Variants of Toll-like Receptor 4 Predict Cardiac Recovery in Patients with Dilated Cardiomyopathy*

Alexander Riad, Henriette Meyer zu Schwabedissen, Kerstin Weitmann, Lars R. Herda, Marcus Dörr, Klaus Empen, Arne Kieback, Astrid Hummel, Marcus Reinthaler, Marcus Grube, Karin Klingel, Matthias Nauck, Reinhard Kandolf, Wolfgang Hoffmann, Heyo K. Kroemer, and Stephan B. Felix

From the Department of Cardiology and Pulmonology, Universitätsmedizin Greifswald, 17475 Greifswald, the Department of Clinical Pharmacology, Universitätsmedizin Greifswald, 17489 Greifswald, the Departments of Community Medicine and Laboratory Medicine, Universitätsmedizin Greifswald, 17487 Greifswald, and the Department of Molecular Pathology, Institute for Pathology and Neuropathology, University of Tübingen, 72076 Tübingen, Germany

Background: The impact of polymorphisms of TLR4 on left ventricular performance in DCM patients is unknown.

Results: Patients carrying TLR4 variants had reduced improvement of left ventricular function and dilation and no increase in NT-pro-BNP level at follow up when compared with wild type genes.

Conclusion: TLR4 variants predict cardiac recovery in DCM.

Significance: This is the first study to investigate the impact of an innate immune receptor on cardiac recovery in human DCM.

The clinical course of patients with dilated cardiomyopathy (DCM) varies from cardiac recovery to end stage heart failure. The etiology of this variability is largely unknown. In this study, we investigated the impact of coding polymorphisms of the innate immune protein Toll-like receptor 4 (TLR4) on left ventricular performance in patients with DCM. Two variants of TLR4 (rs4986790, TLR4 c.1187A→G, p.299D→G and rs4986791, TLR4 c.1487C→T, p.T399I) were investigated in 158 patients with DCM. Other reasons for heart failure were excluded by coronary angiography, myocardial biopsy, and echocardiography. Risk factors, age, gender, or treatment did not differ among the groups. At the follow-up evaluation (median 4.0–5.4 months), patients carrying the TLR4 wild type gene displayed cardiac recovery under intense medical heart failure therapy indexed by reduced left ventricular dilation, improved left ventricular ejection fraction, and reduced NT-pro-BNP natriuretic peptide blood level when compared with the initial evaluation. In contrast, patients carrying both the rs4986790 and the rs4986791 variant showed significantly reduced improvement of left ventricular ejection fraction (p = 0.006) and left ventricular dilation (p = 0.015) at the follow-up evaluation when compared with carriers of the wild type gene under the same treatment conditions. In addition, NT-pro-BNP natriuretic peptide level in carriers of both TLR4 variants did not change significantly at the follow up when compared with the first evaluation. Among patients with DCM, the presence of the TLR4 variants rs4986790 and rs4986791 predicts impaired cardiac recovery independently of medical treatment or cardiac risk factors.

Dilated cardiomyopathy (DCM) is a relevant cause of heart failure leading to increased morbidity and mortality (1, 2). The etiology of this disease is currently under intense investigation. Genetic predisposition has been reported as an etiological factor; about 20–35% of DCM cases have been reported as familial (3). In addition, a recent study showed in patients with recent onset DCM an association between clinical outcome and race (4). Clinical data indicate that in the majority of DCM patients, viral infection and inflammatory processes are involved in the disease process (3, 5). In particular, autoimmune activation is assumed to contribute to the development and/or progression of DCM (1, 6, 7), but the role of both entities is poorly understood. Myocardial inflammation indexed by immune cell infiltration in patients with suspected myocarditis has been shown to be an independent risk factor, which suggests a causal role of immune activation under this condition (8). In this regard, activation of the innate immune system resulting in cytokine activation may be a possible pathophysiological mechanism of DCM (9).

Toll-like receptor 4 (TLR4) is the best characterized immune protein of the Toll-like receptor family (9). It is located in the cell membrane and is known to play a key role in recognizing pathogen-associated molecular patterns and in initiating cytokine activation in a large number of cell types, including cardiac cells (10). Loss of TLR4 in knock-out mouse models showed the protective effects in several types of heart failure to include myocardial ischemia, pressure overload, viral myocarditis, and toxic cardiomyopathy (11–15). Moreover, pharmacological inhibition of TLR4 in mouse models of myocardial ischemia exerts beneficial therapeutic effects (16). In patients with coronary artery disease, the peripheral contents of TLR4-positive monocytes were significantly increased during acute myocardial ischemia (17). Despite the association between TLR4
expression and cardiovascular diseases, two genetic variants of this receptor (rs4986790, TLR4 c.1187A→G, p.299D→G and rs4986791, TLR4 c.1487C→T, p.T399I) have been investigated in a number of studies focusing on atherosclerosis to include coronary artery disease. These genetic variants of TLR4 have been demonstrated to be associated with coronary events and efficacy of statin therapy (18). However, other studies did not show such an impact of TLR4 mutation on atherosclerosis (for detailed review see Ref. 10). Whereas TLR4 has been studied in patients with atherosclerosis, including acute or chronic myocardial ischemia, the role of TLR4 in patients with DCM is still unknown. We therefore analyzed the relationship between two TLR4 variants and the course of early stage DCM.

MATERIALS AND METHODS

Study Population—We screened a total of 454 patients with DCM during 2005 and 2010. Of these, 156 matched the inclusion criteria and were seen in a follow-up medical investigation in our clinic (median 4.2 (3.4;6.6) months after first admission). The inclusion criteria of DCM included increased left ventricular end-diastolic diameter (LVEDD >58 mm) and reduced ejection fraction (LVEF <50%) as derived by echocardiography. Disease duration was less than 1 year. In all patients, significant coronary artery disease or heart valve diseases were ruled out by angiography and echocardiography, respectively, as described previously (19). Active infectious diseases, pulmonary diseases, cancer, chronic alcoholism, or heart failure due to known origins were excluded. In all patients, acute myocarditis was excluded by myocardial biopsy from the right ventricular septum, performed at first hospital admission, according to Dallas criteria as described previously (20). Follow-up evaluation of LVEDD and LVEF was performed by two-dimensional echocardiography. All patients gave informed and written consent. The study was approved by the local ethics committee of the Ernst-Moritz-Arndt University, University Hospital Greifswald, Germany.

Echocardiography—Two-dimensional echocardiography was performed by experienced physicians on all patients at initial hospital admission and at follow-up evaluation according to the American College of Cardiology/American Heart Association guidelines (21). In brief, LVEDD, LV end-systolic diameter, systolic interventricular septum thickness, and left atrium size were calculated from the parasternal longitudinal axis. We calculated LVEF in a biplane manner from the apical two- and four-chamber view according to the Simpson rule. All parameters were quantified three times within the session, and a mean was calculated for all patients.

Spirometry—Spirometry was used for the quantification of lung performance as described previously (19). Lung function was indexed by forced vital capacity, forced expiratory volume in 1 s (lung), forced expiratory volume in 1 s/forced vital capacity ratio (%), total lung capacity, and residual volume (lung).

Analyses of Endomyocardial Biopsies—We performed histopathological, immunohistochemical, and molecular biological analyses of endomyocardial biopsies as described previously (8). In brief, to identify myocarditis, myocardial tissues were stained with hematoxylin and eosin, Masson’s trichrome, and Giemsa following examination by light microscopy. Histological analyses were performed according to the Dallas criteria (22, 23). Immunohistological analyses were used to investigate cardiac inflammation by treating the paraffin-embedded tissue sections with an avidin-biotin-immunoperoxidase method according to the manufacturer’s protocol (Vectastain Elite ABC kit, Vectastain®) (8). Monoclonal antibodies were used to evaluate cardiac cell infiltration of CD3+ T-lymphocytes (Novocastra Laboratories, UK), CD68+ macrophages (DAKO, Denmark), and HLA class II expression (DAKO, Germany) according to the World Health Organization/International Society and Federation of Cardiology Task Force on the Definition and Classification of Cardiomyopathies (1). As described previously, enterovirus species (including coxsackieviruses and echoviruses), parvovirus B19, adenoviruses, Epstein-Barr virus, and human herpesvirus type 6 were evaluated by nested PCR/RT-PCR from deep-frozen or RNAlater-fixed endomyocardial biopsy specimens (8). A biopsy was considered positive for viral infection if viral genome was detected by PCR, and specificity was confirmed by automatic DNA sequencing of viral amplification products.

Quantification of NT-pro-BNP and C-reactive Protein—We measured NT-probrain natriuretic peptide (NT-pro-BNP) plasma levels at first admission and during the follow up using an enzyme-linked immunosorbent assay (ELISA; Biomedica, Vienna, Austria) as described previously (24). C-reactive protein (CRP) was determined at the same time points using an immunoassay (Siemens, Germany).

DNA Analyses—Prior to genotyping, genomic DNA of each individual was extracted as described previously (18, 25). Subsequently, genotyping was performed using pre-developed TaqMan® assays (Applied Biosystems), namely C__11722238_20 and C__11722237_20, to assess for genetic variability of rs4986790 (c.TLR4 1187A>G) and rs4986791 (TLR4 1487C>T), respectively. In detail, reactions were carried out in a 5 μl volume containing 1 μl of genomic DNA, 0.25 μl of Primer/Probe mixture, 2.5 μl of Genotyping Master Mix, and 1.25 μl of water (Applied Biosystems). Fluorescence was assessed for using the fast real time-PCR system 7900 HT (Applied Biosystems) and the Sequence Detection Software SDS 2.3. All failing samples were repeated at least twice.

Statistical Analyses—Data were analyzed using the statistical programs SAS Version 9.1, SAS Institute Inc., Cary, NC, and STATA, Intercooled Stata/SE 10.1. Values were expressed as median and interquartile range (25th percentile; 75th percentile). Frequencies were calculated for categorical data. Patient characteristics were assessed by the Kruskal-Wallis test (non-normally distributed variables) and the Fisher’s exact test (categorical data). A two-tailed p value of <0.05 was considered to indicate statistical significance. Pairwise comparisons of significant results from the Kruskal-Wallis test were performed using the Mann-Whitney U-test. Adjustment for multiple testing was performed by the Bonferroni method (0.05/3 = 0.017). p values of paired group comparisons were calculated with the signed rank test (non-normally distributed variables) or the Bowker test (categorical values).

RESULTS

Frequency of TLR4 rs4986790 and rs4986791 Polymorphisms—A total of 156 patients were included in the statistical analysis
Variants of TLR4 and Cardiac Recovery in Patients with DCM

TABLE 1

| Haplotype frequencies |
|-----------------------|
| rs4986790 | rs4986791 | No. of individuals | Frequency |
| Haplotype 1 | AA | CC | 129 | 0.83 |
| Haplotype 2 | AG | CT | 13 | 0.08 |
| Haplotype 3 | AG | CC | 5 | 0.03 |
| Haplotype 4 | AA | CT | 9 | 0.06 |

reported below. Haplotypes were defined based on genetic information on both TLR4 polymorphisms, namely rs4986790 and rs4986791. Overall, 129 individuals carried neither variant allele and were summarized in haplotype 1. Haplotype 2 was identified in 13 individuals, which summarized heterozygote carriers of both tested polymorphisms. A total of 14 patients carried haplotype 3 and 4. Details on the haplotype frequencies are summarized in Table 1.

Base-line Characteristics—Base-line characteristics are shown in Table 2. The patient groups classified by the different TLR4 haplotypes did not differ with regard to gender, age, disease duration, or time from initial admission to follow-up evaluation. Except for diuretics, the frequency of heart failure medication at base line did not differ significantly. Whereas 100% of the haplotype 2 patients group received diuretics, the frequency of diuretics was 60.5% in the haplotype 1 group and 71.4% in the combined group of haplotype 3 and 4. Furthermore, lung function parameters showed no statistical differences. Tables 3 and 4 show analyses of the doses of the most common heart failure drugs at first admission and at follow up, and evaluations of our patients are shown in Table 3 and 4, respectively. Doses of the angiotensin-converting inhibitor ramipril, the aldosterone antagonist spironolactone, the beta blockers metoprolol, bisoprolol, and carvedilol, and the diuretic drug torasemide at both evaluation time points did not differ significantly. Other drugs did not reach a frequency high enough for grouped analyses. The frequency of positive viral genomes in endomyocardial biopsies did not differ significantly among the groups (data not shown).

Left Ventricular Performance—Data from echocardiography derived parameters are shown in Table 5 and Fig. 1. At first admission, neither LVEF nor LVEDD differed significantly among patients carrying normal TLR4, rs4986790, and/or the rs4986791 variants (Table 4). In contrast, at the follow-up evaluation of the rs4986790 variant in the presence of the rs4986791 variant of TLR4 demonstrated significantly reduced ΔLVEF and increased ΔLVEDD when compared with patients carrying the TLR4 wild type genotype (Fig. 1). Patients carrying either the rs4986790 or the rs4986791 gene variant did not show a significant alteration of LVEF or LVEDD when compared with patients carrying the TLR4 wild type gene.

NT-pro-BNP Levels—We measured NT-pro-BNP plasma levels as a well established surrogate parameter for the severity of heart failure. In line with our results for LV performance, NT-pro-BNP levels did not differ significantly between the patients groups at first admission. However, patients carrying the TLR4 wild type gene showed significantly reduced BNP levels at the follow-up evaluation when compared with levels upon initial admission (Fig. 2). Patients carrying either the rs4986790 or the rs4986791 gene variant furthermore displayed significantly reduced nt-BNP levels at follow up when compared with initial admission. In contrast, patients carrying haplotype 2 of TLR4 did not exhibit significant changes of NT-pro-BNP levels upon comparison of their levels at first admission and at follow up.

Cardiac Inflammation and Fibrosis—Cardiac inflammation was quantified by analyzing the infiltration of CD3$^+$ T-lymphocytes, CD68$^+$ macrophages, and the semi-quantitative evaluation of HLA II-positive epitopes in myocardial biopsies upon initial admission of our patients (Fig. 3). Patients carrying the TLR4 wild type gene showed no statistically significant content of CD3$^+$ T-lymphocytes or CD68$^+$ macrophages when compared with patients carrying the rs4986790 and/or the rs4986791 variant. Representative pictures of transendothelial migration of CD3$^+$ T-lymphocytes and CD68$^+$ macrophages. Fibrosis was not different at base line as shown in the representative pictures. Patients with DCM revealed significant amounts of interstitial fibrosis. To provide a peripheral surrogate parameter for the degree of inflammation, we measured CRP levels at initial admission and at follow up. Levels of CRP did not differ statistically significant between the groups neither at base line nor at follow up (Fig. 4).

DISCUSSION

In this study, we investigated the influence of two genetic variants of TLR4, rs4986790 and rs4986791, on changes of left ventricular performance in a carefully defined cohort of patients with early stage DCM. We revealed an association between TLR4 polymorphisms and changes in LV function over the course of time. Because base-line LV performance did not differ among the groups, our results demonstrated that TLR4 variants are associated with the disease progression. It is worthy of note that we observed no differences regarding left ventricular performance upon initial admission. However, patients carrying both the rs4986790 and the rs4986791 variant of TLR4 displayed slighter improvement of LVEF and LV dilation when compared with TLR4 wild type carriers at the follow-up evaluation. Quantification of nt-BNP strengthen our data. In line with improved LVEF and LVEDD, TLR4 wild type carrier patients exhibited a significant reduction of NT-pro-BNP blood levels at the follow-up evaluation. However, carriers of both rs4986790 and rs4986791 variants did not show a significant reduction of NT-pro-BNP levels under same conditions. At base line and at follow up, left ventricular performance in patients carrying only either rs4986790 or rs4986791 was similar to patients carrying the TLR4 wild type gene.

Patient genotyping revealed that the frequency of the rs4986790 variant was 3% and that of the rs4986791 variant was 6%. The frequency of the common appearance of rs4986790 and rs4986791 was 8%. To our knowledge, data on frequencies of the herein reported TLR4 gene frequencies in other cohorts with DCM are not available at this time. With regard to cohorts for investigation of atherosclerosis, the gene frequency of the rs4986790 variant or the isolated rs4986791 variant in the Brunck study was 6 and 1%, respectively (26). Other population studies investigating atherosclerosis showed a frequency of 6.6, 7.9, and 3.3% (18, 27). In the REGRESS study that investigated patients with coronary artery disease, the overall frequency was 5.9% (18). Thus, the genotype of TLR4, which was associated...
with reduced left ventricular recovery as observed in our study, did not occur at a significantly lower gene frequency compared with other study populations.

In this study, we were also interested in cardiac cell infiltration because this type of cardiac inflammation has been shown to represent an independent risk factor for suspected myocarditis, under the assumption that immune activation may also mediate the course of DCM in patients (8). Even if the underlying mechanisms are currently unclear, it could be hypothesized at this time that immune receptor signaling may play a role in progression of DCM. TLR4 is a transmembrane immune protein expressed in nearly all human immune and cardiac cells. The receptor can be activated by exogenous viral or bacterial ligands and likely by endogenous ligands resulting in cytokine activation (28). To obtain insights into the potential involvement of TLR4 single nucleotide polymorphisms and the cardiac inflammation, we quantified cardiac cell infiltration (8). We did not find associations between TLR4 variants associated with changes in LV performance in DCM patients and/or the degree of cardiac immune cell infiltrations at the first presentation of the patients in our hospital. Our findings must be interpreted carefully. Because of ethical reasons, we did not perform endomyocardial biopsies merely for study purposes at the follow-up evaluation of our patients. We therefore cannot demonstrate whether cardiac immune cell infiltration was associated

### TABLE 2

**Base-line characteristics and heart failure treatment**

|                         | Wild type (n = 129) | Variant 1 and 2 (n = 13) | Variant 1 or 2 (n = 14) | p value |
|-------------------------|--------------------|--------------------------|-------------------------|---------|
| Age, year               | 52.5 (47.2; 61.5)  | 55.3 (51.2; 59.9)        | 51.5 (43.2; 58.4)       | 0.53a   |
| Female                  | 25 (19.4%)         | 4 (28.6%)                | 1 (7.7%)                | 0.37b   |
| **Cardiovascular risk factors** |                |                          |                         |         |
| Body mass index (kg/m²) | 28.9 (25.6; 31.8) | 28.0 (26.4; 29.8)        | 30.2 (26.3; 32.1)       | 0.71a   |
| Systolic blood pressure (mm Hg) | 120 (110; 132)   | 112 (102; 130)           | 118 (108; 140)          | 0.60a   |
| Diastolic blood pressure (mm Hg) | 70 (65; 80)      | 70 (65; 80)              | 77 (70; 85)             | 0.26a   |
| Current smoker          | 66 (51.2%)         | 6 (46.2%)                | 7 (50%)                 | 0.95a   |
| Diabetes mellitus       | 23 (17.8%)         | 2 (15.4%)                | 3 (21.4%)               | 0.92a   |
| Arterial hypertension   | 69 (53.5%)         | 7 (53.9%)                | 7 (50.0%)               | 1.00a   |
| Disease duration (month) | 2.3 (0.6; 9.4)    | 6.7 (2.1; 44.8)          | 2.3 (0.6; 3.8)          | 0.20a   |
| Time until follow-up evaluation (month) | 4.0 (3.3; 6.5) | 5.4 (4.2; 7.2)           | 4.2 (3.3; 5.9)          | 0.10a   |
| ICD devices             | 19 (14.8%)         | 4 (33.3%)                | 6 (42.9%)               | 0.02a   |
| Pacemaker devices       | 5 (3.9%)           | 1 (7.7%)                 | 1 (7.1%)                | 0.36a   |

**Medications**

|                         | Wild type          | Variant 1 and 2         | Variant 1 or 2          | p value |
|-------------------------|--------------------|-------------------------|-------------------------|---------|
| ACE inhibitors          | 106 (82.2%)        | 13 (100%)               | 13 (92.9%)              | 0.21a   |
| AT1-antagonist          | 105 (81.4%)        | 8 (61.5%)               | 10 (71.4%)              | 0.18a   |
| Aldosterone antagonists | 44 (34.1%)         | 4 (30.8%)               | 7 (50.0%)               | 0.46a   |
| Diuretics               | 78 (60.5%)         | 13 (100%)               | 10 (71.4%)              | 0.008a  |

**Echocardiography**

|                         | Wild type          | Variant 1 and 2         | Variant 1 or 2          | p value |
|-------------------------|--------------------|-------------------------|-------------------------|---------|
| Left atrium (mm)        | 48 (44.51)         | 45 (41.53)              | 49 (47.55)              | 0.23a   |
| Left ventricular end systolic diameter (mm) | 58 (52.65) | 62 (56.64)              | 62 (56.67)              | 0.73a   |
| Diastolic interventricular septum wall thickness (mm) | 11 (9.12) | 10 (9.13)               | 11 (10.13)              | 0.82a   |

**Statistical analyses were performed using the Fisher’s exact test.**

**Statistical analyses were performed using the Kruskal-Wallis test.**

### TABLE 3

**Analyses of medication doses at first admission**

Analyses were performed for the most frequent drugs in the patients population at first admission. Data are shown as median (1st quartile; 3rd quartile). Statistical analyses were performed using the Kruskal-Wallis test. Wild type, TLR4 wild type gene; variant 1 and 2, presence of rs4986790 and rs4986791; variant 1 or 2, presence of rs4986790 or rs4986791.

|                         | Wild type          | Variant 1 and 2         | Variant 1 or 2          | p value |
|-------------------------|--------------------|-------------------------|-------------------------|---------|
| Ramipril (mg)           | 5.0 (2.5; 7.5), n = 76 | 5.0 (2.5; 10.0), n = 7    | 2.5 (1.25; 10.0), n = 10 | 0.35    |
| Spironolactone (mg)     | 25.0 (25.0; 50.0), n = 26 | 25.0 (25.0; 25.0), n = 3   | 25.0 (25.0; 62.5), n = 4 | 0.69    |
| Metoprolol (mg)         | 95.0 (47.5; 142.5), n = 26 | 95.0 (47.5; 142.5), n = 5    | 72.5 (50.0; 117.5), n = 4 | 0.99    |
| Bisoprolol (mg)         | 5.0 (2.5; 7.5), n = 59 | 2.5 (2.5; 6.25), n = 4     | 2.5 (2.5; 5.0), n = 5    | 0.60    |
| Carvedilol (mg)         | 20.0 (12.5; 25.0), n = 18 | 25.0 (12.5; 37.5), n = 3    | 18.75 (12.5; 31.9), n = 4 | 0.73    |
| Torasemide (mg)         | 10.0 (10.0; 20.0), n = 51 | 10.0 (5.0; 17.5), n = 8     | 10.0 (10.0; 10.0), n = 5  | 0.48    |

**Statistical analyses were performed using the Fisher’s exact test.**

**Statistical analyses were performed using the Kruskal-Wallis test.**

### TABLE 4

**Analyses of medication doses at follow-up**

Analyses were performed for the most frequent drugs in the patients population at first admission. Data are shown as median (1st quartile; 3rd quartile). Statistical analyses were performed using the Kruskal-Wallis test. Wild type, TLR4 wild type gene; variant 1 and 2, presence of rs4986790 and rs4986791; variant 1 or 2, presence of rs4986790 or rs4986791.

|                         | Wild type          | Variant 1 and 2         | Variant 1 or 2          | p value |
|-------------------------|--------------------|-------------------------|-------------------------|---------|
| Ramipril (mg)           | 5.0 (5.0; 10.0), n = 85 | 5.0 (5.0; 10.0), n = 7    | 5.0 (2.5; 10.0), n = 13  | 0.23    |
| Spironolactone (mg)     | 25.0 (25.0; 25.0), n = 40 | 25.0 (25.0; 25.0), n = 5    | 25.0 (25.0; 25.0), n = 5  | 0.59    |
| Metoprolol (mg)         | 95.0 (47.5; 142.5), n = 29 | 142.5 (142.5; 166.3), n = 4 | 84.4 (48.8; 130.6), n = 4 | 0.8    |
| Bisoprolol (mg)         | 5.0 (2.5; 7.5), n = 75 | 3.3 (2.5; 5.0), n = 5     | 5.0 (2.5; 10.0), n = 6   | 0.77    |
| Carvedilol (mg)         | 25.0 (12.5; 50.0), n = 19 | 12.5 (9.4; 37.5), n = 3    | 15.6 (12.5; 18.8), n = 4  | 0.29    |
| Torasemide (mg)         | 10.0 (10.0; 20.0), n = 55 | 15.0 (5.0; 20.0), n = 6    | 10.0 (5.0; 30.0), n = 7   | 0.93    |
Variants of TLR4 and Cardiac Recovery in Patients with DCM

Left ventricular performance at first admission

Left ventricular performance at first admission in our hospital was indexed by ejection fraction and end-diastolic diameter derived from two-dimensional echocardiography. Lung function was evaluated by spirometry. Data are shown as median (1st quartile; 3rd quartile). Statistical analyses were performed using the Kruskal-Wallis test. Wild type, TLR4 wild type gene; variant 1 and 2, presence of rs4986790 and rs4986791; variant 1 or 2, presence of rs4986790 or rs4986791. TLC, total lung capacity; FVC, forced vital capacity; FEV1, forced expiratory volume in 1 s; RV, residual volume.

| Wild type | Variant 1 and 2 | Variant 1 or 2 | p value |
|-----------|----------------|----------------|---------|
| Ejection fraction (%) | 31.0 (26.0; 36.0) | 30.0 (22.0; 34.0) | 28.0 (23.0; 6.0) | 0.43 |
| Left ventricular diastolic diameter (mm) | 69.0 (63.0; 75.0) | 72.0 (67.0; 74.0) | 68.5 (63.0; 72.0) | 0.51 |
| FVC (lung) | 3.58 (3.06; 4.16) | 4.03 (2.78; 4.6) | 2.75 (2.3; 3.53) | >0.05 |
| FEV1 (lung) | 2.93 (2.52; 3.6) | 3.34 (2.56; 4.1) | 2.6 (1.82; 2.95) | >0.05 |
| FEV1/vital capacity | 0.86 (0.76; 0.96) | 0.89 (0.77; 0.95) | 0.85 (0.74; 0.95) | >0.05 |
| TLC (lung) | 6.4 (5.66; 7.39) | 7.17 (6.09; 7.49) | 5.95 (4.93; 6.27) | >0.05 |
| RV (lung) | 2.65 (2.02; 3.22) | 3.03 (2.57; 3.48) | 2.46 (2.01; 3.1) | >0.05 |

FIGURE 1: Changes of left ventricular function and dilation at the follow-up evaluation. Left ventricular ejection fraction and end-diastolic diameter were measured in the same manner as evaluation at first admission. Data are expressed as box plots. Statistical analyses were performed using the Kruskal-Wallis test followed by Mann-Whitney U-test (post hoc) and adjusted for multiple testing (Bonferroni method). The horizontal lines within the boxes represent the medians; the lower and upper bounds of the boxes represent the 1st quartile and the 3rd quartile; the I bars indicate upper (largest data value that is less than or equal to the 3rd quartile + 1.5 × interquartile range) and lower (smallest data value that is greater than or equal to the 1st quartile − 1.5 × interquartile range) adjacent limits, and o represents outliers. Wild type, TLR4 wild type gene; variant 1 and 2, presence of rs4986790 and rs4986791; variant 1 or 2, presence of rs4986790 or rs4986791.

FIGURE 2: NT-pro-BNP levels at first admission and follow-up evaluation. Blood samples were used to quantify NT-pro-BNP at first admission and follow-up evaluation. Statistical analyses were performed using the signed rank test. Data are expressed as box plots. The horizontal lines within the boxes represent the medians; the lower and upper bounds of the boxes represent the 1st quartile and the 3rd quartile, the I bars indicate upper (largest data value that is less than or equal to the 3rd quartile + 1.5 × interquartile range) and lower (smallest data value that is greater than or equal to the 1st quartile − 1.5 × interquartile range) adjacent limits, and gray circles or gray diamonds represent outliers. Wild type, TLR4 wild type gene; variant 1 and 2, presence of rs4986790 and rs4986791; variant 1 or 2, presence of rs4986790 or rs4986791.

with changes in LV performance and NT-pro-BNP levels during the course of time of DCM. Therefore, we cannot exclude that TLR4 mutations influence the progression of DCM by alterations of cardiac cell infiltration over time. However, we may deduce from our data that TLR4 may not be pivotal for cardiac leukocyte and/or monocyte recruitment at least in the early phase of DCM.

Very little is known about the functional consequences of the two TLR4 variants investigated in this study. The rs4986790 variant results in replacement of a conserved aspartic acid residue with glycine at amino acid 299 and alters the extracellular domain of the receptor (29). This mutation has been shown in vitro to be associated with defective TLR4 signaling after stimulation by the well known activator LPS (27). In contrast, other in vitro studies did not show significant TLR4 dysfunction in the presence of the TLR4 variant rs4986790 (28, 30). This contradiction may be explained by the large heterogeneity of the investigated study populations and cell lines. Furthermore, until now, the key ligand of TLR4 in cardiovascular diseases, including DCM, has not yet been identified. On the basis of our findings, it could be speculated that TLR4 mutations alter the function of infiltrated immune cells but not recruitment of this cell population, at least upon initial presentation of the patients in our clinic.

TLR4 is highly expressed in several circulating immune cells participating both the innate and the adaptive immune system (31). After activation of the TLR4 pathway, gene expression of pro- and anti-inflammatory cytokines bridges interaction of the innate and adaptive immune system (9). It can be speculated that TLR4-modulated immune cells, which performed transendothelial migration into the heart tissue, alter myocyte contractility and extracellular matrix regulation leading to altered LV function and dimension.
In addition, data showing a role of the TLR4 signaling pathway in cardiac resident cells (32) suggest that TLR4 mutations may not only alter the function of immune cells but also that of myocytes, cardiac endothelial cells, and fibroblast cells. Assumption of a cardiac-specific immune effect of the TLR4 mutations would also be strengthened by our finding that CRP, a well established marker of systemic inflammation and shown to be increased in DCM patients (33), was not statistically different among the groups in our study.

In summary, we showed that in patients with DCM, the combined presence of both TLR4 variants (rs4986790 and rs4986791), but not the isolated presence, was associated with a less pronounced improvement in LV function and dilation in the early stage of the disease during follow up. In contrast, upon initial admission, none of the variant combinations was associated with significant changes in left ventricular performance. Analysis of NT-pro-BNP blood levels revealed a significant reduction during the course of time in wild type and isolated variant carriers but not in patients carrying both TLR4 polymorphisms. These observations were independent of heart failure medication doses. The changed cardiac phenotype, which was associated with TLR4 mutations, seems not to be contributed by the initial cardiac infiltration of immune cells at initial admission. Data from experimental studies have shown that TLR4 can relevantly modulate several types of ischemic and nonischemic heart failure. However, this has not been shown for DCM most likely due to the lack of an adequate animal model. In addition, growing clinical evidence suggests that TLR4 and its genetic variants can modulate atherosclerosis, including coronary artery disease. However, the role of TLR4 in DCM remains unknown. To the best of our knowledge, we were able to show for the first time that TLR4 variants have a prognostic value on cardiac performance in a cohort of early stage DCM.

Our study includes several potential limitations. The cohort size is relatively low upon comparison with other studies investigating TLR4 gene polymorphisms. In the light of cardiovascular diseases, human genetic studies exist only for patients with

FIGURE 3. Inflammation in myocardial biopsies from the right ventricle. Statistical analyses of the amounts of CD3$^+$ and CD68$^+$ cells in biopsies from the right ventricle at first admission were performed using the Kruskal-Wallis test. Data are expressed as box plots (A). The horizontal lines within the boxes represent the medians; the lower and upper bounds of the boxes represent 1st and 3rd quartiles; the I bars indicate upper (largest data value that is less than or equal to the 3rd quartile + 1.5 × interquartile range) and lower (smallest data value that is greater than or equal to the 1st quartile − 1.5 × interquartile range) adjacent limits, and gray circles represent outliers. Statistical analyses from semiquantitative measurements of HLA-positive epitopes were performed using the Fisher’s exact test. Cardiac fibrosis was evaluated by trichrome staining (A). Representative pictures from a patient with DCM are shown for transendothelial migration of CD3$^+$ and CD68$^+$ cells (B). Wildtype, TLR4 wild type gene; variant 1 and 2, presence of rs4986790 and rs4986791; variant 1 or 2, presence of rs4986790 or rs4986791.

FIGURE 4. C-reactive protein level at first admission and follow-up evaluation. Levels of CRP were measured at first admission (1st) and at the follow-up evaluation (2nd). Data are shown as frequency of proportion of CRP levels (<5, 5–10, and >10 mg/liter). p values of paired group comparisons were calculated with the signed rank test (non-normally distributed variables) or Bowker test (categorical values). Wildtype, TLR4 wild type gene; variant 1 and 2, presence of rs4986790 and rs4986791; variant 1 or 2, presence of rs4986790 or rs4986791.
peripheral, central, or coronary atherosclerosis. These diseases occur with much higher incidence in comparison with DCM, which could explain the high number of included patients (23). Nevertheless, to the best of our knowledge, there is no larger well defined patient cohort of DCM that has investigated gene polymorphisms of innate immune proteins. The relatively small population size accordingly reflects the incidence of the investigated disease of DCM. Although the group size of the variant carrier is relative small, careful statistically significant results were demonstrated. Importantly, TLR4 variants showed an impact on three independent gold standard surrogate parameters of heart failure, which are the most important in clinical routine. At first, LVEF derived from bialane analyses from two-dimensional echocardiography is the major parameter to quantify systolic LV function (34). In addition, LVEDD is the major parameter for quantification of LV dilation (34). Although this parameter is also derived from two-dimensional echocardiography, it is independent of LVEF. However, NT-pro-BNP is the major laboratory parameter regarding both development and progression of heart failure, and its measurement method is independent from LVEF and LVEDD measurements (34). In this study, we showed by three independent parameters that LV performance is contributed by TLR4 genetic variants, which clearly demonstrated valid results. The disease duration showed a high variation at least in the group carrying bot TLR4 genetic variants. However, there was no statistically significant difference among the patients groups. Finally, the minor allele frequencies of both TLR4 variants are rather low as reported previously (16, 23, 24). Consequently, the frequency of the rs4986790 and rs47986791 haplotype is even lower. Appearance of both the rs4986790 and rs4986791 variants but not the isolated rs4986790 variant in patients with atherosclerosis showed worsened heart failure during the course of time. We investigated the influence of both variants on the development and the course of DCM. However, we cannot make any conclusions about the low frequency of the combinations of genetic variants.

REFERENCES

1. Richardson, P., McKenna, W., Bristow, M., Maisch, B., Mauthner, B., O’Connell, J., Olsen, E., Thiene, G., Goodwin, J., Gyafas, I., Martin, I., and Nordet, P. (1996) Report of the 1995 World Health Organization/International Society and Federation of Cardiology Task Force on the Definition and Classification of cardiomyopathies. Circulation 93, 841–842
2. Sheppard, R., Bedi, M., Kubota, T., Semigran, M. J., Dec, W., Feldman, A. M., Rosenblum, W. D., McTiernan, C. F., and McNamara, D. M. (2005) Myocardial expression of fas and recovery of left ventricular function in patients with recent-onset cardiomyopathy. J. Am. Coll. Cardiol. 46, 1036–1042
3. Maron, B. J., Towbin, J. A., Thiene, G., Antzelevitch, C., Corrado, D., Arnett, D., Moss, A. J., Seidman, C. E., and Young, J. B. (2006) Contemporary definitions and classification of the cardiomyopathies. An American Heart Association Scientific Statement from the Council on Clinical Cardiology, Heart Failure and Transplantation Committee; Quality of Care and Outcomes Research and Functional Genomics and Translational Biology Interdisciplinary Working Groups; and Council on Epidemiology and Prevention. Circulation 113, 1807–1816
4. McNamara, D. M., Starling, R. C., Cooper, L. T., Boehmer, J. P., Mather, P. J., Janosko, K. M., Gorcsan, J., 3rd, Kip, K. E., Dec, G. W., IMAC Investigators (2011) Clinical and demographic predictors of outcomes in recent onset dilated cardiomyopathy results of the IMAC (Intervention in Myocarditis and Acute Cardiomyopathy)-2 study. J. Am. Coll. Cardiol. 58, 1112–1118
5. Feldman, A. M., and McNamara, D. (2000) Myocarditis. N. Engl. J. Med. 343, 1388–1398
6. Herda, L. R., Felix, S. B., and Staudt, A. (2009) Immunoadsorption in patients with dilated cardiomyopathy. Atheroscler. Suppl. 10, 126–128
7. Cooper, L. T., Jr. (2009) Myocarditis. N. Engl. J. Med. 360, 1526–1538
8. Kindermann, I., Kindermann, M., Kandolf, R., Klingel, K., Bültmann, B., Müller, T., Lindinger, A., and Böhm, M. (2008) Predictors of outcome in patients with suspected myocarditis. Circulation 118, 659–668
9. O’Neill, L. A., and Bowie, A. G. (2007) The family of five: TIR-domain-containing adaptors in Toll-like receptor signaling. Nat Rev Immunol. 7, 353–364
10. Mann, D. L. (2011) The emerging role of innate immunity in the heart and vascular system. For whom the cell toils. Circ. Res. 108, 1133–1145
11. Riad, A., Bien, S., Gratz, M., Escher, F., Westermann, D., Heimesaat, M. M., Bereswill, S., Krieg, T., Felix, S. B., Schultheiss, H. P., Kroemer, H. K., and Tschöpe, C. (2008) Toll-like receptor-4 deficiency attenuates doxorubicin-induced cardiomyopathy in mice. Eur. J. Heart Fail. 10, 233–240
12. Riad, A., Jäger, S., Sobirey, M., Escher, F., Yauelma-Riss, A., Westermann, D., Karatas, A., Heimesaat, M. M., Bereswill, S., Dragun, D., Pauschinger, M., Schultheiss, H. P., and Tschöpe, C. (2008) Toll-like receptor-4 modulates survival by induction of left ventricular remodeling after myocardial infarction in mice. J. Immunol. 180, 6954–6961
13. Kim, S. C., Ghanem, A., Stapel, H., Tiemann, K., Knueffermann, P., Hoeft, A., Meyer, R., Grohé, C., Knowlton, A. A., and Baumgarten, G. (2007) Toll-like receptor 4 deficiency. Smaller infarcts, but no gain in function. BMC Physiol. 7, 5
14. Oyama, J., Blais, C., Jr., Liu, X., Pu, M., Kobzik, L., Kelly, R. A., and Bourcier, T. (2004) Reduced myocardial ischemia-reperfusion injury in toll-like receptor-4-deficient mice. Circulation 109, 784–789
15. Stapel, H., Kim, S. C., Osterkamp, S., Knueffermann, P., Hoeft, A., Meyer, R., Grohé, C., and Baumgarten G. (2006) Toll-like receptor 4 modulates myocardial ischemia-reperfusion injury. Role of matrix metalloproteinases. Eur. J. Heart Fail. 8, 665–672
16. Shimamoto, A., Chong, A. J., Yada, M., Shomura, S., Takayama, H., Fleisig, A. J., Agnew, M. L., Hampton, C. R., Rothnie, C. L., Spring, D. J., Pohlman, T. H., Shimoto, H., and Verrier, E. D. (2006) Inhibition of Toll-like receptor 4 with eritoran attenuates myocardial ischemia-reperfusion injury. Circulation 114, Suppl. 1, 1270–1274
17. Methe, H., Kim, J. O., Koller, S., Weiss, M., Nabauer, M., and Koglin J. (2005) Expansion of circulating Toll-like receptor 4-positive monocytes in patients with acute coronary syndrome. Circulation 111, 2654–2661
18. Boekholdt, S. M., Agema, W. R., Peters, R. J., Zwinderman, A. H., van der Wall, E. E., Reitsma, P. H., Kastelein, J. J., Jukema, J. W., and Regression GRowth Evaluation Statin Study Group (2003) Variants of toll-like receptor 4 modify the efficacy of statin therapy and the risk of cardiovascular events. Circulation 107, 2416–2421
19. Herda, L. R., Trimpert, C., Nauke, U., Landsberger, M., Hummel, A., Beug, D., Kieback, A., Dörr, M., Empen, K., Knobel, F., Ewert, R., Angelow, A., Hoffmann, W., Felix, S. B., and Staudt, A. (2010) Effects of immunoadsorption and subsequent immunoglobulin G substitution on cardiopulmonary exercise capacity in patients with dilated cardiomyopathy. Am. J. Cardiol. 159, 809–816
20. Staudt, A., Schäper, F., Stangl, V., Wallukat, G., Lindinger, A., and Böhm, M., Merkal, K., Wallukat, G., and Böhm, M. (2001) Immunohistological changes in dilated cardiomyopathy induced by immunoadsorption and subsequent immunoglobulin G substitution. Eur. J. Heart Fail. 3, 665–672
21. Chetin, M. D., Alpert, J. S., Armstrong, W. F., Aurigemma, G. P., Beller, G. A., Bierman, F. Z., Davidson, T. W., Davis, J. L., Douglas, P. S., and Gillam, L. D. (1997) ACC/AHA Guidelines for the Clinical Application of Echocardiography. A report of the American College of Cardiology/American Heart Association Task Force on Practice Guidelines (Committee on Clinical Application of Echocardiography). Circulation 105, 86–216
Variants of TLR4 and Cardiac Recovery in Patients with DCM

AUGUST 3, 2012 • VOLUME 287 • NUMBER 32

JOURNAL OF BIOLOGICAL CHEMISTRY

27243

Fenoglio, J. J., Jr., Olsen, E. G., and Schoen, F. J. (1987) Myocarditis. A histopathological definition and classification. Am. J. Cardiovasc. Pathol. 1, 3–14

23. Yilmaz, A., Kindermann, I., Kindermann, M., Mahfoud, F., Ukena, C., Athanasiadis, A., Hill, S., Mahrholdt, H., Voehringer, M., Schieber, M., Klingel, K., Kandolf, R., Böhm, M., and Sechtem U. (2010) Comparative evaluation of left and right ventricular endomyocardial biopsy. Differences in complication rate and diagnostic performance. Circulation 122, 900–909

24. Staudt, A., Staudt, Y., Hummel, A., Empen, K., Dörre, M., Trimpert, C., Birkenmeier, K., Kühl, U., Noutsias, M., Russ, D., and Felix, S. B. (2006) Effects of immunoabsorption on the nt-BNP and nt-ANP plasma levels of patients suffering from dilated cardiomyopathy. Ther. Apher. Dial. 10, 42–48

25. Lorenz, E., Frees, K. L., and Schwartz, D. A. (2001) Determination of the TLR4 genotype using allele-specific PCR. BioTechniques 31, 22–24

26. Kiechl, S., Lorenz, E., Reindl, M., Wiedermann, C. J., Oberhollenzer, F., Bonora, E., Willeit, J., and Schwartz, D. A. (2002) Toll-like receptor 4 polymorphisms and atherogenesis. N. Engl. J. Med. 347, 185–192

27. Arbour, N. C., Lorenz, E., Schutte, B. C., Zabner, J., Kline, J. N., Jones, M., Frees, K., Watt, J. L., and Schwartz, D. A. (2000) TLR4 mutations are associated with endotoxin hypersensitivity in humans. Nat. Genet. 25, 187–191

28. Erridge, C. (2010) Endogenous ligands of TLR2 and TLR4. Agonists or assistants? J. Leukocyte Biol. 87, 989–999

29. Garantziotis, S., Hollingsworth, J. W., Zaas, A. K., and Schwartz, D. A. (2008) The effect of toll-like receptors and toll-like receptor genetics in human disease. Annu. Rev. Med. 59, 343–359

30. Erridge, C., Stewart, J., and Poxton, I. R. (2003) Monocytes heterozygous for the Asp-299 → Gly and Thr-399 → Ile mutations in the Toll-like receptor 4 gene show no deficit in lipopolysaccharide signaling. J. Exp. Med. 197, 1787–1791

31. Kawai, T., and Akira, S. (2008) Toll-like receptor and RIG-I-like receptor signaling. Ann. N.Y. Acad. Sci. 1143, 1–20

32. Riad, A., Westermann, D., Zietsch, C., Savvatis, K., Becher, P. M., Bereswill, S., Heimesaat, M. M., Lassner, D., Dörner, A., Poller, W., Busch, M., Felix, S. B., Schultheiss, H. P., and Tschöpe, C. (2011) TRIF is a critical survival factor in viral cardiomyopathy. J. Immunol. 186, 2561–2570

33. De Gennaro, L., Brunetti, N. D., Cuculo, A., Pellegrino, P. L., and Di Biase, M. (2008) Systemic inflammation in nonischemic dilated cardiomyopathy. Heart Vessels 23, 445–450

34. Dickstein, K., Cohen-Solal, A., Filippatos, G., McMurray, J. J., Ponikowski, P., Posse-Wilson, P. A., Strömberg, A., van Veldhuisen, D. J., Atar, D., Hoes, A. W., Keren, A., Priori, S. G., Swedberg, K., ESC Committee for Practice Guidelines (CPG) (2008) ESC guidelines for the diagnosis and treatment of acute and chronic heart failure 2008. The Task Force for the diagnosis and treatment of acute and chronic heart failure 2008 of the European Society of Cardiology. Developed in collaboration with the Heart Failure Association of the ESC (HFA) and endorsed by the European Society of Intensive Care Medicine (ESICM). Eur. J. Heart Fail. 10, 933–989