 $\pm$ 0.50 pg/ml in controls compared to 6.8 $\pm$ 0.56 in AMI; $p=0.01$) was significantly up-regulated, while CXCL10 (255.1 $\pm$ 47.2 pg/ml in control vs. 162.6 $\pm$ 20.3 in AMI; $p=0.002$) was down-regulated in AMI compared to controls (Figure 1). After covariate adjustment for smoking both CXCL8 and CXCL10 remained significantly changed ($p=0.02$ and $p=0.04$ respectively). All other measured chemokines were not differentially regulated during AMI (Table 3). In concurrence with earlier findings by Herder *et al.*, adjustment of the analysis for additional cardiovascular risk factors (sex, age, hypertension, diabetes mellitus, total cholesterol and IL-6) attenuated the observed associations as they became non-significant¹⁵. Differential expression of CCL3, CCL5, CXCL8 and CXCL10, although related to myocardial infarction, do therefore not seem to be useful biomarkers in this specific setting. The CXC chemokines CXCL8 and CXCL10 are already known for their inflammatory and angiogenic role in ischemia/reperfusion¹⁶⁻¹⁹. Recently we have shown that CCL5 is transiently raised during severe ischemic symptoms. More importantly we provided evidence that CCL5 is a specific marker of refractory UAP²⁰. As CCL3 has not yet been implicated as marker for cardiac ischemia, we therefore mainly focused on the expression of this chemokine in UAP.

APRAIS

To assess if elevated CCL3 levels are directly related with AMI and/or with myocardial ischemia rather than reflecting an indirect acute phase response secondary to AMI, we assessed CCL3 levels of patients with unstable angina pectoris in the APRAIS cohort as previously described²¹.

Unstable Angina Pectoris (N=54)	
Age (years)	65.4 ± 11.0
Male/Female	40/14
Diabetes Mellitus	9 (16.4%)
Hypertension	23 (42%)
Smokers	23 (42%)
Elevated Troponin-T levels (>0.1 ng/ml)	7 (16%)
Medication	
Statins	8%
Nitrates	28%
β-blockers	39%
Calcium antagonists	34%
Aspirin	36%
Anti-coagulant	17%
Laboratory parameters:	
Total cholesterol	6.0 ± 1.5 mmol/l
HDL	1.14 ± 0.4 mmol/l
CRP	2.36 mg/l *
ESR	16.44 mm/hr *
Fibrinogen	3.56 g/l *
History of:	
Myocardial infarction	45%
PTCA	26%
CABG	23%

Table 2: APRAIS patient characteristics. Values are mean ± SD when appropriate, * denotes geometric mean.

Although direct statistical comparison between MISSION! and APRAIS patient data is not legitimate, inter-study analysis did show that UAP and AMI patients displayed similar CCL3 plasma levels (70.7 pg/ml in APRAIS vs. 55.7 pg/ml in MISSION!), supporting the notion that the observed elevated CCL3 levels might indeed be directly associated with cardiac ischemia. Elevated levels of Troponin T, present in a small percentage of patients (16%), did not correlate with CCL3 levels (data not shown). Next, we performed a temporal analysis of circulating CCL3 levels in the APRAIS cohort. As not all 54 patients responded to donate blood after 180 days, analysis at this point was performed for 47 patients, but the baseline characteristics of this subcohort matched with those of the original cohort (data not shown). Plasma samples from baseline (t=0), t=2 and t=180, analyzed by ELISA, revealed a significant progressive decline in CCL3 levels during follow-up (t=0 45.4 pg/ml; t=2 38.9 pg/ml; t=180 25.9 pg/ml, $p<0.001$, Figure 2A). Comparing baseline values for both techniques revealed a highly significant cor-

relation ($R=0.92$, $p<0.001$), underlining the validity of the ELISA measurements. Next, we sought to assess if baseline CCL3 plasma levels could predict any primary end point. Given the cohort size, multiplex CCL3 $t=0$ plasma levels were therefore categorized into quartiles (Q1 <41 ng/ml; Q2 >41 and <53 ng/ml; Q3 >53 and <83 ng/ml; Q4 >83 ng/ml). Upper quartile levels of CCL3 were highly predictive for the occurrence of ACS during follow-up (number of recurrent ACS 35; Likelihood Ratio (LR) 11.52; $p<0.01$, Figure 2B). Cardiac death during follow-up also showed a significant, albeit slightly weaker, association (number of cardiac deaths 5; LR 7.92; $p<0.05$). No associations were found between baseline CCL3 levels and coronary revascularisation during follow up. Finally, CCL3 did neither correlate with any of the inflammatory parameters, nor did its levels correlate with any of the other chemokines (data not shown). However, sCD40L levels revealed a significant negative correlation with CCL3 levels ($R=-0.44$; $p=0.001$), suggestive of a feedback response upon platelet activation.

	Control	AMI		<i>p</i>	<i>p</i> *
IL-2	0.07 ± 0.06 pg/ml	0.65 ± 0.28 pg/ml	↑	0.003	0.047
IL-6	9.8 ± 4.1 pg/ml	23.8 ± 8.0 pg/ml	↑	0.04	0.07
TNFα	0.6 pg/ml, (0-1.6)	1.4 pg/ml, (0.5-2.4)	↑	0.03	0.01
sICAM-1	476 ± 80.7 ng/ml	714 ± 50.0 ng/ml	↑	0.045	<0.001
CCL2	305 ± 81 pg/ml	522 ± 77 pg/ml	=	0.08	0.14
CCL3	39.8 pg/ml (21.3-50.6)	47.7 pg/ml, (39.6-67.2)	↑	0.02	0.025
CCL5	13.4 ng/ml (6.4-29.2)	33.3 ng/ml, (19.8-45.3)	↑	0.001	0.006
CCL11	15.9 pg/ml, (12.7-22.0)	21.2 pg/ml, (13.6-29.8)	=	0.27	0.33
CCL17	16.4 pg/ml, (10.5-21.4)	16.6 pg/ml, (8.6-28.9)	=	0.46	0.26
CCL18	555 ± 186 ng/ml	681 ± 160 ng/ml	=	0.18	0.85
CCL22	356 pg/ml, (264-409)	371 pg/ml, (296-549)	=	0.11	0.08
CXCL8	3.5 pg/ml, (1.9-4.3)	5.1 pg/ml, (3.5-7.4)	↑	0.004	0.02
CXCL9	163 ± 51 pg/ml	155 ± 25 pg/ml	=	0.16	0.87
CXCL10	255 ± 47.4 pg/ml	120 ± 20.3 pg/ml	↓	0.001	0.004

Table 3: Mean Cytokine and Chemokine values MISSION! cohort. Reference (IL-2, IL-6, TNF-α and sICAM-1) and chemokine panel of measured parameters containing *p*-value and corrected *p*-value (*p**) after adjustment for smoking. Values are expressed as mean ± SEM or median with IQR when appropriate.

Murine Myocardial Infarction

The obtained results from the MISSION! cohort suggest an important role for CCL3, CCL5, CXCL8 and CXCL10 in ischemic myocardial injury. To investigate whether the chemokines were elevated in response to ischemia we determined chemokine levels in a mouse model of myocardial infarction. AMI was induced by permanent ligation of the left anterior descending coronary artery in C57Bl/6 mice. Since the chemokines CCL5 and CXCL8 have previously been studied regarding acute cardiovascular syndromes we turned our interest to CCL3 and CXCL10^{4, 22-25}. CCL3 levels were, in concurrence with the earlier MISSION! findings, significantly elevated 3 hours after AMI (33.2 ± 1.5 vs. 76.4 ± 37.4 pg/ml in ligated animals; $p=0.02$, Figure 3B). As a control for the AMI model, levels of the ischemia related cytokine IL-6 were measured^{26, 27}. IL-6 levels were significantly up-regulated after ligation (0.67 ± 0.26 in sham vs. 1.34 ± 0.46 ng/ml in ligated animals; $p=0.007$, Figure 3A). The levels of CXCL10 were, contrary to the MISSION! findings, significantly enhanced after AMI (157.3 ± 64.8 in sham operated compared to 310.6 ± 86.6 pg/ml in ligated animals; $p=0.03$, Figure 3C).

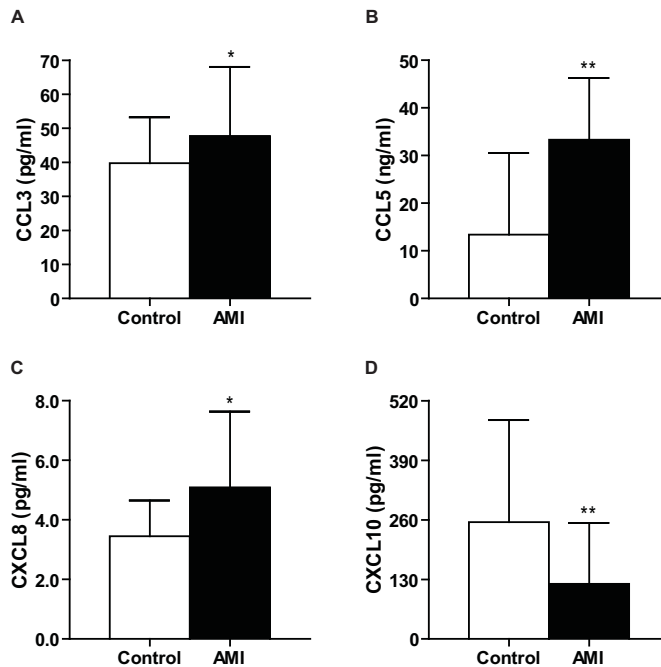


Figure 1: Plasma levels of CCL3 (A), CCL5 (B) and CXCL8 (C) were significantly elevated in AMI patients (black bars) versus controls (white bars), whereas CXCL10 (D) showed an opposite pattern. Panels A,B and C depict median with IQR, panel D depicts mean $\pm$ SD. * $p < 0.05$, ** $p < 0.01$.

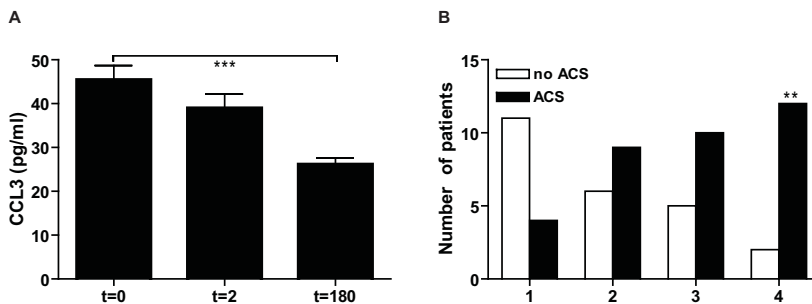


Figure 2: Temporal CCL3 monitoring clearly shows the transient increase of CCL3 during ischemia (A), since levels were significantly lowered at t=180 compared to t=0. Upper quartile levels of CCL3 at baseline are predictive for the occurrence of ACS during follow-up (B). ** $p = 0.01$ and *** $p < 0.001$.

Furthermore, PBMCs were harvested 3 hours after ligation and analyzed for chemokine receptor expression on different cell subsets. Total T cell numbers were enhanced in the circulation after ligation (14.1 ± 3.8 % in controls vs. 32.8 ± 14.4 % in ligated mice; $p = 0.04$, Figure 4A), which was attributable to an increase in $CD4^+$ T cells, while no effects were seen on total PBMC numbers ($0.96 \times 10^6/\text{ml}$ in sham versus $1.01 \times 10^6/\text{ml}$ in ligated animals). No effects were seen on splenic T cells ($p = 0.9$, Figure 4D). Moreover, the number of both circulating as well as splenic macrophages was not altered after ischemic injury (data not shown). CCL3 binds several receptors including CCR5. To assess specific responses to the increased CCL3 levels we determined circulating and splenic CCR5 expressing T cells, revealing an enrichment of $CCR5^+$ T cells ($8.0 \pm$

2.0 % in controls compared to 11.4 ± 1.4 % in ligated animals; $p=0.02$, Figure 4B). The enrichment in circulatory CCR5⁺ T cells is accompanied by a reduction in splenic CCR5⁺ T cells (19.95 ± 0.5 % vs. 14.1 ± 3.1 %; $p=0.004$, Figure 4E). These data suggest CCR5 dependent release of T cells from the secondary lymphoid organs towards the site of ischemic injury. In addition expression of the CXCL10 receptor CXCR3 was determined on the circulating T cells as well. In concurrence with the enhanced CXCL10 levels, the number of circulating CXCR3⁺ T cells was significantly increased after LAD ligation (29.1 ± 1.9 % vs. 43.5 ± 5.7 %; $p=0.04$, Figure 4C). However no effects on CXCR3⁺ splenic T cells were apparent ($p=0.78$, Figure 4F).

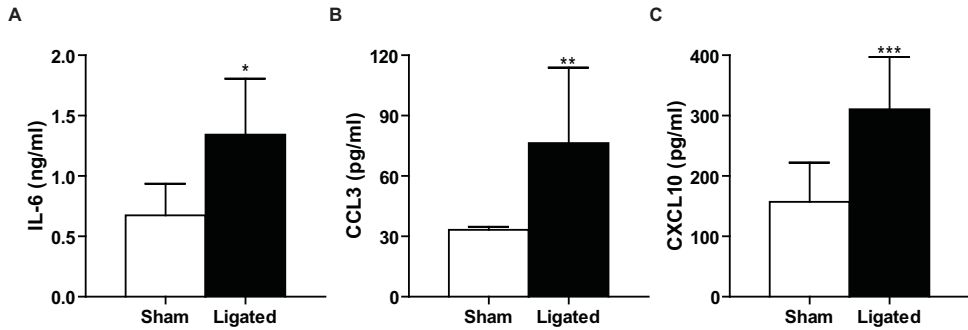


Figure 3: Assessment of IL-6, CCL3 and CXCL10 levels in LAD ligated or sham operated mice. Cardiac ischemia induced significantly elevated levels of IL-6 (A), CCL3 (B) and CXCL10 (C). * $p<0.05$, ** $p<0.01$ and *** $p<0.001$.

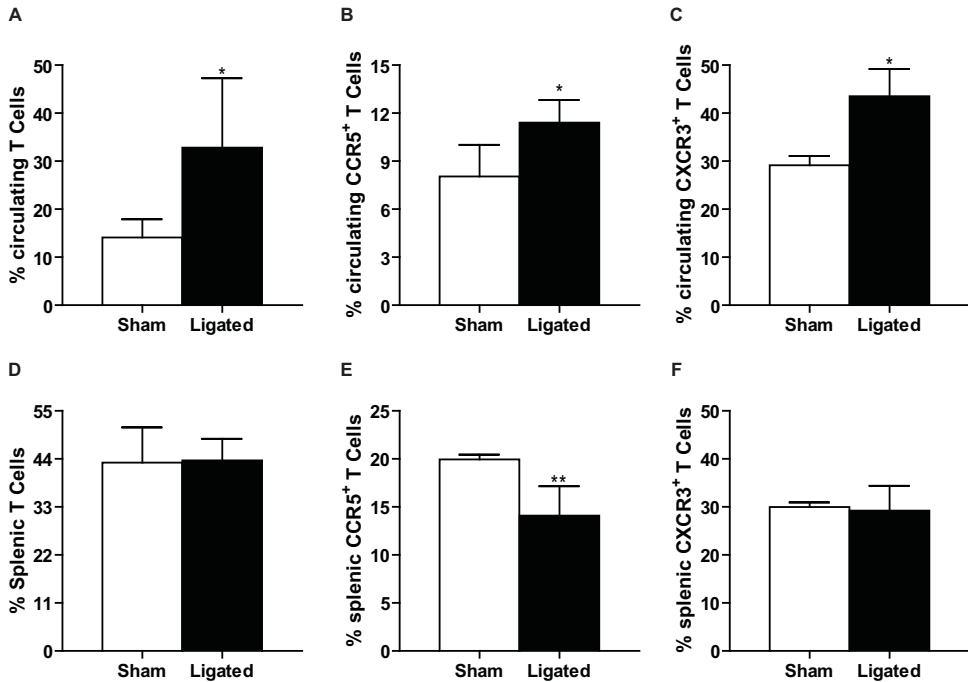


Figure 4: Ligated mice displayed a significant increase in the percentage of circulating T-cells with a concomitant enrichment in the CCR5⁺ and CXCR3⁺ subsets (A-C), while the total number of circulating cells was not changed between sham and ligated animals. The increase in circulating T-cells was accompanied by a decrease in CCR5⁺ splenic T-cells, whereas no effects on total or CXCR3⁺ splenic T-cells was apparent (D-F). * $p<0.05$, ** $p<0.01$.

Discussion

In this study, we have profiled plasma for a broad panel of chemokines in a cohort of patients with AMI. We were able to identify three chemokines (CCL3, CCL5 and CXCL8) that were significantly up-regulated and one chemokine (CXCL10) whose plasma levels were lowered in patients with AMI versus age-, sex-, and risk factor matched control subjects. Furthermore, AMI patients in our MISSION! cohort also displayed altered levels of four reference cytokines, which have previously been linked to myocardial ischemia²⁸⁻³¹, establishing the validity of our cohort. Interestingly the increased levels of CCL3 were confirmed in the APRAIS cohort of UAP patients, since CCL3 was transiently raised during ischemia and showed prognostic power. This finding was corroborated experimentally in a mouse coronary ligation experiment, clearly showing that CCL3 has a distinct role in the ischemic process associated with ACS, independent of platelet activation.

The aforementioned four chemokines were differentially regulated in the MISSION! cohort, of which CCL5 and CXCL8 have been previously associated with AMI^{4, 22-25}. CCL5 levels were significantly elevated during myocardial infarction in the MISSION! study. Recent experiments by Mause *et al.* show that plasma CCL5 in ACS likely originates from activated platelets and thus is a marker of the platelet activation status³². Furthermore, CXCL8 has been shown to be up-regulated during myocardial infarction and most likely provokes neutrophil migration to ischemic tissue via its cognate receptor CXCR2^{4, 10}. In concurrence with these findings, we found CXCL8 levels to be up-regulated during myocardial infarction. All other chemokines in the multiplex panel were not differentially regulated during AMI and are therefore probably not directly associated with myocardial infarction in our cohort.

The angiostatic chemokine CXCL10 was recently proposed to be an early indicator of cardiac injury, peaking within the first few hours and rapidly declining at a later stage possibly to allow angiogenesis¹⁰. Surprisingly, CXCL10 levels were lowered within 6 hours after AMI in our study; whereas those of the angiogenic CXCL8 were elevated at this time point, suggestive of a shifted angiogenic balance within the first 6h after AMI. During murine myocardial infarction however, levels of CXCL10 were strongly induced after three hours, which is in concordance with earlier findings¹⁰. Conceivably this apparent discrepancy is attributable to the dynamic and rapid regulation of CXCL10; being up-regulated immediately after ischemic injury (murine infarction model) and already down-regulated within 6 hours after ischemia (MISSION! cohort). On the other hand these findings might be due to species differences, but further studies will be needed to fully address that. Furthermore we also found an increase in cognate receptor CXCR3 expression on circulating T-cells, which is concordance with observations from Waeckel *et al.*¹⁸, underscoring that this specific subpopulation of T-cells is involved in the acute post-ischemic repair mechanism.

The increased levels of CCL3 during ACS and their rapid decline to baseline in the follow-up period further illustrates the profoundly altered chemokine homeostasis in ACS. Still our study leaves unanswered whether increased CCL3 levels represent a risk factor for the development of or a direct response to coronary artery disease ischemic symptoms. Previously, we reported the transiently increased levels of CCL5 and CCL18 in UAP²⁰, while other groups demonstrated elevated MCP-1 and fractalkine levels in UAP^{33, 34}. CCL3 release was seen to co-occur with cardiac ischemia-repair injury and within two days after ischemia CCL3 levels in UAP patients did not statistically differ from baseline levels. This phenomenon has already been alluded to by Parissis *et al.*, reporting CCL3 levels 24 hours post infarct to correlate not only with creatine kinase levels, but also inversely with left ventricular ejection fraction. This points to a key role of this chemokine in injury and/or repair responses²⁴. Furthermore, CCL3 levels were negatively correlated with those of sCD40L, a marker for platelet activity^{35, 36}, excluding that elevated CCL3 levels reflect thrombosis related processes. Finally, although statins

have been shown to decrease levels of other circulating chemokines such as MCP-1 and CXCL8³⁷⁻⁴¹, the APRAIS samples were gathered in the pre-statin era and only 8% of patients were in fact on statins. Thus we can safely exclude that the decreased CCL3 levels at t=180 were the resultant of statin treatment.

The most relevant clinical observation is that CCL3 shows strong predictive power with a Likelihood Ratio of 11.52 to identify patients who are at increased risk for a new episode of ACS during follow up. This association was even stronger than that for CCL5 and CCL18²⁰. Even though it still remains unclear if and how CCL3 contributes to future cardiovascular events, our study shows that its prognostic power is sufficiently promising to warrant examination in larger scaled trials. To further gain insight in the origin and possible role of CCL3 during myocardial ischemia we performed coronary artery ligation experiments in mice, in order to specifically study chemokine involvement in ischemic injury. CCL3 was similarly and significantly up-regulated after ligation and subsequent myocardial infarction. This is in agreement with previous results which clearly showed an up-regulation of CCL3 mRNA in ischemic myocardium⁷. In addition, already three hours after myocardial infarction we did observe a significant increase circulating CCL3 responsive CCR5⁺ T cells. This was accompanied by a reduced splenic CCR5⁺ T cell content, reflective of a CCL3 driven migration from the secondary lymphoid organs to the site of ischemia. To address a potential direct contribution of coronary ischemia itself to plasma CCL3 levels without the underlying substrate of atherosclerosis, we performed coronary ligation studies in normolipidemic, non-atherosclerotic C57Bl/6 mice. Although the outcome of mouse studies cannot be directly extrapolated to the human situation and this experimental set-up has its limitations, our data are supportive of an ischemic rather than atherogenic origin of the CCL3 chemokine response. Taken together our data indicate that CCL3 is transiently secreted during cardiac ischemia, where it potentially functions in the ischemia/repair mechanism by recruitment of CCR5⁺ T cells, which has also been suggested previously⁷.

A few limitations of this study should be addressed. First, both study populations were relatively small. Therefore, the strong association in two independent ACS cohorts of CCL3 with cardiac ischemia and future cardiovascular event prediction needs to be verified in larger scaled trials. Moreover the significant results seen in the MISSION! cohort disappeared after correction for multiple confounding factors which is in agreement with previous observations and precludes the use of these chemokines as markers of cardiac ischemia¹⁵. Third, the APRAIS cohort only allowed for a temporal analysis as it did not include a control population. However, as patients were clinically stable at t=180, we believe that t=180 levels reliably mimic pre-ischemia. Finally, while cardiovascular patients per definition suffer from atherosclerosis, we did not perform our coronary ligation experiments in atherosclerotic mice which might display alternative chemokine patterns.

In summary, we show that the CC chemokine CCL3 is strongly related to myocardial ischemia as it is highly elevated not only in patients with AMI (MISSION!) but also in UAP (APRAIS). Moreover this chemokine is shown to be up-regulated during myocardial ischemia in a murine myocardial infarction model inducing T-cell migration to the site of injury. Finally, we show that CCL3 is a prognosticator for future cardiovascular events and therefore might prove to be a useful biomarker in identifying high-risk patients.

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