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Toll-like receptor-mediated inflammation markers are strongly induced in heart tissue in patients with cardiac disease under both ischemic and non-ischemic conditions

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ABSTRACT

Background: A sustained low grade inflammatory state is a recognized feature of various diseases, including cardiovascular disease. This state of chronic inflammation involves activation of Toll-like receptor (TLR) signaling. However, little is known regarding the genetic profile of TLR components in cardiac tissue from patients with cardiac disease.

Methods: In this study we investigated the genetic profile of 84 TLR markers in a unique set of cardiac tissue from patients that had undergone either coronary artery bypass grafting (CABG) or aortic valve replacement (AVR). In addition, we compared the gene data from the cardiac tissue with the same gene profile in blood as well as circulating cytokines to elucidate possible targets in blood that could be used to estimate the inflammatory state of the heart in cardiac disease.

Results: We found a marked upregulation of TLR-induced inflammation in cardiac tissue from both patient groups compared to healthy controls. The inflammation appeared to be primarily mediated through TLR1, 3, 7, 8 and 10, resulting in a marked induction of mediators of the innate immune response. Furthermore, the gene expression data in combination with unbiased multivariate analysis suggested a difference in inflammatory response in ischemic cardiac tissue compared to non-ischemic cardiac tissue. Serum levels of IL-13 were significantly elevated in both CABG and AVR patients compared to controls, whereas other cytokines did not appear to coincide with cardiac TLR-induced inflammation.

Conclusions: We propose that cardiac disease in humans may be mediated by local cardiac TLR signaling under both ischemic and non-ischemic conditions.

1. Introduction

The underlying causes of cardiovascular disease (CVD) with subsequent hearth failure (HF) span over a wide range, including ischemic injury as well as non-ischemic causes such as hypertension and metabolic syndrome [1]. A shared common denominator for both ischemic and non-ischemic causes is a correlation between elevated serum pro-inflammatory cytokines and adverse clinical outcomes [2]. The nature of this phenomenon is a low-grade chronic inflammation, significantly less than is observed in cases of autoimmune diseases or acute infection.

Whereas short-term inflammatory activation exerts a protective effect on the heart (reviewed in [3]), we know from studies of chronic inflammation in other tissues, that a reduction in inflammation is a precursor for tissue repair to occur [4,5]. Indeed, loss-of-function studies, primarily targeting Toll-like receptors, have shown that sustained inflammation is maladaptive also in the heart, contributing to cardiac remodeling [6,7]. Many approaches have been successfully used in animal models for their ability to limit inflammation and thus minimize cardiac damage and prevent cardiac disease. These range from the blockade of pro-inflammatory cytokines and chemokines, such as TNF-α and monocyte chemoattractant protein (MCP) 1 to stem cell–based therapies, all of which induce signals for tissue repair [8–10].

Abbreviations: AVR, Aortic valve replacement; CABG, Coronary artery bypass grafting; CVD, Cardiovascular disease; DAMPs, Damage-associated molecular patterns; PAMPs, Pathogen-associated molecular patterns; PCA, Principal Component Analysis; PRRs, Pattern recognition receptors; SIMCA, Soft Independent Modelling by Class Analogy; TLR, Toll-like receptors.

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[1] This author takes responsibility for all aspects of the reliability and freedom from bias of the data presented and their discussed interpretation.

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Table 1
Fold regulation & p- values compared to control group.

	Fold change: CABG	*p*-value: CABG	Fold change: AVR	p-value: AVR
Toll-like receptors				
TLR1	3.8593	0.000921	3.2712	0.036706
TLR2	1.0218	0.996857	−1.5036	0.338373
TLR3	3.3756	0.000024	2.7432	0.005446
TLR4	2.1769	0.001408	1.4335	0.277133
TLR5	2.3677	0.017519	1.4099	0.381766
TLR6	2.6776	0.008755	2.0395	0.08155
TLR7	12.9851	0.000936	11.6259	0.057402
TLR8	4.2492	0.040304	5.4106	0.030155
TLR9	1.3351	0.41895	2.7534	0.099247
TLR10	4.3627	0.005011	2.5785	0.08482
CD180	8.1768	0.004424	5.2467	0.018746
SIGIRR	7.427	0.000408	5.8487	0.01049
Toll-like receptor signaling MYD88-independent				
TAB1	1.9024	0.004872	1.5501	0.036054
NR2C2	3.1024	0.000102	3.0247	0.004146
PELI1	1.5432	0.041788	1.1755	0.403942
TBK1	−1.0665	0.27706	−1.1429	0.151298
TICAM1	2.8211	0.000168	2.0142	0.001127
TICAM2	1.2396	0.235168	−1.092	0.619168
TRAF6	1.2037	0.129509	−1.2379	0.327299
Toll-like receptor signaling MYD88-dependent				
IRAK1	9.9237	0.000161	5.3108	0.013401
IRAK2	4.0993	0.003694	2.1794	0.26033
IRAK4	1.636	0.000048	1.1619	0.346407
MYD88	1.5294	0.049761	1.4128	0.05557
TIRAP	17.8787	0.000148	9.8432	0.000071
NFkB signaling				
BTK	3.8029	0.00726	4.8462	0.029579
CASP8	1.3618	0.107064	1.09	0.51056
CHUK	2.1632	0.000111	1.5589	0.052108
ECSIT	2.5378	0.003236	2.1149	0.018408
FADD	3.6988	0.000095	2.5646	0.021306
IKBKB	1.6117	0.014954	1.4462	0.065697
NFKB1	3.4926	0.00008	2.3945	0.015514
NFKB2	3.758	0.000485	2.2083	0.033969
NFKBIA	−1.0178	0.908358	−1.6123	0.33527
NFKBIL1	4.4224	0.000294	4.2424	0.001927
NFRKB	7.4953	0.000167	6.2635	0.000468
PPARA	3.9196	0.000486	3.5661	0.000731
REL	3.7057	0.000024	2.8966	0.00356
RELA	2.3582	0.001139	1.7535	0.051476
TNFRSF1A	3.2886	0.000465	1.9829	0.030998
UBE2N	2.5857	0.000227	2.2974	0.001504
JNK/p38 signaling				
ELK1	14.6102	0.000049	13.0496	0.000063
FOS	4.8875	0.015452	−2.847	0.786632
JUN	2.2237	0.023457	−1.0402	0.893114
MAP2K3	−1.9589	0.018802	−2.4718	0.00837
MAP2K4	3.021	0.000067	2.4474	0.002611
MAP3K1	2.6352	0.00009	1.8338	0.018848
MAP3K7	1.6726	0.001237	1.7892	0.022426
MAP4K4	2.7102	0.000193	1.6764	0.046495
MAPK8	1.7326	0.000062	1.3665	0.083129
MAPK8IP3	5.6449	0.00077	5.4033	0.00005
Interferon regulatory factor (IRF) signaling				
CXCL10	5.2826	0.003207	8.5817	0.00182
IFNA1	1.0629	0.668194	−1.1018	0.658075
IFNB1	1.7197	0.138643	1.2034	0.488061
IFNG	−1.7147	0.208067	−1.3038	0.337073
IRF1	5.4853	0.000005	3.635	0.004174
IRF3	1.0661	0.304755	1.1405	0.153478
Cytokine/Chemokine signaling				
CCL2	2.4797	0.049343	−1.4214	0.792913
CSF2	−1.1984	0.78897	−1.7177	0.148032
CSF3	−3.7453	0.14592	−3.3981	0.14428
IL1A	2.0489	0.238841	1.5715	0.276826
IL1B	6.708	0.048774	−1.5729	0.849844

(continued on next page)

Table 1 (*continued*)

	Fold change: CABG	*p*-value: CABG	Fold change: AVR	p-value: AVR
IL2	1.2561	0.394919	1.3382	0.316475
IL6	1.4839	0.125655	−17.7562	0.00005
IL8	3.7018	0.090636	−2.4517	0.531907
IL10	3.357	0.252921	−1.8781	0.223193
IL12A	1.2412	0.714702	−1.1574	0.355234
TNF	26.0408	0.028191	13.4032	0.077581
Other Toll-like receptor interacting proteins and adapters				
CD14	15.7566	0.000708	7.592	0.070333
CD80	−1.3593	0.391571	−1.5784	0.199171
CD86	6.379	0.000678	6.4441	0.01436
HMGB1	2.442	0.000045	2.5792	0.000104
HRAS	1.9414	0.003224	1.7625	0.013268
HSPA1A	−2.1988	0.030811	−4.2729	0.008775
HSPD1	1.6054	0.005743	1.0852	0.499561
LY86	7.8399	0.000802	7.4459	0.003273
LY96	−1.0878	0.718252	1.2191	0.506376
RIPK2	1.3779	0.001807	1.0893	0.449283
SARM1	7.446	0.000014	4.5428	0.000588
TOLLIP	6.1325	0.000202	4.8175	0.003864
Other pathogen-specific responses				
CLEC4E	−1.6656	0.232405	−1.7131	0.261774
EIF2AK2	2.0392	0.000192	1.8091	0.003083
LTA	1.3175	0.282062	1.6609	0.248861
PRKRA	1.8286	0.001784	1.4918	0.064065
PTGS2	5.5169	0.031254	1.2371	0.460669

Similar strategies in humans have proven more complex with a significantly lower success rate [11–16].

A key question is whether inflammation precedes cardiac disease and contributes to its initiation or rather is a consequence of cardiac disease, contributing to its maintenance. Recent experimental lines of evidence have suggested that ischemic cardiomyocytes can contribute to the innate immune response besides being a target organ (reviewed in [17]). Activation of the immune response mainly occurs when "danger" signals released from the damaged cardiomyocytes (the so called damage-associated molecular patterns - DAMPs) interact with pattern recognition receptors (PRRs), such as the Toll-like receptors (TLR). Once these DAMPs are recognized by pattern recognition receptors, they activate the components of the innate signaling pathway, including NF-κB, and pro-inflammatory cytokines, that in turn provoke immune cell recruitment and activation. TLRs thus provide a potential link between tissue injury, activation of inflammatory mediators, and the pathogenesis of cardiac disease (reviewed in [18]).

While most studies have been performed in animal models, little is known regarding the genetic profile of inflammatory markers in the cardiac tissue of patients with cardiac disease compared to individuals without cardiac disease. Moreover, nothing has been reported regarding comparison of expression profiles of inflammatory genes in patients with a history of ischemic heart disease and patients with non-ischemic heart disease. Thus, in this study, we investigated the genetic profile of 84 markers of inflammation in a unique set of cardiac tissue from patients that had undergone either coronary artery bypass grafting (CABG) or aortic valve replacement (AVR) and compared with cardiac tissue from individuals without cardiac disease. In addition, we compared the gene data from the cardiac tissue with the same gene profile analysis in blood as well as measured circulating cytokines to elucidate possible gene targets in blood that could be used to estimate the inflammatory state of the heart in cardiac disease.

2. Methods

2.1. Human heart biopsies and blood samples

Biopsies from the right atrium and whole blood samples were obtained from five patients that underwent Coronary Artery Bypass Grafting (CABG) surgery and from five patients that underwent Aortic Valve Replacement (AVR) surgery at the Sahlgrenska University Hospital, Gothenburg, Sweden. Tissue samples were frozen immediately and stored at −80 °C until analysis. Blood samples were collected prior to surgical intervention, aliquoted and stored at −80 °C. Total cardiac tissue RNA from four healthy individuals was used as control samples (AMS Biotechnology Europe Ltd. - Abingdon, U.K.). Control blood samples for gene analysis were obtained from blood donors at the blood donor center at Sahlgrenska University Hospital, Gothenburg.

Blood from matched controls for cytokine ELISA analysis were obtained from the Swedish CArdioPulmonary bioImage Study (SCAPIS). To only include healthy research subjects as controls, a number of exclusion criteria were employed, listed in Supplementary Table 1. Briefly, subjects with a known disease background were excluded. In addition, to ensure that no subjects with cardiac ischemia were included, all subjects with coronary calcium score ≫ 0 were excluded. Case-control matching was carried out using SPSS v. 24 (IBM, New York, NY, USA). Matching variables were age (20 years), BMI (1 kg/m2) and sex (0), tolerances in parentheses. As the control subjects generally were younger compared to CABG and AVR patients, a high initial tolerance was set for age matching and up to five matches per case were selected. From these, the controls with least age difference compared to the cases were selected, for each individual case-control match.

The study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki as reflected in approval by the Ethical Committee of Gothenburg University. The study included written informed consent from the patients.

2.2. Gene expression analysis

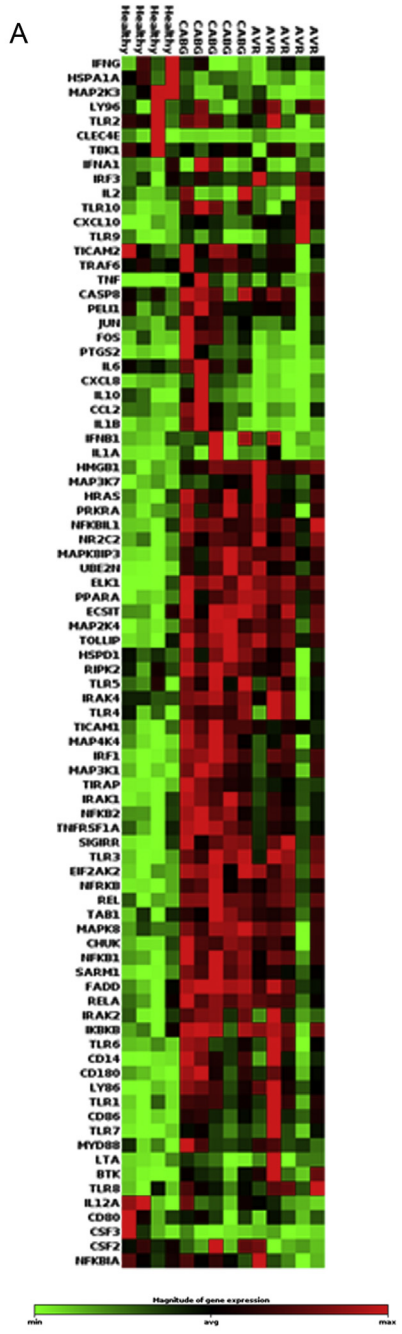
Total RNA was isolated from heart biopsies with the RNeasy Fibrous Tissue Mini kit (Qiagen, Valencia, CA). The tissue was disrupted with 8 mm steal beads and a TissueLyser and treated with Proteinase K for Protease digestion (Qiagen, Valencia, CA). RNA from whole blood samples was isolated with the Rneasy Micro kit and Qiazol treatment (Qiagen, Valencia, CA). For the RT reaction, cDNA was synthesized with the RT^2 First Strand kit (Qiagen, Valencia, CA) in a Gene Amp PCR system 9700 (Applied Biosystems, Foster City, California).

For gene expression analyses of TLR-mediated signal transduction and innate immunity, the human Toll-Like Receptor Signaling Pathway RT^2 Profiler PCR Array (PAHS-018ZA, Qiagen, Valencia, CA) was used. The qPCR reaction was performed with an ABI 7900 HT fast real time 96 well module (Applied Biosystems, Foster City, California). All PCR amplification was performed for 40 cycles.

Data were normalized with an automatic selection of genes from the full plate. Qiagen's Web-based PCR Array Data Analysis Software, available at www.SABiosciences.com/pcrarraydataanalysis.php was used to automatically select an optimal set of internal reference genes for the analysis. The C_T values for these genes were then geometrically averaged and used for the delta delta C_T calculations.

2.3. Protein expression analysis

Levels of cytokines were measured in serum samples using the ultrasensitive multiplex electrochemiluminescence immunoassay (ELISA) (Meso Scale Diagnostics (MSD), Rockville, MD, USA). The human V-PLEX Pro-inflammatory Panel 1 Human Kit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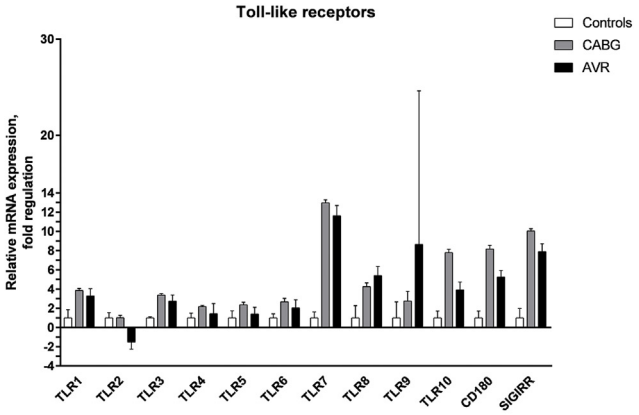
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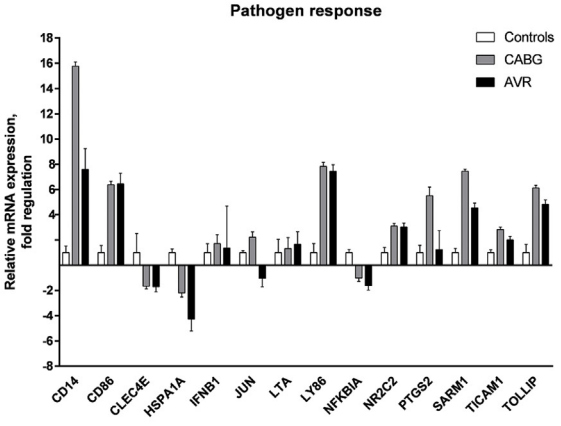
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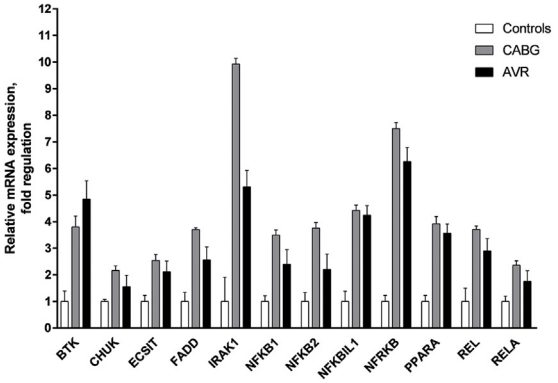
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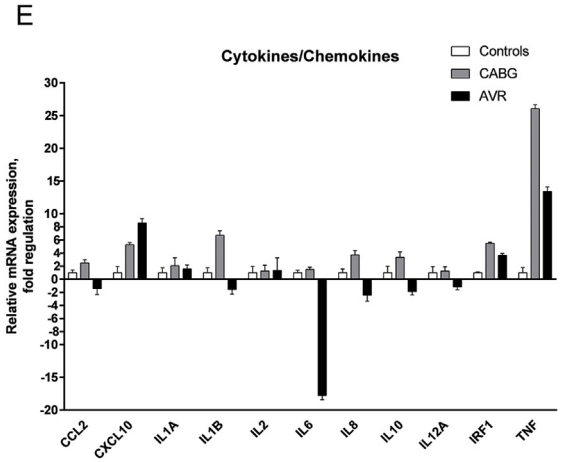
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IL1B
IL2
IL6
IL8
IL10
IL12A
IRF1
TNF

(#K15049) for the precise quantitative determination of interferon gamma (IFN-γ), interleukin (IL)-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12p70, IL-13 and tumor necrosis factor-alpha (TNF-α), was applied. Intensity of the emitted light was measured according to the manufacturer's instructions on an MSD QuickPlex SQ120 plate reader (Meso Scale Diagnostics).

2.4. STRING analysis

STRING is a database of known and predicted protein-protein interactions (https://string-db.org/cgi/about.pl?footer_active_subpage=content), freely available under a 'Creative Commons BY 4.0' license. The interactions include direct (physical) and indirect (functional) associations. These stem from computational prediction, from knowledge transfer between organisms, and from interactions aggregated from other databases. For analysis of our data, STRING version 10.5 (https://version-10-5.string-db.org/) was used.

2.5. Statistics

Statistical analysis of the RT^2 PCR array data was performed with Qiagen's Web-based PCR Array Data Analysis Software, available at www.SABiosciences.com/pcrarraydataanalysis.php. A heat map was created by performing a clustergram of non-supervised hierarchical clustering of the entire dataset. To visualize the differences specifically between the CABG and AVR groups, a volcano plot was created, displaying statistical significance versus fold-change on the y- and x-axes, respectively.

Data for specific sets of genes and for serum cytokine levels were plotted as the mean and standard error of the mean (SEM). The level of significance was calculated using one way ANOVA and GrapPad Prism software version 7 (Graphpad Software, San Diego, California, www.graphpad.com). A p-value of $\ll 0.05$ was considered statistically significant.

Multivariate analyses were carried out by principal component analysis (PCA) using SIMCA v.15.0.2 (Umetrics, Umeå, Sweden). To obtain a more normal-like distribution, data were log transformed prior to statistical analysis. For the circulating cytokine analysis, an additional correction for analysis batch was performed (R Core Team (2018), https://www.R-project.org/) by using the ComBat function in the SVA package (R package version 3.30.0). Group was included as co-variate. Goodness of fit was used to assess explained variation (R2X), while reproducibility of the model was assessed by cross validation (Q2X). A R2X value of 1 represents a model that perfectly explains all variation while a Q2X value of 1 represents a model that is perfectly reproducible. To explore clustering of genes, a hierarchical clustering of the loadings of the PCA model was performed, using Ward algorithm for calculation of distances between clusters. Levels of significance for differences between group means were determined with two-way ANOVA followed by Tukeýs multiple comparison tests. These calculations were carried out using SPSS v. 24.

3. Results

We examined cardiac tissue and whole blood from patients that had undergone coronary artery bypass graft (CABG) and patients that had undergone aortic valve replacement (AVR) and compared with tissue and blood from healthy individuals. A summary of the patient clinical data is provided in Suppl Table 2. Due to protection of individual integrity and restriction regulations, only limited clinical data could be obtained from the control individuals.

3.1. Toll-like receptor signaling is markedly upregulated in heart tissue from patients with cardiac disease

We performed a gene expression profile analysis of 84 genes involved in TLR signaling of cardiac tissue from CABG patients, AVR patients and healthy controls. There was a distinct significant difference in the expression profile of inflammatory genes between controls and the patients with cardiac disease, with approximately 71% of the genes altered in the CABG group and 49% of the genes altered in the AVR group compared to controls (Table 1 and Fig. 1A).

Of the Toll-like receptors, the most pronounced and significant differences were seen for TLR1, 3, 7, 8 and 10 as well as the related receptor CD180 and the IL-1R family member SIGIRR (Fig. 1B). Accordingly, we found marked increased expression of mediators of pathogen response signaling downstream of TLRs, such as CD14, CD86, LY86, SARM1, TICAM1 and TOLLIP (Fig. 1C). In addition, the NFκB pathway was strongly induced (Fig. 1D), accompanied by increased expression of cytokines, including CXCL10, IRF1 and TNFα (Fig. 1E). Taken together, with ~50–70% of the screened genes significantly altered in the groups with cardiac disease compared to healthy controls, our data support a marked and distinct activation of inflammation through the TLRs in the heart in patients suffering from cardiac disease.

3.2. Toll-like receptor signaling is enhanced in ischemic heart tissue compared to non-ischemic heart tissue

Although both the CABG and AVR groups showed increased expression of TLR-induced genes compared to controls, the data in Table 1 demonstrates a difference in the number of altered genes in these groups (71% vs 49% compared to controls). Further investigation revealed 15 genes that were differently expressed between CABG and AVR (Fig. 2A). The most markedly differentially expressed genes were pro-inflammatory cytokines, such as IL-1β, IL-6 and IL-8, which were all markedly upregulated in CABG compared to AVR (Fig. 2A). This finding prompted us to investigate the difference in TLR-mediated gene expression between CABG and AVR in more depth using Principal Component Analysis (PCA), an unbiased multivariate classification model. The PCA showed a separation between the control individuals and the patients with heart disease as expected (Fig. 2B). A more in-depth analysis using three components suggested an additional separation between the ischemic CABG group and the non-ischemic AVR group (Fig. 2C), supporting a difference in inflammatory response in cardiac tissue in ischemic heart disease compared to non-ischemic heart disease with regards to toll-like receptor signaling. Furthermore, hierarchical cluster analysis of PCA loadings revealed a pattern where the majority of the most markedly differentially expressed genes in the cardiac tissue (patients with cardiac disease vs healthy controls) fell into either of two clusters (green and red clusters, Fig. 2D). The most markedly differentially expressed genes between the CABG- and AVR groups fell into a cluster of its own (yellow cluster, Fig. 2D). A STRING-analysis confirmed a strong association of the proteins encoded by the most significantly altered genes within these clusters (Fig. 2E-G). Taken together, multivariate analyses support a unique pattern of increased inflammatory genes in individuals with cardiac disease compared to healthy individuals. Furthermore, using these mathematical tools, we observe a distinct separation between ischemic and non-ischemic patients, supporting our gene data of enhanced cardiac inflammation following ischemia.

3.3. Serum levels of IL-13 are elevated in patients with cardiac disease, whereas other cytokines do not coincide with the marked cardiac TLR-induced inflammation

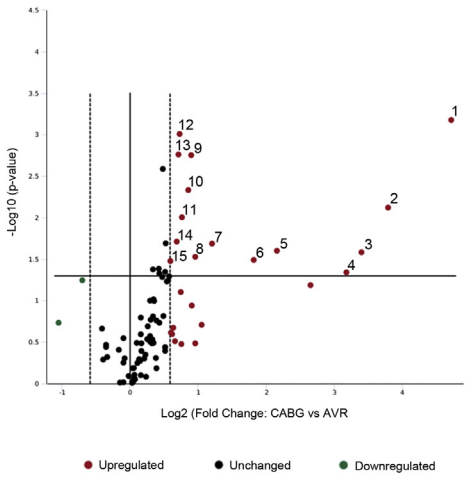
A shared common denominator for both ischemic and non-ischemic CVD is a correlation between elevated serum pro-inflammatory cytokines and adverse clinical outcomes [2]. The markedly increased cardiac inflammation in the patients with cardiac disease in our study thus suggested that the trigger of inflammation was inflammatory mediators in the blood.

RNA from whole blood was extracted from the same CABG- and AVR-patients used for cardiac tissue TLR analysis above, as well as from healthy controls (Suppl Table 2), and the genetic TLR-mediated inflammatory profile was analyzed. In total, 19% of genes showed significant differential expression (Suppl Table 3), but in contrast to cardiac tissue, most of these genes were down-regulated rather than upregulated in the blood compared to controls, with the exception of TLR5-expression in the CABG group, and CXCL10 and IL1A in the AVR group (Suppl Table 3 and Fig. 3A).

Levels of circulating cytokines were investigated using multi-array ELISA for a panel of ten pro-inflammatory cytokines from serum of 20

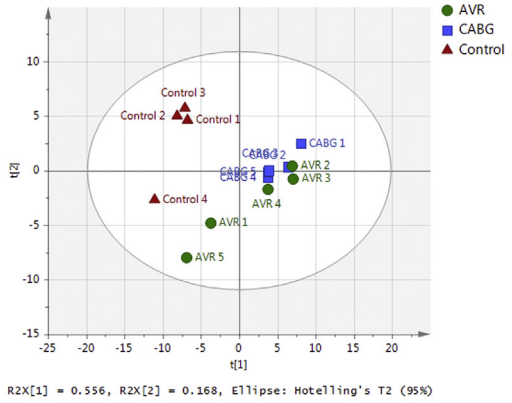
Fig. 1. *Toll-like receptor signaling in human heart tissue from individuals with cardiac disease. (A)* Heat map showing hierarchical clustering of 84 genes involved in TLR-signaling in human heart tissue from patients that have undergone coronary artery bypass grafting (CABG) or aortic valve replacement (AVR) as well as cardiac tissue from individuals without cardiac disease. *(B-E)* Extracted gene expression profiles of differentially expressed signaling pathways in *(A)* (fold regulation). $n = 5$ for each patient group, $n = 4$ for controls.

A



		Fold change	p-value
1	IL6	26.35	0.00067
2	FOS	13.91	0.0075
3	IL1B	10.55	0.0261
4	IL8	9.08	0.0456
5	PTGS2	4.46	0.025
6	CCL2	3.52	0.032
7	JUN	2.31	0.0205
8	HSPA1A	1.94	0.0296
9	IRAK1	1.87	0.0018
10	TIRAP	1.82	0.0047
11	NFKB2	1.7	0.0099
12	TNFRSF1A	1.66	0.001
13	SARM1	1.64	0.0017
14	MAP4K4	1.62	0.0194
15	IRF1	1.51	0.0332

B



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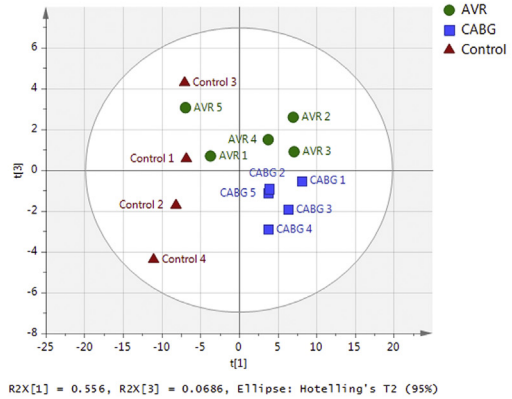
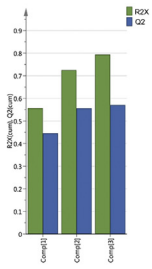
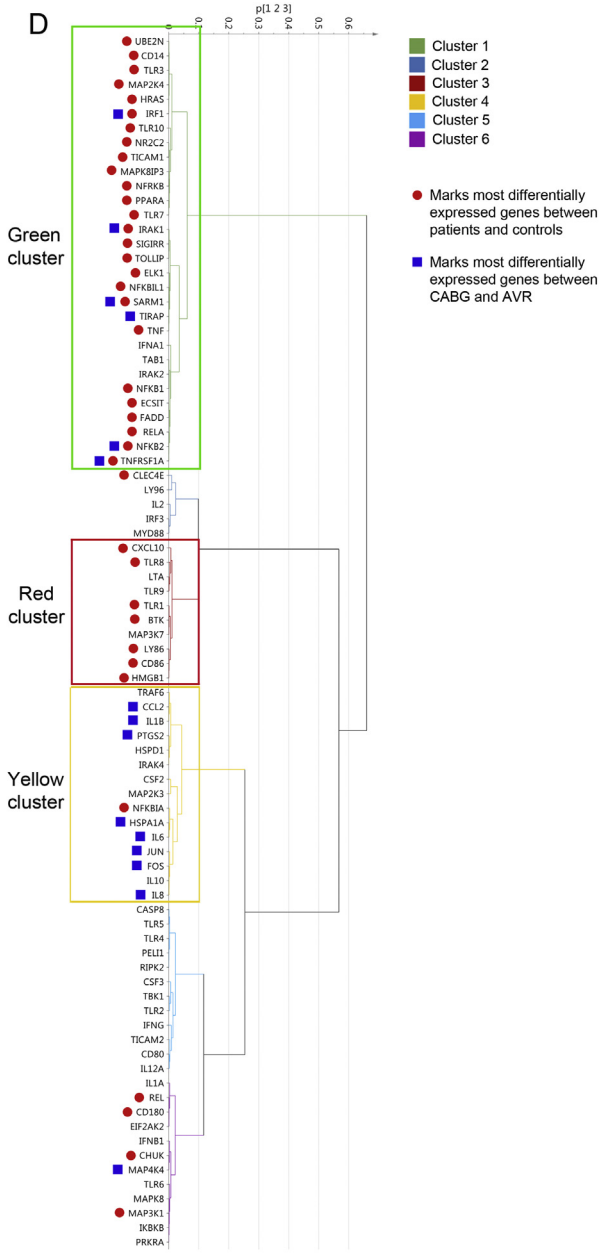




D



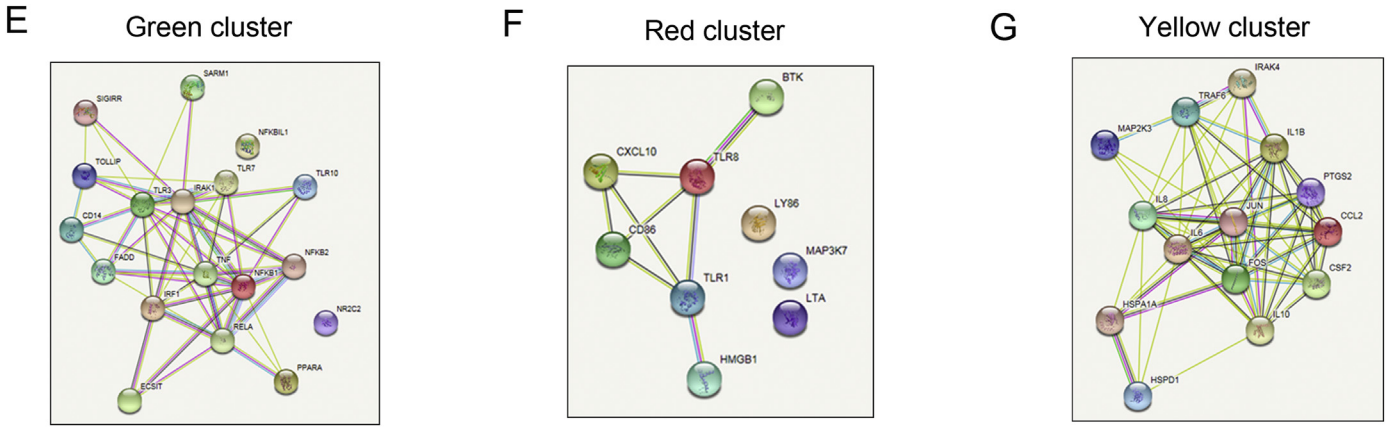


Fig. 2. *Principal Component Analysis (PCA) of cardiac tissue TLR-induced gene expression. (A)* Volcano plot illustrating genes that are differentially expressed between patients that have undergone coronary artery bypass grafting (CABG) and patients that have undergone aortic valve replacement (AVR). Statistical significance versus fold-change on the y- and x-axes, respectively, is shown. *(B)* PCA score plot of the first two components showing a separation between patients with cardiac disease and healthy controls. *(C)* PCA score plot of the first three components showing additional separation between CABG patients and AVR patients. *(D)* Hierarchical Clustering Analysis (HCA) showing that the majority of the most markedly differentially expressed genes in the cardiac tissue (cardiac disease vs healthy controls) fall into either of two clusters (green and red clusters) and the most markedly differentially expressed genes between the CABG- and AVR groups fall into a cluster of its own (yellow cluster). n = 5 for each patient group, n = 4 for controls *(A-D)*. *(E-G)* STRING-analysis, confirming predicted strong association of the proteins encoded by the most significantly altered genes within the green *(E)*, red *(F)* and yellow *(G)* clusters.

CABG patients, 20 AVR patients and 34 matched controls (Fig. 3B-I). Patient clinical data are shown in Suppl Table 4. We observed increased levels of circulating IL-13 in the CABG- and AVR group compared to controls (Fig. 3H), whereas IL-8 was significantly decreased (Fig. 3E). IFNγ, IL-2, IL-6, IL-10, IL-12 p70 and TNFα were not significantly changed (Fig. 3) and IL-1β and IL-4 were undetectable in virtually all individuals (data not shown). Unlike the PCA of the cardiac tissue (Fig. 2), controls and heart patients were not separated with regards to circulating cytokines, nor were CABG patients and AVR patients (Fig. 3J). Taken together, the patients with cardiac disease in our study have higher circulating levels of IL-13 compared to healthy controls. However, overall, circulating inflammatory markers in the blood do not appear to coincide with the marked inflammatory state of the cardiac tissue in the patients with cardiac disease in our study.

4. Discussion

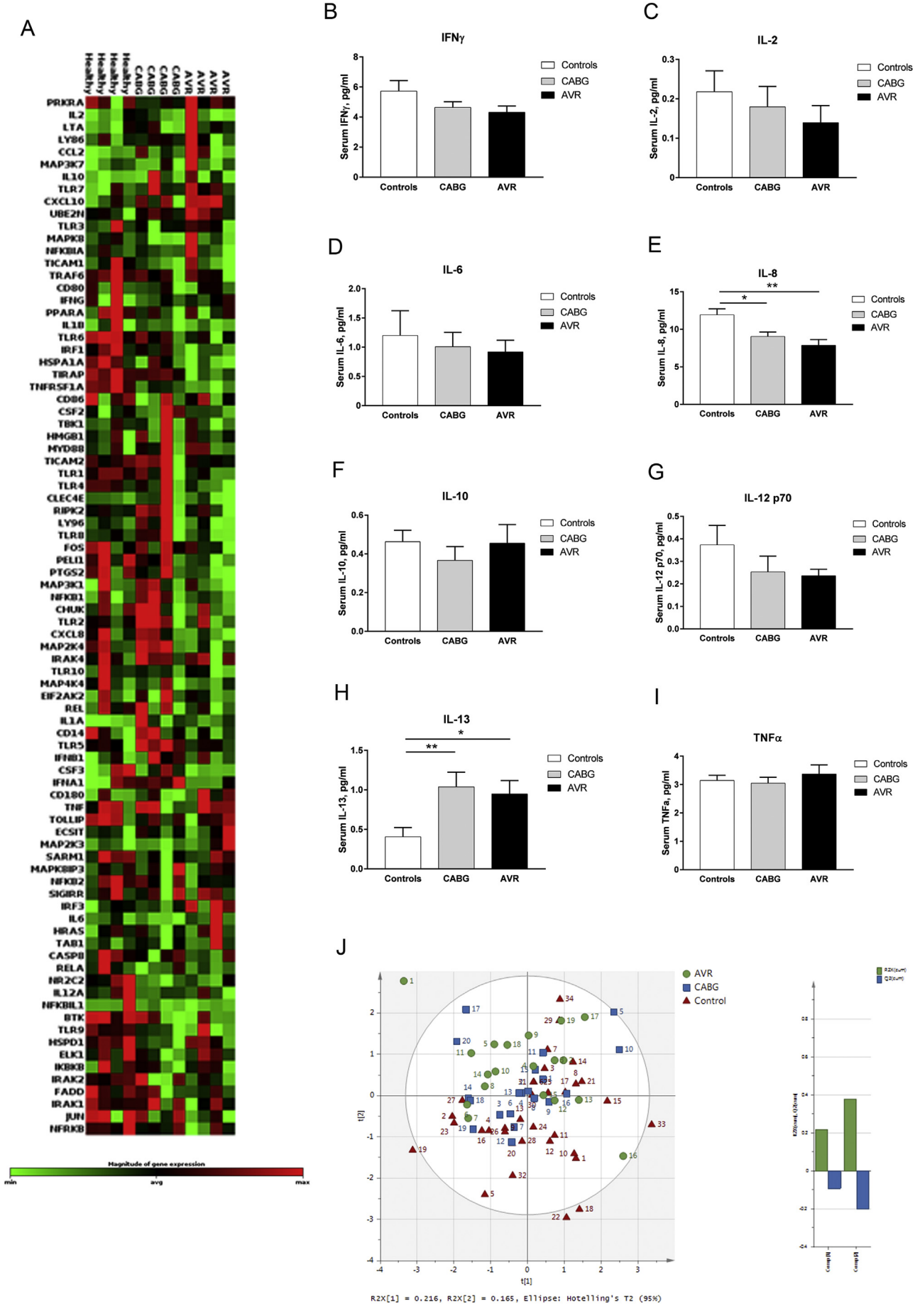
TLRs are one of the most ancient, conserved components of the immune system, displaying three general categories of TLR ligands: proteins (TLR5), nucleic acids (TLR3, TLR7, TLR8, TLR9) and lipid-based particles (TLR1, TLR2, TLR4, TLR6, TLR2/TLR6) [19]. The exact ligands that activate TLR signaling in the heart are not known, but studies suggest that short-term activation of TLR signaling confers cytoprotective responses in the heart, whereas long-term signaling is maladaptive and can lead to adverse cardiac remodeling [18].

The most pronounced and significant differences between patients and healthy controls in our study were seen for TLR 1, 3, 7, 8 and 10 and the related receptor CD180 as well as the IL-1R family member SIGIRR. TLR3, 7 and 8 are found in the endosomal membrane and recognize nucleic acids derived from viruses, resulting in production of inflammatory cytokines and type 1 interferons. TLR1 forms heterodimers with TLR2, recognizing pathogen-associated molecular patterns (PAMPs), particularly from gram-positive bacteria, such as triacyl lipoproteins [20]. CD180 appears to have ambiguous roles, working in concert with TLR4 in promoting B cell activation on the one hand, and inhibiting LPS-induced inflammatory cytokine responses in dendritic cells on the other (reviewed in [21]). TLR10 and SIGIRR both suppress inflammation by negatively regulating TLR- and ILR- mediated activation [22–25].

The majority of the investigated toll-like receptor interacting proteins and adapters were markedly and significantly upregulated in the hearts of the patients with cardiac disease compared to controls as were downstream signaling mediators involved in NF-kB signaling and JNK/p38 signaling. IL-6 gene expression was unexpectedly markedly decreased in the AVR group compared to both controls and the CABG group (17-fold and 26-fold decrease, respectively). This might reflect an immunological defect in the IL-6 production in the AVR group, similar to what Mandi et al. reported for certain subsets of patients with cardiac pathophysiological conditions [26] or polymorphisms in the IL-6 gene, similar to what we have previously reported for genes involved in the inflammasome in patients with cardiac disease [27].

Overall however, our data strongly support a marked and distinct activation of inflammation through the TLRs and their downstream signaling pathways in the heart in patients suffering from cardiac disease, possibly initiated through viral signaling pathways given the particular receptors involved.

Involvement of enhanced innate immune signaling in the pathogenesis of cardiac disease in humans is supported by other studies. Barr et al. reported significant overlap among TLR, T-cell receptor (TCR) and B-cell receptor (BCR) signaling in asymptomatic atherosclerosis, acute ischemic stroke, and myocardial infarction patients, possibly coordinated through epigenetic regulation [28]. A study by Vianello et al. showed that patients with coronary artery disease displayed epicardial adipose tissue (EAT) hypertrophy with an increased EAT pro-inflammatory profile and higher TLR2 and TLR4 expression [29]. Mann et al. used a mathematical modelling approach to compare innate immune gene expression patterns in explanted hearts from patients with heart failure [30]. They found unique gene expression profiles with increased expression of innate immune genes for all forms of cardiomyopathy investigated. Interestingly, the same study reported decreased TLR2 and TLR4 in all forms of cardiomyopathy examined. TLR2 and TLR4 are the most studied forms of TLRs, and particularly TLR4 has been implicated in cells, animal models and humans to be involved in cardiac immune responses in CVD [3,31,32]. TLR2 expression was not significantly changed in the patient groups in our study and TLR4 was only modestly upregulated (2-fold) in the CABG patient group and not significantly changed in the AVR-group compared to controls. Thus, our data support the findings of Mann et al. with no or small effects on TLR2 and TLR4 in human cardiac disease. Differences in the production of specific DAMPs and PAMPs might partly explain the discrepancies between studies with regard to changes in TRL2 and TLR4 expression.


A
Healthy Healthy Healthy Healthy CABG CABG CABG CABG AVR AVR AVR AVR
PRKRA IL2 LTA LY86 CCL2 MAP3K7 IL10 TLR7 CXCL10 UBE2N TLR3 MAPK8 NFKBIA TICAM1 TRAF6 CD80 IFNG PPARA IL1B TLR6 IRF1 HSPA1A TIRAP TNFRSF1A CD86 CSF2 TBK1 HMGB1 MYD88 TICAM2 TLR1 TLR4 CLEC4E RIPK2 LY96 TLR8 FOS PELI1 PTGS2 MAP3K1 NFKB1 CHUK TLR2 CXCL8 MAP2K4 IRAK4 TLR10 MAP4K4 EIF2AK2 REL IL1A CD14 TLR5 IFNB1 CSF3 IFNA1 CD180 TNF TOLLIP ECSIT MAP2K3 SARM1 MAPKBP3 NFKBI2 SIGIRR IRF3 IL6 HRAS TAB1 CASP8 RELA NR2C2 IL12A NFKBIL1 BTK TLR9 HSPD1 ELK1 IKBKB IRAK2 FADD IRAK1 JUN NFRKB
Magnitude of gene expression
min avg max
B
IFNγ
Serum IFNγ, pg/ml
Controls CABG AVR
C
IL-2
Serum IL-2, pg/ml
D
IL-6
Serum IL-6, pg/ml
E
IL-8
Serum IL-8, pg/ml
F
IL-10
Serum IL-10, pg/ml
G
IL-12 p70
Serum IL-12 p70, pg/ml
H
IL-13
Serum IL-13, pg/ml
I
TNFα
Serum TNFa, pg/ml
J
AVR
CABG
Control
t[1]
t[2]
R2X[1] = 0.216, R2X[2] = 0.165, Ellipse: Hotelling's T2 (95%)

Ischemia has been proposed to be a trigger of cardiac inflammation through NOD-like receptor activation [33] and DAMPs such as HSP60 [31,34,35], HSP27 [36] and HSP72 [37]. Using cardiac tissue from both CABG patients and AVR patients allowed us to investigate whether ischemia is an important factor for TLR-mediated cardiac inflammation in the patients with cardiac disease in our study since the included CABG patients had a history of obstructed or stenosed coronary arteries and thus cardiac ischemia, whereas the selected patients with AVR did not.

Unbiased multivariate PCA of the 84 TLR-mediated inflammatory genes revealed a separation between the CABG and AVR groups. The analysis further revealed an interesting pattern of clustering among the 84 investigated genes. Three of these clusters were particularly interesting. Two clusters (the green and red clusters in Fig. 2D), comprised the most differentially expressed TLRs as well as the majority of the most differentially expressed downstream mediators, suggesting that these receptors and their mediators share more similarities compared to the other TLRs and adapters with regard to cardiac inflammation. The third cluster of interest (yellow cluster in Fig. 2D) comprised the genes that were most differentially expressed between the CABG and AVR groups. Evaluating immunologic markers of pancreatic ischemic damage in mice, Lunsford et al. found an increase in the gene expression of ccl-2, fos, hspia1a, il1b and il-6 as well as nfkbia and traf 6 [38], all of which are included in the yellow cluster in our study and most of which were upregulated in the ischemic CABG group compared to the non-ischemic AVR group. The data of Lunsford et al. thus support our data and suggest that these genes co-variate under ischemic conditions and that ischemia may enhance cardiac inflammation and contribute to its maintenance.

We hypothesized that levels of circulating pro-inflammatory markers would be elevated in patients with cardiac disease compared to controls, based on previously published studies in animals and humans (2, 15). Surprisingly, only IL-13 was significantly upregulated in the blood of the patients with cardiac disease, whereas IL-8 was downregulated and the remaining cytokines were either unchanged (IFN-γ, IL-2, IL-6, IL-10, IL-12 p70, TNFα) or not detectable (IL-1β and IL-4). IL-13 has been identified as a regulator of cardiomyocyte cell cycle entry and has been suggested to be an initiating factor in mouse cardiac regeneration via signals through STAT3 [39]. Furthermore, circulating levels of IL-13 has been shown to be associated with the morphological and functional changes of myocardial tissue during aging [40]. IL-13 thus provides an interesting link between circulating pro-inflammatory markers and cardiac inflammation. However, there is a discrepancy between our results and other studies in the overall number and types of cytokines upregulated in cardiac disease. This was somewhat surprising, particularly since the patients with cardiac disease were older than controls subjects in our study, a factor known to positively correlate with cytokine levels [41]. The discrepancy between our study and other studies might be partly due to the fact that we included control individuals that were tightly matched for BMI, a factor that is known to strongly correlate with circulating levels of inflammatory markers [42,43]. Investigating cytokine expression in separate subcellular fractions of the blood might help elucidate distinct variations between the groups, but was not possible in the current study.

In summary, we found a marked upregulation of TLR-induced inflammation in cardiac tissue from patients that had undergone CABG- and AVR- surgery compared to healthy control tissue. The inflammation appeared to be primarily mediated through TLR1, 3, 7, 8 and 10, resulting in a marked induction of NF-κB signaling, JNK/p38 signaling and other TLR interacting proteins and adapters involved in the innate immune response. The gene expression analysis in combination with unbiased multivariate PCA suggested that ischemia may enhance cardiac TLR-induced inflammation. The cardiac inflammation was not accompanied by increased gene expression of inflammatory markers in the immune cells of the blood or marked elevated levels of circulating pro-inflammatory cytokines with the exception of IL-13. Based on our data, we propose that cardiac disease in humans may be mediated by local cardiac TLR signaling under both ischemic and non-ischemic conditions.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijcard.2019.06.033.

Declaration of Competing Interest

The authors report no relationships that could be construed as a conflict of interest.

Acknowledgements

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Fig. 3. *Toll-like receptor signaling and cytokine levels in blood from individuals with cardiac disease. (A)* Heat map showing hierarchical clustering of 84 genes involved in TLR-signaling in blood from patients that have undergone coronary artery bypass grafting (CABG) or aortic valve replacement (AVR), as well as blood from individuals without cardiac disease. $n = 4$. *(B–I)* Levels of cytokines were measured in serum samples of CABG patients and AVR patients and matched healthy controls. $n = 20$ for CABG, n = 20 for AVR and $n = 34$ for controls. $^{*}p \ll 0.05$, $^{**}p \ll 0.01$ (one way ANOVA). *(J)* PCA score plot of the first two components showing no separation between patients and healthy controls nor any separation between CABG and AVR groups.

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