

Complement and Dilated Cardiomyopathy

A Role of Sublytic Terminal Complement Complex-Induced Tumor Necrosis Factor- α Synthesis in Cardiac Myocytes

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Dilated cardiomyopathy is a syndrome characterized by cardiac enlargement and impaired systolic function of the heart. Tumor necrosis factor (TNF)- α , a pleiotropic cytokine, seems to play a central role in the progression of dilated cardiomyopathy. Recent data suggest that ongoing inflammation in the myocardium may, in many cases, contribute to the development of disease. Chronic generation of autoantibodies to myocardial antigens or, in some cases, viral infection are pathobiologically involved. Although both antibodies and some viruses activate the complement system, the role of innate immunity in dilated cardiomyopathy has as yet not been investigated systematically. In this study we demonstrate by analysis of myocardial biopsies from 28 patients that C5b-9, the terminal membrane attack complex of complement, accumulates in human myocardium in dilated cardiomyopathy. C5b-9 significantly correlates with immunoglobulin deposition and myocardial expression of TNF- α . *In vitro*, C5b-9 attack on cardiac myocytes induces nuclear factor (NF)- κ B activation as well as transcription, synthesis, and secretion of TNF- α . We conclude that chronic immunoglobulin-mediated complement activation in the myocardium may contribute in part to the progression of dilated cardiomyopathy via C5b-9-induced TNF- α expression in cardiac myocytes. (*Am J Pathol* 2002, 161:449–457)

Dilated cardiomyopathy is an important cause of heart failure in humans. It has long been hypothesized that chronic myocardial inflammation might contribute to the pathogenesis of dilated cardiomyopathy.^{1–3}

Generation of autoantibodies,^{4–6} viral infection,^{2,7–11} T-cell-mediated immune responses,^{5,7} and apoptosis¹ are recognized as major pathobiological mechanisms

leading to congestive heart failure. Tumor necrosis factor (TNF)- α , a pleiotropic cytokine contributing to cellular immunity and inflammatory reactions in a range of inflammatory diseases, seems to be intimately involved in the progression of cardiac disease.¹² Soluble and transmembrane forms of the molecule have been described and the latter has been shown to be the prime activating ligand for the 80-kd TNF receptor.¹³ TNF- α exerts negative inotropic effects on the myocardium via immediate (NO-independent) and delayed (NO-dependent) mechanisms.^{14,15} Additionally, the molecule has been shown to trigger apoptosis in cardiac myocytes.^{12,14,16} Originally, it has been suggested that inflammatory cells within the cardiac interstitium were responsible for local cardiac expression and release of TNF- α but recent studies have focused on the role of myocardial cells as a source of TNF- α expression.^{12,14,16} Indeed, the failing human myocardium, but not healthy human hearts, express abundant quantities of TNF- α .^{16,17} The major inflammatory mechanisms inducing TNF- α expression in cardiac myocytes during chronic myocarditis and its progression to congestive heart failure are as yet not well defined.

There are few reports on the potential contribution of complement activation to myocardial inflammation.^{10,18} The complement system is part of the innate mammalian immune system. It is highly conserved during evolution and sequence analyses provided evidence for significant interspecies homology¹⁹ suggesting an origination from a single and ancestral gene.²⁰ The complement system is a cascade of serum proteins that can be activated either by the classical pathway involving binding of antibody or, for example, C-reactive protein^{21,22} to complement component C1, or by the alternative pathway that is triggered by the binding of particles, for example cell walls or aggregates of cholesterol to the component C3. There is also evidence that some viruses interact with

Support by the Deutsche Herzstiftung (to J. T., T. P. Z., and N. M.), the Deutsche Forschungsgemeinschaft (SFB 451, project A4, to J. T.), and the Deutsche Herzstiftung (to Z. K.).

This work contains data from the Ph.D. thesis of Dimitar Manolov and the M.D. thesis of Cüneyt Özdemir.

Accepted for publication April 25, 2002.

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complement and lead to or prevent complement activation, respectively.²³⁻²⁵

Complement activation to completion results in the generation of proinflammatory molecules, for example the anaphylatoxins C3a and C5a as well as the terminal complement complex C5b-9.^{22,26,27} C5b-9 is a pore-forming toxin that has been first described by its ability to lyse erythrocytes. In contrast to erythrocytes, nucleated cells survive complement C5b-9 attack responding by synthesis and secretion of proinflammatory molecules such as cytokines, chemokines, or growth factors.²⁸⁻³² Most of these effects seem to be mediated by C5b-9-induced Ca^{2+} influx into cells. Moreover, C5b-9 exerts effects on signal transduction, for example mitogen-activated protein (MAP) kinase activation and nuclear factor (NF)- κ B activation in nucleated cells.^{28,33}

Although autoantibodies⁴⁻⁶ and viruses are involved in cardiac inflammation and although this would be suggestive for a contribution of innate immunity to myocardial inflammation, the role of complement in dilated cardiomyopathy has not yet been studied systematically.

Based on the knowledge that complement components deposit in inflamed myocardium and that C5b-9 induces signaling mechanisms involved in TNF- α synthesis¹⁴ we hypothesized that: 1) chronic complement activation in the myocardium may occur in dilated cardiomyopathy; 2) complement activation may be mediated by antibodies in the myocardium; and 3) C5b-9 may induce NF- κ B-dependent TNF- α synthesis and secretion in cardiac myocytes, thus contributing to the progression of dilated cardiomyopathy.

Materials and Methods

Endomyocardial Biopsies/Study Group

Left or right ventricular (septal) endomyocardial biopsies, respectively, were obtained from 28 patients presenting with the clinical symptoms of cardiac failure and echocardiographic ventricular dysfunction. Twenty men and 8 women were included (mean age, 51.5 ± 12.4 years). Coronary artery disease was excluded by previous cardiac catheterization. Furthermore, valvular and congenital heart diseases were excluded. Six to eight myocardial biopsies from each patient were analyzed to reduce the sampling error. Myocardial biopsies from eight patients that did not display any histomorphological signs of dilated cardiomyopathy (see below) served as controls (mean left ventricular ejection fraction (LVEF): 46.9 ± 9.6), whereas myocardial biopsies from 20 patients with histomorphological signs of dilated cardiomyopathy served as samples (mean LVEF: 36.8 ± 12.0). All procedures were performed in accordance with ethical standards and with the Helsinki Declaration of 1975. Patients gave informed consent for the invasive studies performed.

Animals

Cardiomyocytes were prepared from 10- to 12-week-old male Wistar rats, type Hannover, body weight 300 to 400 g.

Analysis of Myocardial Biopsies

Myocardial biopsies were subjected to liquid nitrogen and subsequently cryofixed. Histomorphological diagnosis of dilated cardiomyopathy was performed through examination of the following criteria: interstitial fibrosis, cellular infiltrates, cellular hypertrophy, myocardial cell degeneration.³⁴⁻³⁶ Five sections from a single biopsy were immunohistochemically analyzed for immunoglobulin (IgG) deposition using mouse monoclonal antibody (clone MK1A6; Dianova, Germany, Hamburg, Germany) against human IgG(Fc) (dilution 1:100), C5b-9 deposition using monoclonal anti-C5b-9 antibodies (clone 978/394, IgG1; S. Bhakdi, Mainz, Germany) and for TNF- α expression by anti-human TNF- α antibodies (Dianova). A semiquantitative score system [staining intensity from no discernible immunoreactivity to strongly abundant immunoreactivity (0 to 5)] was applied for each antigen. Atherectomy specimens from advanced atherosclerotic lesions prepared by identical procedures were used as positive controls for C5b-9 deposition. Coded slides were analyzed in a blinded manner.

Cell Culture

Cardiac myocytes were prepared from rat hearts.^{37,38} For each experiment two hearts were obtained from adult rats after anesthesia with ether. Excised hearts were transferred to ice-cold saline and then mounted to the double cannula of a Langendorff perfusion system. Hearts were perfused through the aorta with Powell medium containing 57 μ g/ml of collagenase (Biochrom, Berlin, Germany), in a recirculating manner. Consecutively, ventricular tissue was chopped and incubated in 30 ml of recirculation medium for 5 minutes. The incubated tissue was filtered through a 200- μ m mesh nylon gauze and then centrifuged for 3 minutes at 400 rpm ($26 \times g$). The cell pellet was washed and Ca^{2+} tolerance was built by centrifuging cell suspension through a $CaCl_2$ -gradient ($CaCl_2$ in Powell medium) with increasing concentrations beginning with a $CaCl_2$ concentration of 200 μ mol/L followed by a concentration of 400 μ mol/L and 1 mmol/L. The pellet was resuspended in maintenance medium [CCT medium (pH 7.4, M199 + HEPES + creatine, carnitine, taurine)] and then plated in M199 medium (Gibco, Karlsruhe, Germany) (4% fetal calf serum) in precoated, tissue culture dishes. Cytosine β -D-arabino-furanoside (Sigma, Munich, Germany) at a final concentration of 1 mg/L was added to eliminate proliferating cells and thus, to exclude fibroblast contamination.

C5b-9 Formation on Cardiac Myocytes

C5b-9 was generated on cardiac myocytes by a reactive lysis mechanism as follows: C5b-6 was isolated from human yeast-activated acute phase serum by anion exchange and gel filtration.³² C5b-6 activity was assayed by titration with guinea pig erythrocytes (Charles River, Wilmington, MA). One MHD was defined as the amount of C5b-9 needed to produce lysis of 50 μ l 1% erythro-

cytes.^{39,40} C7, C8, and C9 were bought from Sigma Chemical. The dose of C7 equivalent to the dose of C5b-6 was determined by titration with guinea pig erythrocytes. C8 and C9 were added in surplus. C5b-9 complexes were generated on cardiac myocytes by adding C5b-6 to the cells in monolayer culture. Fifteen minutes later equivalent doses of C7 were added and a further 15 minutes later C8 and C9 were added in surplus. Heat-inactivated C5b-9 (1 hour, 56°C) served as a control.

Electrophoretic Mobility Shift Assay

Nuclear extracts of cardiac myocytes were prepared as described.⁴¹ Cells were stimulated for 15 minutes before preparation of nuclear extracts. Oligonucleotides for a consensus NF- κ B site (5'-GTCCCGGAGTTTCCTACC-3') were annealed with a complementary primer and radiolabeled using [α -³²P]deoxycytidine triphosphate.⁴¹ Protein-DNA complexes were separated from free DNA probe by electrophoresis through 6% nondenaturing acrylamide gels in 0.5 \times Tris-borate-ethylenediaminetetraacetic acid and bands visualized by autoradiography.

Endotoxin Analysis in Complement Preparations

During preparation of C5b-6, precautions were taken to avoid endotoxin lipopolysaccharide (LPS) contamination. LPS concentrations in the final C5b-9 complement proteins were found to be <0.5 ng/ml as assessed by Limulus endotoxin assay (E-Toxate test, Sigma). C5b-9 was heat-inactivated at 56°C (60 minutes) for negative control. This procedure inactivates complement without influencing LPS activity.

Isolation of Total RNA and Quantitative Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

Total RNA was extracted using the RNeasy kit (Qiagen). Amplification of TNF- α and GAPDH was performed using Omniscript RT kit (Qiagen, Hilden, Germany). Reverse transcription was performed for 60 minutes at 37°C. TaqPCR Master Mix kit (Qiagen) was used for amplification of PCR products. To quantitatively assess TNF- α mRNA and GAPDH PCR was calibrated by amplification of defined amounts of template DNA (TNF- α and GAPDH) cloned into pGEM-T vector (Promega, Madison, WI). The proportion of synthesized RT-PCR products remained constant with 25, 30, and 35 cycles of RT-PCR. Therefore, 30 cycles in the RT-PCR were chosen for further analysis. PCR product was quantified using direct densitometric analysis of agarose gel. Cloned PCR products were used in all PCRs as an internal quantitative control. PCR was performed for 30 cycles each at 94°C (3 minutes), 55°C (40 seconds), and 72°C (1 minute). The forward primer for TNF- α was 5'-CAA GGA GGA GAA GTT CCC AA-3' and the reverse primer was 5'-CGG ACT CCG TGA TGT CTA AG-3'. The forward primer for GAPDH was 5'-ACG

GAT TTG GCC GTA TTG GC-3' and the reverse primer was 5'-CTC CTG GAA GAT GGT GAT G-3'.

LPS (500 ng/ml) from *Escherichia coli* 0127 (Sigma) was used for positive control to stimulate TNF- α transcription.⁴²

Immunoprecipitation and Western Blotting

Cardiomyocytes were lysed and protein was immunoprecipitated using polyclonal goat anti-TNF- α (Santa Cruz Biotechnology, Santa Cruz, CA) and bound to protein Sepharose A (Boehringer, Mannheim, Germany). The eluted TNF- α anti-TNF- α complex samples were subjected to 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and blotted onto a nitrocellulose membrane (Hybond C extra, Amersham). Proteins were detected using anti-TNF- α and peroxidase-conjugated rabbit anti-goat Ig. A chemiluminescence-based detection system (ECL, Amersham, Freiburg, Germany) was used for detection of immunoreactivity. Again, LPS (500 ng/ml) was used for positive control. Monensin (2 μ mol/L) was used to block TNF- α secretion.

Enzyme-Linked Immunosorbent Assay (ELISA) for the Soluble Form of TNF- α

Cell culture was performed in 100-mm tissue culture dishes as described above. The supernatants of C5b-9-attacked cardiac myocytes were concentrated by Centricon-Plus 20 (Millipore, Bedford, MA) centrifugation and analyzed for TNF- α 12 hours after attack by a commercially available ELISA (Endogen, Woburn, MA) according to manufacturer's instructions. Sensitivity of the assay is 8.4 pg/ml, (range, 31.2 to 2000 pg/ml).

Immunofluorescent Staining of Transmembrane TNF- α on Cardiac Myocytes

Cardiac myocytes were isolated, seeded on glass slides, and C5b-9 attack with 100 MHD was performed. Before assay procedure, cells were fixed in 4% formaldehyde. Cells were incubated with anti-TNF- α polyclonal antibody (Santa Cruz Biotechnology) at a concentration of 4 μ g/ml for 30 minutes. After washing cells with phosphate-buffered saline (PBS) a secondary tetramethylrhodamine B isothiocyanate-labeled antibody (donkey anti-goat IgM tetramethylrhodamine B isothiocyanate, Dianova) was added at a dilution of 1:50 for another 30 minutes and finally, after washing with PBS again, cells were mounted in Mowiol and visualized under an immunofluorescent microscope.⁴³

Statistical Analysis

Results were expressed as mean \pm SEM. Differences were analyzed using analysis of variance and appropriate post hoc test and linear regression analysis. Results were expressed as box plots and scatter plots, respectively.

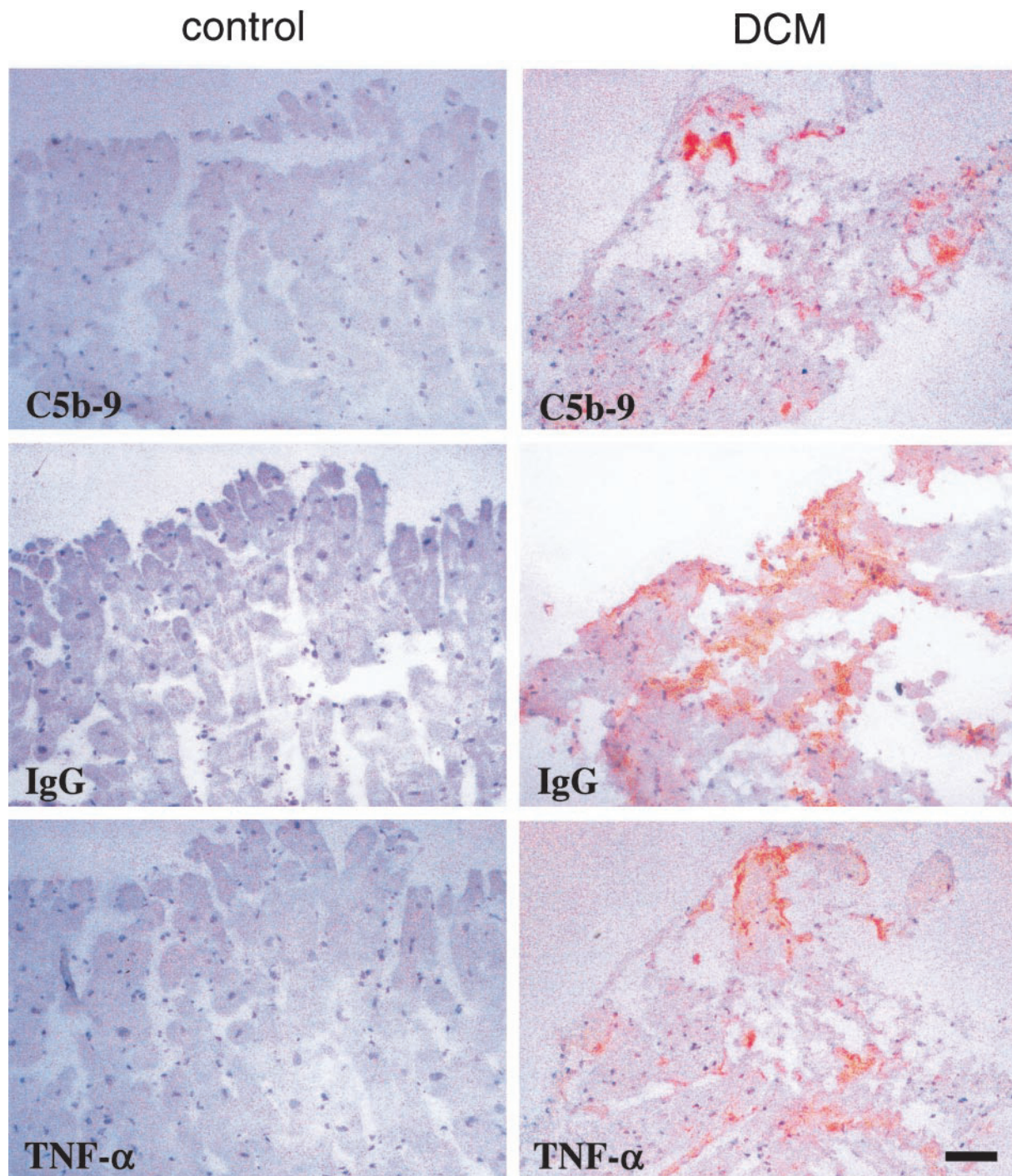


Figure 1. Immunohistochemical staining for C5b-9, IgG, and TNF- α showing sequential sections of myocardial biopsies from two representative patients—control *versus* sample (DCM). Original magnification, $\times 400$; scale bar, 50 μm .

Results

Deposition of IgG and C5b-9 and Expression of TNF- α in Myocardial Tissue

Figure 1 shows sequential sections of two representative patients (sample *versus* control). Sample shows intense deposition of IgG and C5b-9 in the myocardium. Correspondingly, intense TNF- α positivity of myocardial cells was detected in areas of C5b-9 deposition. Control pa-

tient shows no deposition of IgG and C5b-9 or TNF- α positivity of myocardial cells.

Sequential sections of cryofixed myocardial tissue from 28 patients were stained for C5b-9, TNF- α , and human IgG. Eight myocardial biopsies not displaying any histomorphological signs of dilated cardiomyopathy were used as controls. Twenty myocardial biopsies showing histomorphological signs of dilated cardiomyopathy were used as samples. Three groups of antigen

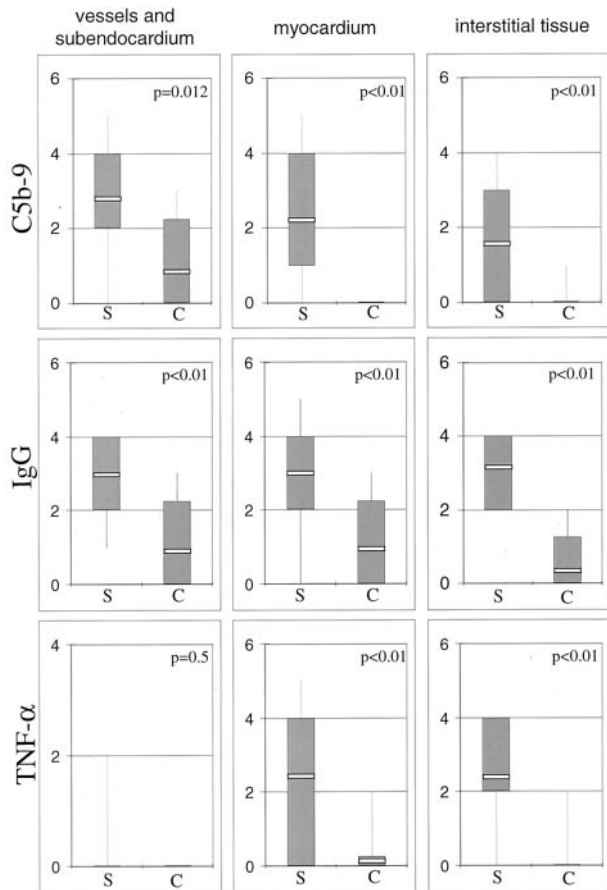


Figure 2. Box plots of semiquantitative analysis of myocardial tissue stained for human C5b-9, IgG, and TNF- α (S, samples, $n = 20$; C, controls, $n = 8$). Three groups of antigen localization were differentiated: vessels and subendocardium, myocardium, and interstitial tissue. Staining intensity (0 to 5) is shown on the y axis.

localization were differentiated: vessels and subendocardium, myocardium, and interstitial tissue (including fibrotic areas) (Figure 2). A score system (staining intensity 0 to 5) was applied to each antigen. Results are expressed as box plots and show significant differences between samples (S) and controls (C) for each antigen except TNF- α in vessels and subendocardium.

Correlation between IgG, C5b-9, and TNF- α

To further evaluate a potential correlation between IgG, C5b-9, and TNF- α linear regression analysis was performed (Figure 3). Results are expressed as scatter plots including linear regression lines. In vessels and subendocardium, no significant correlation between antigens was found. In contrast, correlation between both IgG and C5b-9 as well as C5b-9 and TNF- α was highly significant in the myocardium. In the interstitium only the correlation between IgG and C5b-9 was significant.

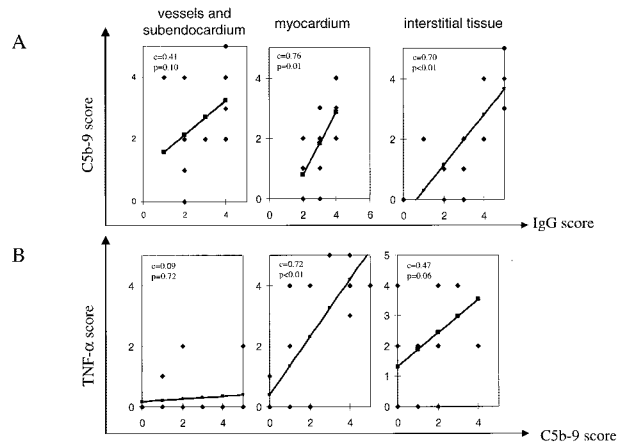


Figure 3. Correlation between stain of IgG, deposition of C5b-9, and expression of TNF- α in different tissue groups of antigen localization. Linear regression analysis expressed as scatter plots.

The Terminal Complement Complex C5b-9 Induces TNF- α mRNA Expression in Cardiac Myocytes

Cardiac myocytes were plated in 100-mm tissue culture dishes and attacked with 100 MHD of C5b-9. Stimulation with LPS (500 ng/ml) was used for positive control, unstimulated cells were used for negative control. mRNA expression was assessed using quantitative RT-PCR. Figure 4A depicts a representative result demonstrating C5b-9-induced stimulation of TNF- α mRNA expression. Densitometric evaluation of RT-PCR products ($n = 3$) revealed significant ($P < 0.05$) stimulation at 100 MHD of C5b-9 and LPS (Figure 4B).

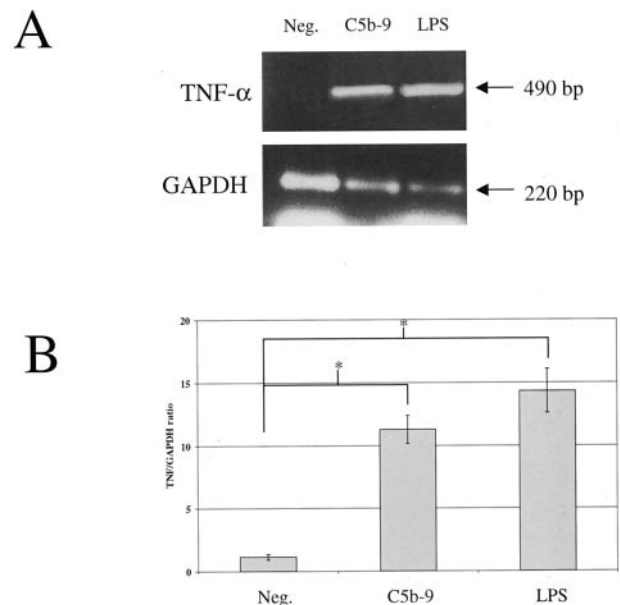


Figure 4. Quantitative RT-PCR evaluation of TNF- α mRNA expression. **A:** TNF- α mRNA expression in cardiomyocyte culture after C5b-9 (100 MHD) attack or LPS (500 ng/ml) stimulation. Unstimulated cells (Neg.) served as negative control and the expression of GAPDH gene as PCR positive control. **B:** Densitometric evaluation of three independent experiments following the setting described in **A**. Bars represent mean \pm SEM, $P < 0.05$.

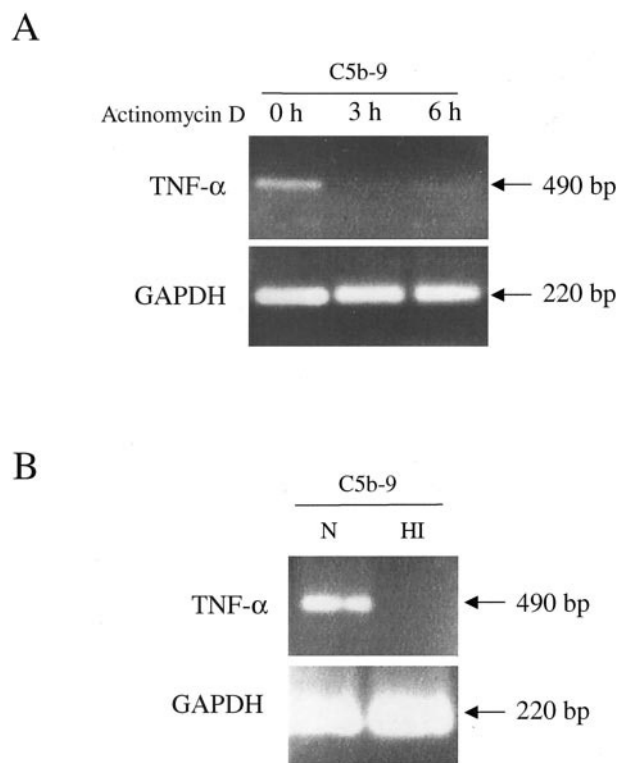


Figure 5. Quantitative RT-PCR assessment of TNF- α mRNA half-life. **A:** Co-incubation of cells with C5b-9 and Actinomycin D, nonspecific transcription inhibitor, shows time-dependent decrease in TNF- α mRNA synthesis. **B:** Heat inactivation (HI) of complement components abolished the TNF- α mRNA expression. N, not heat inactivated.

To exclude that increase in cellular TNF- α mRNA is because of C5b-9-induced mRNA stabilization rather than mRNA transcription experiments with Actinomycin D, a potent nonspecific transcription inhibitor, were performed. The results depicted in Figure 5A demonstrate that the effect of C5b-9 is because of an induction of transcriptional activity as TNF- α mRNA vanishes after a 3- and 6-hour co-incubation with Actinomycin D.

A potential confounding influence of LPS concerning induction of TNF- α mRNA expression in complement samples was excluded: LPS concentrations in the final C5b-9 complement proteins were found to be <0.5 ng/ml as assessed by the Limulus endotoxin assay. LPS concentrations of 2 ng/ml did not reveal any induction of TNF- α mRNA expression (not shown) and heat inactivation of C5b-9 completely (56°C, 60 minutes) abolished induction of TNF- α mRNA (Figure 5B).

The Terminal Complement Complex C5b-9 Induces the 27-kd Transmembrane Form of TNF- α in Cardiac Myocytes

Cardiac myocytes were plated in 100-mm tissue culture dishes and attacked with 20 and 100 MHD of C5b-9. Stimulation with LPS (500 ng/ml) was used for positive control, unstimulated cells were used for negative control. Western blot analysis was performed to detect the TNF- α protein intracellularly. TNF- α -convertase inhibitor

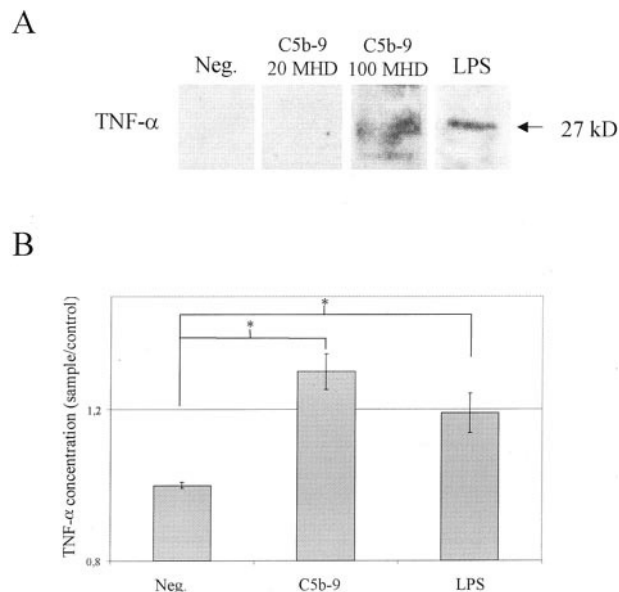


Figure 6. Immunoprecipitation and Western blot analysis of TNF- α in cardiac myocytes. **A:** Synthesis of 27-kd transmembrane TNF- α -protein at 100 MHD of C5b-9 and LPS stimulation, no synthesis at 20 MHD of C5b-9 or by unstimulated cells (Neg.). **B:** ELISA quantification of TNF- α in cell culture supernatants. The soluble TNF- α 19-kd protein was secreted after complement attack at 100 MHD of C5b-9 and stimulation with LPS. Bars represent mean \pm SEM, $P < 0.05$.

Monensin (2 μ g/ml) was used in all samples to block TNF- α secretion. Figure 6A shows the dose-response curve as obtained by Western blot analysis. Because of the relatively small number of cardiac myocytes obtained from a single rat, the different C5b-9 doses were tested in different experiments and compared to LPS (500 ng/ml) and negative control, respectively. The 27-kd membrane-bound form of TNF- α was detected. Corresponding to the results obtained by quantitative RT-PCR the figure depicts significant stimulation of TNF- α protein at 100 MHD of C5b-9.

The Terminal Complement Complex C5b-9 Induces TNF- α Release from Cardiac Myocytes

Cardiac myocytes were plated in 100-mm tissue culture dishes and attacked with 100 MHD of C5b-9. Stimulation with LPS (500 ng/ml) was used for positive control, unstimulated cells were used for negative control. A commercially available ELISA was used to measure TNF- α secretion into the cell supernatants. Figure 6B depicts the results: soluble TNF- α 19-kd protein release significantly increases after C5b-9 attack in comparison to control cells ($P < 0.05$). C5b-9-induced TNF- α secretion exceeds LPS-induced TNF- α secretion ($n = 3$).

TNF- α Is Expressed on Cardiac Myocytes Attacked by C5b-9

To investigate TNF- α expression in cardiac myocytes on C5b-9 stimulation we used immunofluorescent staining of cardiac myocytes with anti-TNF- α . Figure 7B shows in-

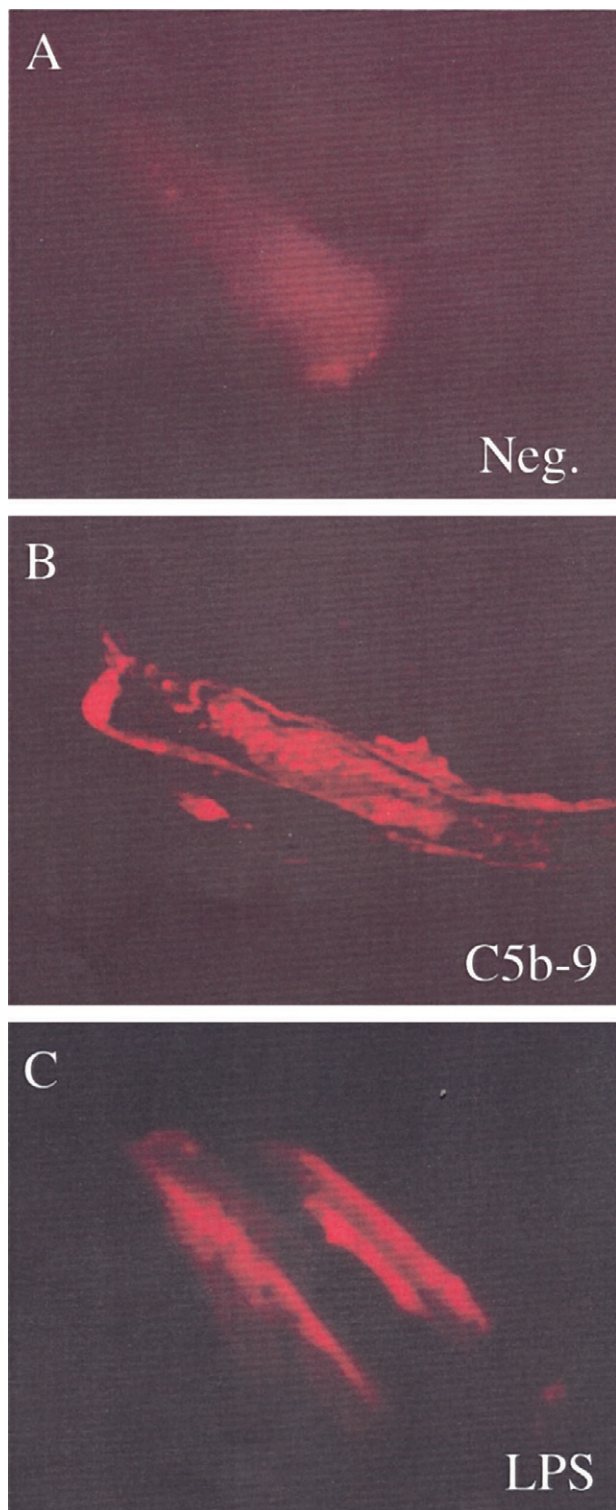


Figure 7. Immunofluorescent staining of TNF- α in cultured cardiac myocytes. Unstimulated cells (Neg.) did not reveal any immunofluorescent staining (A). Intense cell membrane-focused positive staining of cells stimulated with C5b-9 (B) or LPS (C). Original magnifications, $\times 400$.

tense staining focused on the cell membrane of cells stimulated with C5b-9 or 500 ng/ml of LPS, respectively. Unstimulated cells did not reveal any immunofluorescent staining (Figure 7A).

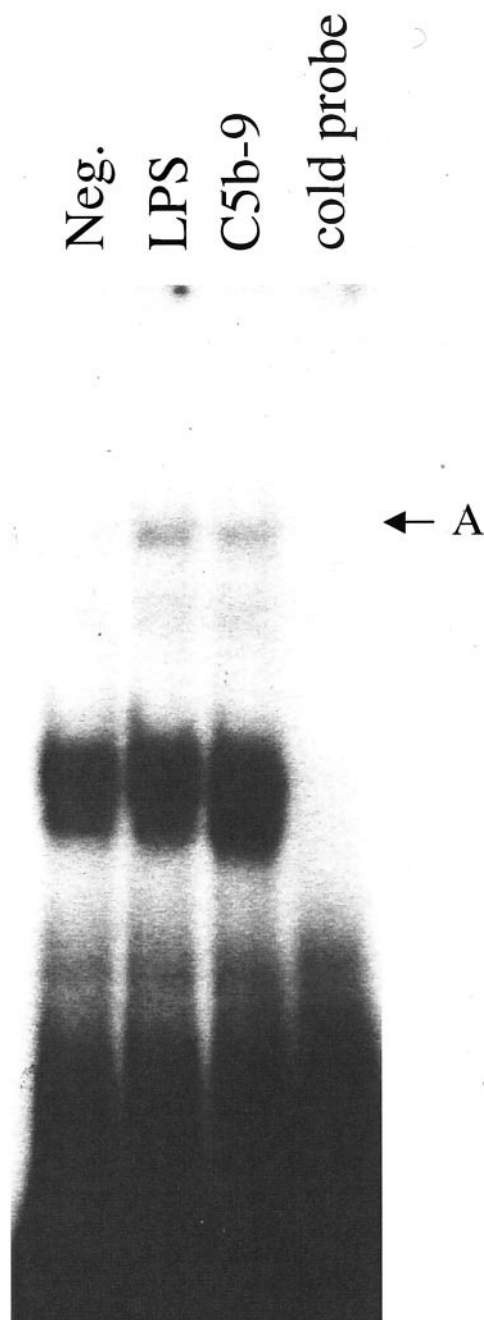


Figure 8. The terminal complement complex C5b-9 induces NF- κ B activation in cardiac myocytes. Unstimulated cells did not show binding of NF- κ B proteins to the oligonucleotide. Stimulation with 100 MHD of C5b-9 and LPS induced DNA-protein complex assembly (arrow, A). Specificity of the detected complexes was determined by addition of 40 ng of unlabeled NF- κ B oligonucleotide (cold probe).

The Terminal Complement Complex C5b-9 Induces NF- κ B Activation in Cardiac Myocytes

To examine whether C5b-9-induced TNF- α mRNA expression was mediated through activation of NF- κ B, we performed gel shift analysis (electrophoretic mobility shift assay) using oligonucleotides corresponding to a consensus NF- κ B site (Figure 8). Unstimulated cardiac myocytes did not show binding of NF- κ B proteins to the

oligonucleotide. In contrast, stimulation of cells with 100 MHD of C5b-9 induced DNA-protein complex assembly, suggesting that C5b-9 activates NF- κ B in cardiac myocytes. LPS-stimulated cells served as a positive control. Specificity of the detected complexes was determined by addition of 40 ng of unlabeled NF- κ B oligonucleotide (cold probe).

Discussion

This is the first study demonstrating terminal complement complex-induced expression of TNF- α in cardiomyocytes. The results of parallel *ex vivo* and *in vitro* experiments tend for us to hypothesize that this mechanism may be essential for the progression of dilated cardiomyopathy.

By immunohistochemical assessment of 20 myocardial biopsies we have demonstrated that C5b-9, the terminal complement complex, deposits in myocardial tissue in dilated cardiomyopathy. C5b-9 staining significantly correlated with IgG deposition as well as TNF- α expression in the myocardium. Control samples including myocardial biopsies of eight patients with no histomorphological signs of dilated cardiomyopathy showed a significantly less intense stain for C5b-9, IgG, and TNF- α , and no correlation between the antigens was found. Furthermore, we have demonstrated in an *in vitro* model that C5b-9 attack on cardiac myocytes induces TNF- α synthesis and release from these cells. The latter was assessed by quantitative RT-PCR, Western blotting, and immunofluorescence as well as ELISA technique. C5b-9-induced TNF- α synthesis was demonstrated to be because of stimulation of TNF- α transcription involving activation of NF- κ B. A potential confounding influence of LPS contamination was excluded.

Dilated cardiomyopathy is a syndrome characterized by cardiac enlargement and impaired systolic function of the heart. In some cases, dilated cardiomyopathy is considered to result from an ongoing inflammatory process in the myocardium because of either generation of autoantibodies, persistence of viral RNA, or T-cell-mediated immune response, respectively.²⁻¹¹

Only a few studies have addressed the significance of complement activation in inflammatory heart disease.^{10,18,44} Recently, it has been demonstrated for the first time that complement is critical for the induction of experimental myocarditis and acts through complement receptor type 1 (CR1) and type 2 (CR2).⁴⁴ Given the fact that both antibodies and some viruses activate complement and that complement activation can also occur through damaged cells, innate immunity is likely to play a significant role in progression of dilated cardiomyopathy. Our data suggest that IgGs may contribute to complement activation in the human heart as there is abundant deposition of IgG in the myocardium of our patients suffering from dilated cardiomyopathy whereas significantly less IgG was detected in control patients.

Complement activation to completion results in the formation of C5b-9. The effect of C5b-9 attack on cardiac myocytes has not yet been investigated. During the past

two decades it has been shown that complement attack on nucleated cells induces a wide range of cellular processes in the absence of cell death.²⁹ Thus, C5b-9 attack stimulates cells to synthesize and release mitogens, cytokines, or growth factors.^{28,30-33} Furthermore, C5b-9 has been demonstrated to induce downstream intracellular signaling events such as NF- κ B activation in smooth muscle cells.²⁸ Interestingly, NF- κ B activation is involved in the signaling cascades of TNF- α synthesis.¹⁴ Thus, C5b-9-induced TNF- α synthesis in cardiac myocytes as observed in our study may be mediated by activation of NF- κ B.

The pivotal role of TNF- α in the progression of congestive heart failure has been extensively confirmed.^{12,14,15} Elevated serum levels of circulating TNF- α have repeatedly been demonstrated in patients with congestive heart failure and animal studies as well as studies in humans suggest that TNF- α exerts negative inotropic effects on the myocardium.¹⁴ TNF- α has also been shown to trigger apoptosis in cardiac myocytes.¹⁴ Accumulating evidence indicates that, in myocardial disease, TNF- α is to a large extent produced by cardiac myocytes themselves and thus, acts as an autocrine contributor to myocardial dysfunction.¹⁴ It is important to note that the transmembrane form of TNF- α (as assessed by Western blot in our experiments) is the prime activating ligand of the TNF- α receptor, whereas the secreted form seems to be less active.¹³ Thus, the transmembrane form is obviously predominant in local inflammatory responses.¹³

Because, in this study, we were able to show that C5b-9 deposition in myocardial tissue of patients with dilated cardiomyopathy correlates with TNF- α expression in cardiac myocytes and because C5b-9 induces TNF- α synthesis and release from cardiac myocytes *in vitro* we suggest that C5b-9 attack on myocardial cells may contribute to autocrine TNF- α synthesis in the failing myocardium *in vivo*. Future studies need to investigate other possible mediators of myocyte damage generated by complement stimulation, as for example nitric oxide, produced either through the mediation of TNF- α or directly by stimulation of nitric oxide synthase.

In summary, the present study linking *ex vivo* and *in vitro*-observations may contribute to the understanding of inflammatory processes leading to the progression of dilated cardiomyopathy.

Acknowledgments

We thank Dr. N. Rose for critical reading and substantial improvement of the manuscript, and M. Bienek and M. Grüb for excellent technical assistance.

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