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2015

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Yildirim, C. (2015). *Immune modulation of bone marrow-derived cells in Ischemic Heart Disease*. [PhD-Thesis - Research and graduation internal, Vrije Universiteit Amsterdam].

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Chapter 2

Systemic toll-like receptor and interleukin-18 pathway activation in patients with acute ST elevation myocardial infarction

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ABSTRACT

Acute myocardial infarction (AMI) is accompanied by increased expression of Toll like receptors (TLR)-2 and TLR4 on circulating monocytes. In animal models, blocking TLR2/4 signaling reduces inflammatory cell influx and infarct size. The clinical consequences of TLR activation during AMI in humans are unknown, including its role in long-term cardiac functional outcome. Therefore, we analyzed gene expression in whole blood samples from 28 patients with an acute ST elevation myocardial infarction (STEMI), enrolled in the EXenatide trial for AMI patients (EXAMI), both at admission and after 4-month follow-up, by whole genome expression profiling and real-time PCR. Cardiac function was determined by cardiac magnetic resonance (CMR) imaging at baseline and after 4-month follow-up. TLR pathway activation was shown by increased expression of TLR4 and its downstream genes, including IL-18R1, IL-18R2, IL-8, MMP9, HIF1A, and NFKBIA. In contrast, expression of the classical TLR-induced genes, TNF, was reduced. Bioinformatics analysis and in vitro experiments explained this non-canonical TLR response by identification of a pivotal role for HIF-1 α . The extent of TLR activation and IL-18R1/2 expression in circulating cells preceded massive troponin-T release and correlated with the CMR-measured ischemic area ($R = 0.48$, $p = 0.01$). In conclusion, we identified a novel HIF-1-dependent non-canonical TLR activation pathway in circulating leukocytes leading to enhanced IL-18R expression which correlated with the magnitude of the ischemic area. This knowledge may contribute to our mechanistic understanding of the involvement of the innate immune system during STEMI and may yield diagnostic and prognostic value for patients with myocardial infarction.

1. INTRODUCTION

Although effective treatment of acute myocardial infarction (AMI) by primary percutaneous coronary intervention (pPCI) and thrombolysis has greatly improved survival and cardiac function, AMI is still a major cause of death and morbidity worldwide. Reperfusion restores tissue oxygenation, but contradictory, also induces adverse effects by reperfusion injury, in which inflammation plays an important role^{1,2}. The innate immune response elicited during AMI through activation of Toll-like receptor (TLR)2 and TLR4 on circulating blood cells negatively influences the course of disease in experimental models, i.e. increases infarct size and worsens ventricular remodeling, reviewed in^{3,4}. TLR4 deficiency, pharmacological inhibition of TLR4 or TLR2 reduces monocyte- and granulocyte influx into the infarcted area, decreases infarct size and ameliorates cardiac remodeling in myocardial infarction models⁵⁻⁷. Transfer of TLR2-deficient bone marrow cells into wild type animals further indicated that the deleterious effect of TLR2 is mediated by circulating immune cells⁸. TLR activation of monocytes results in Myeloid Differentiation primary response gene 88 (MyD88)-dependent nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) signaling, leading to release of pro-inflammatory cytokines, and matrix degrading metalloproteases. During AMI in mice, transfer of NFκB-p50-deficient bone marrow-derived cells indicated that NFκB activation in these cells is responsible for remodeling and dysfunction of the myocardium⁹. The mean expression of TLR2 and TLR4 (mRNA and surface protein expression) by circulating monocytes and granulocytes is increased during AMI, and unstable angina, compared to controls and patients with stable angina¹⁰⁻¹³, indicating that not only leukocyte counts, but also their increased expression of TLR2 and -4 is related to AMI. Several endogenous TLR2 and TLR4 ligands (HSP60, HSP70, HSP90, HMGB1, Fibronectin EDA, the calgranulins S100A8 and S100A9) are expressed in ischemic tissue and also released in the circulation, causing increased TLR expression and TLR activation of circulating cells^{14,15}. Next to TLR ligands, hypoxia may also induce TLR2 expression on monocytes¹². Taken together, although numerous animal studies indicate a harmful effect of the innate inflammatory response on infarct size and cardiac function after AMI, in clinical studies however, the detrimental role of the innate immune system on AMI recovery proves more difficult to unravel^{1,16,17}. Treatment with immunosuppressive corticosteroids¹⁸, anti-CD18 (Integrin beta-2)¹⁹ and anti-CD11/CD18 integrin²⁰ all failed to reduce infarct size and clinical events in STEMI patients, despite the positive effects in animal studies. The functional impact of TLR activation on circulating cells after AMI is largely unexplored in patients. In the present study we aimed at exploring the impact of enhanced TLR expression on circulating leukocytes after STEMI, by analyzing full genome gene expression in circulating cells after STEMI. We hypothesized that this analysis would provide more insight in the contribution of the innate immune system on cardiac function during STEMI.

2. MATERIAL AND METHODS

2.1. Patients

Patients with an acute ST-segment elevation myocardial infarction (STEMI) were enrolled in the EXAMI trial of which the study design, procedures and main results have been reported previously^{21,22}. The study was approved by the institutional ethical committee on human research and is carried out in compliance with the Helsinki Declaration. This study demonstrated that administering high-dose exenatide intravenously in patients undergoing pPCI is safe and feasible. Briefly, informed consent was obtained from patients with STEMI, selected for pPCI, aged between 18 and 80 years. Forty patients were randomized to 5 µg intravenous exenatide or placebo treatment, just prior to PCI, administered intravenously (iv) in 30 min. Treatment was followed by a continuous infusion of 20 µg/24 h for the duration of 72 h. Patients with multivessel disease, Thrombolysis In Myocardial Infarction (TIMI) 3 flow prior to PCI or without a definite culprit lesion were excluded after CAG. Whole blood samples (2.5 ml) were obtained using PAXgene tubes (PreAnalytix, GmbH, Germany) from 33 patients, at the time of admission at the coronary care unit and before administration of exenatide or placebo (T1), directly after the first 5 µg iv bolus of exenatide or placebo (T2, n = 32) and after 4 months (T4, n = 28) for background expression levels. To establish a relation between gene expression with cardiac function, we used samples from 28 patients, for which all time points were available. Peripheral blood cells were counted in whole blood EDTA samples using an automated hematology analyzer (Cell-Dyn Sapphire, Abbott Diagnostics, Santa Clara, CA, USA). Simultaneously drawn EDTA blood plasma was stored at -20 °C until assayed.

2.2. Cardiac Magnetic Resonance (CMR)

Three to 7 days and 4 months after primary PCI, cardiac MRI with delayed contrast enhancement (DCE) was performed to assess left ventricular function and infarction size. The first MRI is performed to visualize myocardial edema, i.e. the area at risk. The second MRI is performed to measure myocardial fibrosis, i.e. infarct area. Doing so, infarct size can be measured as a percentage of the area at risk. Patients were studied on a clinical 1,5 Tesla scanner with a cardiac phased array receiver surface coil placed on the thorax. ECG-gated cine MR images are obtained during repeated breath-holds in the 3 standard long axis views (4-, 3- and 2-chamber view). Additional short axis slices are acquired covering the entire left ventricle to examine regional and global left ventricular function. T2-weighted imaging, using a Short-Tau Inversion Recovery (STIR) sequence, was performed in long and short axis orientations to estimate the area at risk²³. First-pass perfusion images were acquired during intravenous injection of a 0.1 mmol/kg bolus of Gadoteric acid (Dotarem, Guerbet, Villepinte, France) at a rate of 3.0 ml/s, using a single-shot saturation recovery gradient-echo pulse sequence. Three short-axis slices were obtained per heartbeat, covering the infarcted

area as identified with cine and T2-weighted imaging. After the perfusion sequence, an additional dose of 0.1 mmol/kg Dotarem was intravenously injected as a bolus (cumulative dose 0.2 mmol/kg). DCE images were acquired 10 min post-contrast injection to identify the location and extent of myocardial infarction. All images were stored in a database for blinded analysis. The MRI data were analyzed using the Mass software package (Mass, v.5.1 2010-EXP beta Medis, Leiden, The Netherlands). On the short axis cine slices, the endocardial and epicardial borders are outlined manually in end-diastolic and end-systolic images, excluding trabeculae and papillary muscles. Assessment of global left ventricular function is obtained by calculating left ventricular volumes, mass, and ejection fraction using the summation of slice method multiplied by slice distance. For analysis of segmental myocardial function, each short axis slice is divided in 12 equiangular segments, starting at the posterior septal insertion of the right ventricle. Segmental wall thickening is expressed in absolute values (end-diastolic wall thickness subtracted from end-systolic wall thickness [in millimeters]) and relative values (absolute wall thickening divided by end-diastolic wall thickness [%]). Areas of hyperenhancement are outlined, including central dark zones of microvascular obstruction, allowing the calculation of total infarct size by summation of all slice volumes of hyperenhancement. Infarct size and salvage (area at risk - infarct size) were expressed as percentage of the left ventricle. Salvage index was calculated as the ratio of myocardial salvage and area at risk. Both STIR and DCE images were analyzed in consensus by 2 experienced observers (combined experience in Cardiac MRI more than 10 years (FB, AMB), who were blinded to other patient data at the time of the analysis.

2.3. In vitro hypoxia experiments

Monocytes were isolated from six healthy age- and sex-matched donors (3 males, 3 females, average age 59 year) by density centrifugation using ficoll-paque and percoll and were cultured at a concentration of 1×10^6 /ml in IMDM (Gibco Life Technologies) supplemented with 1000 Units/ml penicillin/streptomycin (Lonza), and 5% heat inactivated human AB serum, in the absence or presence of graded doses of LPS (LPS-EB Ultrapure from E. Coli 0111:B4 strain, InvivoGen, San Diego, CA, USA), under hypoxic (1% oxygen) or normoxic (20% oxygen) conditions for 2 or 18 h. Culture medium was equilibrated for 3 h in normoxic or hypoxic conditions before monocyte exposure. Total RNA was isolated using the Rneasy mini kit (Qiagen, Venlo, The Netherlands) for gene quantification by real-time PCR.

2.4. mRNA isolation, real-time PCR and whole genome transcriptome analysis

Total RNA was isolated from peripheral blood using the PAXgene RNA isolation kit according to the manufacturers' instructions including a DNase (Qiagen) step to remove genomic DNA. For specific gene mRNA quantification, real-time reverse transcriptase-polymerase

chain reaction (real-time PCR) measurements were performed on T1, T2 and T4 samples. In brief, 250 ng RNA was reversed transcribed into cDNA, using the RevertAid H Minus First Strand cDNA Synthesis Kit (Fermentas, St. Leon-Rot, Germany). For transcript quantification we used 4% of the generated cDNA for each gene using TaqMan Universal PCR Master Mix (Applied Biosystems) with SYBR green (Applied Biosystems) as a detection agent on an ABI Prism 7900HT Fast Real-Time PCR System. mRNA expression levels of TLR activation-related genes were calculated using an arbitrary standard curve and quantile normalized, which is superior to correction by housekeeping genes²⁴.

List of primer sequences used for real-time PCR:

TLR4	forward 5'-TTCCCAGAACTGCAGGTGCT-3' reverse 5'-TGGCTTAGGCTCTGATATGCC-3'
TLR2	forward 5'-GGCTTCTCTGTCTTGTGACCG-3' reverse 5'-GAGCCCTGAGGGAATGGAG-3'
NFKBIA	forward 5'-ACAGAGGACGAGCTGCCCTA-3' reverse 5'-CCTTTGCGCTCATAACGTCA-3'
IL-18R1	forward 5'-GCTGTGGAGATTTGCCCA-3' reverse 5'-ACAACAGCTCCTCCAGGCAC-3'
IL-18RAP/IL-18R2	forward 5'-CCCAGTCCGTCCTCAACTGAAA-3' reverse 5'-GGTGCCAAGCAGGATGTACAG-3'
IL-18BP	forward 5'-AACCAGGCTTGAGCGTTCC-3' reverse 5'-TCCCATGTCTCTGCTCATTAGTC-3' ²⁵
IL-8	forward 5'-TGAGAGTGGACCACACTGCG-3' reverse 5'-TCTCCACAACCCTCTGCACC-3'
IL-6	forward 5'-TGCAATAACCACCCCTGACC-3' reverse 5'-TGCGCAGAATGAGATGAGTTG-3'
TNF	forward 5'-CCAAGCCCTGGTATGAGCC-3' reverse 5'-GCCGATTGATCTCAGCGC-3'
IL-1B	forward 5'-TTTGAGTCTGCCAGTTCCC-3' reverse 5'-TCAGTTATATCCTGGCCGCC-3'
HIF1A	forward 5'-AGACACAGAAGCAAAGAACCCA-3' reverse 5'-TGACAACTGATCGAAGGAACGT-3'
MMP9	forward 5'-CTTTGGACACGCACGACGT-3' reverse 5'-CCTGGTTCAACTCACTCCGG-3'
SLC2A1/GLUT1	forward 5'-TGACCATCGCGCTAGCACT-3' reverse 5'-GGCCACGATGCTCAGATAGG-3'

For whole genome expression profiling, 23 high quality T1 RNA samples were further processed by ServiceXS (Leiden, The Netherlands). Amplification was performed using the Ambion® Illumina TotalPrep RNA Amplification Kit (Ambion), resulting in biotinylated, amplified cRNA. Labelled RNA samples were hybridized to Sentrix Human HT12v4 Expression bead chip arrays (Illumina, San Diego, CA), followed by scanning and feature extraction.

2.5. Transcriptome data analysis

Bead summary intensities were log₂-transformed and normalized using quantile normalization^{26,27}. For the identification of genes which correlated with TLR4 expression, we used Significance Analysis of Microarrays (SAM)²⁸. For confirmation of TLR4 activation we performed pathway analysis on the 1489 TLR4-correlating genes in the Molecular Signatures Database (MSigDB), using the canonical pathways as genesets²⁹. The 500 bp upstream regulatory region of the TLR4-correlating genes was analyzed for enriched transcription factor binding sites by whole genome rVISTA software at <http://genome.lbl.gov/vista/index.shtml>³⁰, using the Transfac database of all transcription factor binding sites (TFBS) conserved in the human to mouse whole genome alignment of March 2006. Hierarchical clustering³¹ of samples was used to visualize the correlation of co-expressed genes in Treeview (available at <http://rana.lbl.gov/EisenSoftware.htm>).

2.6. Statistical analysis

To determine the change in gene expression over time, we used the paired *t* test on real-time PCR data with normal distribution, and the Wilcoxon matched pairs test for non-Gaussian distributed data. Real-time PCR data were tested for normal distribution by use of a Shapiro-Wilk normality test. A Bonferroni correction for multiple testing was applied when multiple time points were analyzed. To correlate (changes in) gene expression with clinical variables, we used the Spearman's rank correlation test for all data. A two-sided *p*-value < 0.05 was considered statistically significant. Covariate adjustment was performed by multivariate linear regression. Statistical analysis was performed with Statistical Package for Social Sciences software (SPSS 20.0 for Windows, SPSS Inc).

3. RESULTS

3.1. TLR4 expression during STEMI

To analyze TLR expression during STEMI, we studied a subgroup of the patient population which was enrolled in the EXAMI trial. In brief, the EXAMI trial showed that there was no difference between exenatide- and placebo- treated patients with regard to left ventricular function, infarct size or AAR as measured with CMR. The patient's characteristics of the subgroup for which we had whole blood RNA samples available for all time points are

described in the Supplementary data, Table 1. The clinical parameters were comparable between the placebo and the exenatide group, except for the LV EDV after 4 months, although the change in LV EDV did not reach significance. We analyzed the expression of TLR4 and TLR2 by RT-PCR in peripheral blood in paired samples from 28 STEMI patients at 2 time points: at T1; at presentation at the CCU and at T4; after 4 months, considered background expression. None of the patients showed a recurrent coronary event during the 4-month follow-up period. The 4-month samples enabled us to correct for the variable background expression levels in each individual donor. We confirmed an increase in TLR4 mRNA expression at T1 during STEMI, compared to the levels measured after 4 months (Fig. 1). TLR2 expression at T1 was increased in some patients, but overall did not reach statistical significance.

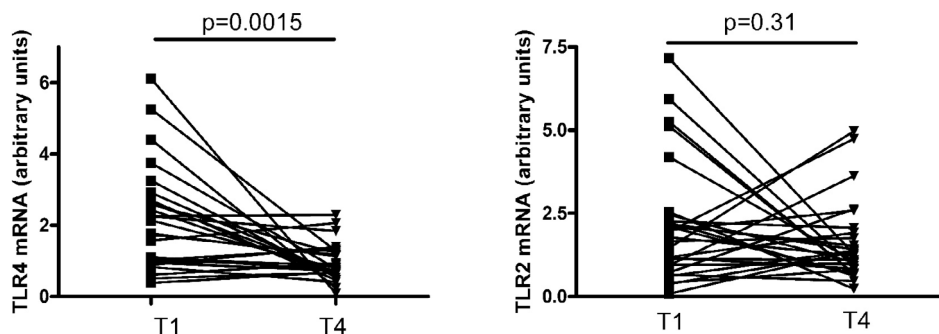


Figure 1. TLR4 mRNA levels are increased during STEMI in circulating cells. Results indicate the mRNA expression levels of TLR4 and TLR2 during STEMI (T1, black squares), and after 4 months (T4, black triangles) per individual patient. p-values were calculated by a Wilcoxon matched pairs test.

3.2. Genome-wide gene expression during STEMI

To determine the functional impact of the increase in TLR4 expression during STEMI, we performed an unbiased whole genome gene expression profile of 23 samples for which high quality RNA was available, obtained at presentation at the CCU (T1). For identification of downstream TLR4 effector genes, we first determined which genes correlated with the expression values of TLR4 across all samples, measured on the BeadChip arrays (using SAM for determination of the false discovery rate per gene). At a false discovery rate of 2% we identified 1489 TLR4-correlating genes (Fig. 2). To confirm that the TLR4-correlating genes are indeed involved in TLR4 signaling, we performed pathway analysis on these 1489 genes in the Molecular Signatures Database. As expected, the pathway; “genes involved in Activated TLR4 signaling” was highly significant (Table 1, $FDR = 2.8 \times 10^{-12}$). Remarkably, typical TLR4-induced genes like TNF and IL-1B expression did not correlate with TLR4 expression in the STEMI patients. For the well known TLR-induced IL-6 gene and for TLR2 we could not establish a correlation with TLR4 because of a low detection signal.

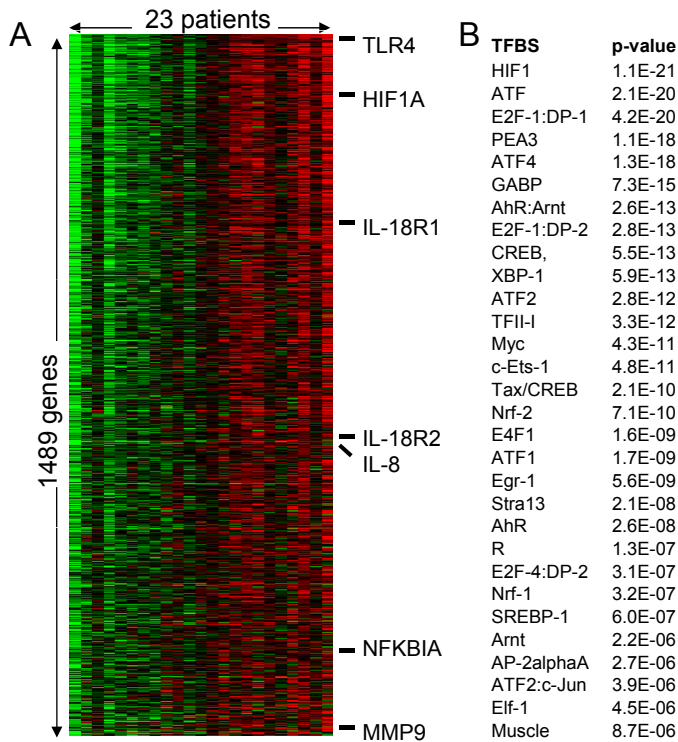


Figure 2. A. Visualization of the genes showing significant correlation with TLR4 expression in circulating cells from STEMI patients at T1 (selected at a false discovery rate of 2%, yielding 1489 genes), determined by whole genome profiling. Genes selected for measurements for real-time PCR at T1 and T4 are indicated. B. Transcription factor binding site analysis. Enriched transcription factor binding sites (TFBS) are shown for 1489 TLR4-correlating genes with p-value < 0.000005 for significantly enriched binding sites.

Table 1. Pathway analysis on 1489 TLR4-correlating genes in the MSig database, identifies specifically activated TLR4 signaling.

Description	# Genes in overlap (k)	p-value	FDR q-value
Genes involved in adaptive immune system	59	0.00E+00	0.00E+00
Genes involved in Immune system	114	0.00E+00	0.00E+00
Genes involved in innate immune system	41	0.00E+00	0.00E+00
Genes involved in cytokine signaling in immune system	39	1.11E-16	3.66E-14
Genes involved in Toll receptor cascades	25	3.77E-15	9.97E-13
Genes involved in MyD88:Mal cascade initiated on plasma membrane	21	1.35E-14	2.76E-12
Genes involved in activated TLR4 signaling	22	1.47E-14	2.76E-12
ErbB1 downstream signaling	22	2.13E-13	3.52E-11
Genes involved in Class I MHC mediated antigen processing & presentation	33	3.38E-13	4.96E-11
Genes involved in membrane trafficking	23	2.20E-12	2.90E-10

3.3. TLR activation during STEMI

A specific group of genes was selected from this large panel for further analysis, based on established induction or inhibition by the TLR4 ligand LPS, using our previously generated whole genome expression dataset in which monocytes from 15 patients with coronary artery disease and 12 controls were stimulated for 3 h with LPS³², and their known involvement in inflammatory processes. NFKBIA, IL-18R1, IL-18RAP, IL-18BP, HIF1A, IL-8, and MMP9 fulfilled these criteria. SLC2A1/GLUT1 is selected because it is a classical HIF1A activation gene involved in anaerobic glycolysis. TLR2, IL-1B, TNF, and IL-6 are selected because of their well-established role in TLR activation.

For these selected genes, including TLR4 itself, we quantified by realtime PCR, the expression at three time points, i.e., at T1 immediately after admission at the CCU, at T2 to establish the acute effect of exenatide treatment immediately after the bolus administration of exenatide, approximately 1 h after T1) and at T4 (after 4 months, Fig. 3).

For the calculations of changes in gene expression over time, we used a paired analysis in which patients from the exenatide and placebo group were treated as a single group as we established that exenatide did not have an effect on gene expression at any of the time points (Supplementary data, Fig. 1). Likewise, the difference between T1 and T4 was unaffected by the exenatide treatment (data not shown).

The genes which were selected because of their correlation with TLR4 expression at T1, all showed a significantly higher expression during STEMI in comparison to background levels at 4 months (Fig. 3; IL-18R1, IL-18R2, MMP9, HIF1A, NFKBIA, and IL-8). The only gene which showed a negative correlation with TLR4, IL-18BP, was not regulated during STEMI. The expression of TNF was lower during STEMI, compared to the expression levels at 4 months. For all genes we observed a slight to significant increase in expression at T2, relative to T1. Therefore, we considered T2 the most relevant time point for further analysis. The increase in TLR4 and IL-18R1 expression at T2 (T2 expression levels were subtracted by the background expression levels at T4), significantly correlated with the increase in TLR2, TLR4, NFKBIA, IL-8, IL-18R1, IL-18RAP, MMP9, and HIF1A expression. In Fig. 4, all correlation coefficients are unsupervised clustered and represented by a color scale for an intuitive overview. The relative transcript levels of IL-6 and of the other typical TLR-4-induced genes, TNF and IL-1B were not related to the relative expression levels of TLR4 or IL-18R1. We thus observed a selective TLR activation during STEMI, with a higher expression of some pro-inflammatory genes and HIF1A, while the prototypical pro-inflammatory TLR-induced gene TNF showed a relative lower expression during STEMI. The TLR response during STEMI does therefore not represent the classical TLR activation program normally observed during inflammatory processes.

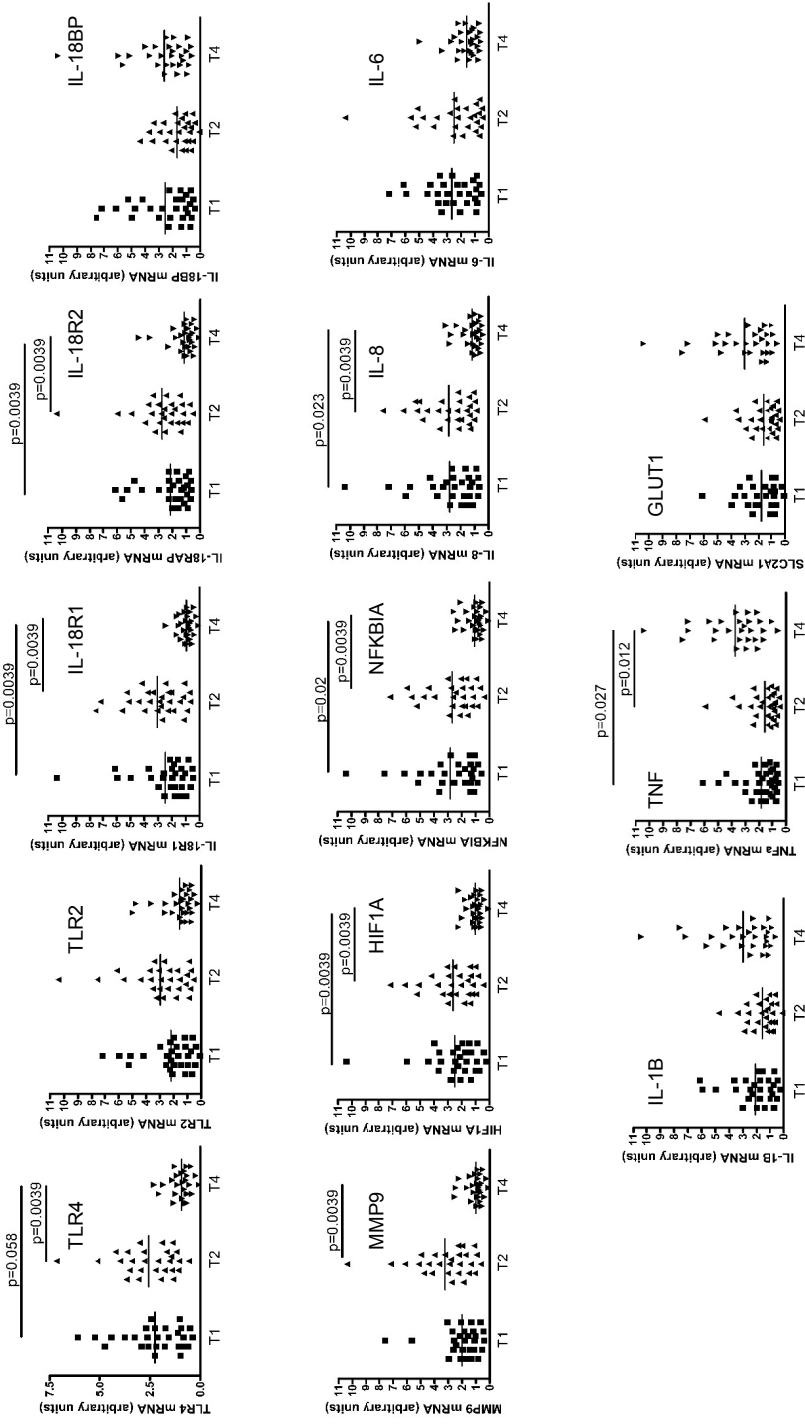


Figure 3. Genes transcribed in circulating cells which showed a correlated expression with TLR4 at T1 (Fig. 2), are increased in expression during STEMI. Gene expression levels are shown for all three time points, including the expression at T2, obtained after the exenatide bolus. We used a paired t test for normally distributed data, and a Wilcoxon matched pairs test for non-normally distributed data. Bonferroni-adjusted p values are shown.

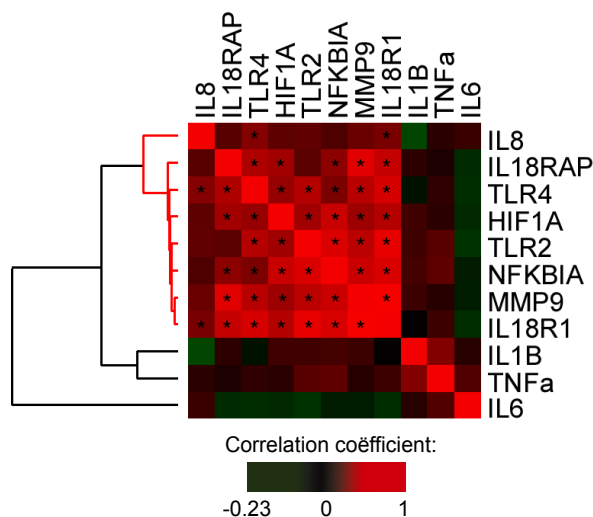


Figure 4. Non-classical TLR activation during STEMI. Unsupervised clustering of spearman Rho correlation coefficients indicates a cluster of TLR4-activated genes with a high level of correlation, with the exception of IL-6, IL-1B and TNF. Colors indicate the degree of correlation between the different relative (T2-T4) gene expression profiles, measured by real-time PCR for 28 patients. * indicates a significant correlation ($P < 0.05$).

Increased TLR4 expression and activity in circulating cells are not significantly related to increased cell numbers, because the relative expression of the thirteen TLR and TLR-activation genes did not correlate with monocyte- or neutrophil counts determined during STEMI (Supplementary data, Table 2), with the exception of a weak correlation of the number of neutrophils and the increase in IL-18R1 expression ($R = 0.39$, $p = 0.04$), and the increase in MMP9 expression with the number of monocytes ($R = 0.45$, $p = 0.02$). In addition, we analyzed the genome-wide gene expression profile for associations with cell counts (monocytes and neutrophils) using SAM. Applying a cut-off for the false discovery rate of 5%, cell numbers was not correlated with any of the expressed genes at T1.

3.4. Transcriptional profile analysis and in vitro hypoxia identifies a role for HIF1A

To identify a mechanism for the non-canonical TLR4-related gene profile, we analyzed which transcription factors might have been involved in our observed gene expression repertoire. Therefore we analyzed the genes with a significant correlation with TLR4 ($n = 1489$, Fig. 2A) and performed a transcription factor binding site analysis using the rVISTA database. The most significant binding site for this set of genes was HIF1 (Fig. 2B, $p = 1.1 \times 10^{-21}$), recognizing the heterodimeric transcription factor HIF-1, consisting of HIF-1 α and the constitutively expressed HIF-1 β . Surprisingly, the classical NF κ B binding sites, normally observed in TLR-responsive genes were not listed as significantly enriched.

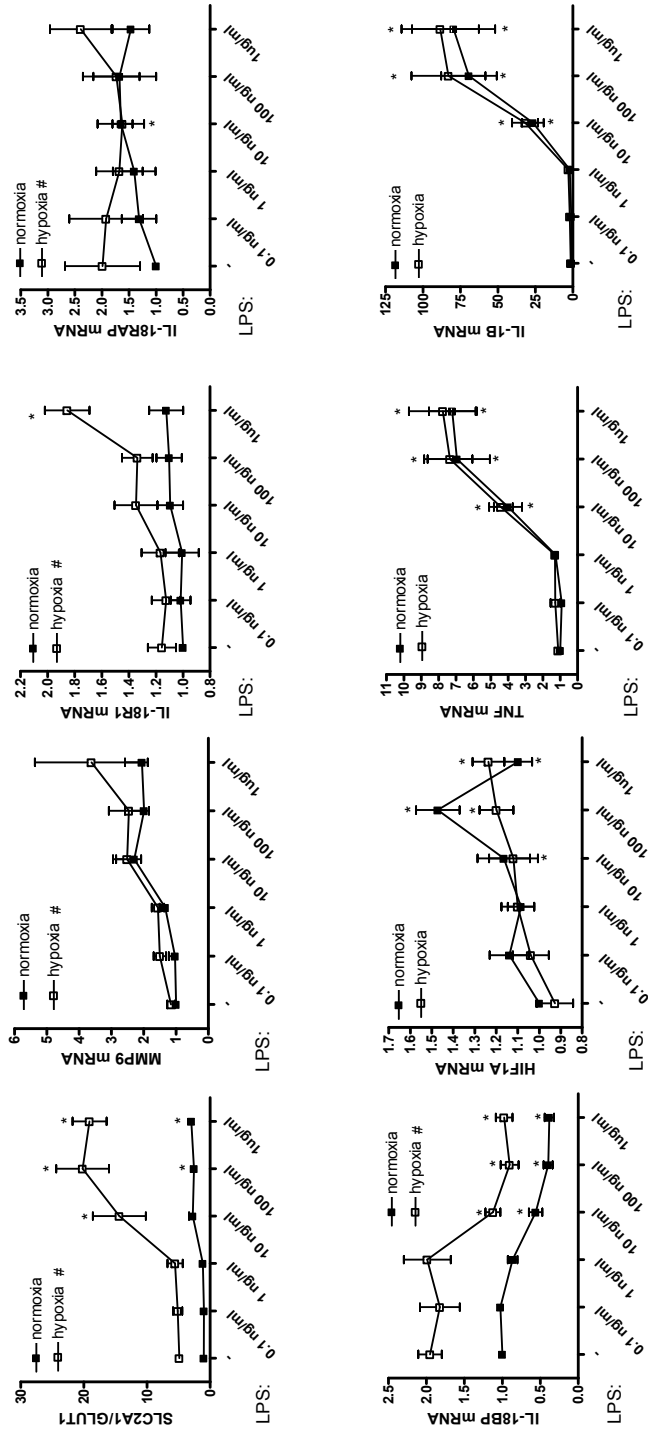


Figure 5. Hypoxia-dependent and -independent gene expression. Monocytes were cultured under normoxic or hypoxic conditions for 18h, in the absence or presence of graded concentrations of LPS. Results are the average expression of six age- and sex-matched donors relative to the expression at normoxia without LPS stimulation for each donor. # the hypoxic response is significantly different from normoxia ($p < 0.05$). * Significant LPS-induced changes compared to the control situation without LPS, either normoxic or hypoxic ($p < 0.05$).

The identification of HIF1 as the most significant binding site in the 1489 TLR4 correlating genes, combined with the increase in expression of HIF1A during STEMI, raised the question whether HIF-1 α acts as an accessory transcription factor during TLR activation.

To test this hypothesis, we cultured monocytes under normoxic (20% O₂) and hypoxic (1% O₂) conditions to identify a potential role for activated HIF-1 α in expression of our selected genes. Additionally, we stimulated monocytes isolated from age- and sex-matched healthy donors for 18 h with LPS as typical TLR4 activating agent. The same set of conditions was also tested at 2 h to check for early induction. The gene expression profile after 2 h of hypoxia stimulation (data not shown), paralleled that of the gene expression after 18 h. Hypoxia indeed induced the expression of the positive control SLC2A1/GLUT1, proving HIF-1 α activity (Fig. 5). HIF1A transcript levels themselves were not increased by hypoxia, but rather by LPS, corresponding with the known mechanism of HIF1A regulation. Interestingly, the genes involved in IL-18 signaling and MMP9 did respond to hypoxia, MMP9 responds to hypoxia when costimulated with LPS, while IL-18R1 transcripts are synergistically induced by LPS and hypoxia, and IL-18RAP/IL-18R2 is induced by both LPS and hypoxia. Hypoxia also strongly induced the expression of IL-18BP, the receptor antagonist of IL-18. On the contrary, the expression of IL-18BP was suppressed by LPS, under normoxic- and hypoxic conditions. The prototypical pro-inflammatory TLR-activated genes TNF and IL-1B, were not affected by hypoxia, but only responded to LPS. Thus our *in vitro* experiments show that the combination of HIF-1 α activation by hypoxia and TLR activation by LPS favors IL-18R expression, while TLR stimulation further augments IL-18 responsiveness because the IL-18 antagonist is suppressed by LPS. In contrast, TLR-induced genes which did not respond to HIF-1 α activity, TNF and IL-1B, were not induced during STEMI, and even showed downregulation, in particular TNF. Our results therefore strongly suggest that HIF-1 α activity may be an important modulator of TLR-induced gene transcription during STEMI.

3.5. TLR/HIF-1 α activation during STEMI

We compared the increase in gene expression at T1 with the earliest appearance of plasma troponin T (> 0.03 ng/ml in 6/29 patients). Among the earliest detectable genes were TLR4 (increased expression compared to T4 in 19/29 patients), HIF1A (22/29 patients), the IL-18R1 (21/29 patients) and IL-18R2 (26/29 patients), which were identified even in patients that were not yet showing increased troponin T levels (Supplementary data, Fig. 2). We subsequently analyzed whether there was an association of the relative expression levels of TLR activation genes with cardiac parameters. We compared the relative increase in expression of TLR-downstream activation genes at T2 with the CMR parameters measured at day 3-7, including infarct size relative to left ventricle mass, and the area at risk (Fig. 6). For both IL-18R1 and IL-18RAP/IL-18R2 mRNA expression levels we identified a significant positive correlation with the area at risk (Figs. 6 A-B, R = 0.44, p = 0.02 and R = 0.41, p = 0.04). Because the expression

of both receptors is highly correlated (Fig. 4), and both are required for IL-18 activity, we averaged the increase in both receptor chains, which again significantly correlated with the area at risk (Fig. 6C, $R = 0.48$, $p = 0.01$). The area at risk was also related to the increase in HIF1A expression (Fig. 6D, $R = 0.43$, $p = 0.03$). Multivariate linear regression analysis indicated that the area at risk was an independent predictor of the averaged increased expression of IL-18R1 and IL-18R2 mRNA ($p = 0.01$) and of the increased HIF1A expression ($p = 0.004$), after correction for chest pain to balloon time and medication (exenatide). After correction for the cardiovascular risk factors, (age, sex, BMI, diabetes, cholesterol, smoking, and blood pressure, using only two possible confounding variables per analysis, due to the limited sample size) the area at risk remained a significant predictor for the increased expression of IL18R1/2 and HIF1A. Neither TLR4 itself, nor one of the other TLR4 pathway related genes showed a significant correlation with these parameters.

Increased gene expression of the IL-18R thus appeared before clear signs of necrosis (increased plasma troponin T levels), strongly suggesting that cardiac ischemia is a sufficient trigger to induce the systemic expression of these genes. This finding is underscored by the observation that there is a significant relation between the increase in IL-18R1- and TLR4 expression with chest pain to balloon time (Figs. 6E-F, $R = 0.44$, $p = 0.03$ and $R = 0.47$, $p = 0.02$).

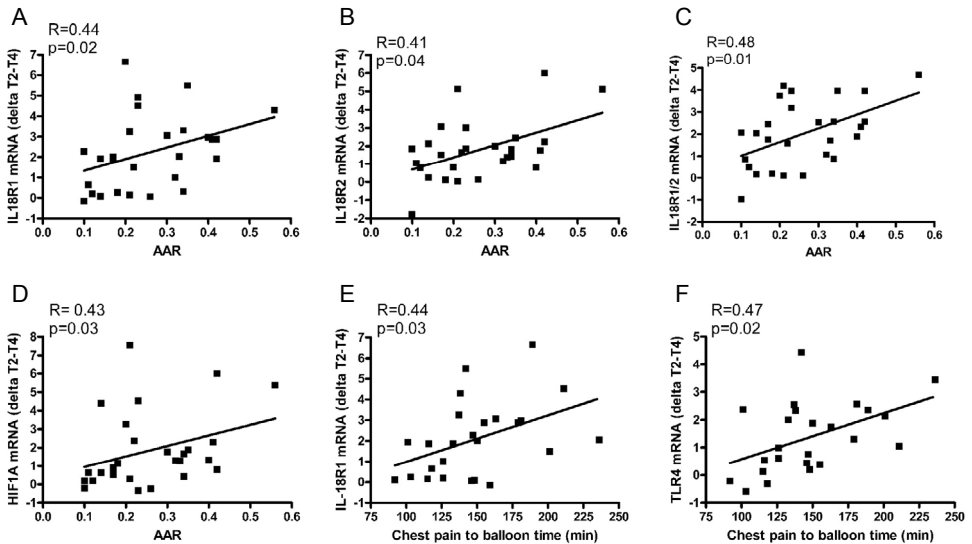


Figure 6. Gene expression is related to cardiac ischemia. Relative gene expression (T2-T4) of TLR-activated and IL-18R genes in peripheral blood cells correlate with the cardiac parameter; area at risk (AAR) shown in A-D, and the chest pain to balloon time, E and F. Spearman rank correlation coefficients are shown with their corresponding p-values.

3.6. TLR4 levels and activation in relation to prognosis

CMR parameters at baseline and at 4-month follow-up were compared, to assess whether our observed gene expression profile in circulating cells has a prognostic value for cardiac outcome. No association was found of TLR2/4 (induction) levels or downstream activation, including HIF1A and IL-18R expression with important endpoints, such as myocardial salvage index or change in LV end diastolic volume and change in LV ejection fraction after 4 months (Supplementary data, Fig. 3).

4. DISCUSSION

Here, we describe for the first time a non-canonically activated systemic TLR response in patients with STEMI with increased TLR2 and TLR4 transcript expression in circulating immune cells, accompanied by induction of a restricted downstream MyD88-, NF κ B- and HIF-1 α -dependent activation program. This response was early detectable, related to the ischemic period, before the release of troponin T, a marker of cardiomyocyte death. This study was not powered for the analysis of clinical endpoints and the non-canonical TLR response we identified in circulating cells in this study was not related to important cardiac functions after 4 months follow-up, such as infarct size, salvage index, and change in end diastolic volume and ejection fraction. However, the TLR response was proportional to the CMR-quantified area at risk, a measure for the ischemic area, and the duration of ischemia. Cardiac ischemia induces the release of endogenous TLR ligands^{14,15}, explaining the systemic response. Larger studies are needed to demonstrate whether TLR activation pathways have prognostic value in patients with myocardial infarction.

4.1. IL-18 pathway activation in acute coronary syndromes

In addition to the release of TLR4 ligands, ischemia also induces expression of IL-18^{33,34} localized in cardiomyocytes and cardiac endothelial cells³⁵, which functions as a chemoattractant for human IL-18R expressing monocytes³⁶ and neutrophils³⁷. The extent of IL-18R activation depends on the level of produced IL-18 and on the presence of IL-18BP, the natural inhibitor of IL-18³⁸. Myocardial ischemia alone is a sufficient trigger to cause an increase in plasma IL-18 levels. The source of IL-18 during AMI is most likely solely the ischemic heart, because circulating immune cells constitutively express IL-18 mRNA and protein, which is hardly influenced by the TLR4 ligand LPS in PBMC and whole blood³⁹.

In several studies circulating IL-18 levels were shown to be increased in patients with AMI⁴⁰. The kinetics show an initial rise after 2.2 h and peak levels after 13 h⁴¹. In patients with acute coronary syndrome, circulating high levels of IL-18 form an independent risk factor for 30-day fatal clinical outcome⁴², in hospital- and 1-year recurrent adverse events^{43,44}, and a lower 60-day event-free survival in STEMI patients⁴⁵. In our patient group, we could detect IL-18

(> 150 pg/ml) in only 4 patients during admission (data not shown), which is due to the short ischemic period of 2.6 h on average. In patients with the reported high circulating levels of IL-18, measurements were performed later than in our study; up to 12 h⁴² and 6 h⁴⁵ of ischemia. Therefore an intervention aimed to block circulating IL-18 may become more effective in patients with prolonged periods of ischemia.

With respect to cardiac function, elevated circulating IL-18 levels measured after 2 days on average, are associated with a decreased LVEF in acute coronary syndrome patients including MI⁴⁶ and after 1 year follow up in STEMI patients⁴⁷. IL-18 may locally further activate monocytes and neutrophils to secrete pro-inflammatory cytokines and superoxide production⁴⁸⁻⁵⁰. Signaling through the IL-18R is, like the IL-1R and most TLR, MyD88-dependent and induces NFκB activation⁵¹, and thus locally induces inflammation which may adversely affect contractility^{33,38}. The IL-18-induced increased expression of extracellular matrix components fibronectin, type I- and type III collagen and collagen gel contraction by cardiac fibroblasts indicates a profibrotic activity, which may also negatively influence myocardial contractility^{52,53}.

We observed a trend for a correlation between an increased IL-18R2 expression with a decrease in LVEF (Supplementary data, Fig. 3, R = -0.37, p = 0.07). Indeed, inhibition of the activity of IL-18 released during experimental MI, by intramyocardial injection of mesenchymal stem cells expressing IL-18BP, improves cardiac function (improved LVEF, increased LV fractional shortening), reduces scar formation indicated by the partially prevented arterial wall thickness thinning and reduces infarct size at day 28 compared to injection with wt MSC³⁴.

4.2. Non-canonical TLR4 activation with HIF-1α activity during STEMI

The reduced IL-1B and TNF transcript levels we observed during myocardial infarction are quite remarkable, but are in agreement with previous findings on lowered TNF mRNA and protein levels in isolated monocytes during AMI from a small group of patients, which was normalized after 1 month⁵⁴. TLR4 stimulation classically induces MyD88-dependent NFκB activation, which induces mRNA expression of pro-inflammatory genes including TNF and IL-1B. Thus the NFκB response is only partially inhibited during STEMI. To unravel this restricted TLR response, with increased expression of HIF1A, NFKBIA, IL-18R1/2, MMP9, and IL-8, indicating NFκB activity⁵⁵, but reduced expression of TNF and IL-1B genes, we performed transcription factor binding site analysis on TLR4-correlating genes, representing TLR4 activation. The highly significant binding site for HIF-1 we identified in TLR4 correlating genes, and the finding that the expression of HIF1A was increased during STEMI provides a strong indication that HIF-1α activity is driving gene expression during STEMI. Normally, hypoxia regulates HIF-1α activity on the protein level by preventing its degradation, reviewed in⁵⁶. However, HIF1A mRNA induction and protein activity in monocytes and macrophages can also be induced by TLR4 activation, *in vitro*^{57,58} and *in vivo*⁵⁹, and plays an important role

in innate immunity and inflammation, reviewed in^{56,60}. In our STEMI patients, HIF-1 α activity is likely induced by endogenous TLR ligands, because of the increased HIF1A mRNA levels, and lack of induction of the typical hypoxia-induced gene GLUT1.

We obtained more insight on the role of HIF-1 α in this differential regulation of NF κ B-dependent gene expression through the hypoxia experiments, which indicated that IL-18R1/2 and MMP9, but not TNF and IL-1B expression is responsive to HIF-1 α activity. HIF-1 α activity may therefore be important to render monocytes responsive to IL-18, allowing them to migrate to the ischemic area and respond to locally released endogenous TLR ligands and IL-18, without a full blown systemic classical TLR-activation profile.

A balanced immune response during AMI is critical for repair of the infarcted area. Pro-inflammatory cell influx has beneficial effects, including removal of hypoxic necrotic and apoptotic cells and matrix debris, followed by the formation of granulation tissue, accompanied by angiogenesis and stimulation of collagen synthesis to facilitate scar formation⁶¹. However, an uninhibited immune response has detrimental effects and causes collateral damage to normal tissue. The non-canonical TLR response we identified during STEMI is consistent with a controlled immune response.

4.3. Limitations

Limitations of this study include a small sample size, which requires that, especially the correlation of TLR-activated gene expression in circulating cells with CMR data (area at risk), needs to be reproduced in larger data sets. Nevertheless, our findings are intuitive and consistent with the fact that cardiac ischemia releases endogenous TLR ligands into the circulation. We corrected for individual differences in background gene expression by subtracting the expression levels of the T4 samples from the T1 samples. The T4 samples may not fully resemble the situation before STEMI, as medication and life-style changes may affect the results and which may be a source of bias. However, controlling for individual differences improved the power to detect correlations of gene expression levels with the area at risk. We cannot rule out that increased numbers of TLR4 expressing cells during STEMI partially explained the higher expression of TLR activation genes.

4.4. Conclusions

We identified the induction of a non-canonical HIF-1 α -dependent TLR response in circulating cells obtained from patients during STEMI, that was associated with the CMR-measured area at risk in these patients. However, the acute TLR response was not predictive of CMR-assessed prognostic LV parameters at 4-month follow-up.

We are the first to show a specific, early induction of IL-18R expression in these circulating leukocytes, related to cardiac ischemia. This might increase homing of leukocytes to the IL-18 producing, ischemic at risk zone. Thus the systemic TLR response is aimed to optimize cell

migration and is different from the classical TLR activation after monocytes have infiltrated the myocardium⁷. The increased IL-18R expression may render the cells more responsive to local cardiac-expressed IL-18, with subsequent pro-fibrotic and pro-inflammatory effects. Potentially, as a specific single target of the TLR response, it may be possible to improve cardiac function by neutralization of IL-18, especially in patients with prolonged ischemic periods and concomitant high production of IL-18. Together with the observed beneficial effects of IL-18BP on cardiac function and infarct size during experimental MI³⁴, this study may serve as a base for future studies aimed to inhibit migration and activation of monocytes and neutrophils during myocardial infarction by administration of IL-18BP.

Acknowledgements

The authors acknowledge Dr. R. Holman and Dr. R. de Menezes (Dept. Epidemiology and Biostatistics, VUmc) for statistical advice.

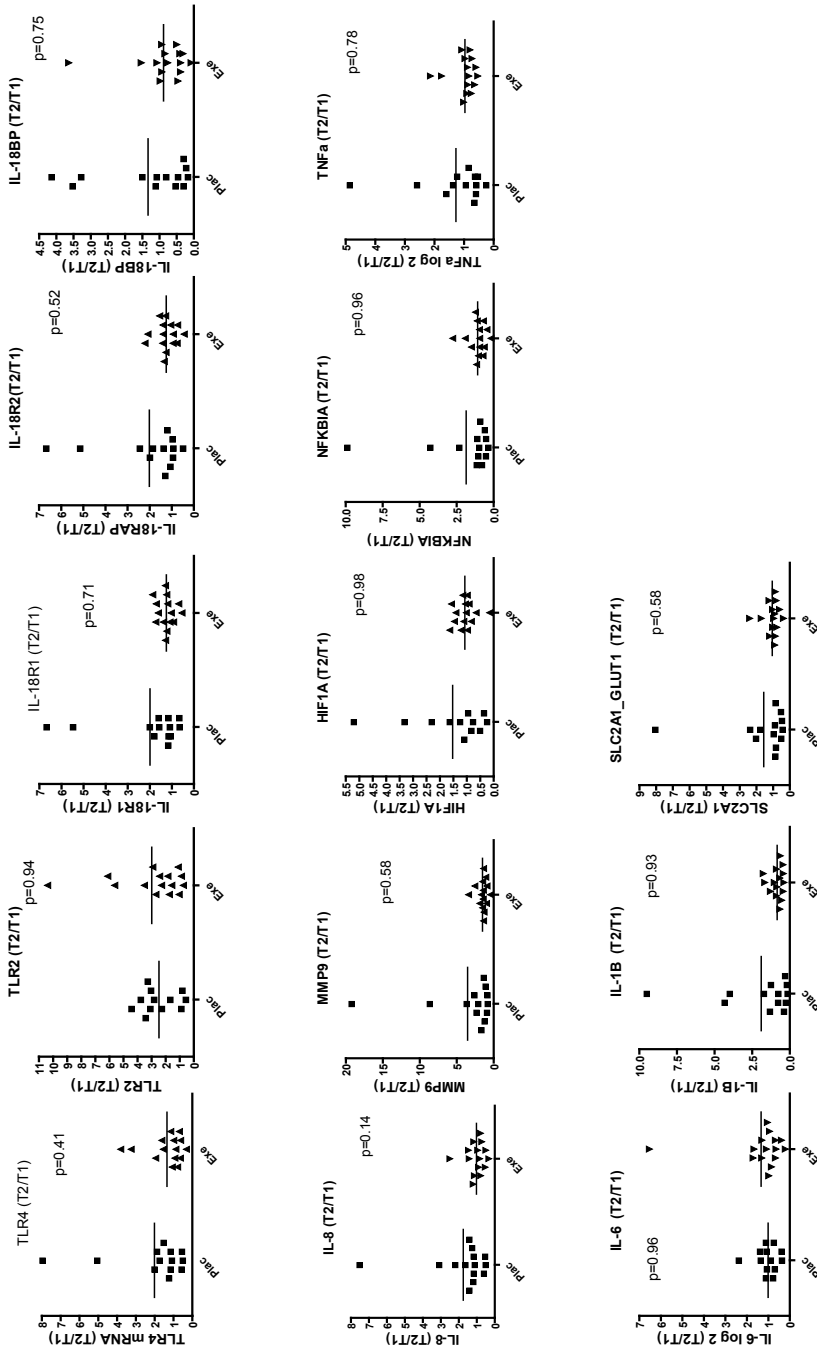
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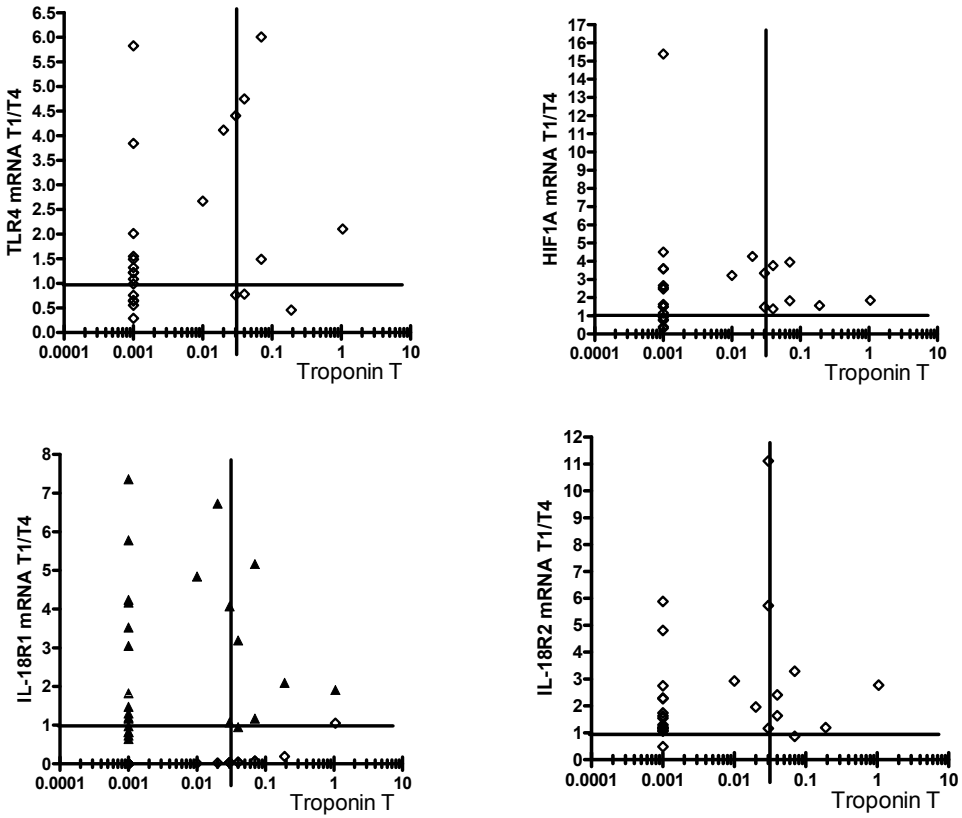
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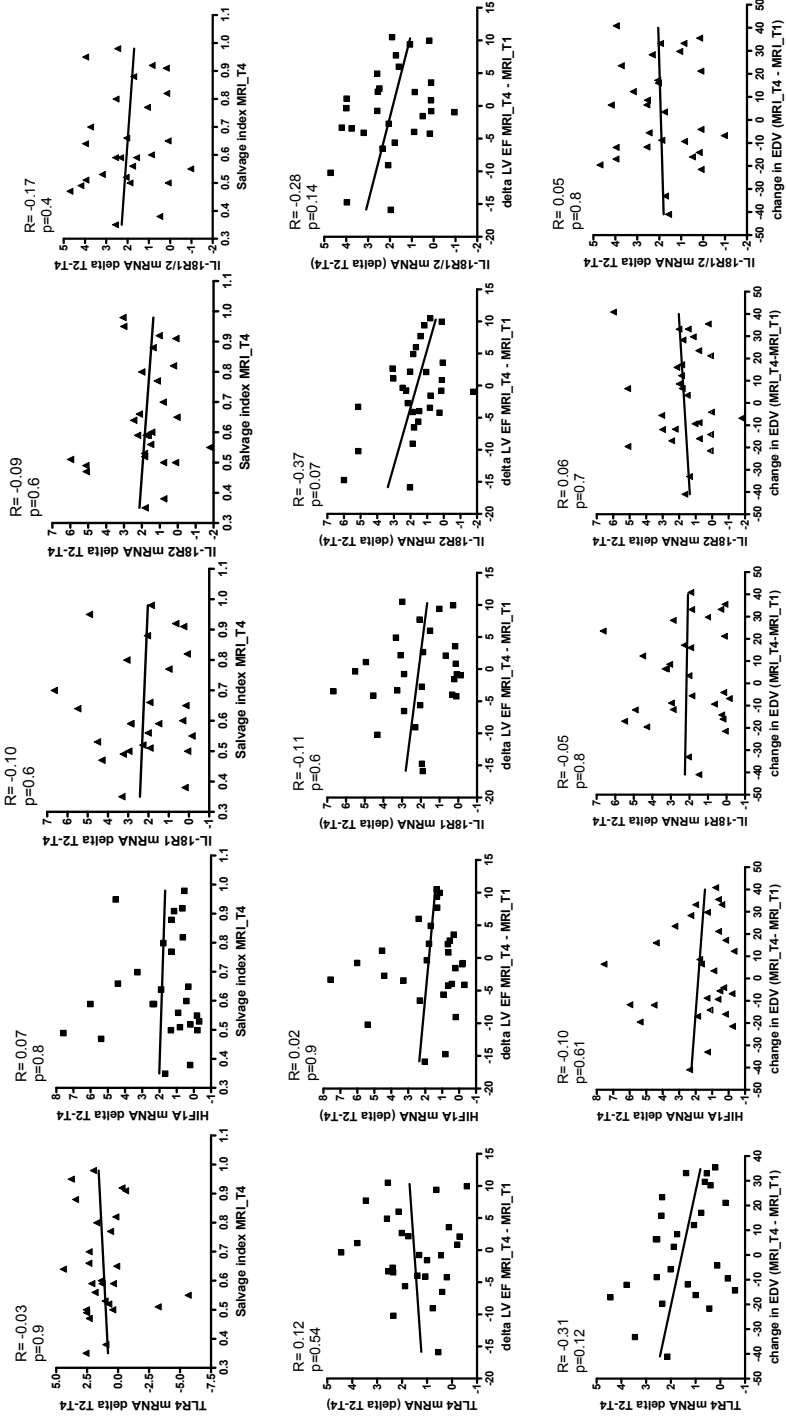
SUPPLEMENTARY INFORMATION



Supplementary Figure 1. Exenatide has no effect on gene expression. Patients treated with exenatide (n=15) were compared to placebo-treated patients (n=13). We calculated the ratio of gene expression before medication relative to the gene expression after medication for each individual patient. A t-test was used for normally distributed data, and a Mann-Whitney U test for non-Gaussian distributed data.



Supplementary Figure 2. TLR activation is an early event. The ratio of gene expression was calculated of T1 relative to T4 (4 months) for each individual patient. A ratio >1 thus identifies an increase in gene expression. Plasma troponin T levels were measured by the 4th generation Troponin T test (Roche) with an applied cut-off of 0.03 ng/ml⁶². TLR activation precedes the appearance of troponin T in the majority of patients, see text.



Supplementary Figure 3. TLR activation and IL-18R expression do not predict cardiac function (Salvage index, change in ejection fraction, and change in EDV) after 4 months. Expression levels are shown of T2 samples minus background expression at T4 for each individual patient in relation to cardiac parameters assessed by CMR. Spearman rank correlation coefficients are shown with corresponding p-values.

Supplementary data, Table 1. Patient characteristics. None of the parameters were significantly different between the placebo- and exenatide-treated group, except for a larger LV EDV at T4 for the exenatide group.

	Placebo	Exenatide	p-value
Female/Male ¹	4/9	2/13	0.37 ³
Age (years)	58.9 ± 9.2	60.1 ± 10.2	0.74 ⁴
AAR (relative to left ventricle mass)	0.27 ± 0.13	0.25 ± 0.11	0.56 ⁴
Infarctsize T1 (relative to left ventricle mass)	0.15 ± 0.10	0.13 ± 0.11	0.51 ⁵
LV EDV T1 (ml)	170.7 ± 38.2	187.2 ± 38.1	0.36 ⁵
LV EDV T4 (ml)	166.1 ± 29.9	197.1 ± 33.3	0.02 ⁴
Change in EDV (T4-T1)	-4.55 ± 22.62	9.90 ± 20.14	0.10 ⁵
Salvage index (Salvage at T4/AAR)	0.62 ± 0.18	0.68 ± 0.17	0.46 ⁴
LV EF T1 (%)	56.1 ± 9.2	54.0 ± 5.2	0.23 ⁴
LV EF T4 (%)	54.8 ± 13.0	53.5 ± 8.2	0.36 ⁴
Change in LVEF (T4-T1)	-1.26 ± 6.74	-0.42 ± 6.97	0.23 ⁴
CKMB peak (U/l)	268.2 ± 279.8	203.1 ± 149.6	0.78 ⁴
Chest pain to balloon time (min) ²	174.9 ± 67.6	141.6 ± 27.8	0.15 ⁶
Total cholesterol	5.84 ± 1.28	5.18 ± 0.91	0.12 ⁴
LDL	4.05 ± 1.19	3.58 ± 0.88	0.25 ⁴
HDL	1.19 ± 0.34	1.24 ± 0.40	0.70 ⁴
systolic blood pressure	121.46 ± 16.07	130.93 ± 18.15	0.16 ⁴
diastolic blood pressure	76.92 ± 11.82	77.40 ± 11.49	0.90 ⁴
BMI	27.16 ± 3.79	26.98 ± 2.66	0.89 ⁴
Diabetes	2	0	0.21 ³

AAR; area at risk, LV EDV; left ventricular end diastolic volume, LV EF; left ventricular ejection fraction, CK-MB; plasma creatin kinase MB levels. Data are means ± SD. ¹ number of individuals; ² n = 11 for the placebo group and n = 14 for the exenatide group. ³ Fisher's exact test; ⁴ t-test; ⁵ Mann-Whitney test. ⁶ t-test, plus Welch-correction for unequal variances.

Supplementary data, Table 2. Correlation analysis of the numbers of circulating neutrophils and monocytes with the increase in gene expression during AMI. R; Spearman Rho correlation coefficients.

Gene expression: delta T2_T4	Neutrophils		Monocytes		Average of mono's and neutro's	
	R	p-value	R	p-value	R	p-value
TLR4	0.18	0.38	0.20	0.32	0.20	0.32
TLR2	0.21	0.28	0.20	0.31	0.21	0.27
IL18R1	0.39	0.04	0.22	0.26	0.39	0.04
IL18RAP	0.32	0.10	0.25	0.19	0.32	0.09
HIF1A	0.15	0.45	0.32	0.10	0.18	0.36
NFKBIA	0.04	0.85	0.22	0.27	0.05	0.82
MMP9	0.35	0.06	0.45	0.02	0.36	0.06
IL8	0.16	0.41	0.14	0.49	0.16	0.41
IL18BP	-0.14	0.47	0.26	0.17	-0.11	0.59
TNFa	-0.28	0.15	0.20	0.31	-0.26	0.18
IL6	0.07	0.72	0.34	0.08	0.10	0.61
IL1B	-0.29	0.13	0.27	0.16	-0.27	0.17
SLC2A1	-0.26	0.18	0.04	0.83	-0.23	0.23

