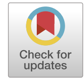
Interleukin-6 inhibition in ST-elevation myocardial infarction: Immune cell profile in the randomised ASSAIL-MI trial



Camilla Huse,[a,k,1] Anne Kristine Anstensrud,[b,k,1] Annika E. Michelsen,[a,k] Thor Ueland,[a,c] Kaspar Broch,[b,d] Sindre Woxholt,[e,h] Kuan Yang,[a,k] Kapil Sharma,[f] Ingvild Maria Tøllefsen,[f] Bjørn Bendz,[b,k] Brage Høyem Amundsen,[e,h] Jan Kristian Damås,[g,l] Erlend Sturle Berg,[b] Elisabeth Bjørkelund,[b] Ana Quiles-Jiménez,[a,k] Vigdis Bjerkeli,[a,k] Christina Bendz,[b] Ola Kleveland,[e,h] Knut Haakon Stensaeth,[h,i] Anders Opdahl,[b] Nils-Einar Kløw,[j,k] Geir Øystein Andersen,[f,m] Rune Wiseth,[e,h] Bente Halvorsen,[a,k] Lars Gullestad,[b,d,k] Ingebjørg Seljeflot,[f,k,m] Pål Aukrust,[k,n] Liv Osnes,[o] and Tuva B. Dahl[a,p,*]

[a]Research Institute of Internal Medicine, Oslo University Hospital Rikshospitalet, Oslo, Norway
[b]Department of Cardiology, Oslo University Hospital Rikshospitalet, Oslo, Norway
[c]K. G. Jebsen Thrombosis Research and Expertise Centre (TREC), The Arctic University of Norway, Tromsø, Norway
[d]K. G. Jebsen Cardiac Research Centre and Centre for Heart Failure Research, University of Oslo, Oslo, Norway
[e]Clinic of Cardiology, St. Olav's Hospital, Trondheim University Hospital, Trondheim, Norway
[f]Department of Cardiology, Oslo University Hospital Ullevål, Oslo, Norway
[g]Department of Infectious Disease, St. Olavs Hospital, Trondheim University Hospital, Trondheim, Norway
[h]Department of Circulation and Medical Imaging, Norwegian University of Science and Technology (NTNU), Trondheim, Norway
[i]Department of Radiology and Nuclear Medicine, St. Olav's Hospital, Trondheim University Hospital, Trondheim, Norway
[j]Department of Radiology, Oslo University Hospital Ullevål, Oslo, Norway
[k]Faculty of Medicine, Institute of Clinical Medicine, University of Oslo, Oslo, Norway
[l]Department of Clinical and Molecular Medicine, Centre of Molecular Inflammation Research, Norwegian University of Science and Technology (NTNU), Trondheim, Norway
[m]Department of Cardiology, Center for Clinical Heart Research, Oslo University Hospital Ullevål, Oslo, Norway
[n]Department of Rheumatology, Dermatology and Infectious Disease, Oslo University Hospital Rikshospitalet, Oslo, Norway
[o]Department of Immunology, Oslo University Hospital Rikshospitalet, Oslo, Norway
[p]Department of Research and Development, Division of Emergencies and Critical Care, Oslo University Hospital, Norway

Summary

Background We recently showed that interleukin (IL)-6 inhibition by tocilizumab improves myocardial salvage in ST-elevation myocardial infarction (STEMI). However, the mechanisms for this effect are not clear.

Methods In this exploratory sub-study of the ASSAIL-MI trial, we examined leukocyte differential counts and their relation to myocardial salvage and peak troponin T (TnT) in STEMI patients randomised to tocilizumab ($n = 101$) or placebo ($n = 98$). We performed RNA-sequencing on whole blood ($n = 40$) and T cells ($n = 20$). B and T cell subpopulations were examined by flow cytometry ($n = 69$).

Findings (i) STEMI patients had higher neutrophil counts at hospitalisation compared with stable angina patients. (ii) After percutaneous coronary intervention there was a gradual decline in neutrophils, which was significantly more pronounced in the tocilizumab group. (iii) The decrease in neutrophils in the tocilizumab group was associated with improved myocardial salvage and lower peak TnT. (iv) RNA-sequencing suggested that neutrophil function was also attenuated by tocilizumab. (v) B and T cell sub-populations changed only minimally after STEMI with minor effects of tocilizumab, supported as well by RNA-sequencing analyses of T cells. (vi) However, a low $CD8^+$ count was associated with improved myocardial salvage in patients admitted to the hospital > 3 h after symptom onset.

Interpretation Tocilizumab induced a rapid reduction in neutrophils and seemed to attenuate neutrophil function in STEMI patients potentially related to the beneficial effects of tocilizumab on myocardial salvage.

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*Corresponding author at: Research Institute of Internal Medicine, Oslo University Hospital Rikshospitalet, PO Box 4950 Nydalen, Oslo 0424, Norway.
E-mail address: tuvad@medisin.uio.no (T.B. Dahl).

[1] These authors contributed equally to this work.

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Keywords: ST-elevation myocardial infarction; Neutrophils; Lymphocytes; Tocilizumab; Interleukin-6; Inflammation

Research in context

Evidence before this study

One study has shown that interleukin-6 inhibition with tocilizumab reduces C-reactive protein and troponin T in patients with NSTEMI. Recently, we showed that a single dose of tocilizumab administered before percutaneous coronary intervention was associated with improved myocardial salvage and reduced levels of circulating neutrophils in patients with STEMI. The effect on myocardial salvage was more pronounced in the patients who received treatment >3 h after symptom onset. However, (i) the kinetics of the effects of tocilizumab in STEMI patients, (ii) whether tocilizumab also modulates other leukocyte subpopulations, and most importantly, (iii) if these effects on leukocytes are related to the beneficial effects of tocilizumab in STEMI are not known.

Added value of this study

We characterize leukocyte levels and subpopulations in STEMI patients treated with tocilizumab in this exploratory sub-study of the clinical ASSAIL-MI trial. Repeated assessments were made from hospital admission to six months into remission. Our major findings were: (i) High neutrophil levels were observed at hospitalisation. Following percutaneous coronary intervention (PCI) there was a gradual decline in neutrophils, which was more pronounced in the tocilizumab group. (ii) A similar decline was also seen for the neutrophil-lymphocyte ratio. (iii) The decrease in neutrophils in the tocilizumab group was associated with improved myocardial salvage and lower peak troponin. (iv) RNA-sequencing and Reactome analysis of whole blood revealed that tocilizumab attenuated the innate immune response and signal transduction. (v) Cell type deconvolution and gene expression imputation analysis revealed several altered pathways relevant for neutrophil function. (vi) B and T cell sub-populations changed only minimally after STEMI and were only slightly affected by tocilizumab. Only minor changes were seen in RNA-sequencing analyses of T cells. (vii) However, the lack of rising in $CD8^+$ T cells in the tocilizumab arm in patients who were admitted > 3 h after symptom onset, previously reported to have the best effect of tocilizumab, was associated with improved myocardial salvage.

Implications of all the available evidence

Our findings provide evidence that tocilizumab markedly affects neutrophil levels and function in patients treated with PCI for acute STEMI. We suggest that the beneficial effects of tocilizumab on myocardial salvage in STEMI patients may, at least partly, be related to the observed effect on neutrophils, emphasising the major importance of these cells during STEMI.

Introduction

Inflammation plays a crucial role in atherosclerotic disease and might be a therapeutic target in acute coronary syndrome (ACS).[1] Both local and systemic inflammation have been documented in patients with myocardial infarction (MI), and can potentially contribute to plaque destabilization and myocardial damage.[1] However, following MI, inflammation also plays an important role in infarct healing, in which both too much and too little inflammation could potentially be harmful.[2] Several inflammatory cytokines are upregulated in coronary artery disease and in particular during acute MI[2,3] and, from a therapeutic point of view, much focus has been put on interleukin (IL)-1.

In the landmark "Canakinumab Antiinflammatory Thrombosis Outcome Study" (CANTOS), canakinumab, a monoclonal antibody against IL-1β, significantly reduced the hazard of cardiovascular events in patients with previous MI.[4] Interestingly, the beneficial effect of canakinumab in the CANTOS study was particularly strong in those who obtained a reduction in IL-6.[4] IL-6 is an inflammatory cytokine that is upregulated during MI and affects both plaque destabilization and myocardial remodeling.[4] We have previously shown that a single dose of tocilizumab, a humanized monoclonal antibody that blocks IL-6 signalling by binding to the soluble and membrane-bound IL-6 receptor (IL-6R), significantly reduced C-reactive protein (CRP) and troponin T (TnT) in patients with non-ST segment elevation MI (NSTEMI).[5] In our recently published randomised trial "Assessing the effect of Anti-IL-6 treatment in MI" (ASSAIL-MI), patients with ST-elevation MI (STEMI) had an improved myocardial salvage and reduced CRP (area under the curve during

hospitalisation) when receiving a single intravenous dose of tocilizumab (280 mg) just prior to percutaneous coronary intervention (PCI).[6]

Tocilizumab reduced the absolute neutrophil count in STEMI and NSTEMI.[5,6] However, the kinetic and the molecular consequence of this effect and its clinical relevance is not clear. IL-6 is known to have pleiotropic effects on other leukocyte subpopulations, in particular lymphocyte subpopulations. These effects might have relevance for the development of myocardial injury after acute MI.[7] However, whether tocilizumab affects these cells in patients with STEMI is still not known. In this predefined exploratory sub-study of the ASSAIL-MI trial we examined the effect of tocilizumab on a broad spectrum of leukocyte subpopulations.

Methods

Ethics

The trial protocol was approved by the regional ethics committee (REK South-East 2016/1223) and all participants provided written informed consent. An independent Data and Safety Monitoring Board oversaw the safety of the trial. The trial was approved by The Norwegian Medicines Agency and was conducted in compliance with the Declaration of Helsinki and the rules outlined in the guidelines for Good Clinical Practice.

Patients and study design

In the phase 2 ASSAIL-MI trial (Clinicaltrials.gov: NCT03004703), we investigated the hypothesis that a single dose of intravenous tocilizumab would be superior to placebo in improving myocardial salvage in patients admitted with acute STEMI. The study design and participants have been described previously and the groups were found to be well balanced after inclusion.[6,8] The trial was conducted at three high-volume PCI centres in Norway (Oslo University Hospital Rikshospitalet, Oslo University Hospital Ullevål, and St. Olav's Hospital, Trondheim). Briefly, 200 patients were randomised in the ASSAIL-MI trial and stratified according to the three PCI centres. One patient withdrew consent, leaving 199 patients for analyses in this investigation. The trial was double-blinded, placebo-controlled, and the patients were allocated in a 1:1 fashion in the period from March 2017 until February 2020. The key inclusion criteria were STEMI and symptom onset less than 6 h before PCI. Patients with previous MI; chronic infection, or chronic autoimmune or inflammatory disease; uncontrolled inflammatory bowel disease; ongoing infectious or immunologic disease; major surgery within the past eight weeks; or treatment with immunosuppressants other than low-dose steroids (equivalent to a systemic exposure to 5 mg prednisone per day) were excluded. The full inclusion and exclusion criteria, as well as the study design, have been published elsewhere.[8] Baseline characteristics of the study population are described in Table 1.

The primary endpoint of the ASSAIL-MI trial was the myocardial salvage index (MSI; %). MSI is defined as: [(area at risk- infarct size): area at risk] x 100, assessed by magnetic resonance imaging (MRI) 3–7 days after intervention.

For this predefined explorative sub-study, blood samples were also obtained from 20 patients admitted to Oslo University Hospital Rikshospitalet for elective coronary angiography due to stable angina pectoris. Except for previous MI, these patients had the same exclusion criteria as in the main study population. These patients were recruited in the autumn of 2020 providing written consent. Baseline characteristics for these patients are described in Supplemental Table S3.

Blood sampling protocol

The trial participants received double antiplatelet therapy and unfractionated heparin (5000–7500 IE) intravenous before PCI. Most of the patients (76%) had also received unfractionated heparin (5000 IE) in the ambulance before arrival at the hospital. Arterial blood samples were collected at admission, just before PCI, before intra-arterial unfractionated heparin and intravenous study medication were administrated at the catheterisation laboratory. Thereafter, venous blood samples were collected at 14–33 h (24 h), at 3–7 days, at 3 months, and at 6 months. For RNA isolation of whole blood, BD PAXgene™ Blood RNA tubes (BD, Franklin Lakes, NJ) were collected at admission before PCI,at 3–7 days and at 6 months. These tubes were used for RNA isolation in whole blood. Leukocytes and differential counts were analysed on Sysmex XN-10 (Sysmex, Kobe, Japan) per routine. The neutrophil-lymphocyte ratio (NLR), an independent risk factor for mortality after cardiac events, was calculated by dividing the absolute neutrophil count by the absolute lymphocyte count. High-sensitivity TnT was measured by electrochemiluminescence immunoassay (Elecsys 2010 analyzer, Roche Diagnostics, Basel Switzerland). In a subgroup of patients, EDTA-blood was sampled for flow cytometry at hospital admission and at three time-points post-treatment; 14–33 h, at 3–7 days and at 6 months. Venous blood samples from patients with stable angina pectoris were collected on the day before PCI. These patients had received platelet inhibitors but not heparin.

Isolation of T cells

Peripheral blood mononuclear cells (PBMCs) were obtained from sodium-heparin-anticoagulated blood by Isopaque-Ficoll (Lymphoprep; Axis-Shield, Oslo, Norway) gradient centrifugation. PBMCs were then

	Tocilizumab (*n* = 101)	Placebo (*n* = 98)
Demographics		
Age, years	62 ± 10	60 ± 9
Men	80 (79)	87 (89)
Body mass index, kg/m^2	27.1 ± 4.5	27.5 ± 4.3
Caucasian	99 (98)	94 (96)
Smoking status		
Never smokers	38 (38)	36 (37)
Previous smokers	33 (33)	24 (24)
Current smokers	30 (30)	38 (39)
Prior conditions		
Other vascular disease	6 (6)	6 (6)
Aortic disease	0	2
Angina pectoris	1	1
Cerebrovascular disease	4	2
Peripheral vascular disease	1	1
Diabetes mellitus	8 (8)	6 (6)
Hypertension	33 (33)	30 (31)
Treatment		
ACE inhibitor or ARB	22 (22)	25 (26)
Aldosterone antagonist	1 (1)	0 (0)
Oral anticoagulants	5 (5)	2 (2)
Platelet inhibitor	12 (12)	5 (5)
Beta-blocker	8 (8)	3 (3)
Calcium antagonist	13 (13)	10 (10)
Diuretic	8 (8)	8 (8)
Statin	19 (19)	9 (9)
Up-front DAPT	101 (100)	98 (100)
Time from symptom onset to arrival at PCI centre, min	151 ± 71	149 ± 72
Door-to-balloon time, min	23 ± 10	23 ± 11
Laboratory values		
Haemoglobin, g/l	143 ± 13	144 ± 12
Platelet count, 10^9/l	253 ± 59	260 ± 62
Total white blood cell count, 10^9/l	11.6 ± 3.4	11.6 ± 3.4
Aspartate transaminase, U/l	28 (22–37)	30 (24–37)
Troponin T, ng/l	44 (22–163)	49 (28–95)
CK-MB, μg/l	5.0 (2.6–14.0)	5.3 (3.0–10.0)
NT-proBNP, ng/l	79 (50–178)	63 (50–146)
Creatinine, mmol/l	74 ± 17	78 ± 20
Glucose, mmol/l	9 ± 3	9 ± 3
HbA1c, mmol/mol	37 (34–41)	37 (34–40)
Total cholesterol, mmol/l	5.3 ± 1.2	5.2 ± 1.0
HDL cholesterol, mmol/l	1.2 (0.9–1.3)	1.1 (0.9–1.3)
LDL cholesterol, mmol/l	3.7 ± 1.1	3.7 ± 0.9
C-reactive protein, mg/l	2.4 (0.9–5.0)	2.9 (1.4–5.0)
Albumin, g/l	42 ± 3	42 ± 3

***Table 1*: Baseline characteristics for the STEMI population before treatment and study drug administration.**

Values are mean ± SD, *n* (%), or median (interquartile range). Note, all laboratory values including total white blood cell counts and CRP reflect values before the administration of tocilizumab.

ACE = angiotensin-converting enzyme; ARB = angiotensin receptor blocker; CK-MB = creatine kinase myocardial band; DAPT = dual anti-platelet therapy; HDL = high-density lipoprotein; LDL = low-density lipoprotein; PCI = percutaneous coronary intervention; NT-proBNP = N-terminal pro-B-type natriuretic peptide.

resuspended in autoMACS® rinsing buffer (Miltenyi Biotec, Bergisch Gladbach, Germany) for negative selection of pan-T cells with Pan-T cell Isolation Kit (Miltenyi Biotec) following manufacturer's instructions. Pelleted T cells were stored at -80 °C before RNA isolation.

RNA isolation and RNA-sequencing

Total RNA was isolated from BD PAXgene™ Blood RNA tubes using MagMAX™ for Stabilized Blood Tubes RNA Isolation Kit (Invitrogen™, Waltham, MA) following the manufacturer's instructions. Total RNA from T cells was isolated under RNase-free conditions using Allprep DNA/RNA/Protein Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. The isolated RNA samples were sent to Novogene (UK) Company Limited. rRNA depletion library preparation was used for the RNA isolated from whole blood, while mRNA enrichment method was used for T cell RNA. The fastp (v0.23.0) was used to remove contaminated adapters and low-quality reads with phred score below 30 in the pair-end mode.[9] Filtered reads were mapped to the human transcriptome (Gencode Human Release H37), and transcripts were quantified with 200 bootstrap iterations by Salmon (v1.5.2).[10,11] The Salmon outputs were summarised to gene-level and imported into DESeq2 (v1.34.0) via tximeta (v.1.12.3).[12,13] For better accuracy, haemoglobin mRNAs were removed from the neutrophils before the analysis of differentially expressed genes (DEGs).[14] Raw counts were uploaded to the Reactome Pathway Knowledgebase[15] for pathway analyses. For neutrophil deconvolution and gene expression imputation, the CIBERSORTx high-resolution mode and its build-in LM22 reference matrix were used.[16] Differentially regulated imputed genes with more than 50 counts and a *p*-value < 0.01 were uploaded to Metascape[17] for gene annotation analyses. Cytoscape[18] was used for network plot visualization. For gene set enrichment analysis (GSEA), all imputed gene were imported into GSEA software (version 4.2.2) and analysed against the gene sets "Neutrophil degranulation" Reactome pathway and "MAPK cascade" GO term, respectively (MSigDB version 7.5.1).[19,20]

Flow cytometry

In a subgroup of 69 patients treated with tocilizumab (*n* = 37) or placebo (*n* = 32) recruited at Oslo University Hospital Rikshospitalet, we performed an extended flow cytometry analysis of lymphocyte subpopulations at the Department of Immunology. Patient characteristics are provided in Supplemental Table S3 showing no significant differences in baseline characteristics between the two treatment arms. Routine analyses of absolute counts for B-, T-, and NK-cells were analysed in Trucount tubes (BD) on a FacsCanot II instrument and

analysed in BD FACSCanto^{TM} Clinical Software according to the manufacturer's instructions. Instrument settings were standardised as recommended and daily quality run with CS&T-Beads (BD) and 7-color Setup Beads (BD) ensuring high reproducibility. The laboratory follows standard operation procedure and also have ISO (International Standard Organization) certification. Further sub-classification of B- and T-cells was performed on a Gallios Flow cytometer (Beckman Coulter, San Diego, CA). For B-cell analysis, the blood samples were washed twice before incubation with antibodies. T-cell analysis was performed in unwashed blood samples. Briefly, EDTA-blood was incubated with optimally titrated antibodies for 15 min at room temperature, followed by erythrocyte lysis (BD FACSLysing Solution, Beckman Dickinson, CA). Data acquisition was performed using Kaluza Software (Beckman Coulter). For T-cells, 1×10^5 cells was acquired; for B-cells, 1×10^6 cells if possible. The antibodies that were used and their RRID tags are provided in Supplemental Table 1. B-cell were gated as $CD19^+$ and further sub-classified as naive (IgD^+, IgM^+, $CD27^-$), IgM memory ($CD27^+$, IgD^+, IgM^+), class switched ($CD27^+$, IgM^-, IgD^-), plasmablasts ($CD19^+$ dim, $CD27^{++}$, $CD38^{++}$), transitional (IgM^{++}, $CD38^{++}$, $CD24^+$) and CD21 low B cells (CD38 low, CD21 low). T-cells were gated as $CD3^+$ and further as naive $CD4^+$ ($CD4^+$, $CD45RA^+$), recent thymic emigrants ($CD4^+$, $CD45RA^+$, $CD31^+$), $CD4^+$ memory ($CD4^+$, $CD45RO^+$), follicular like $CD4^+$ ($CD4^+$, $CD45RO^+$, $CCR5^+$), regulatory T-cells ($CD4^+$, $CD25^{++}$, $CD127^-$), naive $CD8^+$ ($CD8^+$, $CD27^+$, $CD28^+$), $CD8^+$ early effector memory ($CD8^+$, $CD27^+$, $CD28^-$), $CD8^+$ late effector memory ($CD8^+$, $CD27^-$, $CD28^-$). Gating strategy is provided in Supplemental Figure 1. Reference values are 5−95 percentile for 65 normal controls (blood donors).

Statistics

Continuous data are presented as mean (standard deviation or standard error of the mean) or median (interquartile range) if distributions were skewed. Categorical data are reported as numbers and percentages. One-way ANOVA with Dunnett's multiple comparison test was performed to investigate significant differences between the stable angina pectoris group compared with the STEMI-group. To investigate differences between the intervention groups for counts or imputed gene expression, or between patients admitted ≤ 3 or > 3 h after the symptom onset, we either used mixed effect analysis with Bonferroni's multiple comparison test or unpaired two-tailed t tests. Correlations were calculated using Spearman's correlation coefficient. The percent change from admission to 24 h, that takes into account the differences in admission levels, was calculated as: [absolute neutrophil count (24 h) − absolute neutrophil count (admission)]: absolute neutrophil count (admission). *P*-values less than 0.05 were considered statistically significant. For RNAseq we performed FDR adjustment and report adjusted *p*-values. Patient number (given as *n*) might vary over time for both counts and RNA analyses due to missing samples or quality issues. The amount of missing data was evenly distributed between the treatment groups and the missing values are assumed to be missing at random. Statistical analyses were performed in SPSS version 25 (IBM Corp., Armonk, New York) and in GraphPad Prism 8.3.0 (GraphPad Software, La Jolla, CA).

Restrictions to availability of material and data

Ethical restrictions from the Regional Committee for Medical and Research Ethics in South−East Norway prohibits data from individual patients to be made available on a publicly available repository. However, an institutional data transfer agreement can be established and data can be shared if the aims of data use are covered by ethical approval and patient consent. The procedure will involve an update to the ethical approval as well as a review by legal departments at both institutions, with the process typically taking 2 to 4 months from the first contact.

Role of funders

The funders of the study had no role in the study design, data collection, analysis, interpretation, or writing of the report.

Results

Neutrophil and neutrophil-lymphocyte ratio after STEMI: downregulatory effects of tocilizumab

Patients in the placebo arm of the ASSAIL-MI trial had higher neutrophil counts at admission and after 24 h than patients with stable angina pectoris ($p < 0.0001$ and $p < 0.0001$, respectively) (Figure 1a, showing the placebo arm only). Following hospital admission and PCI, there was a gradual decline in the level of circulating neutrophils. However, while there was no difference in neutrophils between the two treatment arms at admission, the decrease in the number of neutrophils during hospitalisation was more pronounced in the tocilizumab arm ($p < 0.0001$ at 24 h, $p < 0.0001$ at 3−7 days) (Figure 1b). The STEMI patients also had higher neutrophil-lymphocyte ratios (NLR) at admission ($p < 0.0001$) and after 24 h ($p = 0.0068$) than patients with stable angina pectoris (Figure 1c, showing the placebo arm only). Tocilizumab significantly reduced the NLR at 24 h ($p < 0.0001$) and day 3-7 ($p < 0.0001$) after admission compared with placebo (Figure 1d). The decrease in NLR was driven mainly by the decrease of neutrophils. Meanwhile, the lymphocyte counts did not change significantly over time or differ between the intervention groups (Supplemental Figure S2). The

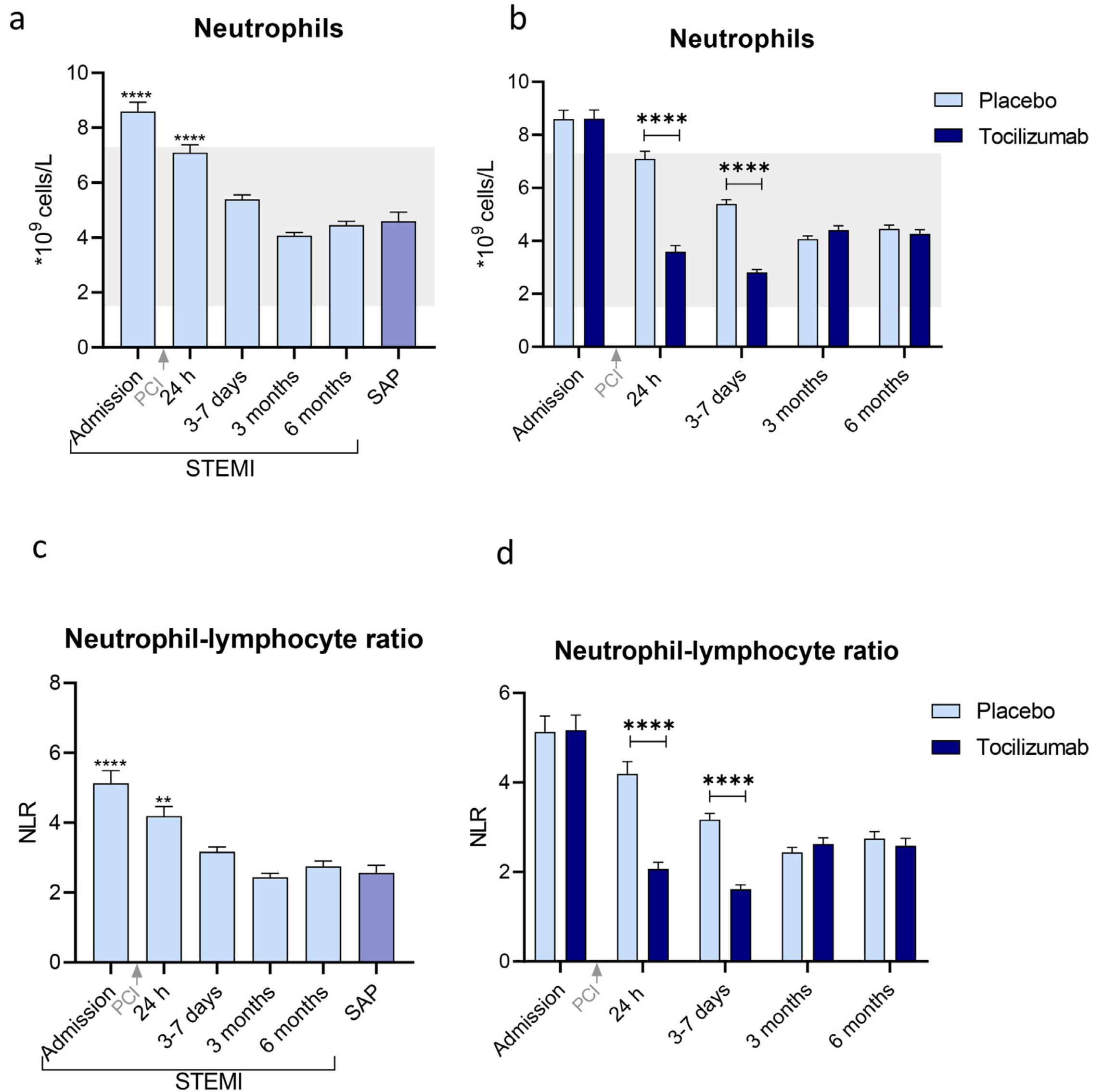

Figure 1. Downregulatory effects by tocilizumab treatment on neutrophils and neutrophil-lymphocyte ratio (NLR) following STEMI. *Note, Panel A and C show only the placebo group to compare the pattern in STEMI patients with levels in stable angina pectoris.* Panel a shows the level of circulating neutrophils in STEMI patients treated with PCI (*only placebo*) over time compared with patients with stable angina pectoris (SAP) ($n = 20$) as mean with SEM. Grey-shaded area shows normal range. ****$p < 0.0001$ versus SAP (One-way ANOVA with Dunnett's multiple comparisons test). Panel b shows a comparison of the effect of tocilizumab treatment at all time-points between the neutrophil counts in the tocilizumab arm and in the placebo arm as mean with SEM. ****$p < 0.0001$ comparing the two treatment groups (Mixed effect analyses with Bonferroni's multiple comparisons test). Panel c shows NLR in STEMI patients treated with PCI (*only placebo*) over time compared with patients with stable angina pectoris (SAP) ($n = 20$) as mean with SEM. ****$p < 0.0001$ and **$p < 0.01$ versus SAP (Dunnett's multiple comparisons test). Panel d shows a comparison of the effect of tocilizumab treatment at all time-points between the tocilizumab arm and the placebo arm as mean with SEM. ****$p < 0.0001$ comparing the two treatment groups (Mixed effect analyses with Bonferroni's multiple comparisons test). Data are given as mean and SEM. Hospital admission was within 6 h after symptom onset. Placebo ($n = 98$): at hospitalisation ($n = 93$), 24 h ($n = 98$), 3-7 days ($n = 96$), 3 months ($n = 94$) and 6 months ($n = 96$). Tocilizumab ($n = 101$): at hospitalisation ($n = 98$), 24 h ($n = 99$), 3-7 days ($n = 100$), 3 months ($n = 99$) and 6 months ($n = 99$).

effect of tocilizumab on the MSI was more pronounced in patients undergoing PCI more than 3 h after symptom onset. The decrease in neutrophil counts and NLR in the tocilizumab group was, however, not dependent on the time since symptom onset (Supplemental Figure S3).

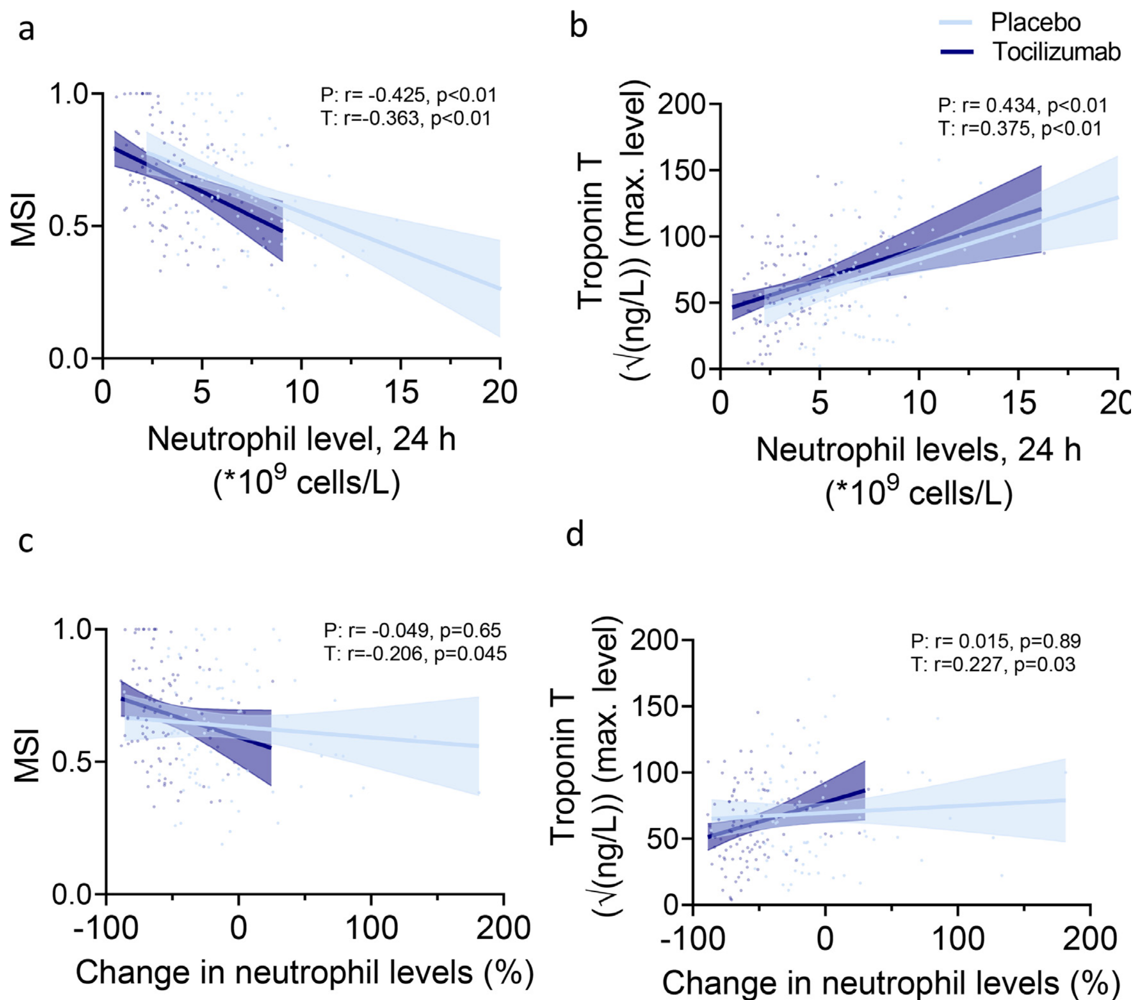

Figure 2. Correlation between neutrophil counts *and* myocardial salvage index (MSI) and maximum troponin T (TnT) levels in STEMI patients. Panel a and b shows correlation between absolute neutrophil counts at 24 h *and* MSI (a) and maximum TnT levels (b) in the tocilizumab (T) and the placebo (P) group. Panel c and d show correlation between percent neutrophil change from admission to 24 h *and* MSI (c) and maximum TnT levels (d) in the tocilizumab (T) and the placebo (P) group. Trend line is indicated for easier visualization. Correlations were calculated using Spearman's correlation coefficient (r). Placebo (n = 98): at hospitalisation (n = 93), 24 h (n = 98). Tocilizumab (n = 101): at hospitalisation (n = 98), 24 h (n = 99).

The decrease in neutrophils after tocilizumab treatment: potential effects on MSI and TnT release

The absolute neutrophil counts at 24 h correlated inversely with MSI (placebo: r = -0.425, $p < 0.01$; tocilizumab: r = -0.363, $p < 0.01$) and positively with maximum TnT levels (placebo: r = 0.434, $p < 0.01$; tocilizumab: r = 0.375, $p < 0.01$). This relationship was independent of treatment allocation (Figure 2a, b). When examining the percentage changes in neutrophil counts after 24 h, we found that the marked and rapid decrease in neutrophil counts in the tocilizumab group (mean 56% decline) was significantly associated with increased MSI (r = -0.206, p = 0.045) and decreased peak TnT (r = 0.227, p = 0.03) (Figure 2c, d). However, such correlations were not seen in the placebo-treated patients, potentially reflecting that the percentage decrease in this group was modest (MSI: r = -0.049, p = 0.65; TnT: r = 0.015, p = 0.89) (mean 8% decline). Thus, it seems that the decrease in neutrophils in the tocilizumab arm compared with the placebo arm may be associated with a beneficial effect on MSI and TnT release.

Gene expression in whole blood: attenuated innate immunity response after treatment with tocilizumab

We extracted and sequenced RNA from whole blood drawn from 20 patients who were allocated to placebo in the ASSAIL-MI trial and from 20 patients allocated to tocilizumab. Patients were selected to obtain equal distribution of age and gender as well as levels of HbA1C, LDL and HDL cholesterol, and triglycerides between the

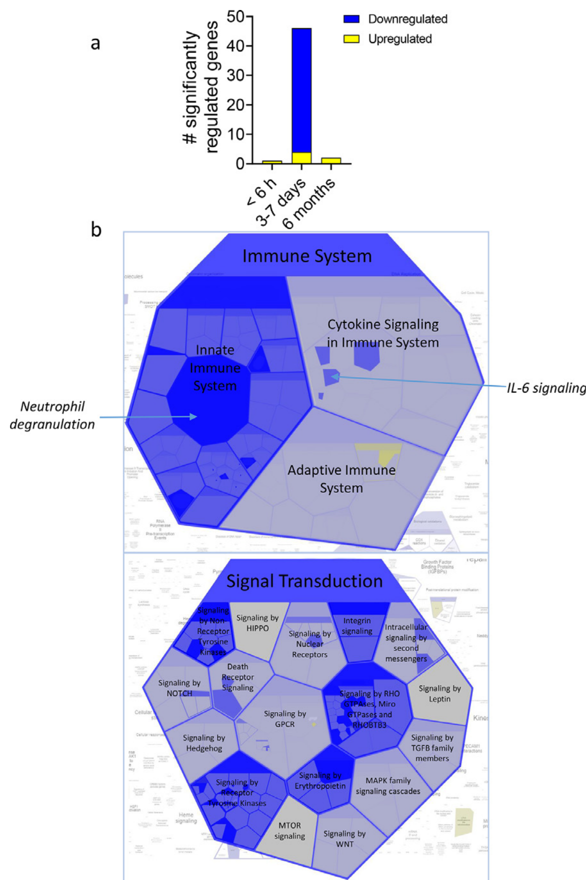

Figure 3. Comparison of gene expression in whole blood after 3-7 days between placebo and tocilizumab treated STEMI patients. Panel a shows the number of significantly differentially regulated genes (adjusted p-value < 0.05) at hospitalisation (placebo [p]: $n = 18$, tocilizumab [t]: $n = 19$), 3-7 days after hospitalisation (p: $n = 19$, t: $n = 20$) and after 6 months (p: $n = 18$, t: $n = 20$) between the two treatment arms. Panel b shows the pattern of differential regulation between placebo and tocilizumab treated patients of the two Reactome pathways "Immune system" (R-HSA-168256) and "Signal transduction" (R-HSA-162582), when including all genes with a p-value < 0.01. Dark blue is significantly downregulated in the tocilizumab-treated compared with the placebo-treated patients, while bright yellow is significantly upregulated in the same groups.

treatment groups. Few differences were observed at hospitalisation and after 6 months between the treatment groups. However, after 3-7 days, a total of 46 genes were significantly differentially expressed between treatment groups. Importantly, 42 of these genes were expressed at lower levels in the tocilizumab arm than in the placebo arm (Figure 3a). Reactome analysis of all genes revealed an attenuated expression of the pathway "Immune system" (R-HSA-168256). It is worth noticing that the "Innate immune system" (R-HSA-168249) pathway and the underlying "Neutrophil degranulation" (R-HSA-6798695) pathway were downregulated in the tocilizumab arm compared with the placebo arm (Figure 3b). In addition, the "Signal transduction" (R-HSA-162582) pathway along with several relevant sub-pathways (e.g., "Signalling by Receptor Tyrosine Kinases" (R-HSA-9006934) and "RHO GTPase cycle" (R-HSA-9012999)) were reduced in patients treated with tocilizumab compared with patients allocated to placebo (Figure 3B). The "IL-6 signalling" pathway was also downregulated in the tocilizumab arm, in accordance with tocilizumab treatment.

Downregulation of genes related to neutrophil function and signal transduction after tocilizumab

To estimate neutrophil gene expression, we used CIBERSORTx for deconvolution of whole blood RNA-sequencing data and to impute the gene expression profile of neutrophils. The enrichment analysis of significantly differentially expressed genes revealed that "Neutrophil degranulation" (R-HSA-6798695) was the most significantly regulated pathway between the treatment groups 3-7 days after hospitalisation (Figure 4a). "MAPK cascade" (GO:0000165), "signalling by Receptor Tyrosine Kinases" (R-HSA-9006934), "RHO GTPase cycle" (R-HSA-9012999), and the "immune response-regulating signalling pathway" (GO:0002764) were also regulated differently between the treatment groups. All these pathways are important for signal transduction in immune cells. In addition, gene ontology (GO) terms related to neutrophil function and cell development, like "positive regulation of cell migration" (GO:0030335), "organelle localization" (GO:0051640) and "regulation of cell development" (GO:0060284) were significantly altered (Figure 4A). We plotted the fold change of genes associated with these pathways. Most of them were downregulated, suggesting a dampened function of these GO terms (Figure 4b, c). To determine the association between the patient groups and genes involved in the two most pronounced GO terms "Neutrophil degranulation" and Reactome pathways "MAPK cascade" we ran a gene set enrichment analysis (GSEA). The results from this clearly showed that the genes in these two terms are most highly expressed in the placebo group, indicating a downregulation in the tocilizumab arm (Figure 4d, e).

After performing corrections for multiple testing, 86 genes were significantly altered (adjusted p-value < 0.05). Of these, 4 were higher and 82 were lower in the tocilizumab arm compared with the placebo arm (Supplemental Table S2). Several of these genes may be of importance for cell migration, exosome function and inflammation (Supplemental Table S2).

Minor changes in lymphocyte subpopulation composition following STEMI: no effect of tocilizumab

In a subgroup of 69 patients from the ASSAIL-MI trial and in 20 patients with stable angina, we performed a

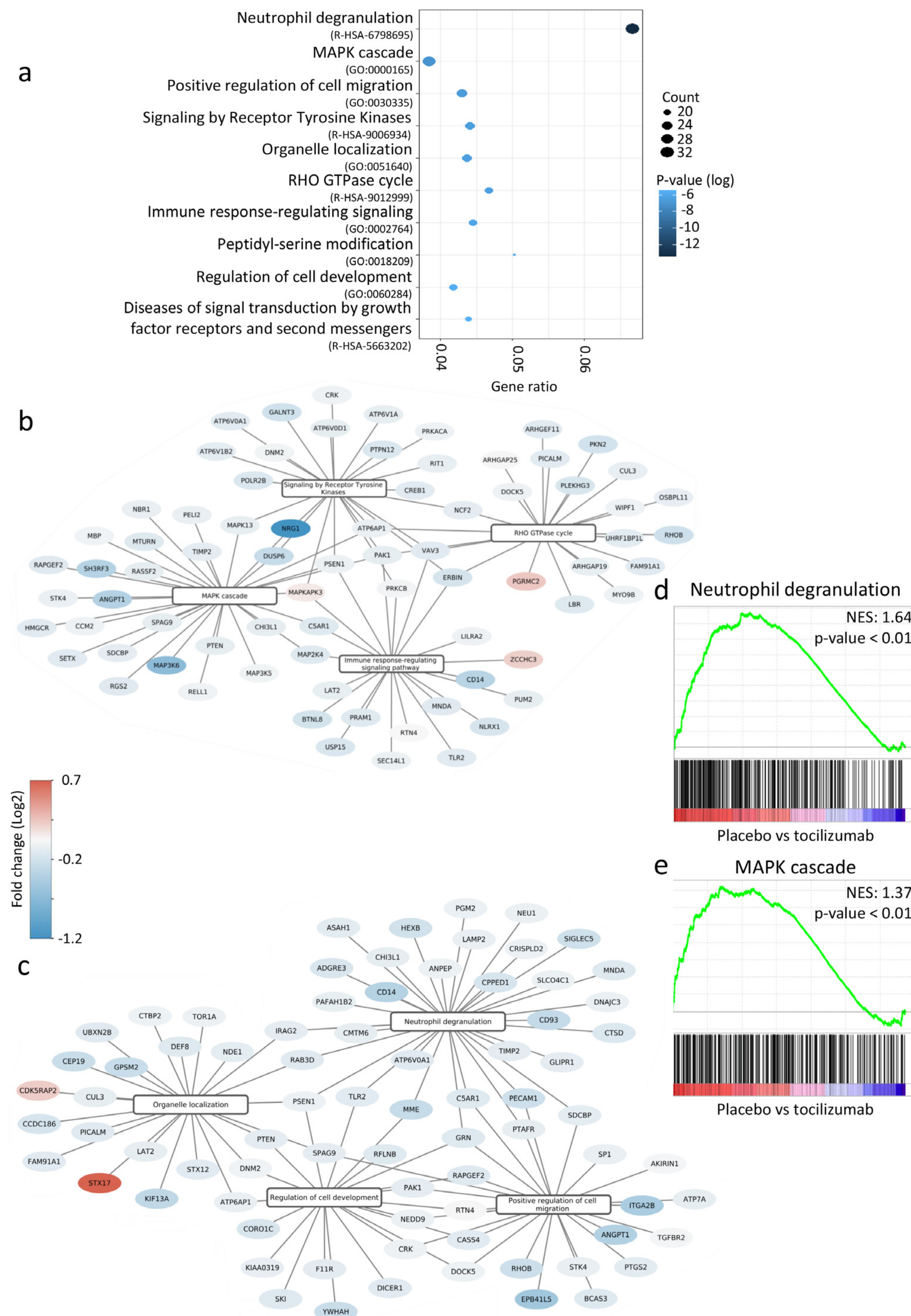

Figure 4. Gene expression imputation in neutrophils derived from whole blood analyses. Panel a shows the 10 most enriched pathways (Reactome) and Gene Ontology (GO) terms from analysis with the gene expression level of the 423 genes belonging to neutrophils that had more than 50 average counts and a *p*-value < 0.01 (unpaired t test) after the deconvolution analysis performed on RNA-sequencing results from whole blood by CIBERSORTx. Panel b shows a network plot for the four most relevant Reactome pathways and GO terms for signal transduction, and Panel c shows pathways relevant for neutrophil function and cell development. Blue shows lower gene level (log2 fold change), while red shows higher gene level in the tocilizumab group compared with placebo treated patients. Panel d shows GSEA analysis for genes related to the Reactome pathway "Neutrophil degranulation", and Panel e shows the GSEA analysis for the GO term "MAPK cascade".

more thorough analysis of lymphocyte populations by flow cytometry. Patient characteristics are presented in Supplemental Table S3. The level of NK cells was higher immediately after hospital admission than at all later assessments, but the level only slightly exceeded the normal range (grey area), and the difference did not reach statistical significance (p = 0.079) (Figure 5a). There were only minor and mostly non-significant changes in the other lymphocyte subpopulations ($CD19^+$ B-cells, $CD4^+$ T-cells and $CD8^+$ T-cells) following STEMI (Figure 5B−D). B and T cell subpopulations barely differed from normal values at time of hospitalisation and changed little during follow-up after STEMI. In the patients with STEMI, plasmablasts peaked at 3-7 days (p = 0.04) compared with stable angina patients (Supplemental Table S4). Importantly, for all these subpopulation analyses there were no differences between the tocilizumab arm and the placebo arm (Supplemental Table S4). A RNA-sequencing on isolated T cells revealed few differences between the treatment groups when looking at differentially expressed genes (adjusted p-value < 0.05, average gene expression > 50), with 4 regulated genes at time of hospitalisation, 6 at 24 h, none after 3-7 days and 1 after 6 months (Figure 5e). The fold change of the regulated genes was in general modest and the net effects of these changes are uncertain (Supplemental Table S5).

The effect of tocilizumab on $CD8^+$ T cells is dependent on the time from symptom onset

We found significantly lower levels of $CD4^+$ T cells (p = 0.02) and $CD8^+$ T cells (p = 0.02), but no NK cells (p = 0.2) or B cells (p = 0.06), in patients admitted > 3 h after symptom onset than in patients admitted ≤ 3 h after symptom onset (Figure 6A). Notably, in the tocilizumab arm, the levels of $CD8^+$ T cells, but not the $CD4^+$ T cells (Supplemental Table S6), remained low in the patients admitted to the hospital > 3 h after symptom onset compared with patients receiving tocilizumab ≤ 3 h after symptom onset. The placebo-treated patients arriving > 3 h after symptom onset had an increase in $CD8^+$ T cells after hospital admission (p = 0.02), not found in the tocilizumab group (Figure 6B). Moreover, tocilizumab significantly reduced the absolute number of late effector/memory $CD8^+$ T cells (p = 0.01 at 24 h), but not the numbers of naive $CD8^+$ T cells (p = 0.2 at 24 h) and early effector/memory $CD8^+$ T cells (p = 0.08 at 24 h) in patients arriving to the hospital > 3 h after symptom onset (Supplemental Figure S4). In the patient group as a whole, we found an inverse correlation between $CD8^+$ T cell counts and MSI in those hospitalezed later (> 3 h after symptom onset) (24 h: r = -0.6, p = 0.002) (Table 2). This correlation was only seen in the placebo group (24 h: r = -0.64, p = 0.02) and not in the tocilizumab group (24 h: r = -0.49, p = 0.13) (Table 2), either for TnT (24 h, whole population: r = 0.366, p = 0.07) (Supplemental Table S7).

Discussion

We have previously shown that tocilizumab improved myocardial salvage in STEMI, and that tocilizumab reduced TnT levels in NSTEMI.[5,6] In both studies, we observed lower neutrophil counts during hospitalisation in the tocilizumab group than in the placebo group. In this exploratory and predefined sub-study, we present novel data on associations between neutrophils and MSI and TnT in STEMI patients. Our results may suggest that the beneficial effects of tocilizumab in these patients could at least partly be mediated through an attenuation of neutrophil functions. Our data show that tocilizumab not only reduces the number of circulating neutrophils but also mitigates the inflammatory potential of the remaining circulating neutrophils. However, these associations do not prove any causal relationship and it could be claimed that the decrease in neutrophils in the tocilizumab group could be caused by a lower degree of myocardial damage and not *vice versa*.

Acute STEMI and the subsequent ischaemia/reperfusion (I/R) injury promotes an inflammatory cascade that contributes to the replacement of damaged tissue and tissue repair. However, this inflammatory reaction also has detrimental effects if, in particular, the inflammatory responses are too strong or persist for too long. Experimental and clinical studies have suggested that neutrophils contribute to tissue damage, including cardiomyocyte apoptosis, following MI.[21] During I/R, neutrophils are major contributors to myocardial damage, at least partly through oxidative stress.[21] Moreover, clinical studies have shown that high neutrophil counts predict both acute and chronic cardiovascular disease (CVD), and a high neutrophil count is proposed as a clinical predictor of poor outcomes after coronary events.[22] Interestingly, an elevated NLR, reflecting the balance between the innate and the adaptive immune responses, has been associated not only with the presence of CVD but also with short-term adverse outcomes, including mortality, in patients with CVD.[23] In accordance with previous reports,[24,25] we found higher levels of neutrophils and NLR in STEMI patients at admission compared with patients with stable angina. However, herein we showed that there was a significant decrease in neutrophil counts and NLR, mainly driven by a reduction in neutrophil counts, during hospitalisation in the tocilizumab arm compared with placebo. A reduction in neutrophils has been suggested to be a side effect that predisposes patients treated with IL-6 inhibitors to infectious complications.[26] However, we found that a high number of neutrophils in STEMI patients 24 h after hospital admission correlated inversely with MSI and positively with maximum levels of TnT. Moreover, we found that the rapid decrease in neutrophils

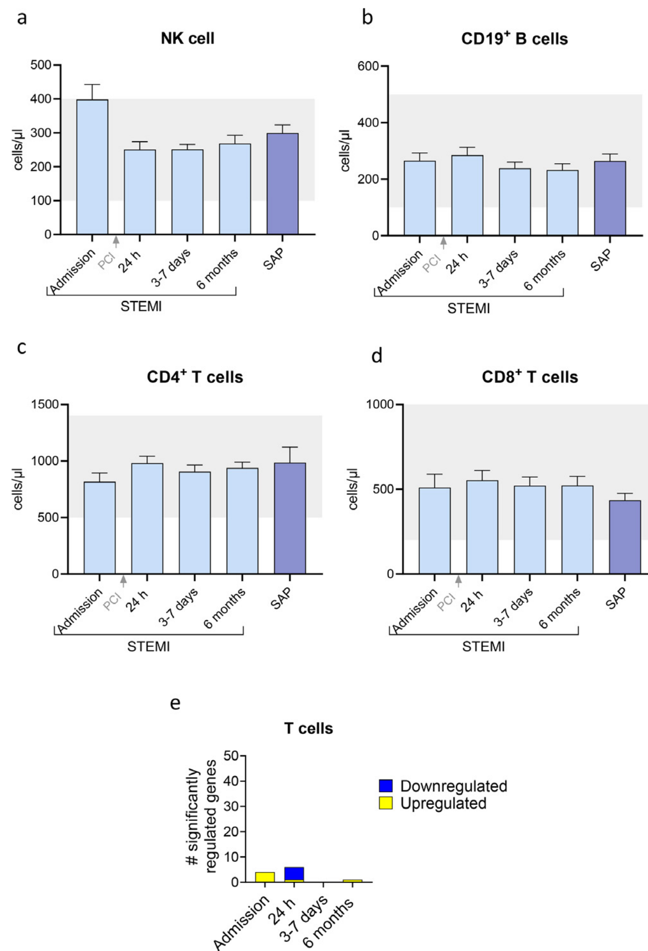

Figure 5. B cells, T cells and NK cells following STEMI. Levels of (a) NK cells, (b) B cells (CD19), (c) $CD4^+$ T cells and (d) $CD8^+$ T cells in STEMI patients (*only placebo*) at hospitalisation ($n = 30$), 24 h ($n = 31$), 3-7 days ($n = 29$) and 6 months ($n = 30$) compared with stabile angina pectoris (SAP) patients ($n = 20$). Data are presented as mean with SEM. Grey-shaded area: normal range. $^*p < 0.05$ versus SAP (One-way ANOVA with Dunnett's multiple comparisons test). Data are given as mean and SEM. Hospital admission was within 6 h after symptom onset. Panel e shows significantly DEGs (adjusted p-value < 0.05, average gene expression > 50) in T cells between the treatment groups at hospitalisation (placebo [p]: $n = 10$, tocilizumab [t]: $n = 6$), 24 h after hospitalisation (p: $n = 8$, t: $n = 8$), after 3-7 days (p: $n = 8$, t: $n = 8$) and after 6 months (p: $n = 10$, t: $n = 9$).

from admission to 24 h in the tocilizumab treated patients was associated with increased MSI and decreased peak TnT. These data suggest that the beneficial effect of tocilizumab in STEMI may involve an attenuated neutrophil level.

Our data suggests that a sustained high number of neutrophils may be harmful during STEMI and may contribute to myocardial damage. However, we do not know whether the reduced neutrophil counts in the tocilizumab group are due to increased migration to the infarct site, increased neutrophil apoptosis or, more likely, due to a reduced influx to the circulation from the bone marrow or spleen, or due to a combination thereof. IL-6 stimulation is suggested to mobilize neutrophils from the bone marrow into the circulation, possibly through the CX_3CR1 receptor (fractalkine receptor).[27,28] It could be hypothesized that the reduced neutrophil level in the tocilizumab treated patients is due to a direct inhibition of the IL-6 receptor of the cells in the bone marrow. In the RNA-sequencing of whole blood 3–7 days after tocilizumab treatment, we found no difference in the CX_3CR1 receptor gene expression between the two treatment arms. Further data on this subject is needed. Notably, it has also been suggested that the decrease in lymphocytes during MI could reflect enhanced myocardial infiltration of these cells at least partly involving increased expressing of CX_3CR1 on these lymphocytes.[29,30] In addition, neutrophils have been found to change their phenotype over time to regulate not only tissue damage but also the resolution of the inflammation following MI.[31] To elucidate these important issues, forthcoming studies should clarify

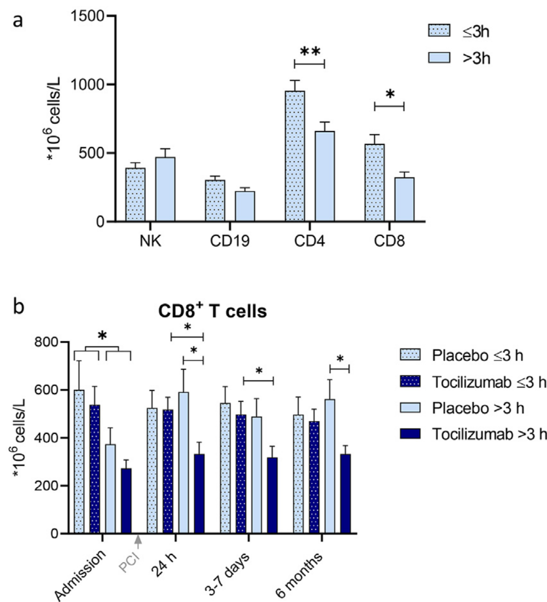

Figure 6. Effect of tocilizumab on numbers of NK cells, B (CD19) cells, $CD4^+$ T cells and $CD8^+$ T cells according to time since symptom onset. Panel a shows numbers of NK cells, B (CD19) cells, $CD4^+$ T cells, and $CD8^+$ T cells at admission (before drug administration and PCI) according to time since symptom onset ($\leq$ 3 h, n = 40 and $>$ 3 h n = 24) as mean with SEM. Panel b shows $CD8^+$ T cells counts for STEMI patients in the placebo and tocilizumab group based on time since symptom onset as mean with SEM. *$p < 0.05$ (Unpaired t test between relevant groups). Data are given as mean and SEM. Placebo $\leq$ 3 h (admission: n = 18, 24 h: n = 18, 3-7 days: n = 17, 6 months: n = 18); placebo $>$ 3 h (admission: n = 12, 24 h: n = 13, 3-7 days: n = 12, 6 months: n = 12); tocilizumab $\leq$ 3 h (admission: n = 22, 24 h: n = 21, 3-7 days: n = 25, 6 months: n = 23); tocilizumab $>$ 3 h (admission: n = 12, 24 h: n = 12, 3-7 days: n = 12, 6 months: n = 12). Hospital admission was within 6 h after symptom onset.

which neutrophil subtypes are decreased by tocilizumab.

Attenuation of neutrophil responses due to tocilizumab treatment was reflected at the RNA level. The innate immunity response was dampened and signal transduction was reduced 3–7 days after treatment with tocilizumab. Further, pathways related to neutrophil activity, like neutrophil degranulation and several of the signalling pathways, were downregulated in the tocilizumab arm compared with the placebo arm. The same tendency was also observed at the estimated gene expression level in neutrophils, where the downregulated “Neutrophil degranulation” pathway and the “MAPK cascade” GO term potentially promote a dampened inflammatory phenotype in the tocilizumab-treated patients. Genes associated with the RHO GTPase cycle, the MAPK cascade and signalling by Receptor Tyrosine Kinases were also downregulated. Signal transduction through these three pathways have roles in neutrophil recruitment, including polarization, trans-endothelial migration and chemotaxis, as well as neutrophil activation.[32–34] Thus, a downregulation of these signalling pathways might contribute to an attenuated neutrophil function. Moreover, one of the few upregulated genes by tocilizumab was *Syntaxin 17*. Syntaxin 17 is involved in the initiation of autophagy, a process of importance for maintaining cardiac homeostasis and a survival mechanism that is upregulated during myocardial stress.[35] Interestingly, a proteomic sub-study of the LoDoCo2 study, using low dose colchicine-treatment to reduce the risk of cardiovascular events after MI also revealed an attenuation of neutrophil degranulation-related proteins thought to be beneficial in reducing atherogenesis.[36,37] Finally, we have recently shown that tocilizumab may induce the formation of neutrophil extracellular traps (NETs) in NSTEMI.[38] NETS may have a harmful effect during plaque destabilization and their potential role in STEMI needs clarification.

In the present study, we analysed a wide range of lymphocyte subpopulations in the ASSAIL-MI study. However, except for a peak at 3-7 days for plasmablasts, these cell populations remained surprisingly stable and did not differ across treatment groups. Thus, whereas IL-6 is known to have pronounced effects on B cells,[39] we found no substantial differences in B cell subpopulations between the two treatment groups. It has previously been reported that an immediate reduction in T cells within the first 24 h after PCI is associated with poor outcomes for STEMI patients,[29,40] but we do not have data on the immediate T cell levels after PCI in our intervention trial. Moreover, in contrast to neutrophils, we observed no pronounced differences in gene expression of T cells in the tocilizumab arm compared with the placebo treated patients, indicating that tocilizumab treatment has few effects on both T cell subpopulations and T cell function. Cormark *et al.* showed that a single dose of ciclosporin induced a significant and incidental decrease in T cell counts.[41] Importantly, nevertheless, the mechanisms of action differ widely between ciclosporin and tocilizumab affecting mainly IL-2 and IL-6, respectively. In patients who were admitted > 3 h after symptom onset, however, $CD8^+$ T cell counts did not increase during hospitalisation in the tocilizumab arm, whereas a pronounced increase in the placebo arm was observed. Inhibition of IL-6-mediated differentiation of $CD8^+$ T cells,[42] which affects the late effector/memory $CD8^+$ T cells in particular, could be operating in the tocilizumab arm in this subgroup of patients. In patients admitted to the hospital > 3 h after symptom onset, there was a negative correlation between $CD8^+$ T cell counts and the MSI in the placebo group, suggesting harmful effects of $CD8^+$ T cells in this subgroup. However, although $CD8^+$ T cells have been suggested to contribute to myocardial damage following MI, their roles in this context are far from clear. Further studies

		Total population					
		≤ 3 h			**> 3 h**		
		r	***P* value**	***n***	***r***	***P* value**	***n***
Absolute count of circulating CD8+ T cells	24 h	0.09	0.57	39	-0.60	0.002	24
	3-7 days	0.13	0.41	42	-0.60	0.002	23
		Placebo					
		≤ 3 h			> 3 h		
		r	*P* value	*n*	*r*	*P* value	*n*
Absolute count of circulating CD8+ T cells	24 h	0.09	0.73	18	-0.64	0.02	13
	3-7 days	0.07	0.79	17	-0.76	0.004	12
		Tocilizumab					
		≤ 3 h			> 3 h		
		r	*P* value	*n*	*r*	*P* value	*n*
Absolute count of circulating CD8+ T cells	24 h	0.15	0.51	21	-0.49	0.13	11
	3-7 days	0.17	0.41	25	-0.39	0.24	11

***Table 2*: Correlations between CD8+ T cell counts and MSI according to time of hospitalisation after symptom onset.**
Significant values are marked in bold. *r* is the Spearman's correlation coefficient.

are needed to clarify if the effect of tocilizumab on CD8+ T cells is beneficial in STEMI.

Our study has some limitations. Associations do not necessarily imply causal relationships, and association between the circulating numbers of neutrophils and the myocardial salvage does not prove that a reduced number of neutrophils *causes* improved salvage. Further, due to the strict inclusion criteria, the study participants are presumably not representative of the whole STEMI population. However, this was a proof-of-concept trial where we knowingly "purified" the population, and any between-group difference must be regarded as a treatment effect due to the randomization. Forthcoming studies should evaluate tocilizumab treatment in an unselected study population. Moreover, the number of patients who underwent extended flow cytometry was limited and the data derived from these analyses should be interpreted with caution. This is particularly important in the interpretation of the analyses looking at time between symptom onset and hospitalisation, where the groups are small. The cells we investigated were from peripheral blood. Our findings may therefore not necessarily reflect the processes that occur within the infarcted myocardium and we do not know if the reduced number of circulating neutrophils in the tocilizumab arm might reflect less mobilization from the bone marrow or a less likely migration to the myocardium. Single-cell mRNA-sequencing, as well as proteomic analyses and measurements of neutrophil-derived mediators in plasma, would have strengthened the results. Studies in animal models and *in vitro* studies would have also strengthened our conclusion and given more mechanistic insight into the molecular mechanisms of action of tocilizumab in STEMI patients and its effect on the myocardium. Forthcoming studies should include such experiments.

In conclusion, tocilizumab treatment reduces the number of circulating neutrophils and seemingly also dampens neutrophil function when administered prior to PCI in patients with STEMI. Finally, our data suggest that these effects could be related to the beneficial effects of tocilizumab on myocardial salvage in these patients.

Declaration of interests

Kaspar Broch has received lecture fees from Pharmacosmos, AstraZeneca, Boehringer Ingelheim, Pfizer, Orion Pharma, and Vifor Pharma, and has been on advisory boards for Pfizer and AstraZeneca. Lars L. Gullestad has received lecture fees from AstraZeneca, Boehringer Ingelheim, Novartis, and Amgen. He has also been a member of the local advisory board in AstraZeneca and Boehringer Ingelheim. Annika E. Michelsen is a stock holder in Pfizer. Bente E. Halvorsen has a patent on fish derived fatty acids, is a SAB member in CircM, Linkøping, Sweden, and is a leader of the scientific evaluation committee, Svensk Hjerte Lung Fonden.

Contributors

Study conception and design: PA, BH, LO, CH, AKA, TBD, LG, KB, RW, GÄA, IS, JKD, TU, BB

Recruitment of patients, laboratory analysis and collection of data. CH, AKA, TBD, AM, TU, KY, LO, RW, AQJ, VB, GÄA, SW, KB, KS, IMT, BB, BHA, ESB, EB, CB, OK, KHS, AO, NEK

Contributed to analysis and interpretation of data. TBD, CH, PA, AKA, AM, TU, LO

Verification of all data; Clinical data verification. CH, AKA, TU. RNA-sequencing data verification. CH, KY, TBD. Flow cytometry data verification. CH, LO, TBD

Contributed to the original drafting of the article. CH, AKA, KY, TBD, PA, TU

All authors contributed to review and editing of the article and has given final approval of the version to be submitted.

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Data sharing statement

Because of ethical restrictions from the Regional Committee for Medical and Research Ethics in South−East Norway, the data from the individual patients will unfortunately not be made available to other researchers for purposes of reproducing the results or replicating the procedure. However, researchers and others can contact the corresponding authors for more information. Regarding RNA-sequencing data, an institutional data transfer agreement can be established, and data can be shared if the aims of data use are covered by ethical approval and patient consent. The procedure will involve an update to the ethical approval as well as review by the legal departments at both institutions, and the process will typically take 2−4 months from the firts contact.

Supplementary materials

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.ebiom.2022.104013.

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