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Untersuchungen zur Rolle der angeborenen Immunität bei kardiovaskulären Erkrankungen

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Vorwort

In der vorliegenden Dissertation wird die Rolle der angeborenen Immunität in der Genese kardiovaskulärer Erkrankungen behandelt. An zwei Beispielen wird exemplarisch dargestellt, in welcher Weise die angeborenen Immunität in die Entstehung kardiovaskulärer Erkrankungen involviert ist. Beleuchtet wird die Rolle des Komplementsystems, und hierbei insbesondere des terminalen Komplementkomplexes C5b-9, in der Pathogenese der Dilatativen Kardiomyopathie einerseits, und die Bedeutung des C-reaktiven Proteins in der Pathogenese der Arteriosklerose andererseits.

Die Untersuchungen zur Rolle des Komplementsystems in der Genese der Dilatativen Kardiomyopathie wurden bereits im *American Journal of Pathology* (August, 2002) publiziert. Dieser Arbeit wurde vom *Am. J. Pathol.* ein „Commentary“ gewidmet. Die Ergebnisse der Untersuchungen werden deshalb, unter Hinzufügung der Publikation und des Kommentars, in einer kurzen Zusammenfassung präsentiert.

Die Untersuchungen zur Bedeutung des C-reaktiven Proteins in der Pathogenese der Arteriosklerose, die den Schwerpunkt der Dissertation darstellen, fokussieren auf der Interaktion des C-reaktiven Proteins mit seinem vermuteten Rezeptor FcγRIIa. Die Untersuchungen werden detailliert dargestellt und diskutiert. Das entsprechende Manuskript wurde inzwischen zur Publikation eingereicht und ist ebenfalls in die Dissertation eingefügt.

Abkürzungsverzeichnis

A	Adenosine
BSA	Bovine serum albumine
CD	Cluster of differentiation
CRP	C-reactive protein
dd H ₂ O	Didestilled water
DMEM	Dulbecco's modified Eagle Medium
DNA	Desoxyribonucleic acid
dNTPs	Desoxynucleoside triphosphates
EDTA	Ethylene diamine tetraacetic acid
Fab	Fragment antigen binding
FACS	Fluorescent activated cell sorting
Fc	Fragment crystallizable
FCS	Foetal calf serum
FITC	Fluorescein isothiocyanate
G	Guanosine
ICAM	Intercellular adhesion molecule
Ig	Immunoglobulin
LB	Luria-Bertrani Medium
LDL	Low density lipoprotein
LPS	Lipopolysaccharide
mRNA	messenger-Ribonucleic acid
PAB	PBS mit 0.05% Azide und 0.1% BSA
PBS	Phosphate buffered saline
PCh	Phosphocholine
RT-PCR	Reverse transcriptase - Polymerase chain reaction
PE	Phycoerythrine
SD	Standard deviation
SAP	Serum Amyloid P
SDS/PAGE	Sodium Dodecyl Sulphate Polyacrilamide Gel Electrophoresis
TE	Tris-EDTA
TNF- α	Tumor Necrosis Factor- α
VCAM	Vascular cell adhesion molecule

**1. Komplement und Dilatative Kardiomyopathie:
Der terminale Komplement-Komplex C5b-9 induziert
die Synthese von Tumor Necrosis Factor- α in
Kardiomyozyten**

1.1 Zusammenfassung der Publikation

Die **Dilatative Kardiomyopathie (DCM)** ist eine Erkrankung unklarer Genese. Die Erkrankung geht mit einer Vergrößerung der Herzkammern einher, und die Folge ist eine eingeschränkte myokardiale Pumpfunktion. Die Leistungsfähigkeit des Herzmuskels ist vermindert. Eine Entzündung der Herzmuskelatur, hervorgerufen von Bakterien, Pilzen oder Viren, wird als Myokarditis bezeichnet. Die Persistenz einer solchen Entzündung wird als mögliche Ursache der DCM vermutet.

Möglicherweise spielt die Aktivierung des Komplementsystems in der Genese der DCM eine wesentliche Rolle, da verschiedene Komplementkomponenten im entzündeten Myokard nachweisbar sind.

Der **terminale Komplementkomplex – C5b-9** wurde ursprünglich durch seine Fähigkeit definiert, Erythrozyten zu lysieren. Im Gegensatz zu Erythrozyten überleben kernhaltige Zellen einen C5b-9-Angriff. Sie synthetisieren und setzen als Reaktion auf einen C5b-9-Angriff proinflammatorische Moleküle wie Zytokine, Chemokine und Wachstumsfaktoren frei. Es wurde zudem demonstriert, dass Signaltransduktionsprozesse wie MAP Kinase- und NF κ B-Aktivierung in kernhaltigen Zellen durch C5b-9-Angriff verursacht werden.

Das pleiotrope Zytokin – **Tumor Necrosis Factor- α (TNF- α)** wird im Rahmen der DCM im Myokard exprimiert und übt dort negative inotrope Effekte aus.

In dieser Arbeit wurde die Frage gestellt, ob der terminale Komplementkomplex-C5b-9 zu einer TNF- α -Expression im Myokard führt und eine Rolle in der Progression von akuter Myokarditis zu DCM spielt.

Um diese Hypothese zu untersuchen, wurden sowohl *ex vivo* als auch *in vitro* Experimente durchgeführt.

Die **ex vivo Versuche** umfassen immunhistochemische Färbungen von menschlichen Myokardbiopsien. Endomyokardbiopsien wurden von DCM- und Kontrollpatienten entnommen. Sequentielle Schnitte aus kryofixiertem Myokardgewebe wurden gegen C5b-9, IgG und TNF- α immunhistochemisch gefärbt. **C5b-9 akkumuliert bei DCM-Patienten und kolokalisiert mit IgG. Diese Areale zeigen eine deutliche Überexpression von TNF- α .** In den Kontrollgruppen wurden signifikant geringer Ablagerungen dieser drei Substanzen nachgewiesen.

Basierend auf diesen Beobachtungen – wurden 3 Gruppen von Antigen-Lokalisation unterschieden: Gefäße und Subendokardium, Myokardium und Interstitium. Bei DCM-Patienten findet man deutlich mehr C5b-9, IgG und TNF- α Ablagerungen auf Myokardzellen und im Interstitium als bei den Kontrollgruppen. Das Ausmaß der C5b-9 Ablagerungen korreliert mit der Menge an IgG. Auf Kardiomyozyten korreliert die Expression von TNF- α mit der Menge an abgelagertem C5b-9.

Die ***in vitro* Versuche** beinhalten die Stimulierung von erwachsenen Ratten-Kardiomyozyten-Kulturen mit dem terminalen Komplementkomplex. Die C5b-9-induzierte Transkription, Translation und Sekretion von TNF- α wurde untersucht. C5b-9 wurde durch die sequentielle Zugabe von C5b-6, C7, C8 und C9 auf den Zellen gebildet.

C5b-9 induziert *in vitro* die Synthese von TNF- α in Kardiomyozyten. Durch quantitative RT-PCR wurde gezeigt, dass TNF- α nach Stimulation der Kultur mit C5b-9 auf Transkriptionsebene induziert wird. Als Positivkontrolle diente die Stimulation mit LPS (Lipopolysaccharide).

C5b-9 induziert die Expression der transmembranen Form des TNF- α Proteins auf Kardiomyozyten. Protein Lysate von stimulierten Zellen wurden mit anti-TNF- α Antikörper immunopräzipitiert und mittels Western blot Analyse untersucht. Die 27kD Bande ist sowohl bei den C5b-9- als auch bei den LPS-stimulierten Zellen zu sehen. Die transmembrane TNF- α Form wurde zusätzlich durch Immunfluoreszenz auf Kardiomyozyten nachgewiesen.

C5b-9 induziert die TNF- α -Freisetzung (als 19kD protein). Dies wurde mittels ELISA in Zellkulturüberständen ermittelt. Als Positivkontrolle diente erneut LPS. Als möglicher Mechanismus wurde durch Gel-Shift Analyse aus Zellkernextrakten demonstriert, dass die **Stimulation mit C5b-9 zu einer NF κ B-Aktivierung führt.**

Dies ist die erste Studie, die demonstriert, dass der terminale Komplementkomplex eine Expression von TNF- α in Kardiomyozyten induziert. Die C5b-9 Ablagerung im Myokardgewebe von DCM-Patienten korreliert mit der TNF- α -Expression. Die *in vitro* Stimulation der Kardiomyozyten mit C5b-9 führt zu einer vollständigen TNF- α Synthese, da TNF- α als RNA-Transkript, als transmembranes Protein und freigesetztes Protein nachgewiesen wurde.

Diese Studie, die *ex vivo* und *in vitro* Versuche beinhaltet, kann zum besseren Verständnis der Pathogenese der dilatativen Kardiomyopathie beitragen.

1.2 Poster:

Complement and Dilated Cardiomyopathy: A role of syblytic terminal complement complex-induced Tumor necrosis factor- α synthesis in cardiac myocytes.

Präsentiert auf dem 2002 *International Symposium „New aspects in inflammatory Cardiomyopathy – diagnosis and therapy“*,

Berlin, 18-20 Januar 2002

Complement and dilated cardiomyopathy: Role of sublytic terminal complement complex-induced Tumor Necrosis Factor- α synthesis in cardiac myocytes

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Abstract

Dilated cardiomyopathy is a disease of unknown etiology. Recent data suggest that it may result from ongoing inflammation in the myocardium following acute myocarditis. The underlying immunological mechanisms leading from acute myocarditis to dilated cardiomyopathy are as yet not well defined. Tumor Necrosis Factor- α (TNF- α), a pleiotropic cytokine, seems to play a central role in the progression of cardiac disease. Chronic generation of autoantibodies to myocardial antigens or, in some cases, viral infection are considered to be pathobiologically involved in dilated cardiomyopathy. Although both, antibodies and some viruses activate the complement system, the role of innate immunity in myocarditis and dilated cardiomyopathy has as yet not been investigated systematically.

In this study we demonstrate that C5b-9, the terminal membrane attack complex of complement, accumulates in human myocardium in dilated cardiomyopathy. Complement activation in dilated cardiomyopathy seems to be mediated by immunoglobulins (IgGs) deposited in the failing myocardium. C5b-9 deposition is associated with cardiac expression of TNF- α . *In vitro*, C5b-9 attack on cardiac myocytes induces transcription, synthesis and secretion of TNF- α as assessed by qRT-PCR, immunofluorescence and ELISA.

We conclude that chronic IgG-mediated complement activation in the myocardium may contribute to the progression from myocarditis to dilated cardiomyopathy via C5b-9-induced TNF- α expression in cardiac myocytes.

Materials and methods

Endomyocardial biopsies / study group: 6-8 endomyocardial biopsies were taken from 28 patients (20 men, 8 women, age 51.5 ± 12.4 years).

Myocardium of 20 patients showed the histomorphological signs of DCM. 8 patients without histomorphological features of DCM served as controls (K).

Cardiomyocytes: Cardiomyocytes were isolated from 10 – 12 weeks old Wistar-rats and investigated in primary culture.

Analysis of endomyocardial biopsies: Frozen sections of myocardial biopsies were immunohistochemically stained using monoclonal antibodies to IgG, C5b-9 and TNF- α . Staining intensity was assessed by a semiquantitative score system (0 – 5).

Generation of C5b-9 complexes on cardiomyocytes: C5b-9 was generated on cardiomyocytes *in vitro* using a “reactive lysis” system by sequential addition of C5b6, C7, C8 and C9 to the cells.

Electrophoretic mobility shift assay: Gelshift-analysis was performed using oligonucleotides for a consensus NF κ B-site from nuclear extracts of cardiomyocytes.

qRT-PCR: RNA isolation from cardiomyocytes and reverse transcription was followed by quantitative PCR for TNF- α . GAPDH was used as an internal standard.

Immunoprecipitation and Western blot: Total protein from cardiomyocytes was immunoprecipitated using a monoclonal antibody to TNF- α . TNF- α was detected by Western blot technique.

ELISA: TNF- α in the cell supernatant was measured using commercially available ELISA.

Immunofluorescence: Immunofluorescence using TRITC conjugated monoclonal antibodies was performed to directly assess TNF- α on cardiomyocytes.

Results: Deposition of IgG and C5b-9 and expression of TNF- α in myocardial tissue

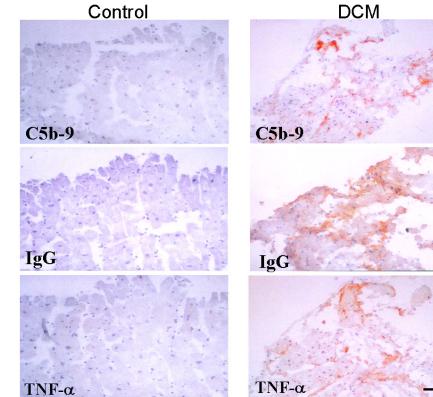


Figure 1: Sequential sections (control versus sample). Control shows no deposition of C5b-9 or IgG, or TNF- α positivity of myocardial cells. Sample shows intense deposition of IgG and C5b-9 in the myocardium. Correspondingly, intense TNF- α positivity of myocardial cells is seen in areas of C5b-9 deposition (Bar=50 μ m).

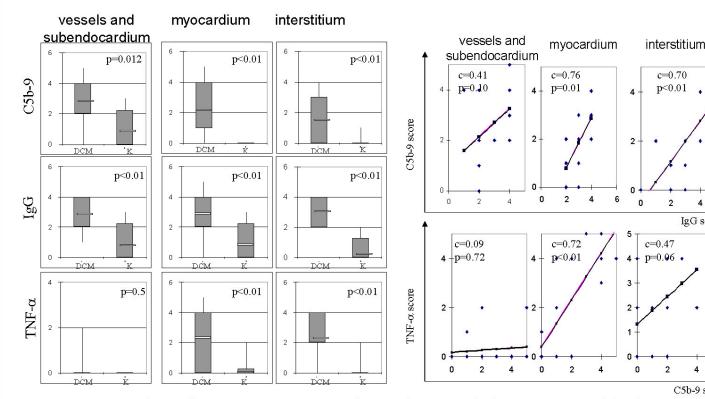


Figure 2: Boxplots of semiquantitative analysis of myocardial tissue stained for human C5b-9, IgG and TNF- α .

Controls (C, n=8): Myocardial biopsies without any histomorphological signs of dilated cardiomyopathy. Samples (S, n=20): Myocardial biopsies showing histomorphological signs of dilated cardiomyopathy.

Three groups of antigen localization were differentiated: vessels and subendocardium, myocardium and interstitial tissue. Staining intensity (0-5) is shown on the y-axis. Results demonstrating significant differences between samples and controls for each antigen except TNF- α in vessels and subendocardium.

Results: C5b-9 induces the synthesis and secretion of TNF- α in cardiomyocytes in vitro

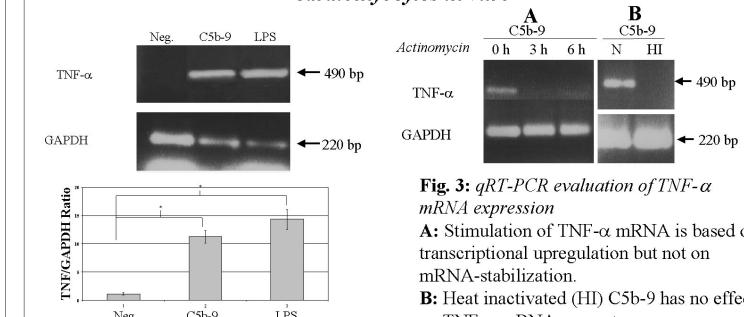


Fig. 3: qRT-PCR evaluation of TNF- α mRNA expression

A: Stimulation of TNF- α mRNA is based on transcriptional upregulation but not on mRNA-stabilization.

B: Heat inactivated (HI) C5b-9 has no effect on TNF- α mRNA amount.

Fig. 4: qRT-PCR of TNF- α
C5b-9 increases the amount of TNF- α mRNA in cardiomyocytes.

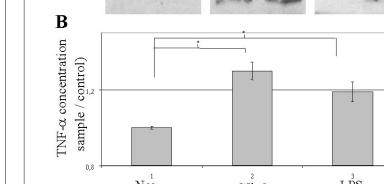
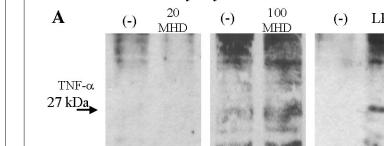


Fig. 5: Immunoprecipitation and ELISA for TNF- α

A: C5b-9 increases the amount of TNF- α protein in cardiomyocytes.

B: C5b-9 increases the amount of TNF- α in cells culture supernatant.

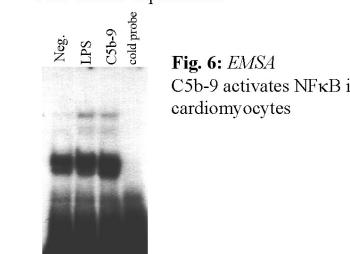


Fig. 6: EMSA
C5b-9 activates NF κ B in cardiomyocytes

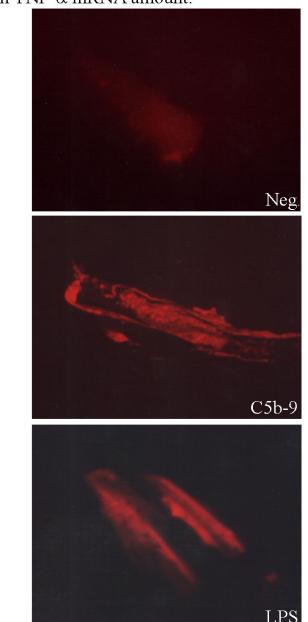


Fig. 7: Immunofluorescence
C5b-9 increases the amount of membranous TNF- α in cardiomyocytes.

1.3 Publikation:

Complement and Dilated Cardiomyopathy:
A role of syblytic terminal complement complex-induced
Tumor necrosis factor- α synthesis in cardiac myocytes.

American Journal of Pathology. 2002. 161: 449-457

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

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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 autoantibodies^{4–6} 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 $\mu\text{g}/\text{ml}$ 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- μm 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 $\mu\text{mol}/\text{L}$ followed by a concentration of 400 $\mu\text{mol}/\text{L}$ 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 μl 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'-GTCCCGGAGTTCTACC-3') were annealed with a complementary primer and radio-labeled 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× 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 μmol/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 μg/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 ± 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.

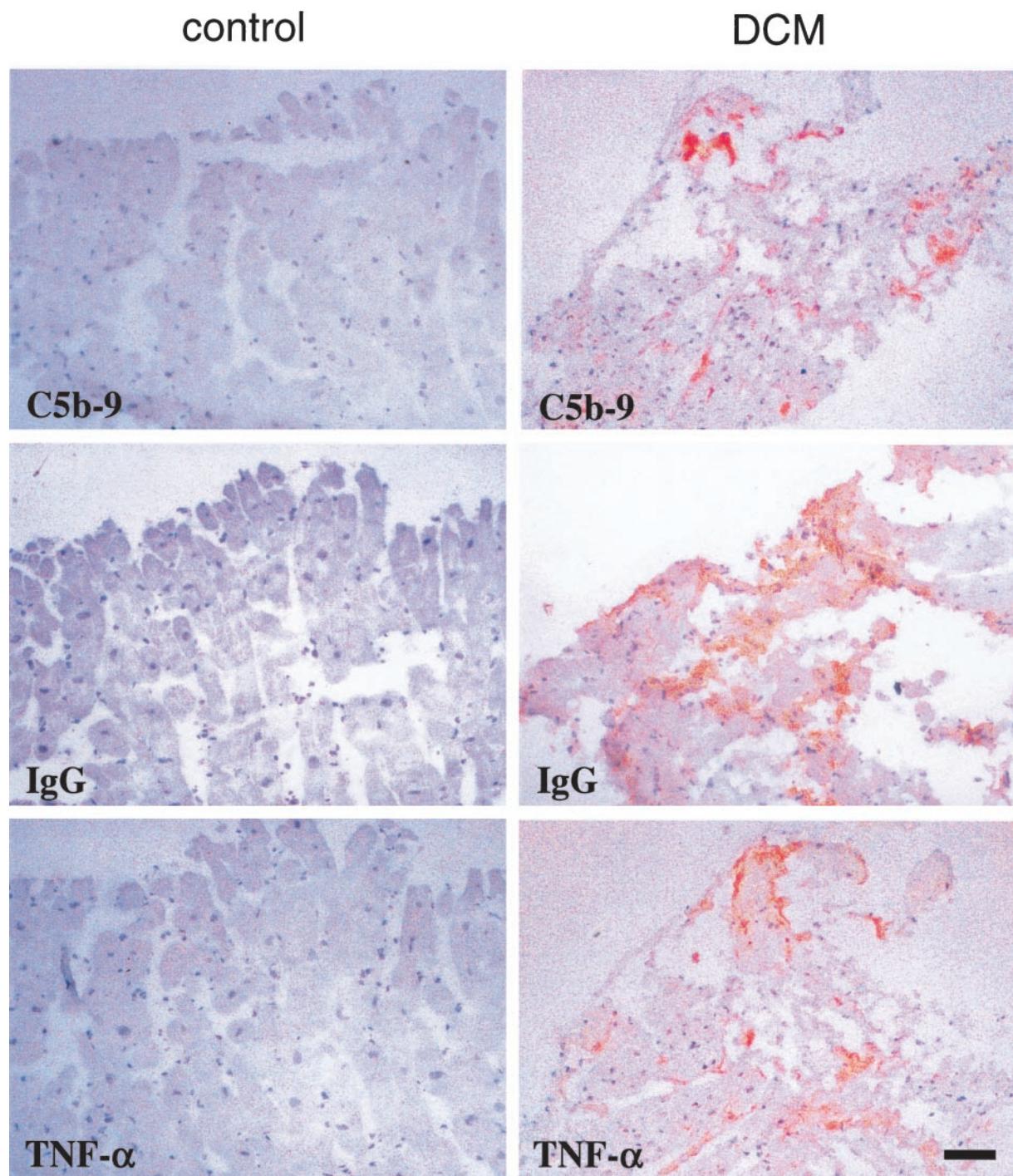


Figure 1. Immunohistochemical staining for C5b-9, IgG, and TNF- α showing sequential sections of myocardial biopsies from two representative patients—control versus sample (DCM). Original magnification, $\times 400$; scale bar, 50 μm .

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

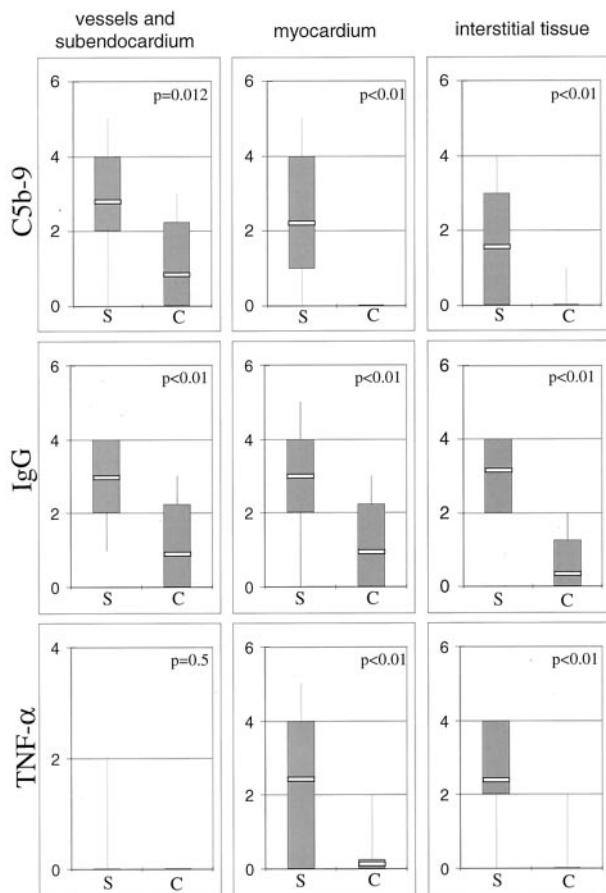


Figure 2. Box plots of semiquantitative analysis of myocardial tissue stained for human C5b-9, IgG, and TNF- α (S, samples, $n = 20$; C, controls, $n = 8$). Three groups of antigen localization were differentiated: vessels and subendocardium, myocardium, and interstitial tissue. Staining intensity (0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

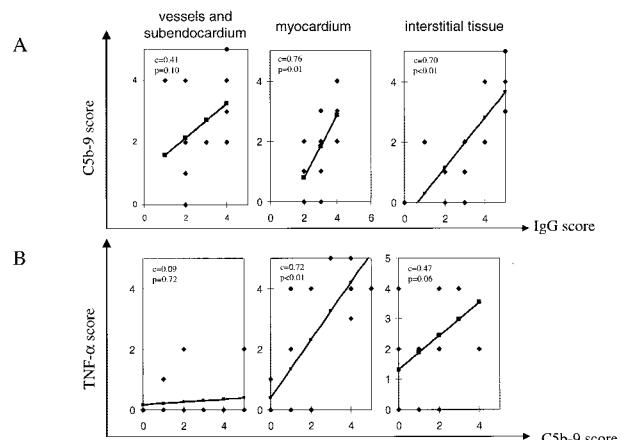


Figure 3. Correlation between stain of IgG, deposition of C5b-9, and expression of TNF- α in different tissue groups of antigen localization. Linear regression analysis expressed as scatter plots.

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).

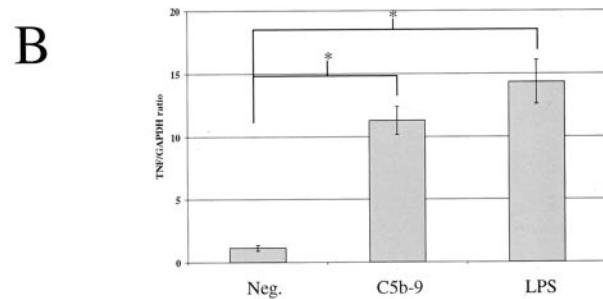
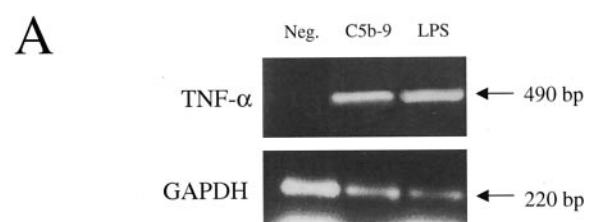


Figure 4. Quantitative RT-PCR evaluation of TNF- α mRNA expression. **A:** TNF- α mRNA expression in cardiomyocyte culture after C5b-9 (100 MHD) attack or LPS (500 ng/ml) stimulation. Unstimulated cells (Neg.) served as negative control and the expression of GAPDH gene as PCR positive control. **B:**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

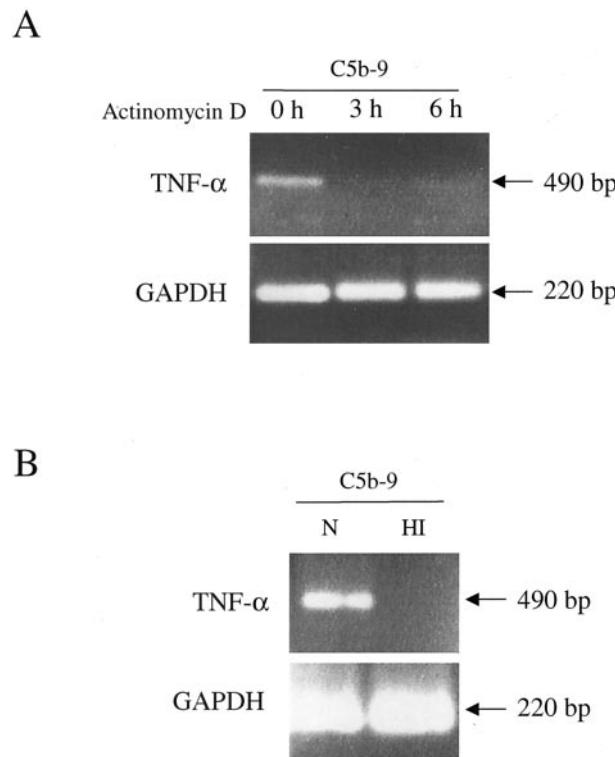


Figure 5. Quantitative RT-PCR assessment of TNF- α mRNA half-life. **A:** Co-incubation of cells with C5b-9 and Actinomycin D, nonspecific transcription inhibitor, shows time-dependent decrease in TNF- α mRNA synthesis. **B:** Heat inactivation (HI) of complement components abolished the TNF- α mRNA 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

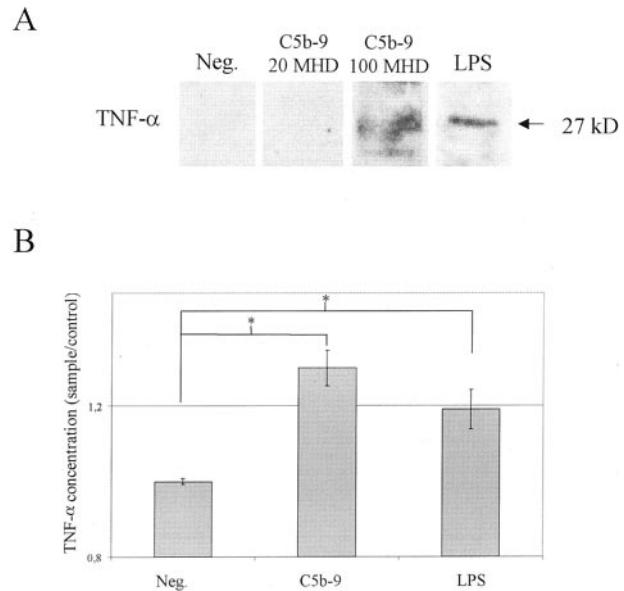


Figure 6. Immunoprecipitation and Western blot analysis of TNF- α in cardiac myocytes. **A:** Synthesis of 27-kd transmembrane TNF- α -protein at 100 MHD of C5b-9 and LPS stimulation, no synthesis at 20 MHD of C5b-9 or by unstimulated cells (Neg.). **B:** ELISA quantification of TNF- α in cell culture 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-

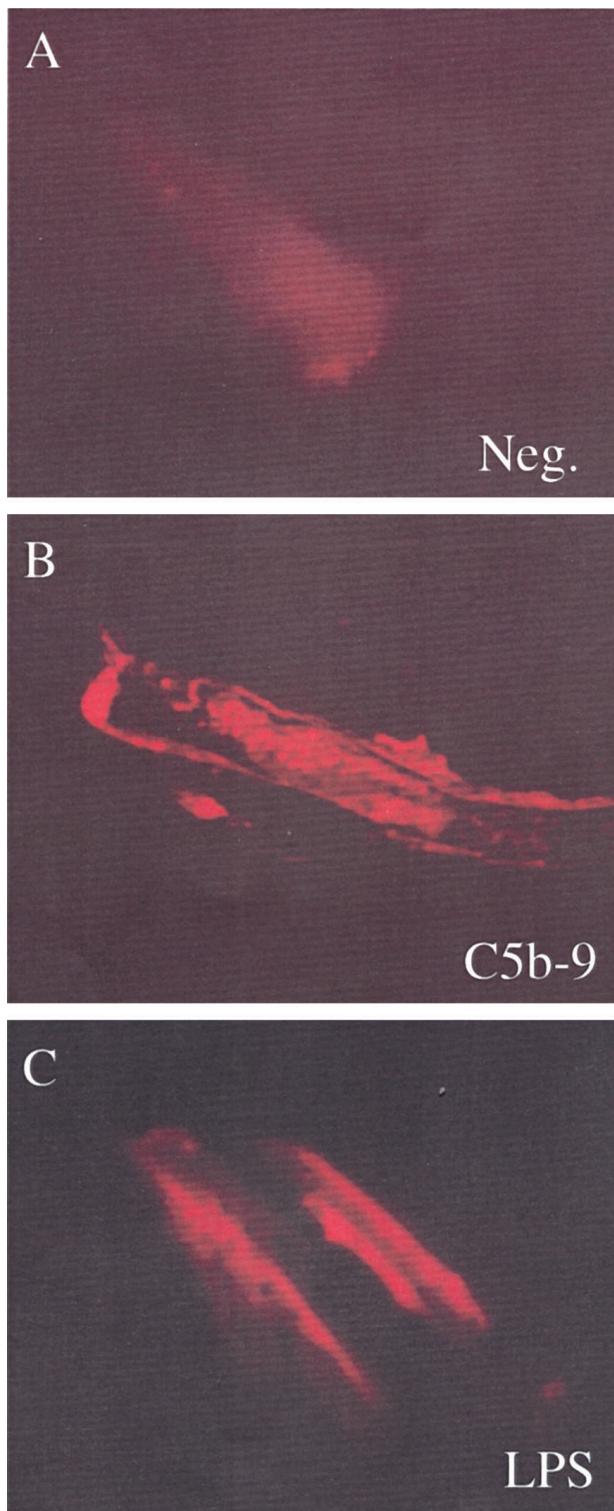


Figure 7. Immunofluorescent staining of TNF- α in cultured cardiac myocytes. Unstimulated cells (Neg.) did not reveal any immunofluorescent staining (**A**). Intense cell membrane-focused positive staining of cells stimulated with C5b-9 (**B**) or LPS (**C**). Original magnifications, $\times 400$.

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).

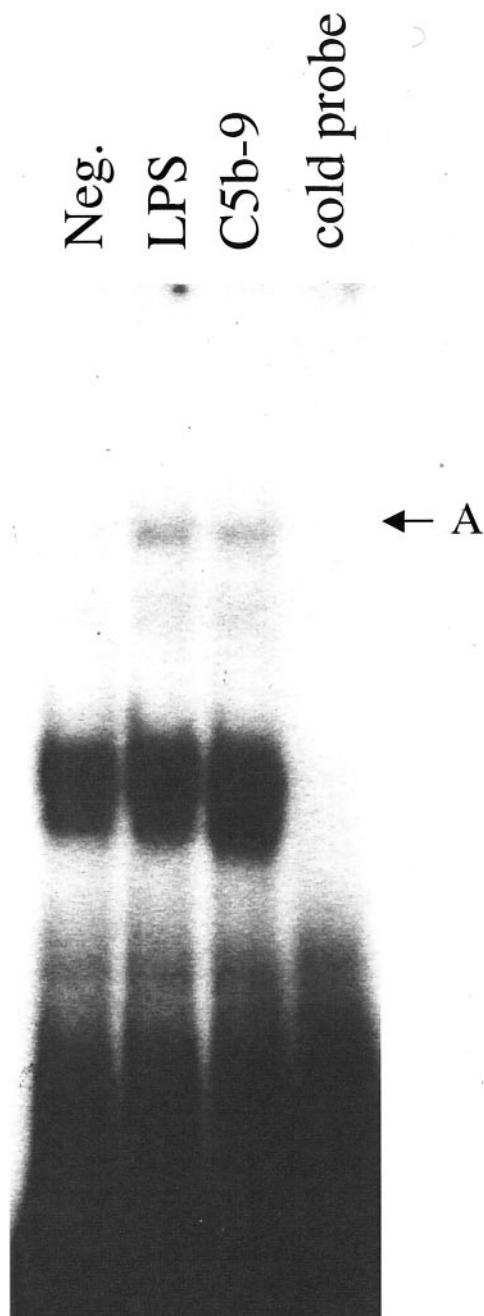


Figure 8. The terminal complement complex C5b-9 induces NF- κ B activation in cardiac myocytes. Unstimulated cells did not show binding of NF- κ B proteins to the oligonucleotide. Stimulation with 100 MHD of C5b-9 and LPS induced DNA-protein complex assembly (**arrow, A**). Specificity of the detected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

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1.4 Commentary:

Cardiomyopathy is linked to complement activation

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Commentary

Cardiomyopathy Is Linked to Complement Activation

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Cardiomyopathies are traditionally divided into dilated cardiomyopathy (DCM), restrictive cardiomyopathy, and hypertrophic cardiomyopathy. DCM is characterized by depressed systolic function and an increase in the size of the left ventricle usually involving dilatation of all four chambers.¹ DCM represents a common cause of congestive heart failure and frequently leads to cardiac transplantation.² Approximately 30 to 40% of patients with DCM have a familial form of the disease involving mutations in genes coding for cytoskeletal and sarcomeric proteins.^{2,3} The remaining cases are idiopathic, many of which are believed to be due to preceding myocarditis, particularly virus-induced. The detection of a cardiotropic virus, such as coxsackievirus and adenovirus, in endomyocardial biopsy specimens from patients with DCM supports this hypothesis.^{4,5} The presence of a virus in the myocardium has been shown to be associated with a poorer prognosis in DCM.^{6,7}

Animal Models

The ability of cardiotropic viruses to cause myocarditis and subsequently trigger an autoimmune response to autologous cardiac tissue has been demonstrated in animal models.^{8–10} In susceptible mouse strains such as BALB/c, coxsackievirus B3 (CB3) induces myocarditis which consists of an early, viral phase followed by a late, autoimmune phase.¹⁰ On the other hand, C57BL/6 mice develop only early, viral disease, suggesting the importance of the genetic constitution of the host in determining the progression from an infectious to an autoimmune process. In BALB/c mice, the autoimmune phase of myocarditis (which typically occurs 28–56 days after infection) is characterized by the absence of infectious virus in the heart; however, the viral genome may persist in a small proportion of cardiomyocytes.⁹ Similar disease can be induced in mice by murine cytomegalovirus¹¹ and encephalomyocarditis virus,¹² also leading to a late, autoimmune phase, in BALB/c but not in C57BL/6.

Many cases of myocarditis in humans have been associated with autoantibodies to cardiac myosin (CM).^{13,14} CM immunization of mice induces autoimmune myocarditis,¹⁵ which resembles both human autoimmune myocarditis and the autoimmune phase of murine virus-induced myocarditis. Interestingly, mice that are susceptible to CB3-induced myocarditis also exhibit susceptibility to CM-induced disease. Similarly, mice that are resistant to CB3-induced myocarditis demonstrate resistance in the CM model. The advantage of the CM-induced model is the ability to study the effects of different interventions on a purely autoimmune process without the associated complication of a viral infection. Moreover, CM immunization might provide a better model for those myocarditides that are not associated with viral infections, such as giant cell myocarditis.

Similar to humans, mice with myocarditis can progress to DCM and end-stage heart failure. Some mice, either infected with a cardiotropic virus or immunized with CM, develop enlarged hearts with dilated left and right ventricular cavities.^{12,16} Such morphological presentation of DCM is accompanied by impairment of cardiac function. Echocardiographic examination reveals increased left-ventricular chamber dimensions, decreased fractional shortening, and global left-ventricular wall hypokinesis, classic hallmarks of DCM.¹⁷ Pressure-volume relations obtained by means of left-ventricular catheterization further confirm the diagnosis of DCM in a subgroup of mice immunized with CM (Figure 1). These mice demonstrate increased left-ventricular volumes, decreased end-systolic pressure, decreased cardiac output, pronounced depression of systolic function manifested by reduced slope and rightward shift of the end-systolic pressure-volume relation (ESPVR), reduced maximal rate of pressure development (dP/dt_{max}), and reduced stroke work-end diastolic volume relation. Systolic dysfunction is accompanied by impairment of diastolic function with significantly increased passive stiffness (β), prolonged time constant of pressure relaxation (τ), decreased peak filling rate, and increased end-diastolic pressure

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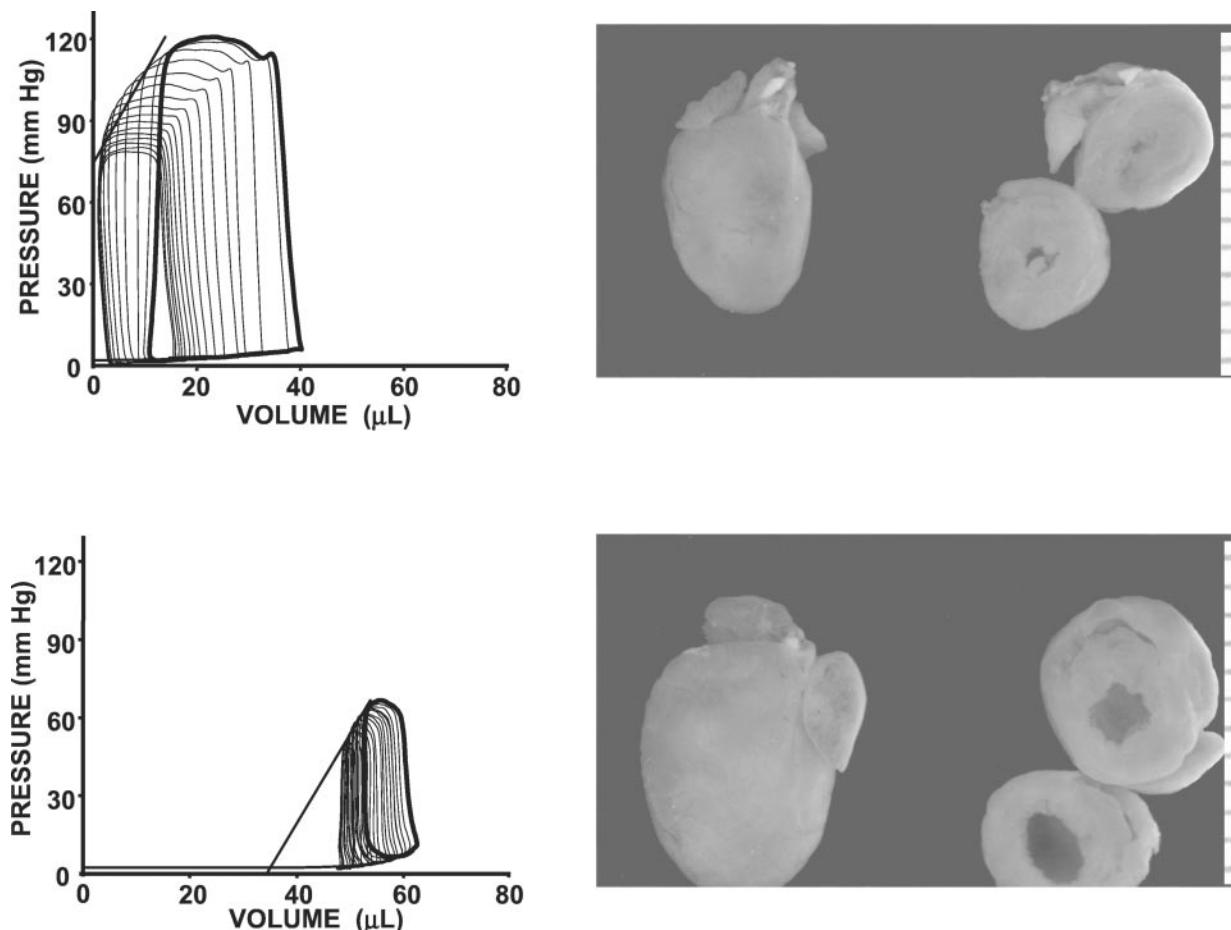


Figure 1. DCM in murine model of experimental autoimmune myocarditis. Multiple pressure-volume loops were derived by acutely reducing end-diastolic volume through transient occlusion of the inferior vena cava. **Top:** Normal mouse heart and corresponding pressure-volume relations. **Bottom:** A heart and pressure-volume relations represent day 32 after immunization with CM. DCM is manifested by the enlarged cavity of the left ventricle. The pressure-volume relations demonstrate large volumes, reduced end-systolic pressure, depressed and rightward shifted end-systolic pressure-volume relations (ESPVVR), and reduced stroke volume (the width of a loop). The line connecting the upper left corners of the loops represents ESPVVR. The slope of this line represents a load-independent measure of systolic function.

(Figure 1).¹⁷ These functional changes are virtually identical to human DCM. Thus, experimental autoimmune myocarditis in the rodent provides an excellent model to study both immunological and hemodynamic aspects of inflammatory heart disease, including DCM.

Complement: An Overview

Complement (C), or alexin, was first described in the second half of the 19th century as a heat-labile component of blood with bactericidal and hemolytic properties.¹⁸ Currently, complement is known to represent a complex system consisting of over 30 proteins.¹⁹ These proteins interact with one another to initiate and propagate a cascade of enzymatic activation leading to the non-enzymatic assembly of the membrane attack complex on the surface of target cells causing cell lysis. Three modes of complement activation have been described: the classical, lectin, and alternative pathways. The classical pathway, the first to be described, is initiated by IgM or IgG antibody bound to its cognate antigen. It is now known that some other substances, including C-reactive

protein (CRP),²⁰ can also initiate classical pathway. The cascade starts with activation of the C1 complex. Antibody, or sometimes other substances, binds to C1q and induces its conformational change leading to subsequent activation of the two enzymes, C1r and C1s; the latter then sequentially cleaves C4 and C2. The lectin pathway, the last to be described, is initiated in an antibody-independent fashion by mannose and N-acetyl glucosamine residues present abundantly in bacterial cell walls.²¹ These residues are recognized by mannose binding lectin (MBL), which has a structure similar to that of C1q and which activates two associated serine proteases, MASP-1 and MASP-2. These proteases, which are homologous to C1r and C1s, cleave C4 and C2. Thus, MBL and the associated proteases represent a C1-like complex that initiates events similar to that of the classical pathway. Other lectins can trigger complement activation in a fashion similar to MBL.

Both the classical and lectin pathways result in cleavage and activation of C3 and, at this step, they converge with the alternative pathway. Antibody-independent alternative pathway does not involve C1, C2, or C4, but starts

with an activation of C3. This activation step is a result of spontaneous hydrolysis of the thioester in C3 producing C3b. C3b may deposit on host cells, but this deposition normally does not lead to further activation of the complement cascade because factor B does not bind to C3b and surface-bound C3b is degraded by factors I and H. However, C3b deposition on microorganisms or foreign cells favors binding of factor B and initiates subsequent events in the cascade leading to cleavage of factor B by factor D, formation of C3 convertase (C3bBb) and further cleavage of C3. The classical and lectin pathways involve C4b2a as C3 convertase, which acts like C3bBb. All three pathways result in cleavage and activation of C5 involving somewhat different C5 convertases. Cleavage of C3 and C5 produces the anaphylatoxins, C3a and C5a, which act as strong chemoattractants. C5a is the more potent chemoattractant, recruiting neutrophils, monocytes, basophils, and eosinophils.¹⁸ Engagement of the C5a receptor on monocytes activates NF- κ B and triggers production of interleukin (IL)-1, IL-6, IL-8, and tumor necrosis factor (TNF)- α .^{19,22-25}

All pathways may eventually lead to the assembly of the membrane attack complex. C5b combines sequentially with terminal complement components, C6, C7, C8, and C9, resulting in a formation of a pore in the plasma membrane of a susceptible target cell. If sufficiently high densities of membrane attack complex are assembled on the cell surface, the cell is lysed. However, the terminal complement complex does not typically lyse allogeneic cells because of the presence of complement regulators, such as protectin (CD59) on the surface of these cells. In this case, sublytic amounts of membrane attack complex induce activation of the target cells.¹⁹ The liver represents the primary source of circulating complement components. Other sources of certain complement components include monocytes, macrophages, fibroblasts, endothelial cells, mucosal epithelial cells, and adipocytes.^{26,27} A simplified diagram of the three complement pathways is provided in Figure 2.

The Role of Complement in Heart Disease

Early Complement Components

The role of the adaptive immune system in the development of experimental autoimmune myocarditis has been extensively studied. It is now clear, however, that mediators generated during the innate immune response modulate the initiation of the subsequent adaptive immune reaction. Components of the complement system provide an important link between the innate and adaptive immune systems. Regardless of the pathway, activation of the complement cascade results in the formation of the active products of C3 cleavage. To better understand the role of complement system in the development of inflammatory heart disease, studies were performed addressing the effects of C3 on the autoimmune response to CM and subsequent development of disease in a murine model of CM-induced myocarditis.²⁸ C3 de-

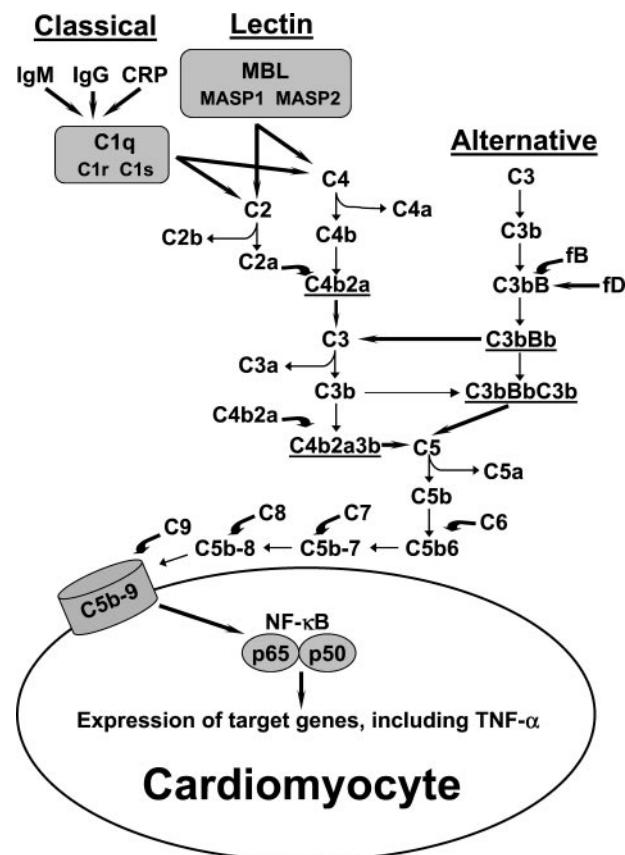


Figure 2. Pathways of complement activation and effects of membrane attack complex on cardiomyocytes. Enzymatic cleavages and activation are indicated by **thick arrows**. fB, factor B; fD, factor D. C3 and C5 convertases are **underlined**.

pletion through activation by cobra venom factor resulted in impaired IgG antibody responses to CM and prevention of myocarditis. It was critical to deplete C3 at the time of initiation of the immune response since multiple injections of cobra venom factor between days 1 and 9 after immunization, but not between days 10 and 18, were effective in preventing myocarditis. Blockade of the C3 receptors, complement receptor 1 (CR1), or CD35, and complement receptor 2 (CR2), or CD21, with a monoclonal antibody (mAb) that binds to the extracellular domain shared by the two receptors, led to the abrogation of disease and dramatically reduced the production of CM-specific IgG. CM immunization of mice genetically deficient in both CR1 and CR2 further confirmed that these complement receptors are required for the development of autoimmune myocarditis. These studies involved A/J mice, which harbor a spontaneous deletion in the gene encoding C5 rendering them deficient in the functional C5 protein.²⁹ Due to this deficiency, A/J mice enable the separation of the effects of early complement components from those exerted by the membrane attack complex, C5b-9. Binding of the products of C3 cleavage, C3d in particular, to CR1 and CR2 has been shown to be important for antigen presentation to B cells and subsequent B cell activation and antibody production.³⁰ Additionally, it was demonstrated that these receptors are present on a subset of activated/memory

CD44^{high}CD62L^{low} T cells and their engagement triggers T cell responses.²⁸ T cell expression of CR1/CR2 was required for optimal up-regulation of CD69 or CD25 in response to *in vitro* stimulation with antigen in the presence of either macrophages or B cells.²⁸ Recently, Pratt and colleagues³¹ further confirmed the observation that murine T cells up-regulate CR1/CR2 on activation and that these receptors are important for effective T cell responses. They found that CR1/CR2-expressing T cells belong to CD4⁺ population, whereas CD8⁺ T cells do not express these receptors.

In addition to being involved in exacerbation of myocarditis, complement mediates myocardial damage associated with other heart diseases, such as myocardial infarction. It has been shown that complement deposits on injured cardiomyocytes within the infarcted area.³² The pathogenic role of complement in mediating ischemic injury and increasing infarct size has been demonstrated in a number of experimental studies.³³⁻³⁸ CRP, which activates complement through initiation of the classical cascade by binding to C1q,¹⁹ has been shown to colocalize with complement in infarcted myocardium.³² Griselli and colleagues³⁹ have demonstrated that CRP-induced complement activation contributed to myocardial damage in a rat model of acute myocardial infarction. Furthermore, complement has been implicated as a mediator of reperfusion injury.^{40,41}

Membrane Attack Complex C5b-9

In this issue of *The American Journal of Pathology*, Zwaka and colleagues⁴² explore the role of the terminal membrane attack complex of complement, C5b-9, in the development of DCM. They report that myocardial biopsy specimens from patients with DCM stained positively for IgG, C5b-9, and TNF- α by immunohistochemistry. The diagnosis of DCM was based on clinical and echocardiographic features of heart failure unrelated to coronary artery disease, valvular or congenic defects, and supported by histological evidence of myocardial fibrosis, infiltration, hypertrophy, and myocyte degeneration. Control specimens, obtained from patients with similar clinical and echocardiographic manifestations but no histological evidence of myocardial disease, were negative for IgG, C5b-9, or TNF- α . The authors further demonstrated that the amount of C5b-9 found in the myocardium correlated with TNF- α production. They suggest that sublytic amounts of C5b-9 attached to the surface of cardiomyocytes trigger intracellular signaling which leads to TNF- α production thereby promoting the development of cardiomyopathy and heart failure. To test this hypothesis, the authors undertook a series of *in vitro* experiments using rat cardiomyocytes and found that C5b-9 induces both mRNA and protein synthesis of TNF- α . Using the electrophoretic mobility shift assay, Zwaka and colleagues also observed that C5b-9 induces NF- κ B activation, an important mediator of TNF- α gene expression.

The importance of TNF- α in heart disease has been demonstrated in a number of animal models as well as in clinical studies.^{43,44} Being a potent proinflammatory cy-

tokine, TNF- α is involved in the development of inflammatory processes in the heart leading to myocarditis and its sequelae, such as DCM. Using a murine model of CM-induced myocarditis, Smith and colleagues⁴⁵ demonstrated that anti-TNF mAb reduced the severity of myocarditis and Bachmaier and colleagues⁴⁶ showed that mice genetically deficient in the TNF receptor subunit p55 are resistant to the induction of the disease. The ability of splenocytes from immunized mice to produce TNF- α on *in vitro* stimulation correlates with the severity of CM-induced myocarditis.⁴⁷ The pathogenic role of TNF in the development of cardiac inflammation has also been confirmed in CB3-induced murine myocarditis.^{48,49} In addition to its proinflammatory effects, TNF- α exerts direct negative inotropic effects and promotes cardiomyopathy.⁵⁰⁻⁵² Patients with congestive heart failure have increased levels of circulating TNF- α and greater expression of this cytokine in the myocardium.⁵³ Increased myocardial expression of TNF- α has been observed in response to increases in wall stress caused by either volume or pressure overload.^{54,55} Chronic volume unloading by means of a left ventricular assist device in patients with congestive heart failure significantly reduced TNF- α levels in the myocardium as assessed by immunostaining of myocardial samples.⁵⁶ Similarly, decreasing the intraventricular pressure gradient with septal reduction therapy in patients with obstructive hypertrophic cardiomyopathy resulted in a significant decrease in TNF- α levels in the myocardium.⁵⁷ Reducing TNF- α levels with either a nonspecific agent, pentoxifylline, or the recombinant TNF- α receptor fusion protein, etanercept, has been successful in improving symptoms in patients with heart failure.⁵⁸⁻⁶⁰ Thus, the finding by Zwaka and colleagues⁴² that C5b-9 induces TNF- α production in cardiomyocytes provides an important link between complement activation and cardiac dysfunction in DCM.

The role of sublytic C5b-9 in producing acute metabolic and proinflammatory changes in target cells has also been studied in disorders other than heart muscle disease. The proinflammatory effects of the sublytic C5b-9 have been implicated in the pathogenesis of atherosclerosis and transplant rejection.^{19,27,61-64} C5b-9 has been shown to promote vascular injury and inflammation by activating vascular smooth muscle and endothelial cells. In response to C5b-9, endothelial cells release von Willebrand factor (vWF), P-selectin, and CD63 from the Weibel-Palade granules, produce IL-8, monocyte chemoattractant protein-1, fibroblast growth factor, and platelet-derived growth factor.^{19,65,66} Additionally, the terminal complement complex potentiates TNF- α -induced up-regulation of E-selectin and ICAM-1 on the surface of endothelial cells.⁶¹ These events lead to leukocyte adhesion, platelet aggregation, and endothelial cell injury, thereby facilitating the formation of atherosclerotic lesions and allograft rejection. Sublytic amounts of C5b-9 also induce proliferation of vascular smooth muscle cells resulting in neointimal thickening and vascular remodeling, characteristic of both atherosclerotic lesions and accelerated graft arteriosclerosis.⁶² The importance of the late complement components in mediating both

acute and chronic allograft rejection has been demonstrated using C6-deficient rats.^{27,64} Viedt and colleagues⁶³ demonstrated that C5b-9 induces IL-6 production in vascular smooth muscle cells. Consistent with the observation by Zwaka and colleagues⁴² that C5b-9 induces NF-κB in cardiomyocytes, the authors also found that C5b-9 signaling triggers activation of NF-κB. Viedt and colleagues⁶³ further found that NF-κB activation was required for IL-6 production, since blocking NF-κB activity either with its pharmacological inhibitor, pyrrolidine dithiocarbamate (PDTC), or with decoy oligonucleotides completely inhibited IL-6 release. NF-κB is a dimeric transcription factor formed by hetero- or homodimerization of protein members of Rel family, which include p65, RelB, cRel, p50, and p52.⁶⁷ The authors demonstrated that p65 and p50 subunits participated in DNA binding on induction by C5b-9. Furthermore, C5b-9 has been shown to activate NF-κB in endothelial cells leading to production of IL-8 and monocyte chemoattractant protein-1.⁶⁶ These findings suggest that C5b-9-induced NF-κB activation represents a general phenomenon independent of the target cells.

Concluding Remarks

Evidence has been rapidly emerging that inflammation and its mediators are involved in the pathogenesis of a number of heart diseases and facilitate the deterioration of heart function. Inflammatory mediators are capable of altering cardiomyocyte homeostasis leading to the activation of multiple signaling cascades, ultimately affecting myocardial function. The role of complement and C5b-9 in particular has been extensively studied in models of atherosclerosis and transplant rejection. Very little data, however, existed about the role of the terminal complement complex in inflammatory heart disease. The findings described by Zwaka and colleagues⁴² in this issue are the first demonstration of the ability of C5b-9 to induce NF-κB activation and TNF-α production in cardiomyocytes. These findings draw our attention to the importance of complement in DCM and provide a basis for new therapies targeting components of the complement system that may one day be included as a part of the usual regimen in the fight against the devastating and usually fatal course of heart failure.

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**2. C-reaktives Protein und Arteriosklerose:
Die Bedeutung der Interaktion von C-reaktivem Protein
mit dem Immunglobulinrezeptor Fc γ RIIa**

2.1 Einleitung

2.1.1 Atherogenese

Die Entstehung der Arteriosklerose wird als Atherogenese bezeichnet. Die Arteriosklerose ist die Grundlage kardiovaskulärer Erkrankungen, wie z. B. Myokardinfarkt, ischämischer Insult und periphere arterielle Verschlusskrankheit. Das pathophysiologische Merkmal ist die arteriosklerotische Läsion. Die arteriosklerotische Läsion entsteht, vereinfacht formuliert, durch Lipidablagerung, Einwanderung von Blutmonozyten/Makrophagen in die Gefäßwand und Proliferation glatter Muskelzellen in der Gefäßwand, insbesondere in der Tunica intima.

Die arteriosklerotischen Läsionen werden als Läsionen vom Typ I bis VI klassifiziert (1, 2). In den Initialstadien der Arteriosklerose sind in der Gefäßwand Lipidablagerungen und Monozyten/Makrophagen-Infiltrationen zu beobachten. Durch Aufnahme der abgelagerten Lipide wandeln sich die Makrophagen in Schaumzellen um. Fortlaufende Akkumulation von Lipiden, Schaumzellen und Proliferation glatter Muskelzellen kennzeichnen die späteren Stadien der Arteriosklerose. Durch die Proliferation glatter Muskelzellen wird die Struktur der Gefäßwand aus Intima, Media und Adventitia unterbrochen. Zu klinischen Komplikationen der Arteriosklerose kommt es, wenn entweder das Gefäßlumen durch Wachstum der Läsion kritisch eingeengt wird, oder wenn die arteriosklerotische Läsion aufbricht (Ruptur) und sich Thromben auf der rupturierten Läsion ablagern.

Es existieren verschiedene Hypothesen zur Entstehung der Arteriosklerose.

Russel Ross postulierte 1973, dass die Proliferation glatter Gefäßmuskelzellen das entscheidende Merkmal arteriosklerotischer Läsionen ist (3). Später formulierte er die sogenannte *response to injury Hypothese*, die die endotheliale Dysfunktion als Auslöser der Arteriosklerose betrachtet (4, 5). Eine Schädigung des Endothels wird nach dieser Hypothese durch die führenden kardiovaskulären Risikofaktoren, wie Nikotinabusus, Hypertonie, Diabetes mellitus und Hyperlipoproteinämie, verursacht. An den Orten der endothelialen Verletzungen ist die Expression von Adhäsionsmolekülen auf Endothelzellen erhöht, was eine Infiltration von Leukozyten und Thrombozyten und eine Ablagerung atherogener

Faktoren, wie Lipoproteine, zur Folge hat. Diese Hypothese versteht die Atherogenese als Entzündungsprozess der Arterienwand.

Seit plasmatische LDL (*Low density lipoprotein*) - Erhöhungen als entscheidender Risikofaktor für die Arteriosklerose identifiziert wurden (6), werden die Mechanismen, über die LDL die Atherogenese induziert, systematisch untersucht. Dabei steht im Zentrum die Frage der Schaumzellbildung. Da natives LDL nicht zur Schaumzellbildung führt, weil LDL-Rezeptoren einem negativen Feedback unterliegen (7), wurde über alternative Mechanismen der Schaumzellbildung nachgedacht. Auf diese Weise entstanden die Konzepte des *scavenger-receptor pathways*, des *ox-LDL (oxidized LDL) pathways* und des *E-LDL (enzymatically modified LDL) pathways*.

Die sogenannten „*scavenger*“ (*Straßenkehrer*) - *receptors* wurden als Rezeptoren für modifiziertes LDL beschrieben (8, 9, 10). Für diese Rezeptoren sind eine Vielfalt von Liganden, wie Polyribonukleotide und bakterielle Polysaccharide, bekannt (11). Nach Aufnahme von Lipiden in die Zelle, werden diese durch Lysosomen hydrolysiert. Das freigesetzte Cholesterin wird im Zytosol reesterifiziert, wodurch massive Cholesterylester-Ablagerungen entstehen (12).

Die *ox-LDL* Hypothese, entwickelt von D. Steinberg, hebt in der Atherogenese die Schlüsselrolle einer oxidativen Modifikation von LDL, sogenanntes *ox-LDL*, hervor (13, 14). Nach dieser Hypothese wird LDL in Endothelzellen, glatten Muskelzellen und Makrophagen einer radikal-katalysierten Oxidation unterzogen, welche zu strukturellen Änderungen im LDL-Molekül führt. Ox-LDL hat einen entscheidenden Einfluss auf die vasomotorischen Eigenschaften und auf den Beginn und die Progression der Läsionen in der arteriellen Wand (15, 16).

Nach einer anderen Hypothese wird das LDL in der Gefäßwand enzymatisch modifiziert (*E-LDL*) und gewinnt dadurch atherogene Eigenschaften. Es handelt sich um ein dreifach verdautes LDL durch Trypsin, Cholesterinesterase und Neuraminidase. Es wurde nachgewiesen, dass E-LDL das Komplementsystem aktiviert (17) und Monozytenchemotaxis durch Expression von MCP-1 (monocyte chemotactic protein-1) induziert (18). E-LDL wird im Vergleich zu ox-LDL oder acetyliertem LDL (ac-LDL) stärker von Makrophagen aufgenommen. Aufgrund dessen spielt E-LDL nach dieser Hypothese eine große Rolle in der Bildung der subendothelialen Lipidablagerungen und späterer Läsionen.

In den letzten Jahren wurde die Rolle des Komplementsystems und des C-reaktiven Proteins (CRP) als Risikomarker und/oder als Risikofaktoren der Atherogenese untersucht. Ihre Funktion und Bedeutung wird in den kommenden Kapiteln besprochen.

2.1.2 C-reaktives Protein

Das C-reaktive Protein (CRP) ist ein evolutionär sehr altes, hochkonserviertes Molekül des Immunsystems. Man trifft ein analoges Molekül in primitiven Organismen wie den Pfeilschwanzkrebs *Limulus polyphemus* an (19).

CRP spielt eine Schutzrolle in der angeborenen Immunität, da es an Polysaccharide der bakteriellen Zellwand bindet (20) und es die Phagozytose an manche Arten von pathogenen Bakterien vermittelt (21). Es ist ursprünglich durch seine Fähigkeit definiert, an das C-Fragment der Polysaccharidkapsel von *Streptococcus pneumoniae* zu binden - daher der Name *C-reactive protein* (22).

CRP ist prototypisch ein Akute-Phase-Protein des Menschen, und seine Plasmakonzentration steigt als Antwort auf Entzündung, Infektion und Gewebeschaden bis auf das 1000-fach des Normwertes an (23).

Die CRP-Synthese findet in Hepatozyten statt und die Expression wird synergistisch von den Zytokinen Interleukin-1 (IL-1) und Interleukin-6 (IL-6) reguliert (24).

C-reaktives Protein ist ein Pentamer, bestehend aus fünf identischen Untereinheiten (Monomere), wobei jede Untereinheit ein Molekulargewicht von 23 kDa aufweist. CRP gehört zur Familie der Pentraxine (**Abb. 1**), zusammen mit Serum Amyloid P (SAP). Jede Untereinheit bindet Ca^{2+} -abhängig an Phosphocholine (PCh).

CRP bindet an verschiedene Liganden und biologische Partikel. Auf diese Weise wird die Opsonierung dieser Partikel in Analogie zu Immunoglobulinen vermittelt, oder es werden Signaltransduktionsprozesse induziert. Es bindet an beschädigte Zellmembranen und nukleare Strukturen. Durch sein Ca^{2+} -abhängige-PCh Bindungsstelle bindet es an Histone (25) und kleine nukleäre Ribonukleoproteine (snRNPs) (26).

Dank seiner Pentraxin-Struktur hat CRP zwei Flächen (**Abb. 2**) (27). An der Fläche, an der Ca^{2+} -Ionen gebunden sind, bindet es durch PCh an bakterielle

Wand- und Zellmembranstrukturen und auch an Lipoproteine. Die gegenüberliegende Fläche bindet Ca^{2+} -unabhängig an die C1q Komponente des Komplementsystems und möglicherweise an Fc γ -Rezeptoren von Leukozyten.

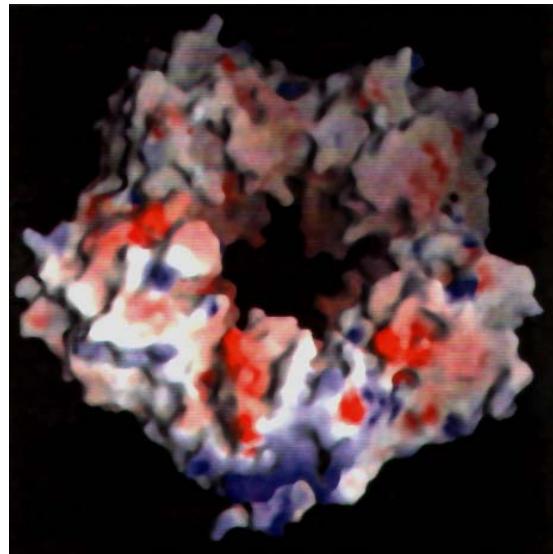


Abb. 1. 3-dimensionale Pentraxin-Struktur des C-reaktiven Proteins (23)

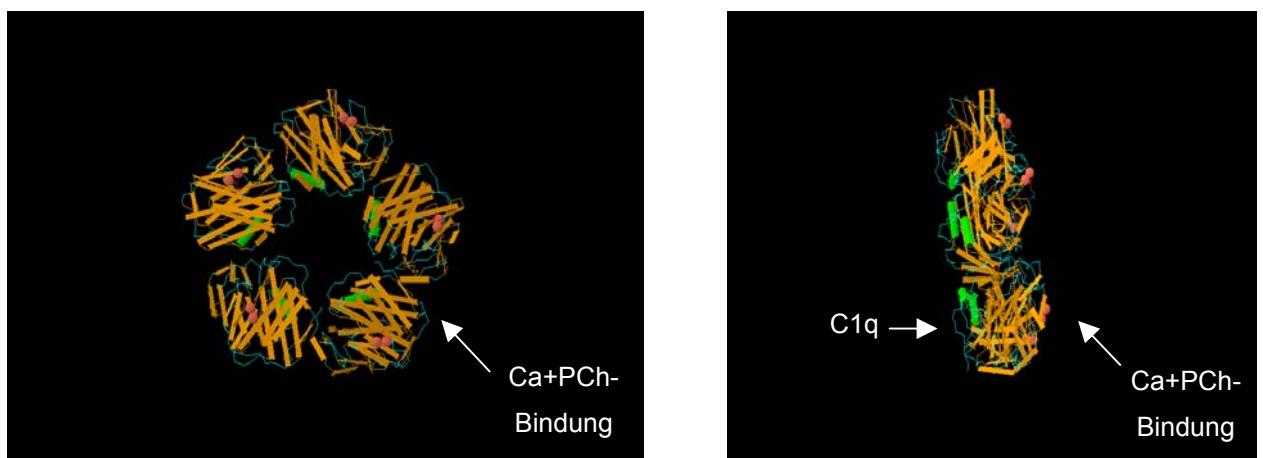


Abb. 2. Die CRP-Flächen: links – frontale Ansicht; rechts – seitliche Ansicht. Durch Ca-PCh bindet CRP von einer Seite an Membranstrukturen und Lipoproteine, von der anderen Seite bindet es an C1q und möglicherweise an Fc γ Rezeptoren.

An der CRP-Bindung von Zellen wird seit längerer Zeit geforscht. Es wurde ursprünglich demonstriert, dass CRP an nekrotischen Zellen oder beschädigte Zellmembranen (28-30) bindet, aber nicht an intakte Zellen. Es zeigte sich eine Bindung von CRP an PCh in beschädigten Zellmembranen. Dieser Prozess fordert

eine partielle Hydrolyse der Membranphospholipide (30). Es wurde auch berichtet, dass CRP an apoptotische Zellen bindet (31, 32).

2.1.3 CRP und Atherogenese

Die Rolle des CRP als Risikomarker für kardiovaskuläre Erkrankungen wurde in zahlreichen epidemiologischen prospektiven Studien bestätigt (33-38).

Wenn sowohl die CRP- als auch die Cholesterol-Spiegel in den obersten Quintilen liegen, ist das Risiko für das erste koronare Ereignis um das 8 bis 9-fache im Vergleich zu den niedrigsten Quintilen erhöht (34) (s. **Abb. 3**). Auch in Abwesenheit von erhöhten Cholesterol-Spiegeln findet sich ein erhöhtes Risiko bei erhöhten CRP-Plasmaspiegeln.

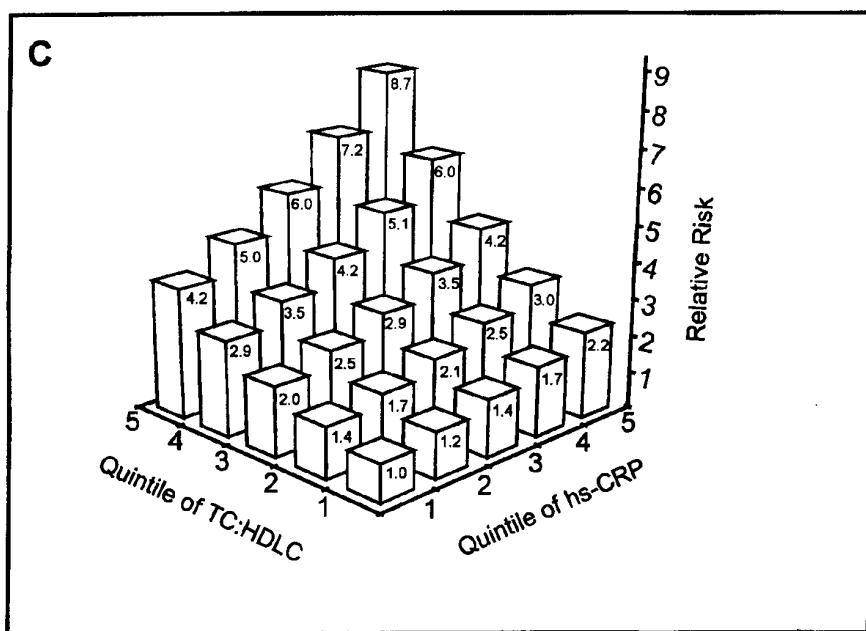


Abb. 3. Kardiovaskuläres Risiko bei erhöhten Cholesterol- und CRP-Plasmaspiegeln (34). Das Risiko für das erste kardiovaskuläre Ereignis ist am höchsten bei Menschen, die zu beiden obersten Quintilen gehören.

Aus diesen epidemiologischen Untersuchungen ergibt sich die Frage, ob CRP ein kardiovaskulärer Risikomarker oder ein Risikofaktor ist. Eine Reihe von Befunden spricht dafür, dass CRP eine aktive Rolle in der Atherogenese spielt.

Eine wichtige Frage im Kontext der Bedeutung von CRP als kardiovaskulärer Risikofaktor ist die Anwesenheit des Moleküls in arteriosklerotischen Läsionen. Da der erste Versuch, das Molekül in arteriosklerotischen Läsionen nachzuweisen, aus technischen Gründen fehlschlug (39), wurde die Hypothese, dass CRP aktiv in die Atherogenese involviert sein könnte, lange nicht beachtet. Einzelne Berichte über einen Nachweis von CRP in der Gefäßwand wurden aufgrund geringer Fallzahlen nahezu ignoriert (40, 41). Erst die epidemiologischen Untersuchungen der letzten Jahre haben erneut die Aufmerksamkeit auf dieses Thema gelenkt. Seit 1998 wurde CRP in mehreren experimentellen Untersuchungen definitiv in arteriosklerotischen Läsionen nachgewiesen (42-44).

Folgende Effekte von CRP, die für eine aktive Rolle des Moleküls in der Atherogenese sprechen, wurden nachgewiesen:

Es wurde gezeigt, dass CRP Ca^{2+} -PCh-abhängig an Apolipoprotein-B-haltige Lipoproteine wie zum Beispiel LDL oder VLDL bindet (45, 46). Unklarheit besteht darüber, ob die Interaktion von CRP in der Gefäßwand mit nativem LDL stattfindet, oder ob das Lipoprotein modifiziert sein muss. Es wurde in jüngster Zeit berichtet, dass CRP an E-LDL (47, 48) oder an ox-LDL bindet (49).

CRP aktiviert das Komplementsystem. Dies ist seit den 70-er Jahren des letzten Jahrhunderts bekannt (50). Tierversuche deuten auf die Rolle des Komplementsystems in der Atherogenese hin, da bei C6-defizienten Tieren im Rahmen einer fettreichen Diät vermindert Arteriosklerose auftritt (51, 52). Zudem wurde eine Ablagerung vom terminalen Komplementkomplexen (C5b-9) in der Gefäßwand bei Tieren mit Hypercholesterinämie nachgewiesen (53). Da eine CRP- und C5b-9 in arteriosklerotischen Läsionen menschlicher Koronararterien kolokalisieren, ist CRP möglicherweise eine wichtige komplementaktivierende Substanz in der Atherogenese (42).

Durch Chemotaxis-Studien wurde *in vitro* eine Migration von Monozyten nach einem CRP-Gradienten demonstriert. CRP und CD68 (Makrophagenmarker) werden in arteriosklerotischen Läsionen kolokalisiert aufgefunden (54). Im Kontext

der Monozyten-Infiltration als zentralem Prozess der Atherogenese (5) spielt CRP möglicherweise neben anderen Molekülen eine Rolle.

CRP vermittelt die Aufnahme von nativem LDL in Makrophagen (55). Daraus leitet sich eine mögliche Bedeutung von CRP im Rahmen der Schaumzellbildung ab. Dies ist von besonderem Interesse, da CRP ein bekanntes Opsonin ist und an Lipoproteine bindet (s.o.)

CRP induziert die Expression der Adhäsionsmoleküle ICAM-1, VCAM-1 und E-selectin in endothelialen Zellen (56). Da die vermehrte Expression von Adhäsionsmolekülen im Gefäßendothel ein wesentliches Merkmal der Anfangsstadien der Atherogenese (5, 57) ist, weisen diese Daten auf die direkte Rolle von CRP im Entzündungsverlauf der Atherogenese hin.

2.1.4 CRP-Rezeptor

Die entscheidende Funktion von CRP, biologische Partikel zu opsonieren, hat im Organismus eine doppelte Bedeutung: Einerseits erkennt und bindet CRP an Pathogene und an apoptotische und nekrotische Zellen des Wirts, um auf solche Weise ihre Eliminierung durch Rekrutierung des Komplementsystems und phagozytierende Zellen zu vermitteln. Andererseits hat CRP neben dieser protektiven Wirkung auch schädliche Effekte: die Evidenzen für die Rolle des CRP in der Atherogenese sind hierfür ein Beispiel.

Sollte CRP eine aktive Rolle in der Atherogenese spielen, könnte die Frage nach dem CRP-Rezeptor auf Leukozyten von klinischem Interesse werden. Eine medikamentöse Intervention durch Rezeptorinhibition wäre denkbar.

Noch in den 60-er und 70-er Jahren des 20. Jahrhunderts wurde über den Effekt von CRP berichtet, Phagozytose zu induzieren (58-60). Eine CRP-Bindung an verschiedene Arten von Leukozyten wurde demonstriert (58-63). Einige Forschungsgruppen, die in dieses Thema involviert sind, wie z. B. die Arbeitsgruppe um R. Mortensen (Columbus, OH, USA), führten zahlreiche Arbeiten aus, welche die Existenz eines eigenen CRP-Rezeptors postulierten (60, 64-68). Obwohl viele funktionelle Evidenzen darüber existieren, dass CRP mit

Leukozyten interagiert, wurde bislang keine klare Definition eines spezifischen CRP-Rezeptors gewonnen.

Andere Forschungsgruppen haben über die Bindung von CRP an Fc-Rezeptoren berichtet.

Die Fc-Rezeptoren (FcRs) sind eine Familie von Molekülen, die spezifisch an den Fc-Teil von bestimmten Antikörper-Isotypen binden (69). Die FcRs-tragenden Zellen werden aktiviert, wenn ihre Fc-Rezeptoren mit Antikörper-Antigen-Komplexen assoziiert sind. Es gibt fünf Klassen von Antikörper-Isotypen – Immunoglobulin (Ig) A, IgG, IgD, IgE und IgM. Sie unterscheiden sich in der Struktur ihrer schweren Ketten, *heavy chains (H-chains)*, ihrer Verbreitung und Funktion. Fc γ -Rezeptoren, die im Kontext der CRP-Bindung diskutiert werden, sind IgG-Rezeptoren. Es sind drei Klassen von Fc γ -Rezeptoren bekannt - Fc γ RI (CD64), Fc γ RII (CD32) und Fc γ RIII (CD16).

Die Arbeitsgruppe von T.W. Du Clos (Albuquerque, NM, USA) berichtete 1999, dass Fc γ RIIa (CD32) der wichtigste Rezeptor für CRP ist (70). Es wurden CRP-Bindungsstudien an Fc γ RIIa-transfizierten COS-7 Zellen durchgeführt. Die Zellen wurden mit CRP inkubiert, mit anti-CRP Antikörpern gefärbt und durch FACS Analysen ausgewertet. Die Ergebnisse sprachen für eine Bindung von CRP an Fc γ RIIa. Die gleiche Arbeitsgruppe berichtete bereits zuvor über eine CRP-Bindung an die U-937 Zelllinie und an Fc γ RI (71, 72). Später wurde eine Bindung von humanem CRP an Maus-Fc γ Rs (73) dargestellt.

Fc γ RIIa ist der niedrigaffine Rezeptor für IgG₁ an Monozyten, Neutrophilen, B-Zellen und Eosinophilen. Er besteht aus zwei extrazellulären, einer transmembranösen und einer intrazellulären Domäne (**Abb. 4**). Es existiert ein Polymorphismus für Fc γ RIIa, der durch einen Austausch von G nach A an DNA-Position 506 definiert ist. Es resultiert ein Austausch der Aminosäure Arginin (R) zu Histidin (H) in Protein-Position 131 des Rezeptormoleküls. Dieser Polymorphismus entscheidet über die Affinität des Rezeptors zu IgG. Die sogenannte „*high responder form*“, Fc γ RIIa-RR-131 besitzt eine hohe Affinität zu

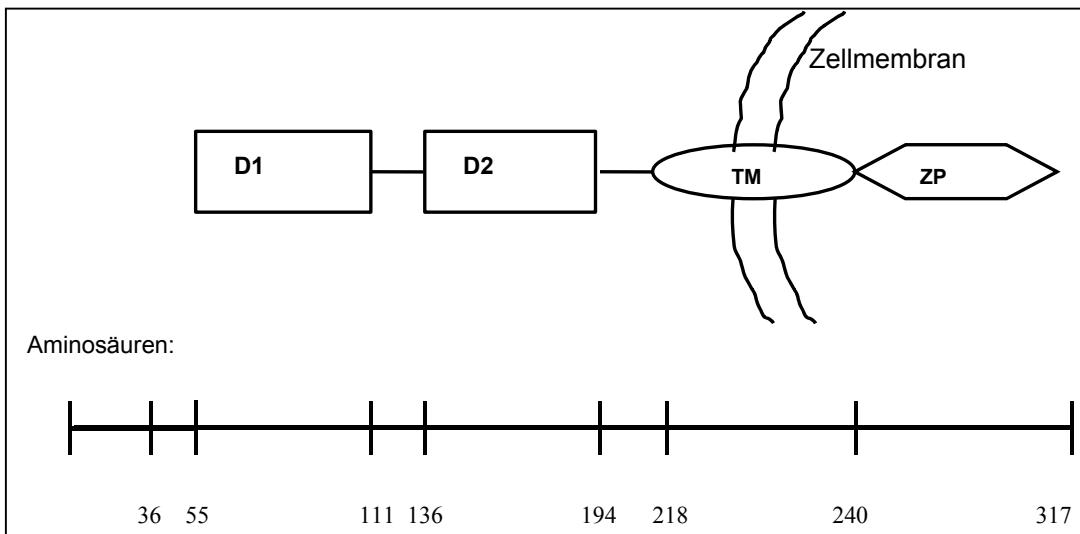


Abb. 4. Fc γ RIIa Domänen Struktur: D1 – IgG-ähnliche C2 Typ Domäne 1; D2 – IgG-ähnliche C2 Typ Domäne 2; TM – transmembranöse Domäne; ZP – zytoplasmatische Domäne

IgG, während die sogenannte „low responder form“ Fc γ RIIa-HH-131 eine niedrige Affinität aufweist (74-77). Die klinische Rolle dieses Polymorphismus als Risikomarker wurde für verschiedene Krankheiten wie zum Beispiel *Lupus erythematosus* sowie für die Heparin-induzierte Thrombozytopenie untersucht (78, 79).

Nachdem Fc γ RIIa als zellulärer CRP-Rezeptor definiert worden war, berichtete die Arbeitsgruppe von T.W. Du Clos im Jahr 2000 über signifikante Unterschiede für die CRP-Bindung an die verschiedenen Fc γ RIIa-Alellen (80). CRP-Bindungsstudien an Monozyten und Neutrophilen von gesunden, freiwilligen Probanden zeigten eine hohe Affinität von CRP für die RR-131 Fc γ RIIa homozygote Form. Die CRP-Bindung an die HH-131 Fc γ RIIa homozygote Form war nach diesen Berichten kaum zu detektieren. Bei der heterozygoten RH-131 Form wurde eine intermediäre Bindung demonstriert. Es wurden große Affinitätsunterschiede aufgezeigt (RR : RH : HH = 290 : 134 : 1).

Die Evidenz, dass Fc γ RIIa der wichtigste Rezeptor für CRP ist (70), unterlag starker Kritik, da in der Orginalarbeit Antikörper (IgGs) verwendet wurden, um eine Bindung an Fc γ Rezeptoren zu detektieren. In CRP-Bindungsstudien an polymorphnukleären Leukozyten und Fc γ RIIa-transfizierten IIA1.6-Zellen mit

anschließender FACS Analyse, wurden F(ab')₂-Fragmente von anti-CRP Antikörper (maus-IgG₁) zur Färbung verwendet (81). Mit diesen F(ab')₂-Fragmenten wurde keine positive Anfärbung detektiert, die einer CRP-Bindung entsprechen würde. Es wurde vermutet, dass die intakte Fc-Region der anti-CRP Antikörper entscheidend für die Detektion einer CRP-Bindung an Fc γ RIIa sei.

Darüberhinaus wurde von anderen Autoren berichtet, dass die beobachtete CRP-Bindung an Fc γ RIIa (80) die Folge einer IgG-Kontamination des verwendeten CRP-Reagenzes ist (82).

Hinweise, dass Fc γ RIIa der CRP-Rezeptor ist, sind nicht nur auf Ergebnisse von direkten Bindungsstudien beschränkt, sondern es gibt zusätzlich funktionelle Nachweise für eine CRP-Fc γ RIIa Interaktion. Durch *in-vitro* Experimente mit verschiedenen Zellarten wurden Fc γ RIIa-vermittelte CRP-induzierte Effekte demonstriert, wie zum Beispiel die Phosphorylierung gewisser Enzyme und ihre Translokation in der Zelle (83), Steigerung des intrazellulären Ca²⁺ (80, 83) und Aufnahme von Lipoproteinen (55). Durch Experimente mit γ -Kette-defizienten Mäusen wurde, im Vergleich zu Wildtypmäusen, das Fehlen einer Phagozytose CRP-opsonierter Liganden demonstriert (84).

Im Kontext der Evidenzen für eine Involvierung des CRP in die Pathogenese verschiedener Krankheiten, einschließlich Arteriosklerose, steigt das Interesse für eine eindeutige Identifizierung des wichtigsten Rezeptors für CRP.

2.1.5 Fragestellung

Das Ziel dieser Arbeit war ursprünglich, die Bindungsstelle von Fc γ RIIa an CRP zu finden. Dazu wurden trunkierte Formen („*truncated forms*“) von Fc γ RIIa (CD32) generiert und in Transfektionsvektoren kloniert. Die trunkierten Fc γ RIIa Formen umfassen einzelne Domänen des vollständigen Rezeptors. An Zellen, die mit diesen Vektoren transfiziert werden sollten, waren CRP-Bindungsstudien geplant, um die Bindungsstelle von CRP auf Domänenebene zu analysieren.

Unsere Bindungstudien mit Fc γ RIIa-transfizierten Zellen deuteten darauf hin, dass CRP nicht an Fc γ RIIa bindet.

In dieser Arbeit wurden daher antikörper-abhängige und antikörper-unabhängige Verfahren entwickelt, um die CRP-Bindung an verschiedenen Fc γ RIIa-Alellen zu untersuchen.

Konkret wurden folgende Fragen gestellt:

In antikörper-abhängigen Methoden:

- Ist CRP die Ursache für die hohe Anfärbung bei der Detektion der CRP-Bindung an Fc γ RIIa, oder ist die Bindung des anti-CRP Antikörpers an Fc γ RIIa für das hohe Fluoreszenzsignal verantwortlich?
- Ist eine IgG-Kontamination des CRP-Reagenzes für die Beobachtung einer CRP-Bindung verantwortlich?
- Wie beeinflussen die Fc γ RIIa-Allelen die Detektion der CRP-Bindung?

In antikörper-unabhängigen Methoden:

- Lässt sich durch radioaktive Markierung oder Fluoreszeinmarkierung von CRP eine Bindung des Moleküls an Fc γ RIIa direkt detektieren?
- Gibt es signifikante Unterschiede für die CRP-Bindung an verschiedenen Fc γ RIIa Allelen in antikörper-unabhängigen Verfahren?

2.2 Material und Methoden

2.2.1 Reagenzien

CRPs:

ppCRP - „ <i>partially purified</i> “	Sigma, Taufkirchen;
hpCRP - „ <i>highly purified</i> “	Aus humaner pleuraler Flüssigkeit, aufgereinigt, freundlicherweise von Prof. Terry W. Du Clos (Universitiy of New Mexico, Albuquerque, NM, USA) zur Verfügung gestellt
rCRP - <i>recombinant</i> CRP	Calbiochem, Bad Soden
F-CRP - <i>Fluorescein</i> -CRP	Markiert in unserem Laboratorium mittels <i>Alexa Fluor 488, protein-</i> <i>labeling kit</i> (s. unten)
125I-CRP - <i>iodinated</i> CRP	Jodiert durch <i>Chloramine-T</i> Methode in der Zentralen Einrichtung für Isotopenanwendung, Universität Ulm

Immunglobuline und Antikörper:

human IgG	Sigma
mouse IgG ₁ -PE	BD Biosciences, Heidelberg
monoclonal anti-CRP, clone 2C10	Freundlicherweise von Dr. Larry Potempa (Immtech, Evanston IL, USA) zur Verfügung gestellt
anti-CD32-FITC, Klon FL8.26 (2003)	BD Biosciences
goat-anti-mouse F(ab) ₂ -PE	Caltag Labs, Hamburg
an-V5-HRP	Invitrogen, Groningen (NL)
anti-human IgG-HRP (Fc-specific)	Dianova, Hamburg

Kits:

pcDNA3.1 Directional TOPO Expression Kit	Invitrogen
Alexa Fluor 488, protein-labeling kit	Mo Bi Tec, Göttingen (Molecularprobes, USA)

RNeasy Mini Kit	QIAGEN, Hilden
Omniscript Reverse Transcriptase Kit	QIAGEN
Pfu DNA Polymerase	Stratagene, Amsterdam (NL)
QIAquick Gel Extraction Kit	QIAGEN
Qiaprep Spin Miniprep Kit	QIAGEN
Hi Speed Plasmid Maxi Kit	QIAGEN

Enzyme:

T4 DNA Ligase, Xho I, Hind III, Nhe I, Pme I	NewEngland Biolabs, Frankfurt am Main
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Zellkultur:

COS-7 Zellen	DSMZ – Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig
Dulbecco's Mod Eagle Medium (DMEM)	Invitrogen
Phosphat Buffer Saline (PBS)	Invitrogen
PolyFect Transfection Reagent	QIAGEN
Fetal Calb Serum (FCS)	PAA Labs, Linz, A
Penicillin/Streptomycin/L-Glutamine	Invitrogen

Gele und Puffer für Western blot:

Lyse-Puffer:

50mM Tris-HCL, pH 7.5
 1% Triton X 100
 5mM EDTA, pH 8.0
 100mM NaCl
 dd. H₂O;
 frisch zugegeben: PMSF – 18µg/ml, Aprotinin 3µM, Leupeptin 4µM

Laemmli Sample-Puffer:

10% Glycerol
375mM Tris, pH8.8
2% SDS
0.02% Bromphenol Blau
4% Mercaptoethanol - frisch zugegeben

SDS-PAGE:

12% Gel:

	ml
H ₂ O	3.3
30% Acrylamide mix	4.0
1.5 M Tris, pH 8.8	2.5
10 % SDS	0.1
10 % APS	0.1
TEMED	0.004

,,Stacking“ Gel 5%:

	ml
H ₂ O	6.8
30% Acrylamide mix	1.7
1.0 M Tris pH 6.8	1.25
10 % SDS	0.1
10 % APS	0.1
TEMED	0.01

Gel-Lauf-Puffer:

0.1% SDS
0.3% Tris
1.4.% Glycine
in dd H₂O

Blocking Solution:

4% Milch Pulver
1% BSA in PBS

Transfer-Puffer, pH 8.3

0.3% Tris base
1.142% Glycine
20% Methanol in dd.H₂O

Wasch-Puffer:

1xPBS

0.1% Tween 20

Andere Reagenzien, Labormittel und Service:

Western-blot Kammer	Biometra, Göttingen
Hybond-C extra	Amersham
Hypercasette	Amersham
Hyperfilm MP	Amersham
ECL Western blotting detection reagent	Amersham
Trans S-Blot SD Semi-dry transfer cell	BIO-RAD, München
Extra thick Blot Papier, Criterion size	BIO-RAD
BenchMark Prestained Protein Ladder	Invitrogen
T3 Thermocycler	Biometra
Primer (Oligonukleotide)	ROTH, Karlsruhe
Sequenzierung	MWG BIOTECH, Ebersberg
FACS Calibur flow cytometer	BD Biosciences
CellQuest software	BD Biosciences
Multigamma counter	LKB Wallac

2.2.2 PCR Synthese der Fc γ RIIa-Sequenz

Die Fc γ RIIa-DNA Sequenz wurde aus der *copy*-DNA der reverse-transkribierten RNA aus Makrophagen generiert.

- Monozyten wurden aus *buffy coats* isoliert, diese wurden eine Woche in 10% AB-Serum/DMEM Medium kultiviert, bis sie zu Makrophagen ausdifferenzierten.
- Gesamt RNA wurde mittels *RNeasy Mini Kit* aus den Zellen isoliert.
- *copy*-DNA wurde mittels *Omniscript Reverse Transcriptase Kit* aus der RNA transkribiert.
- Die Fc γ RIIa Gensequenz wurde aus der *copy*-DNA durch PCR generiert.

PCR:

	µl pro Reaktion
10x <i>Pfu Buffer</i>	5.0
dNTPs (je 5mM)	2.0
*forward Fc γ RIIa-Primer	2.5
*reverse Fc γ RIIa-Primer	2.5
dd H ₂ O	36.0
DNA Matrize (<i>copy</i> DNA - 0.2 µg)	1.0
<i>cloned Pfu DNA Polymerase</i> (2.5 U/µl)	<u>1.0</u>
Gesamtvolumen	50.0

*forward – vorwärts; reverse – rückwärts

forward Fc γ RIIa-Primer:

5'<CAA CAT GGC TAT GGA GAC C>3'

reverse Fc γ RIIa-Primer:

5 '<GTT ATT ACT GTT GAC ATG>3'

PCR Programm:

	Zeit (min)	T°C
Initiation	1	<u>94</u>
Denaturierung	1	94
Annealing	1	55
Elongation	2	<u>72</u>
Finale Elongation	10	72

30 Zyklen

Die PCR Produkte wurden im 1.5%-igen Agarosegel elektrophoretisch aufgetrennt. Das Gel wurde mit Ethidium-Bromid gefärbt, die Banden herausgeschnitten und die DNA mittels *QIAquick Gel Extraction Kit* aufgereinigt.

2.2.3 Seitengerichtete Mutagenese

Die Fc γ RIIa Sequenz, die an Position 506 den Polymorphismus A-506 trägt, wurde durch *side-directed mutagenesis by overlap extension* nach dem Herstellerprotokoll (85) generiert. Die Position 506 wurde aus der Fc γ RIIa-Sequenz der NCBI Datenbank, *accession No M31932*, bestimmt.

Es wurden Mutationsprimer in beide Richtungen synthetisiert, komplementär zu der Fc γ RIIa-Region, welche die Position 506 umfassten. Bei den Primern wurde G durch A an der Position 506 ersetzt. Das Kodon CGT, das für Arginine (R) kodiert, wird in das Kodon CAT, kodierend für Histidine (H), ausgetauscht.

In einem ersten PCR-Schritt wurden Mutationsfragmente hergestellt, die das Allel Fc γ RIIa A-506 einschließen. Als DNA Matrize diente der *pcDNA3.1-Fc γ RIIa-R-131 Vektor* (2.4.), der das Fc γ RIIa-G-506 Allel enthält. Die erhaltenen PCR Produkte 1 und 2 wurden in einem zweiten PCR-Schritt fusioniert.

PCR 1:

PCR 2:

	µl pro Reaktion	
10x <i>Pfu Buffer</i>	5.0	10x <i>Pfu Buffer</i>
dNTPs (je 5mM)	2.0	dNTPs (je 5mM)
<i>forward</i> Primer	2.5	<i>forward-mutation</i> Primer
<i>reverse-mutation</i> Primer	2.5	<i>reverse</i> Primer
dd H ₂ O	36.0	dd H ₂ O
<i>pcDNA3.1- FcγRIIa</i>	1.0	<i>pcDNA3.1-FcγRIIa</i>
<i>cloned Pfu DNA</i>		<i>cloned Pfu DNA</i>
<i>Polymerase</i> (2.5 U/µl)	1.0	<i>Polymerase</i> (2,5 U/µl)
Gesamtvolumen	50.0	Gesamtvolumen

forward-mutation Primer:

5' >TC TCC C**A**T TTG GAT CCC ACC TTC <3'

reverse-mutation Primer:

5' > GAA GGA GGG ATC CAA **ATG** GGA GA <3'

(Die Position 506 ist gekennzeichnet)

PCR Programm:

	Zeit (min)	T°(C°)
Initiation	1	<u>94</u>
Denaturierung	1	94
Annealing	1	57.2
Elongation	2	<u>72</u>
Finale Elongation	10	72

} 30 Zyklen

Die PCR Produkte 1 und 2 wurden in einem 1.5%-igen Agarosegel elektrophoretisch aufgetrennt, die Banden herausgeschnitten und die DNA mittels QIAquick Gel Extraction Kit aufgereinigt.

In einem weiteren Schritt wurden die PCR Produkte 1 und 2 sowohl als Matrizen als auch als Primer benutzt. Da die PCR Produkte 1 und 2 an ihren 3'-Enden bzw. 5'-Enden komplementär sind, kann die *Pfu Polymerase* die beiden Fragmente fusionieren.

PCR 3:

	µl pro Reaktion
10x <i>Pfu Buffer</i>	5.0
dNTPs (je 5mM)	2.0
<i>forward</i> Primer	2.5
<i>reverse</i> Primer	2.5
PCR Produkt 1	1.0
PCR Produkt 2	1.0
dd H ₂ O	35.0
<i>Cloned Pfu DNA</i>	
<i>Polymerase</i> (2.5 U/µl)	<u>1.0</u>
Gesamtvolumen	50.0

PCR Programm:

	Zeit (min)	T°(C°)	
Initiation	1	<u>94</u>	
Denaturierung	1	94	
<i>Annealing</i>	1	57.2	} 20 Zyklen
Elongation	2	<u>72</u>	
Finale Elongation	10	72	

Das PCR Produkt 3 wurde im 1.5%-igen Agarosegel elektrophoretisch aufgetrennt, die Bande herausgeschnitten und die DNA mittels *QIAquick Gel Extraction Kit* aufgereinigt.

2.2.4 Klonierung der Fc γ RIIa-G-506 und Fc γ RIIa-A-506 PCR-Fragmente in einen Transfektionsvektor

Die PCR Fragmente wurden in einen Transfektionsvektor mittels *pcDNA3.1 Directional TOPO Expression Kit* ligiert und transformiert.

Ligation:

	Probe (μl)	Negativ Kontrolle
H ₂ O	-	3.0
<i>Salt Solution</i>	1.0	1.0
<i>Insert-DNA*</i>	3.0	-
<i>pcDNA3.1D/V5-His-TOPO</i>		
(linearisiert)	1.0	1.0
Gesamtvolumen	5.0	6.0

* PCR Produkt - Fc γ RIIa-G-506 bzw. Fc γ RIIa-A-506 (s. 2.2.2 und 2.2.3)

Die Ligation erfolgte bei Raumtemperatur für 25 min.

Transformation:

- 2μl Ligationsansatz pro Tube *One Shot TOP 10* Zellen (von *pcDNA3.1 Directional TOPO Expression Kit*) auf Eis zugegeben
- Hitzeschock – 30 sec/ 42°C
- Proben sofort auf Eis
- 250 μl SOC Medium bei Raumtemperatur zugegeben
- 1h bei 37°C/ 200rpm geschüttelt
- Probe auf einer 37°C vorgewärmten LB/Agarplatte (mit 100μg/ml Ampicillin) ausplattiert
- Über Nacht bei 37°C inkubiert

Vorkultur:

Einzelne Kolonien wurden gepickt. In 3ml LB/Ampicillin (100μg/ml) wurde mit je einer Kolonie eine Vorkultur angeimpft und über Nacht bei 37°C, 220 rpm inkubiert.

Vektor-DNA Isolation:

Die Vektor-DNA mit dem eingefügten Fragment (*Insert*) wurde mittels *Qiaprep Spin Miniprep Kit* aus den Vorkulturen isoliert.

Restriktionsverdau:

Der Erfolg der Klonierung wurde durch Restriktionsverdau der isolierten Vektoren mit *Xho I* und *Hind III* getestet. Das Vorhandensein des *Inserts* wurde im 1.5% Agarosegel elektrophoretisch nachgewiesen.

Bei den *Insert*-positiven Proben wurde mittels Sequenzierung überprüft, ob der Vektor den Polymorphismus A-506 enthält. Die Vektoren dieser Proben wurden mittels *Hi Speed Plasmid Maxi Kit* wiedergewonnen.

2.2.5 Transfektion von COS-7 Zellen

Die Transfektion ist eine Methode, bei der Fremd-DNA in eukaryotische Zellen eingefügt wird.

COS-7 ist eine adhärente, eukaryotische Zelllinie, die aus einem Affen-Nierentumor stammt. Die Zellen exprimieren keine Fc-Rezeptoren und sind gut transfizierbar. Sie wurden bei 37°C/5%CO₂ in DMEM/10% FCS/1% PSG kultiviert.

Als Transfektions-Reagenz wurde das *PolyFect Transfection Reagent* benutzt. Dies ist ein aktiviertes Dendrimer mit verzweigter sphärischer Struktur, dessen Enden positiv geladen sind. PolyFect bindet DNA als kompakte Struktur. Positiv geladene Gruppen des DNA-PolyFect Komplexes binden an negativ geladene Zellmembran-Rezeptoren und dringen mittels Pinozytose in die Zelle ein.

Protokoll:

- COS-7 Zellen wurden auf 6-Well Platten ausgesät, bei 37°C/5%CO₂ inkubiert und bis zur Zelldichte von 2,5 – 5x10⁵ Zellen/Well kultiviert.
- 2µg DNA (FcγRIIa-Vektor) wurde in 100µl Serum- und Antibiotikumfreiem DMEM verdünnt.
- es folgte eine Zugabe von 10µl PolyFect.
- Der DNA-PolyFect Komplex wurde 10 min bei Raumtemperatur inkubiert.
- In der Zwischenzeit wurden die Zellen mit PBS gewaschen.

- Pro Reaktionsansatz wurden 600µl DMEM/10% FCS/1% (Penicillin/Streptomycin-L-Glutamine) zugegeben, gut gemischt und sofort auf die Zellen gegeben.
- Die Zellen wurden für 48h bei 37°C, 5% CO₂ inkubiert.
- Als Negativkontrolle dienten Zellen, die nur mit *PolyFect Transfectant* aber ohne DNA behandelt wurden. Diese werden *mock-transfizierte* Zellen genannt („mock“ – pseudo).

2.2.6 Western-blot

Prinzip:

Proteinproben wurden mittels SDS-PAGE aufgetrennt. Die Proteinbanden wurden vom Gel auf eine Nitrozellulose Membran (Blot-Membran) übertragen und mit einem Enzym-markiertem spezifischen Antikörper inkubiert. Anschließend wurde Substrat dazugegeben, um die Banden, an denen der Antikörper gebunden hat, zur Fluoreszenz anzuregen. Nach Exposition der Blot-Membran auf einem Fotofilm konnten die fluoreszierenden Banden detektiert werden.

Bestimmung der Fc_γRIIa-Expression:

- Proteinlysate von transfizierten Zellen wurden 1:1 mit *Laemmli sample Buffer* gemischt und bei 100°C denaturiert. Die Proben wurden im 12% SDS/PAGE bei 20mA für 1.5 h aufgetrennt.
- Das Gel wurde in *Semidry blot transfer cell* auf eine Nitrozellulose Membran bei 50 V (150 mA) für 1h geblottet.
- Die Nitrozellulose-Membran wurde mit anti-V5-HRP Antikörper in einer Verdünnung von 1:2500 in *Blocking Solution* für 1h inkubiert.
- Nach sechs Waschschritten wurde die Membran für 1 min in *ECL Detektion reagent* inkubiert und dann für 15 min in einer *Hypcasette* auf *Hyperfilm* exponiert.

IgG-Kontaminationstest von CRP

Die Western-blot Analyse von CRP- und IgG-Proben erfolgte bei den obenbeschriebenen Bedingungen. Die Membran wurde mit *anti-human IgG-HRP (Fc-specific)* in einer Verdünnung von 1:10 000 inkubiert.

2.2.7 Fluorescein-Markierung von CRP

Um die Ammoniumionen (Tris) aus der rCRP-Aufbewahrungslösung zu entfernen, wurde diese gegen PBS dialysiert.

Anschließend wurde rCRP mittels *Alexa Fluor 488 protein labeling Kit* nach dem Herstellerprotokoll mit Fluorescein markiert:

- 500 μ l Protein (1mg/ml) wurde mit 50 μ l 1M Na₂CO₃ (pH 8.3) gemischt und in ein Fluorescein (Succinimidyl Ester, dilithium Salz)-Röhrchen überführt.
- Das Reaktionsgemisch wurde 1h im Dunkeln gerührt
- Dieses wurde chromatographisch aufgetrennt, das nichtgebundene Fluorescein blieb in der Säule.
- Das markierte Protein, Fluorescein-CRP (F-CRP), wurde als Eluat aufgefangen.

2.2.8 CRP- Bindungsstudien

Antikörper-abhängige Studien:

- COS-7 Zellen wurden 48 h nach der Transfektion mittels PBS/EDTA von den 6-well Platten abgelöst.
- Die Zellsuspensionen aus 3 Wells (7.5×10^5 bis 10^6 Zellen) wurden gepoolt und für 5 min bei 300 g zentrifugiert.
(Alle weitere Zentrifugationsschritte wurden bei den selben Bedingungen durchgeführt).
- Die Zellen wurden einmal mit PBS und nach einer Zentrifugation einmal mit PAB Buffer (49) (0.1% BSA, 0,05% Azide in Ca²⁺freiem PBS) gewaschen.
- Es folgte eine Zugabe von 100 μ l eiskaltem PAB Buffer und Inkubation mit verschiedenen Konzentrationen an CRP für 1h auf Eis. Anschließend wurde zweimal mit PAB Buffer gewaschen.
- Die Zellen wurden mit *anti-CRP, 2C10, (1:1000)* für 30 min auf Eis inkubiert, danach zweimal mit PAB Buffer gewaschen und mit *goat anti-mouse F(ab')2-PE* für 30 min auf Eis im Dunkeln inkubiert.

- Am Ende wurden die Zellen zweimal gewaschen und in 500 μ l PAB pro Probe resuspendiert und zur FACS Analyse vorgelegt.

Antikörper-unabhängige Studien:

- Abgelöste Zellen wurden mit F-CRP für 1h auf Eis im Dunkeln inkubiert. Nach dem letzten Waschschritt wurden die Zellen der FACS Analyse unterzogen.
- Für die radioaktive Studien wurde die Zellsuspensionen mit I-CRP inkubiert. Nach sechs Waschschritten mit PAB Buffer wurde die Radioaktivität (in cpm) am *Multigamma counter* gemessen.

2.2.9 FACS Analyse

Transfizierte COS-7 Zellen wurden nach Inkubation mit CRP im *FACS Calibur flow cytometer*, ausgestattet mit *CellQuest Software*, analysiert. 30 000 Zellen pro Probe wurden mittels Fluoreszenz-1 und 2 (*FL1-Height und FL2-Height*) ausgewertet.

2.2.10 Statistik

Daten der Fluoreszenz-Färbung aus den durchgeführten FACS Untersuchungen (n=5), in Abbildungen 3B, 3D, 4A und 4B, und Versuche mit ^{125}I -CRP (n=3), in Abbildungen 4C und 4D, sind mit Mittelwert und SD dargestellt. Sie wurden durch gepaartem *Students' t-Test* (*Microsoft Excel 2000*) analysiert, wobei p<0.05 als statistisch signifikant angesehen wurde.

2.3 Ergebnisse

2.3.1 Generierung der Fc γ Rezeptor-Vektoren

2.3.1.1 Fc γ RIIa-RR-131 Vektor

Die Fc γ RIIa-Gensequenz wurde durch RT-PCR aus Makrophagen-mRNA generiert (**Abb. 4**). Sie wurde anschließend in den Transfektionsvektor *pcDNA3.1* kloniert. Das Vorhandensein des *Inserts* in diesem Vektor wurde mittels Restriktionsverdau mit *Hind III* und *Xho I* getestet (**Abb. 5**).

Die Fc γ RIIa-Vektoren wurden nach Sequenzanalyse für die Transfektionsversuche verwendet. Das Fc γ RIIa-*Insert* enthält an Position 506 ein „G“. Die Sequenz kodiert für das R-131 Allel des Fc γ RIIa-Gens.

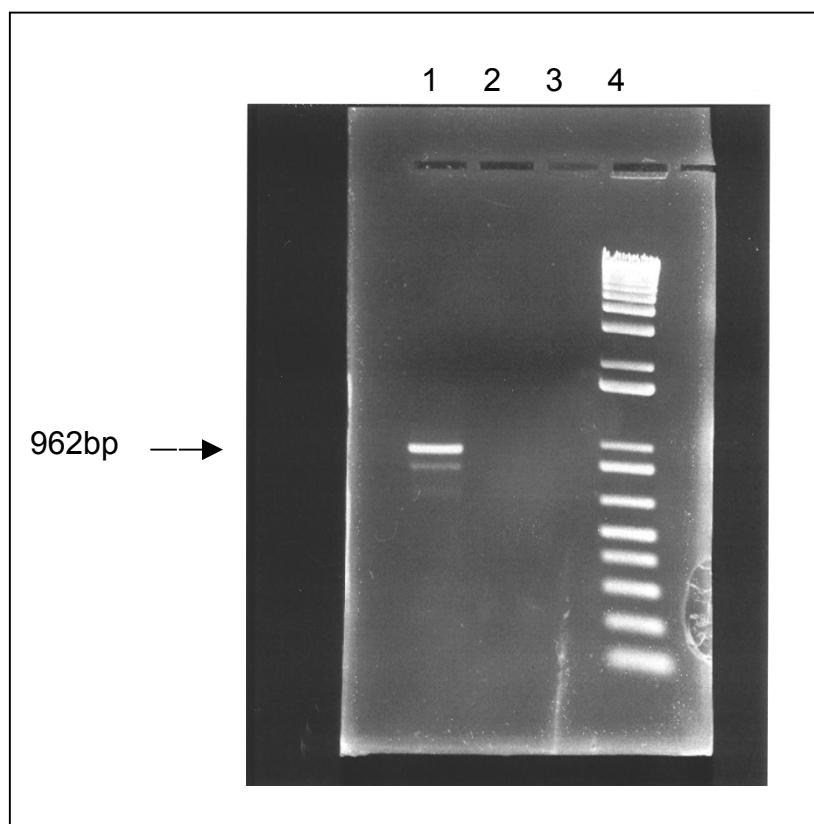


Abb. 4. 1.5%-iges Agarosegel, elektrophoretische Auf trennung des Fc γ RIIa PCR Fragments. 1: Fc γ RIIa-positive PCR-Probe, 962bp Bande = Fc γ RIIa-Gensequenz; 2: RT-negative Probe; 3: PCR-negative Probe; 4: 1kb DNA-Leiter.

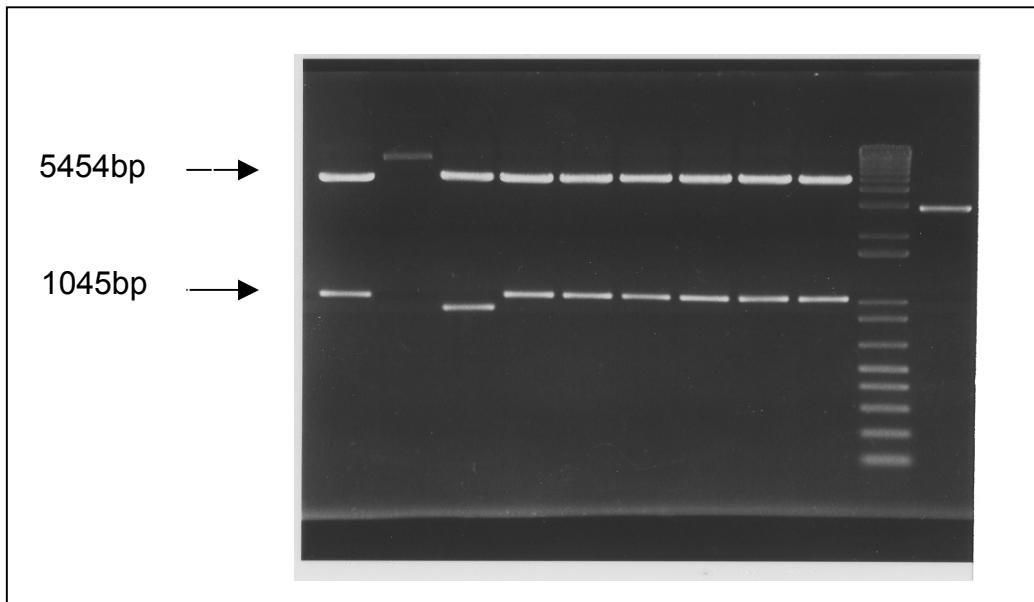


Abb. 5. 1.5%-iges Agarosegel, elektrophoretische Auf trennung des *pcDNA3.1-Fc γ RIIa* Vektors nach einem Restriktionsverdau mit *Hind* III / *Xho* I. 5454bp Bande = Plasmid; 1045bp Bande = Insert (*Fc γ RIIa*).

2.3.1.2 Fc γ RIIa-HH-131 Vektor

Das Fc γ RIIa-A-506 PCR-Fragment wurde durch seitengerichtete Mutagenese erzeugt (2.2.2). Diese Sequenz kodiert für das H-131-Allel des Fc γ RIIa-Gens. Als DNA-Matze diente der Fc γ RIIa-RR-131 Vektor (2.3.1.1.).

Zwei Mutations-PCR-Fragmente wurden mittels *forward* und *reverse-mutation* Primer bzw. *forward-mutation* und *reverse* Primer generiert (Abb. 6).

In einem zweiten PCR-Schritt wurden mit den *forward* und *reverse* Primern die beiden PCR-Produkte 1 und 2 fusioniert und amplifiziert. Sie dienten gleichzeitig als DNA-Matrizen und Primer (Abb. 7). Das entstandene PCR Produkt 3 enthält den gewünschten Polymorphismus A-506.

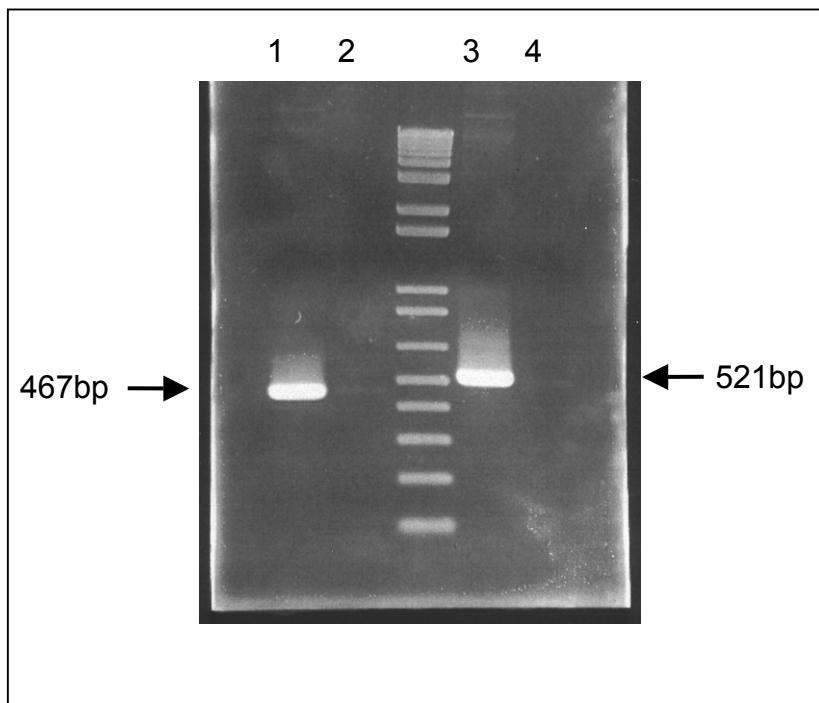


Abb. 6. 1.5%-iges Agarosegel, elektrophoretische Auftrennung von: 1: 467bp Bande = PCR Produkt 1; 3: 521bp Bande = PCR Produkt 2; 2 und 4: PCR Negativkontrollen.

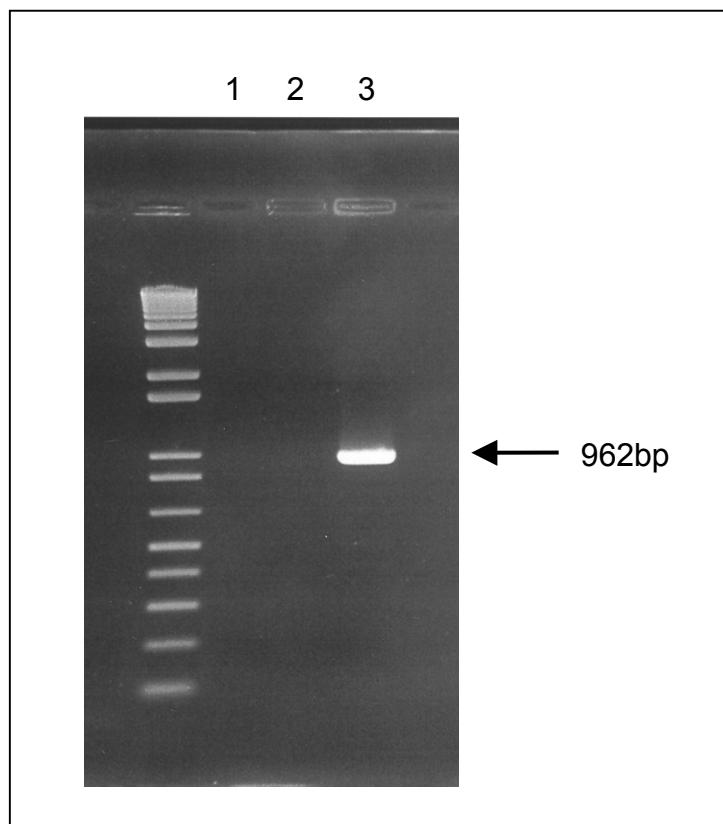


Abb. 7. 1.5% Agarosegel, elektrophoretische Auftrennung von 1 und 2: PCR Negativkontrolle; 3: PCR Produkt 3 - Fc γ RIIa-A-506

Das PCR Produkt 3 wurde in den Transfektionsvektor *pcDNA3.1* kloniert. Der Erfolg der Klonierung wurde durch Restriktion mit *Hind* III und *Xho* I geprüft (**Abb.8**).

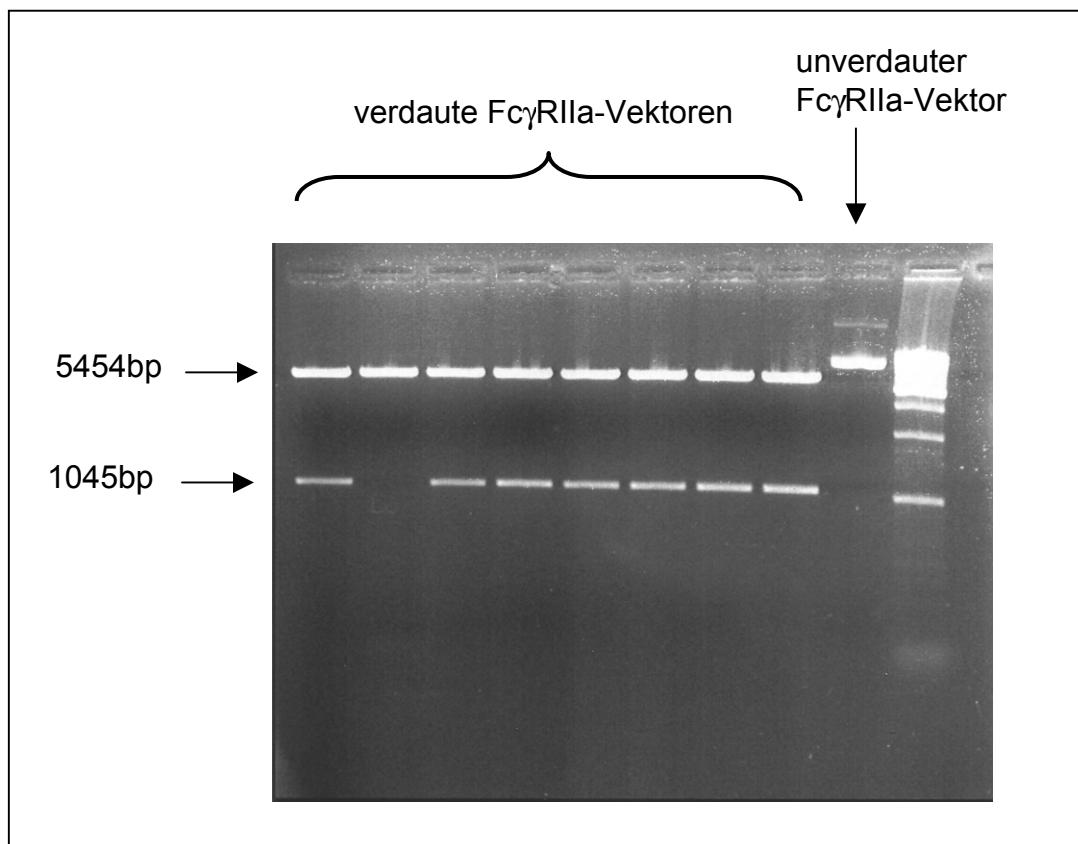


Abb. 8. 1.5%-iges Agarosegel, elektrophoretische Auftrennung von *pcDNA3.1-Fc γ RIIa-A-506* nach Restriktionsverdau mit *Hind* III und *Xho* I. 5454bp Bande = Plasmid; 1045bp Bande = *Insert* (*Fc γ RIIa-A-506*).

Die *Insert*-positiven Vektoren wurden sequenziert und auf Anwesenheit des Polymorphismus A-506 überprüft.

2.3.2 Rezeptor-Expression in transfizierten COS-7 Zellen

2.3.2.1 Fc γ RIIa-Expression

Die Expression von Fc γ RIIa (RR-131) wurde durch Westernblot-Analyse der Proteinlysate aus Fc γ RIIa-RR-131-transfizierten COS-7 Zellen nachgewiesen. Da der *pcDNA3.1* Vektor eine V5-Epitop-Sequenz (welche für ein sog. V5 virale

Epitop kodiert) am 3' Ende des *Fc γ RIIa-Inserts* enthält, kommt es zu einer Expression des V5-Epitops am C' Ende des Rezeptors und ermöglicht dadurch eine Detektion dieses Rezeptors mittels hochspezifischer anti-V5 Antikörper. Diese Färbung weist die 40kDa *Fc γ RIIa*-Bande nach (**Abb. 9**). Als Negativkontrolle dienten Proben von mock-transfizierten Zellen. Sie zeigten keine *Fc γ RIIa*-Expression.

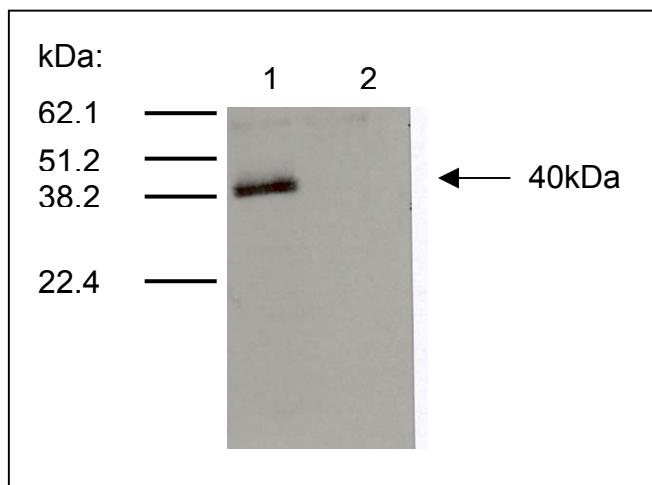


Abb. 9. 12% SDS/PAGE. 1: *Fc γ RIIa*-(RR-131)-transfizierte Zellen; 2: mock-transfizierte Zellen. Färbung mit anti-V5-HRP Antikörper weist *Fc γ RIIa* (40kDa) nach.

2.3.2.2 *Fc γ RIIa*-Allel-Expression

COS-7 Zellen wurden mit *Fc γ RIIa*-Vektoren transfiziert. Die heterozygote RH-131-Form wurde durch Kotransfektion mit *Fc γ RIIa*-RR-131 und *Fc γ RIIa*-HH-131 in den Zellen generiert. Die Expression wurde durch Färbung mit anti-CD32-FITC und anschließender FACS Analyse getestet (**Abb. 10**). Bei allen drei Rezeptorformen konnte eine Färbung von ca. 53% rezeptorpositiven Zellen beobachtet werden. 95% der mock-transfizierten Zellen, behandelt auf gleiche Weise, dienten als Hintergrundfärbung.

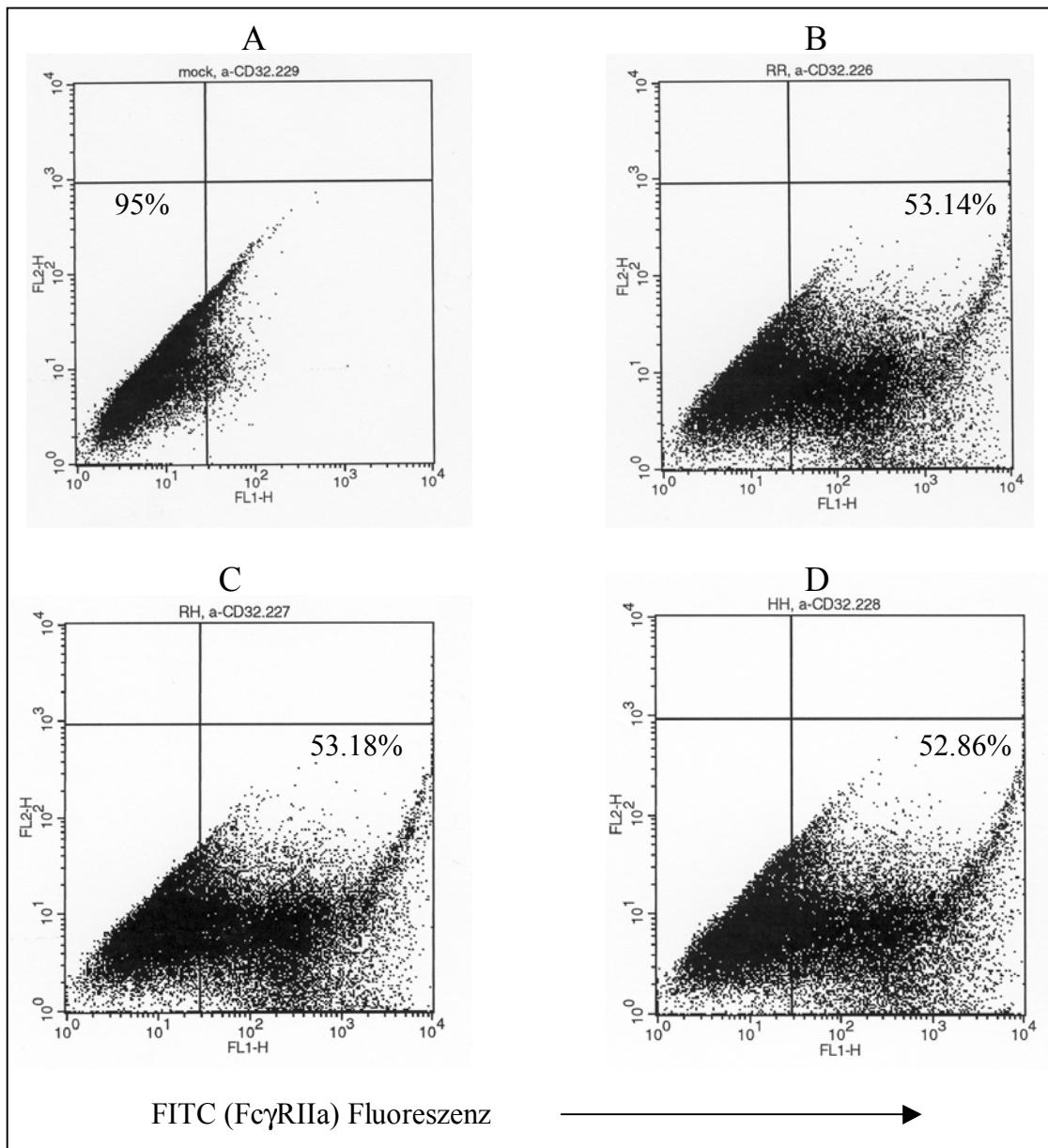


Abb. 10. FACS Analyse der Fc γ RIIa-Allel-Expression nach Färbung mit anti-CD32-FITC. A: mock-; B: Fc γ RIIa RR-131-; C: RH-131-; D: HH-131-transfizierte Zellen.

2.3.3 CRP-Bindungsstudien an Fc γ RIIa-transfizierten COS-7 Zellen

2.3.3.1 Antikörper-abhängige Detektion

2.3.3.1.1 Dosisantwortabhängigkeit

Mock- und Fc γ RIIa (RR-131) transfizierte COS-7 Zellen wurden mit verschiedenen Konzentrationen von rCRP inkubiert und FACS Analysen nach Färbung mit anti-

CRP Antikörpern durchgeführt. 95% der identisch behandelten, mock-transfizierten Zellen wurden als Hintergrundfärbung ausgewertet. In Analogie zu den publizierten Daten (70, 71, 80) zeigte die parallele Färbung mock- und Fc_YRIIa (RR131)-transfizierter Zellen in Anwesenheit von rCRP eine maximale Zunahme der PE-Fluoreszenz bei einer rCRP-Konzentration von 200µg/ml (**Abb.11**).

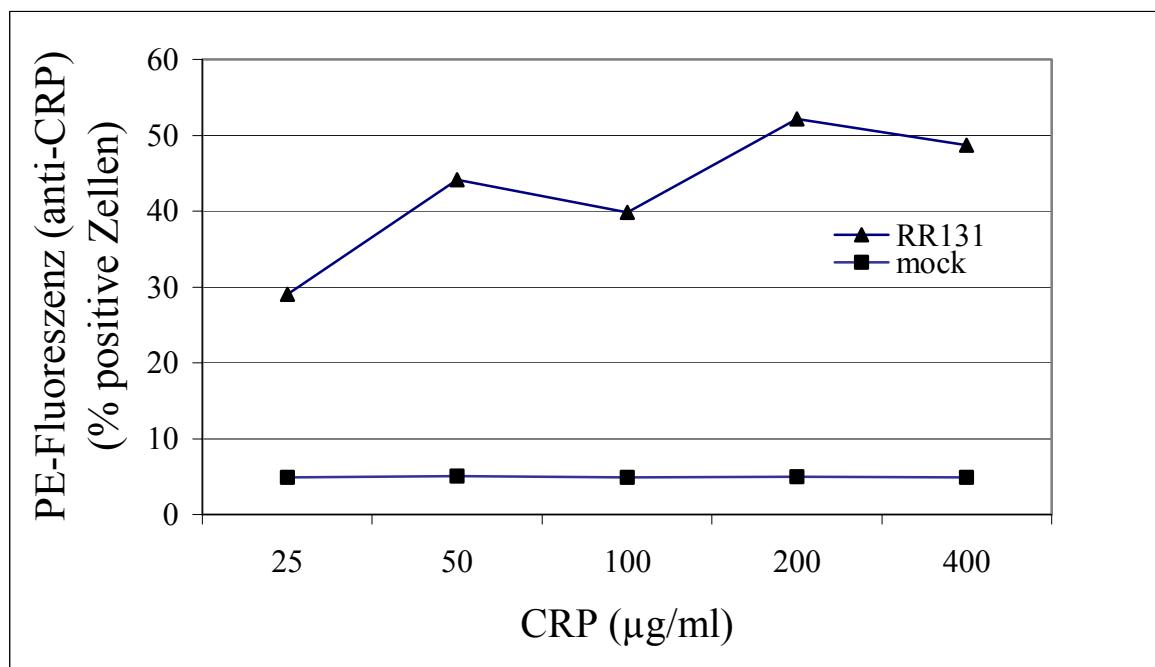


Abb. 11. Mock- und Fc_YRIIa-(RR-131)-transfizierte Zellen, inkubiert mit steigenden rCRP-Konzentrationen. Färbung mit *anti-CRP* (Klon 2C10) und *goat anti-mouse F(ab')₂-PE*. Für jede CRP-Konzentration 95% der identisch behandelten mock-transfizierten Zellen wurden als Hintergrundfärbung ausgewertet. Die Abbildung zeigt eine dosisabhängige Zunahme der PE-Fluoreszenz für Fc_YRIIa-transfizierte Zellen mit einem Maximum bei einer rCRP-Konzentration von 200µg/ml.

2.3.3.1.2 Effekt der IgG-Kontamination der CRP-Reagenzien

Eine eventuelle IgG-Kontamination der verwendeten CRP-Reagenzien wurde durch Westernblot-Analysen getestet. Es wurden im einzelnen untersucht: teilweise gereinigtes (*partially purified*) - ppCRP, hoch gereingtes (*highly purified*) - hpCRP und rekombinantes (*recombinant*) - rCRP. Als Positivkontrolle diente menschliches IgG in abnehmender Konzentration (**Abb. 12**). Die Proben wurden durch SDS-PAGE getrennt und mit *anti-human IgG-HRP (Fc-specific)* gefärbt. Die 50kDa schwere Kette (*H-chain*) des IgGs wurde ausschließlich im ppCRP-

Reagenz detektiert. Es errechnet sich eine IgG-Konzentration von < 0,1%. In hpCRP und rCRP wurde kein IgG detektiert.

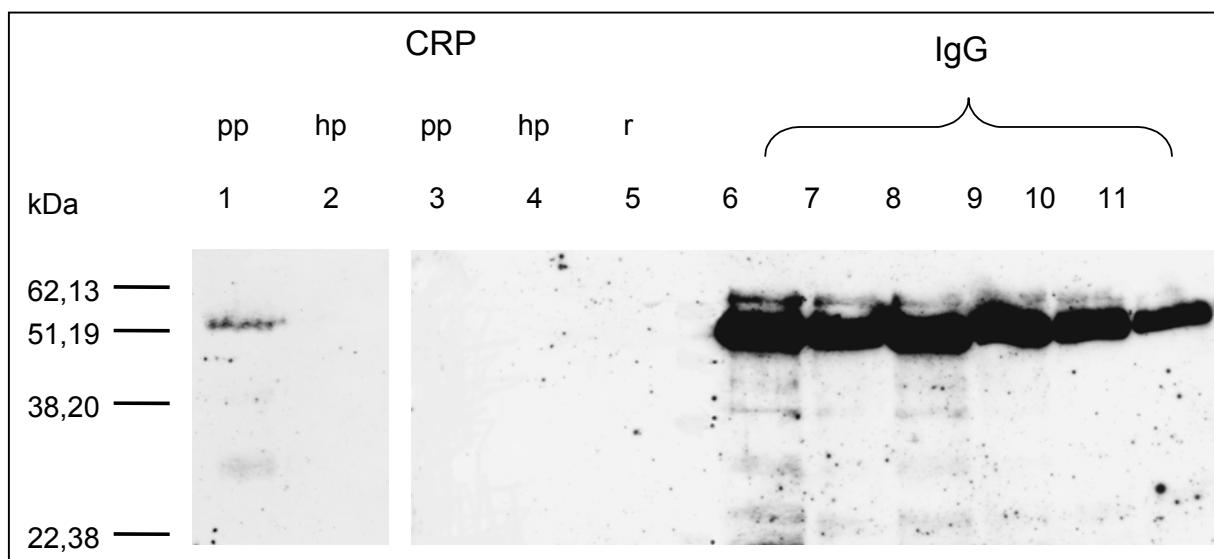


Abb. 12. 12% SDS-PAGE verschiedener CRP-Reagenzien und IgG. Färbung mit anti-IgG. 1: 50 µg ppCRP, 2: 50µg hpCRP, 3: 25µg ppCRP, 4: 25 µg hpCRP, 5: 25 µg rCRP, 6 bis 11: IgG – 2µg, 1µg, 500ng, 250ng, 100ng, 50ng.

50µg von ppCRP beinhalten weniger als 50ng (< 0.1%) IgG.

Um zu prüfen, ob das Ergebnis von antikörper-abhängigen Bindungsstudien von einer IgG-Kontamination des CRP-Reagenzes beeinflusst wird, wurden Fc_γRIIa-transfizierte (RR-131) und mock-transfizierte COS-7 Zellen mit den drei verschiedenen CRP-Reagenzien - ppCRP, hpCRP und rCRP (200µg/ml) inkubiert. 95% der identisch behandelten, mock-transfizierten Zellen wurden erneut als Hintergrundfärbung gewertet. Die Bindung ist IgG-unabhängig, da eine analoge Zunahme in der PE-Fluoreszenz sowohl bei ppCRP (IgG-kontaminiert) als auch bei (IgG-freiem) hpCRP und rCRP beobachtet wurde (**Abb. 13**).

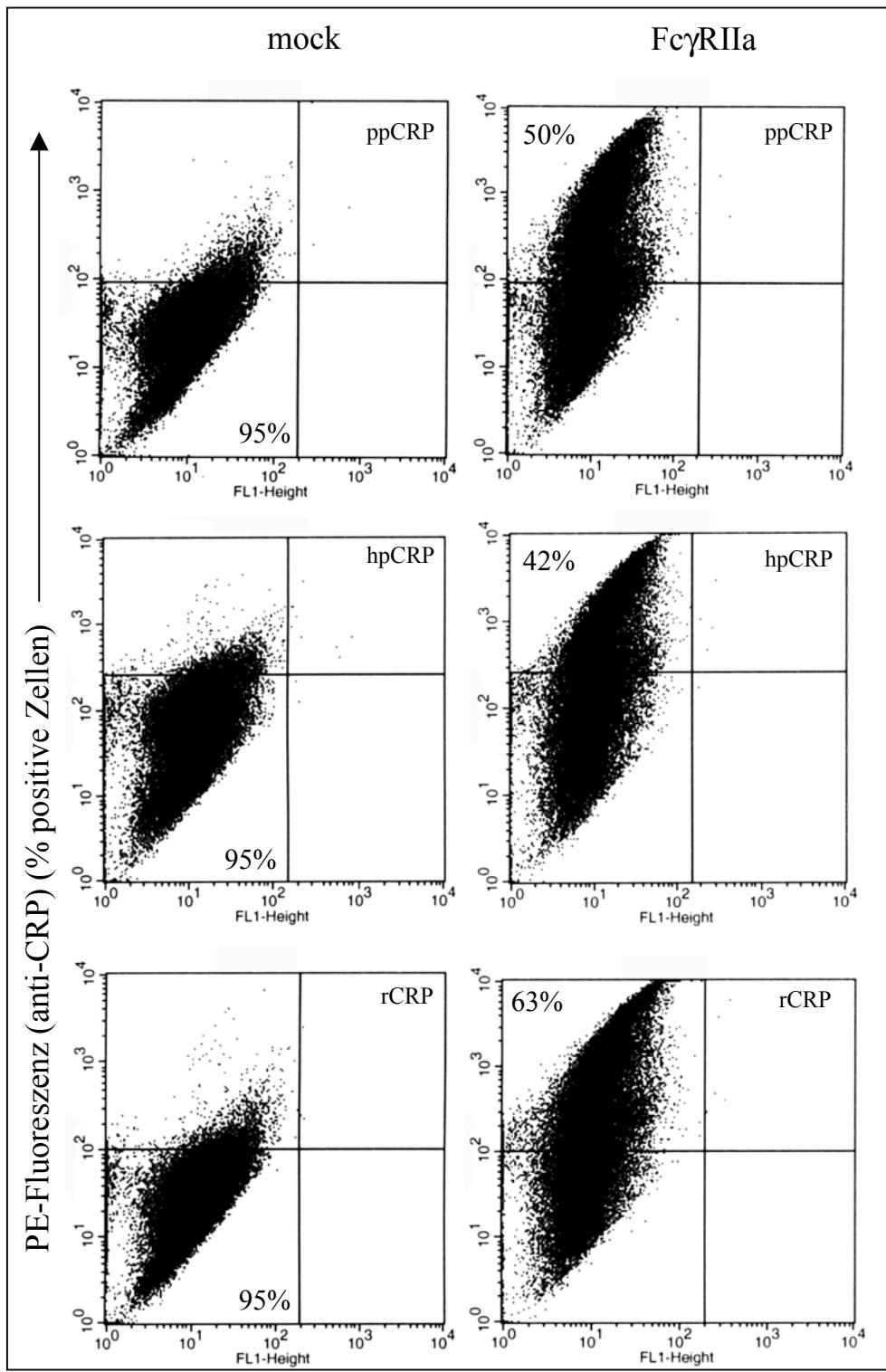


Abb. 13. FACS Analyse der anti-CRP/PE Färbung an mock- (links) und Fc γ RIIa (RR131)- (rechts) transfizierten Zellen, inkubiert mit ppCRP, hpCRP und rCRP. Die Färbung ist unabhängig von einer IgG-Kontamination.

2.3.3.1.3 Fc γ RIIa-Allele und anti-CRP/Fc γ RIIa-Interaktionen

Die CRP-Bindung wurde an mock- und Fc γ RIIa (RR-131) transfizierten Zellen in Anwesenheit und Abwesenheit von CRP untersucht. Erneut wurden 95% der identisch behandelten mock-transfizierten Zellen als Hintergrundfärbung gewertet. In Abwesenheit von CRP zeigten Fc γ RIIa-transfizierte Zellen im Vergleich zu den mock-transfizierten Zellen eine schwache Zunahme der PE-Färbung (9%). Im Gegensatz dazu zeigte die PE-Färbung von Fc γ RIIa-transfizierten Zellen, inkubiert mit rCRP, eine deutliche Zunahme des Signals.

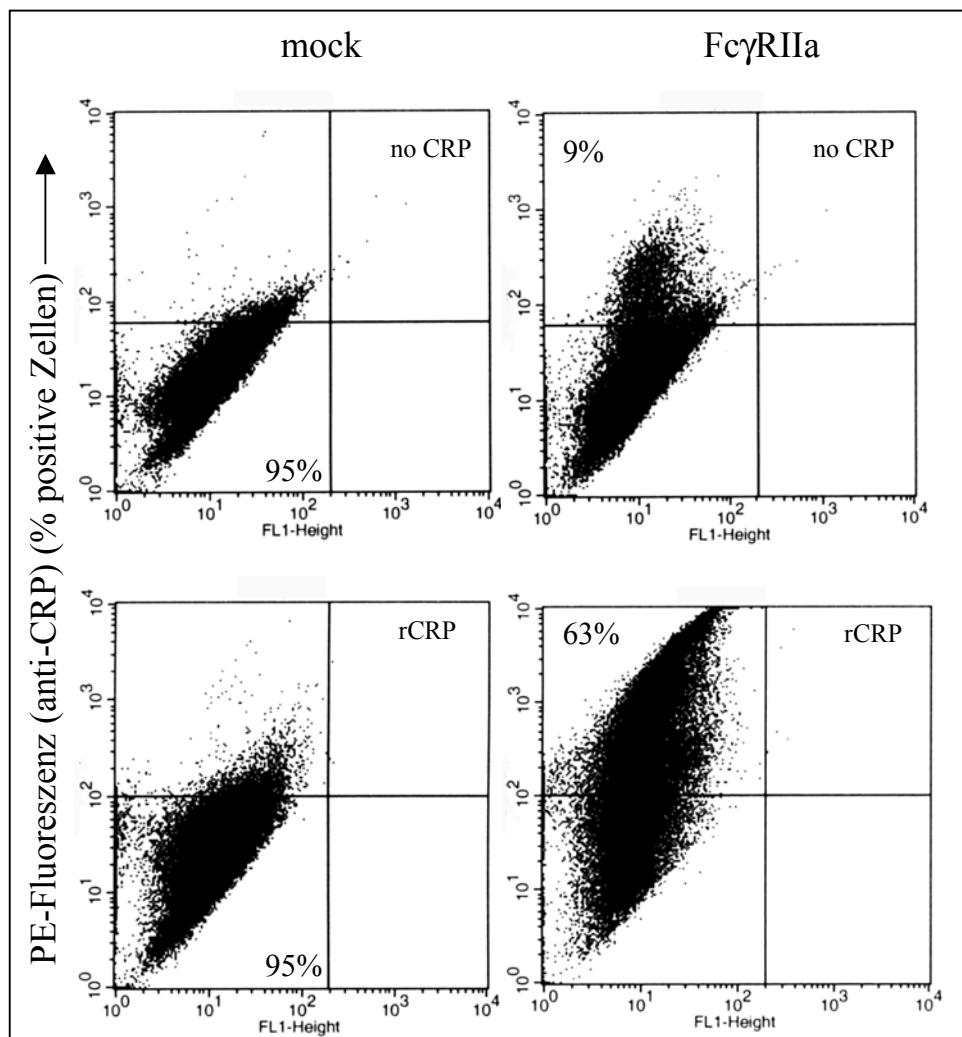


Abb. 14. FACS Analyse der anti-CRP/PE Färbung an mock- (links) und Fc γ RIIa (RR-131)- (rechts) transfizierten COS-7 Zellen mit und ohne CRP (200 μ g/ml). 95% der identisch behandelten, mock-transfizierten Zellen wurden als Hintergrundfärbung gewertet. In Anwesenheit von rCRP zeigten Fc γ RIIa-transfizierte Zellen eine 63%-ige Färbung, in Abwesenheit von CRP eine 9%-ige Färbung.

Um den Effekt der Fc γ RIIa-Allele auf die antikörper-abhängige Bindungsanalyse zu testen, wurden Zellen mit den verschiedenen Fc γ RIIa-131-Alleen transfiziert. Für die verschiedenen Allele wurde eine äquivalente Expression (~50%) festgestellt (s. **Abb. 10**). Es wurden Bindungsstudien mit und ohne rCRP durchgeführt. Wie in **Abb. 14** dargestellt, führte die Inkubation mit CRP zu einer deutlichen Erhöhung der PE-Fluoreszenz (**Abb. 15**). Ein unterschiedliche anti-CRP/PE Färbung wurde für die verschiedenen Allele beobachtet. Das Verhältnis RR : RH : HH der anti-CRP/PE Färbung war in Anwesenheit von CRP 1,6 : 1,2 : 2,0. In Abwesenheit von CRP war das Verhältnis 2,8 : 2,0 : 1,0. Die beobachteten Unterschiede waren statistisch signifikant. Auffällig ist, dass die Tendenz einer Abnahme der Färbung für die verschiedenen Allele (RR : RH : HH) in den Experimenten mit CRP ein ähnliches Verhältnis zeigt wie in den Experimenten ohne CRP.

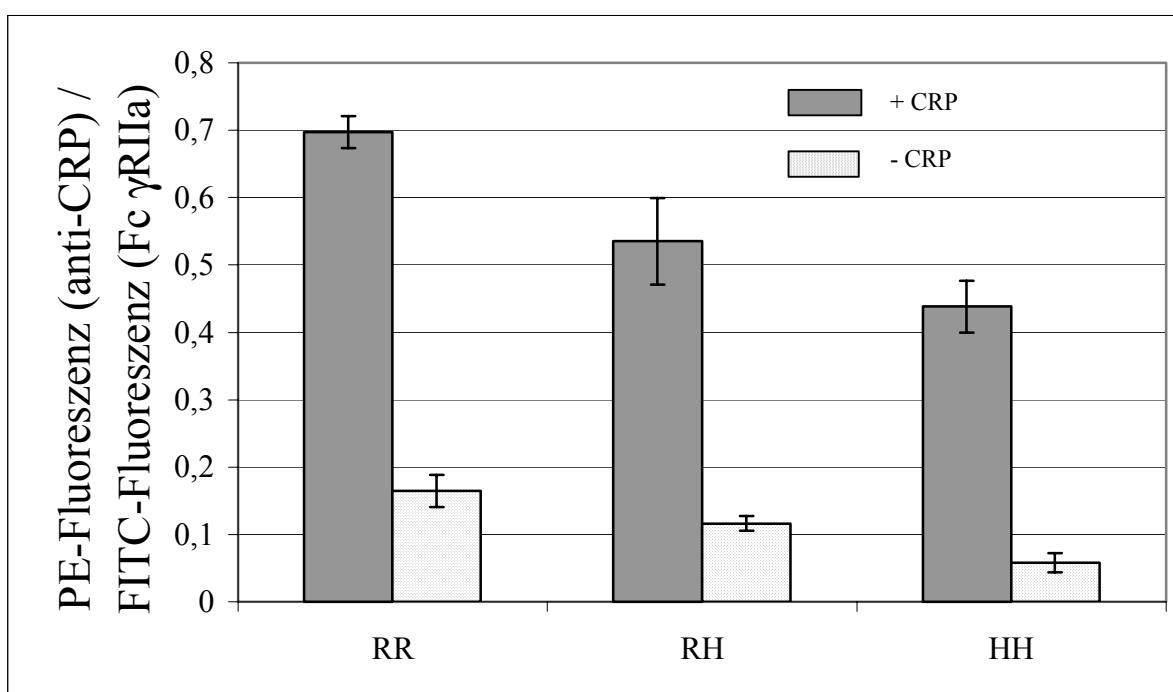


Abb. 15. COS-7 Zellen transfiziert mit verschiedenen Fc γ RIIa-Allelen in Anwesenheit (+ CRP) und Abwesenheit (-CRP) von rCRP (200 μ g/ml). Die anti-CRP/PE Färbung wurde auf die Fc γ RIIa-Allel-Expression, quantifiziert durch anti-CD32-FITC-Färbung, bezogen (s. Abb. 10). Die Unterschiede der PE-Fluoreszenz für die verschiedenen Fc γ RIIa-Allele sind statistisch signifikant (n=5): + CRP: RR/RH p=0.0015, RR/HH p=5.6E-05, RH/HH p=0.041; -CRP: RR/RH p=0.0052, RR/HH p=0.0017, RH/HH p=0.0006.

Analog zur Färbung mit anti-CRP ergab die Färbung mit *mouse-anti-human IgG₁*, Isotypen-Kontrollen ähnliche Ergebnisse (**Abb. 16**). Das Verhältnis von RR : RH : HH in diesen Versuchen war 2,5 : 1,8 : 1,0 (**Abb. 17**). Die beobachteten Unterschiede in Abb. 15 und Abb. 17 sind statistisch signifikant. Eine Zugabe von CRP hatte keinen Einfluss auf das Ergebnis (Daten nicht gezeigt).

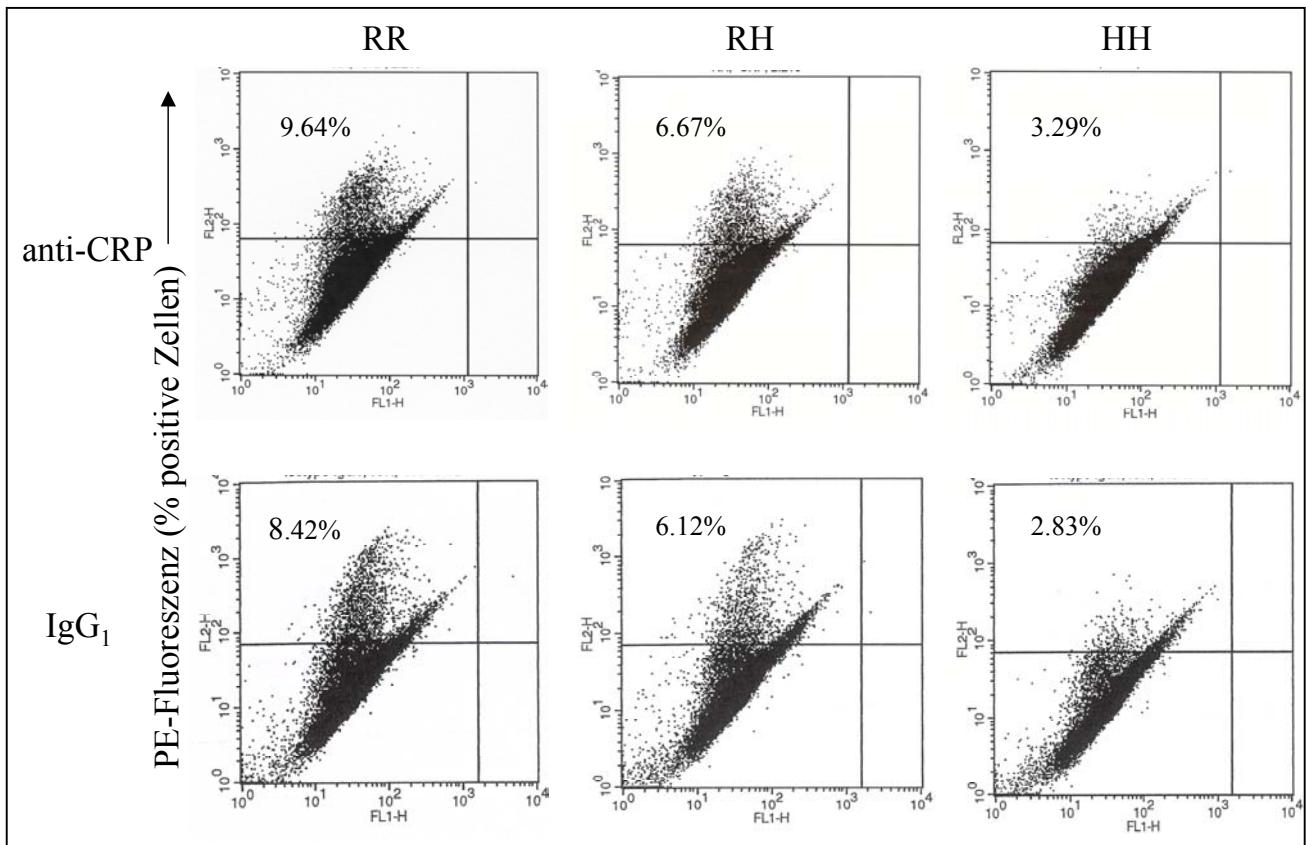


Abb. 16. FACS-Analyse von Fc γ RIIa RR-, RH- und HH-131 exprimierenden Zellen, die mit anti-CRP (obere Reihe) und einer *mouse-IgG₁*-Isotypen-Kontrolle (untere Reihe), in Abwesenheit von CRP, inkubiert wurden. Die Abbildung zeigt ein repräsentatives Experiment.

Schließlich wurde gezeigt, dass eine Inkubation der Fc γ RIIa-(RR-131)-transfizierten Zellen mit dem präformierten Antikörper/Antigen Komplex (anti-CRP/CRP) zu einer PE-Fluoreszenz (**Abb. 18**, rechts) führt, die dem Signal der Zellen entspricht, die zunächst mit CRP und anschließend mit anti-CRP inkubiert wurden (**Abb. 18**, links oder auch Abb. 13 und 14).

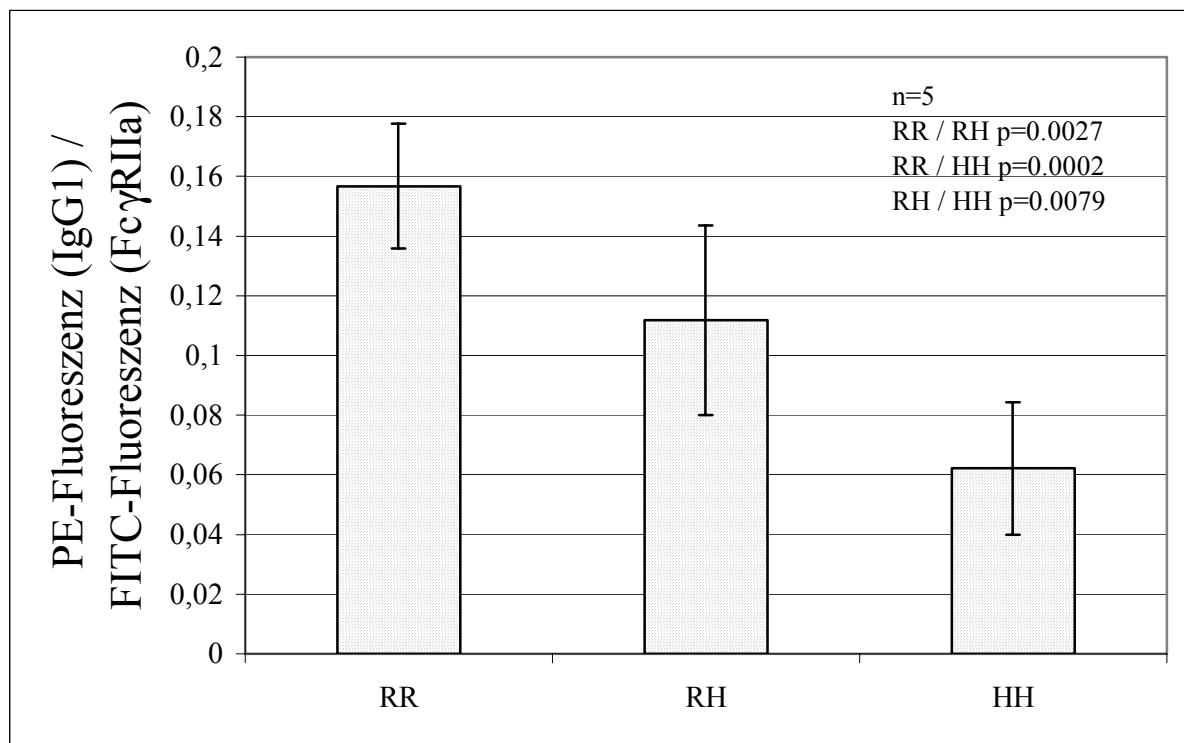


Abb. 17: COS-7 Zellen transfiziert mit verschiedenen Fc γ RIIa-Allelen und gefärbt mit *mouse IgG₁*-Isotyp-Kontrolle. Die IgG₁-PE Färbung wurde auf die Fc γ RIIa-Allel-Expression, quantifiziert durch anti-CD32-FITC-Färbung, bezogen. Das Verhältnis von RR : RH : HH ist 2,5 : 1,8 : 1,0.

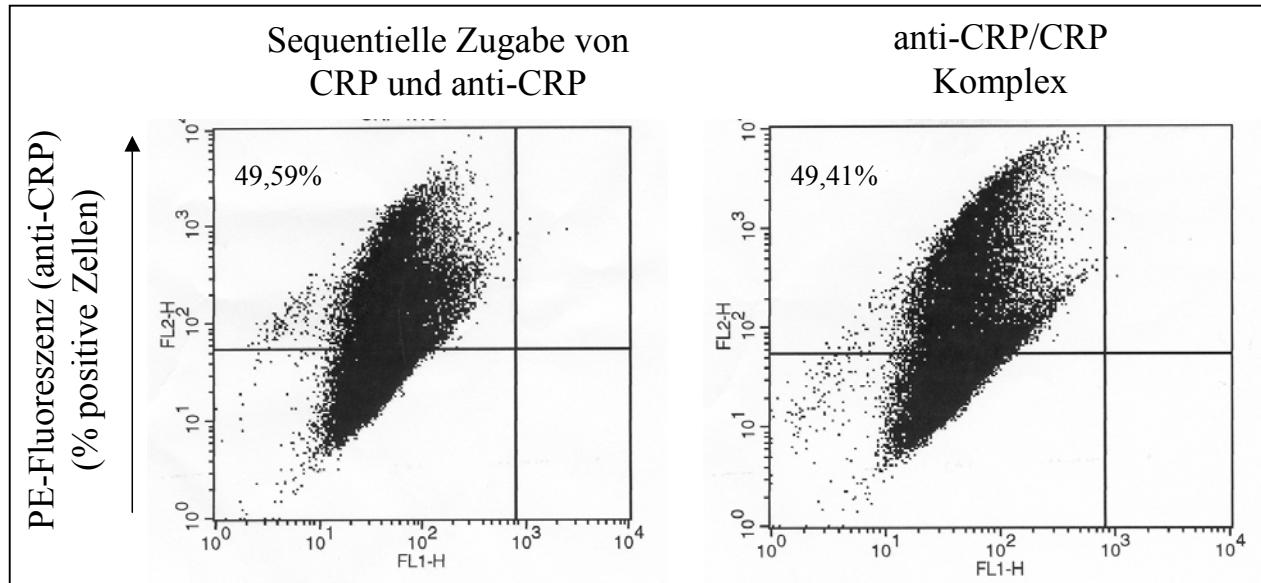


Abb. 18. FACS Analyse von Fc γ RIIa-(RR-131)-transfizierten Zellen inkubiert mit: (links) rCRP (200 μ g/ml) und anschließend anti-CRP Antikörper; (rechts) präformiertem Antikörper/Antigen Komplex anti-CRP/CRP. Es wurden keine Unterschiede detektiert.

2.3.3.1.4. Effekt der Zellalterung

Anti-CRP/PE Färbungen der mock-transfizierten COS-7 Zellen von früheren und späteren Passagen, mit und ohne Inkubation von CRP, wurden verglichen. Eine deutliche Zunahme der Färbung zeigten die Zellen der späteren, 17-ten, Passage, in Anwesenheit von CRP (**Abb. 19**, unten rechts).

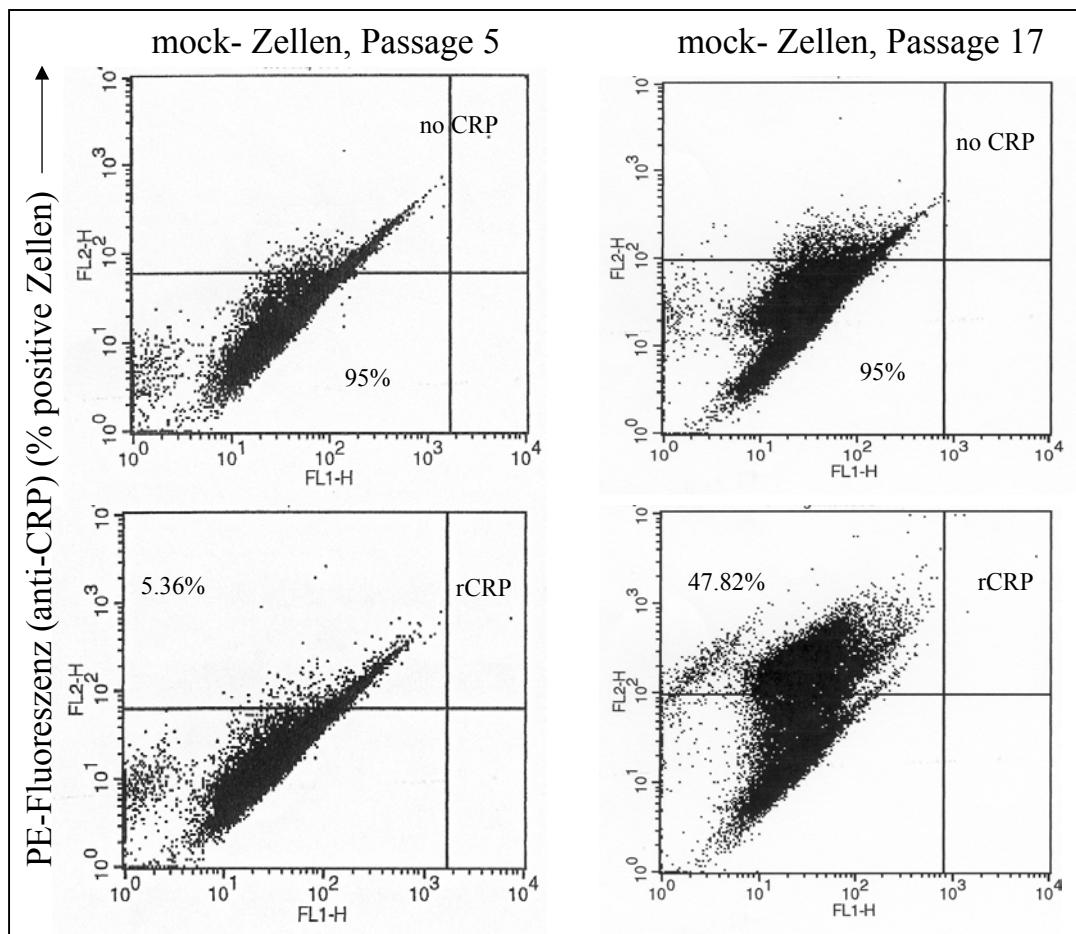


Abb. 19. Effekt der Zellalterung. Vergleich von mock-transfizierten COS-7 Zellen, Passagen 5 und 17, inkubiert mit 200 μ g/ml rCRP. Eine wesentliche Zunahme der anti-CRP/PE-Färbung wurde bei den Zellen von Passage 17 (rechts, unten) beobachtet.

2.3.3.2 Bindungsstudien mit direkt markiertem CRP

Die CRP-Bindung an mock- bzw. Fc γ RIIa-(RR-131)-transfizierte Zellen wurde mittels Fluorescein-markiertem CRP (F-CRP) und Jod-markiertem CRP (125 I-CRP) untersucht.

2.3.3.2.1 Fluorescein-CRP-Bindungsstudie

Es wurde die F-CRP-Dosisabhängigkeit parallel an Fc γ RIIa-(RR-131)- und mock-transfizierten Zellen untersucht. 95% der Zellen, inkubiert mit der niedrigsten F-CRP Konzentration (5 μ g/ml), wurden als Hintergrundfärbung gewertet. **Abb. 20.** zeigt eine Dosisantwortabhängigkeit bei F-CRP Konzentrationen von 5 bis 160 μ g/ml. Das F-CRP-Färbungssignal stieg sowohl für Fc γ RIIa-(RR-131)- als auch für mock-transfizierte Zellen dosisabhängig. Im Vergleich zu mock-transfizierten Zellen zeigten Fc γ RIIa (RR-131)-transfizierte Zellen kein intensiveres Anfärbungssignal.

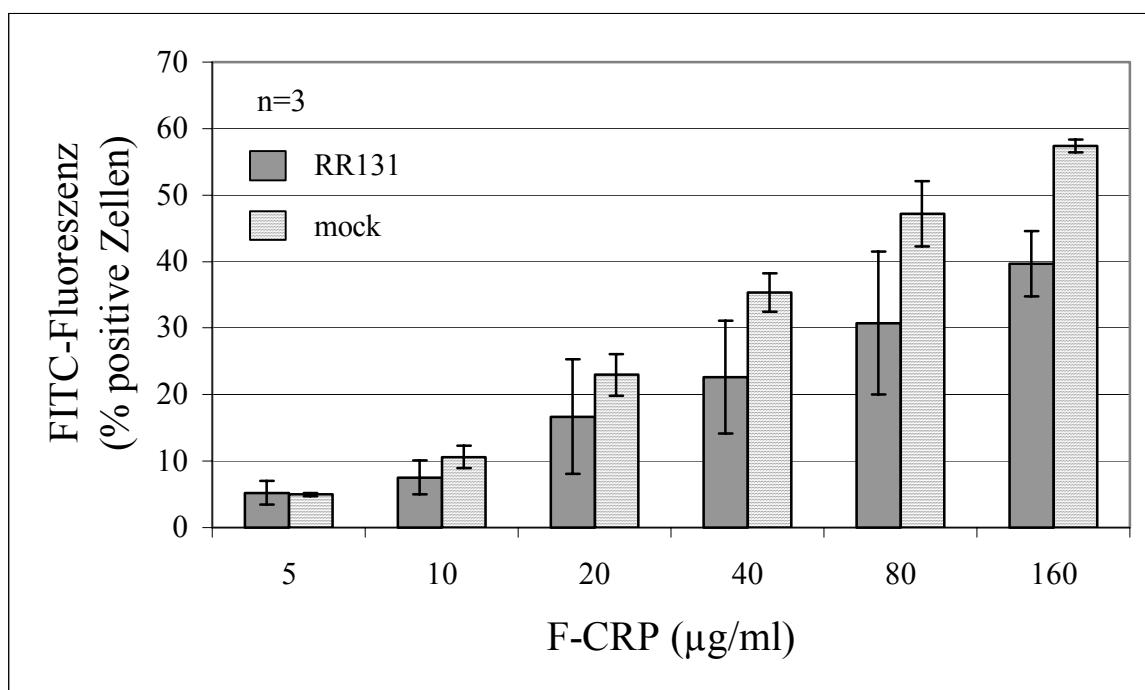


Abb. 20. Fc γ RIIa (RR-131)- und mock-transfizierte Zellen inkubiert mit zunehmenden Konzentrationen von F-CRP. 95% der Zellen inkubiert mit der niedrigsten F-CRP Konzentration (5 μ g/ml) wurden als Hintergrundfärbung gewertet. Das F-CRP-Färbungssignal steigt dosisabhängig sowohl für mock- als auch für Fc γ RIIa (RR-131)-transfizierte Zellen.

Darüber hinaus wurden bei den verschiedenen Fc γ RIIa-Allelen in der F-CRP-Bindung keine statistisch signifikanten Unterschiede festgestellt (**Abb. 21**). Die Experimente wurden mit einer Konzentration von 80 μ g/ml F-CRP durchgeführt.

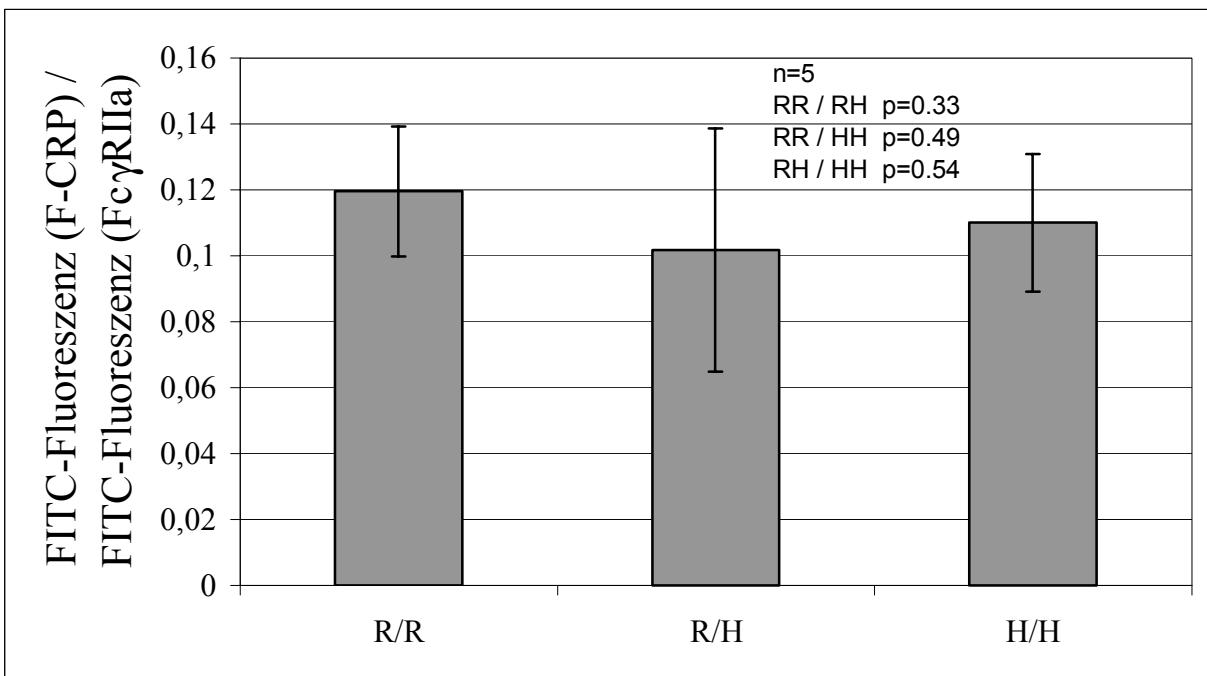


Abb. 21. F-CRP-Bindung für COS-7 Zellen transfiziert mit verschiedenen Fc γ RIIa-Allelen. Die F-CRP-Bindung (FITC-Fluoreszenz) wurde auf die Fc γ RIIa-Allel-Expressionen bezogen. Es wurden keine statistisch signifikanten Unterschiede beobachtet.

2.3.3.2.2. ^{125}I -CRP-Bindungsstudie

Bindungsstudien mit steigenden Konzentrationen von jodiertem-CRP, ^{125}I -CRP, zeigten eine dosisabhängige Zunahme der Radioaktivität (**Abb. 22**). Die Radioaktivität nimmt sowohl bei Fc γ RIIa (RR-131)- als auch bei mock-transfizierten Zellen zu. Fc γ RIIa (RR-131)-transfizierte Zellen zeigten kein intensiveres radioaktives Signal im Vergleich zu mock-transfizierten Zellen. Kompetitive Bindungsstudien mit kaltem Ligand (rCRP) zeigten keine Inhibition der ^{125}I -CRP-Bindung an Fc γ RIIa (RR-131)-Zellen (**Abb. 23**).

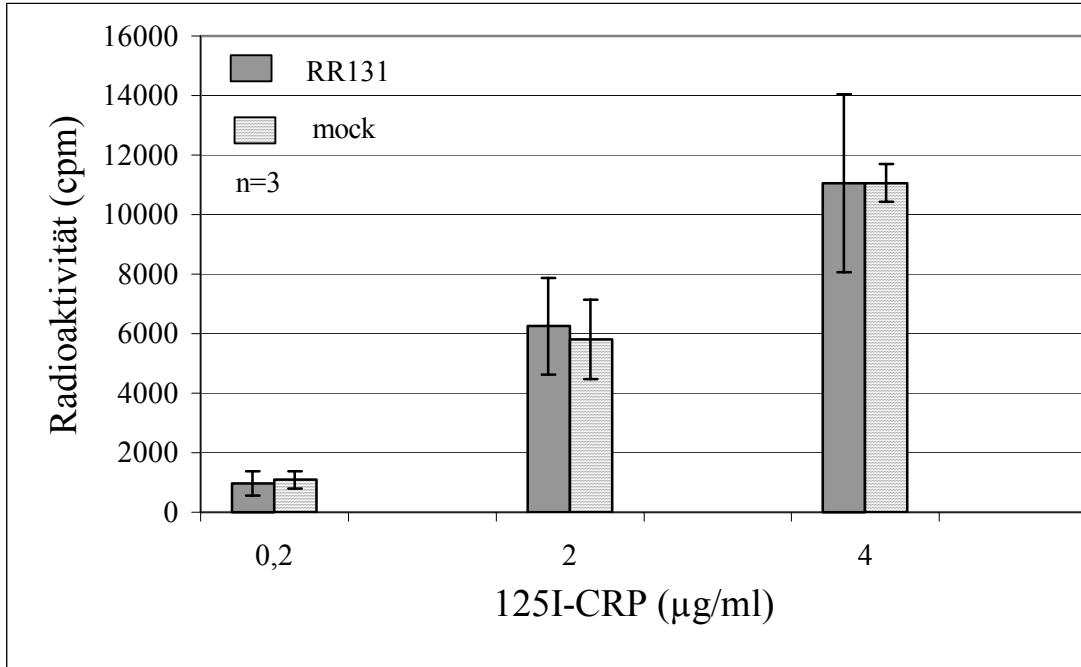


Abb. 22. Bindungsstudien mit verschiedenen Konzentrationen von 125I-CRP an mock- und Fc γ RIIa(RR-131)-transfizierten Zellen. Eine analoge Dosisabhängigkeit wurde sowohl für die Fc γ RIIa(RR-131)-transfizierten als auch für die mock-transfizierten Zellen beobachtet.

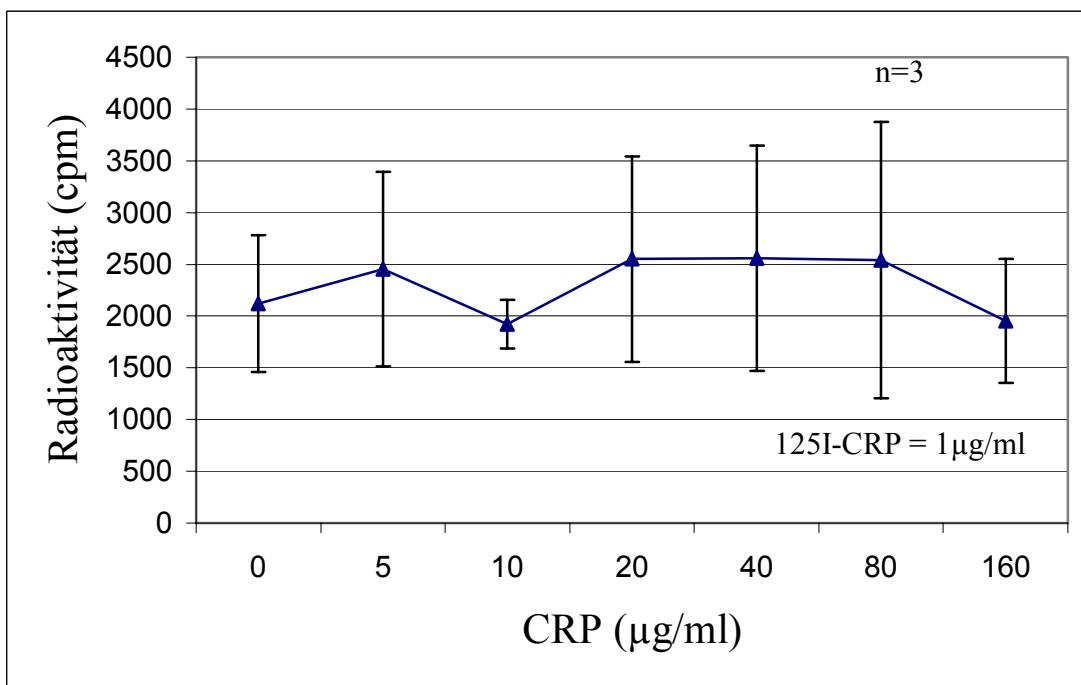


Abb. 23. Kompetitive Bindungsstudien mit kaltem Ligand. Fc γ RIIa (RR-131)-transfizierte Zellen wurden mit verschiedenen Konzentrationen von rCRP bei konstanten 125I-CRP-Konzentrationen (1 μg/ml) behandelt. Eine kompetitive Inhibition wurde für die 125I-CRP-Bindung nicht beobachtet.

2.4 Diskussion

Die vorliegende Dissertation war ursprünglich darauf ausgerichtet, die Bindungsstelle von CRP an Fc γ RIIa zu definieren. In den Arbeiten der Forschungsgruppe um T.W. Du Clos wurde mittels antikörper-abhängiger Methoden demonstriert, dass Fc γ RIIa der wichtigste Rezeptor für CRP an Leukozyten ist (70). Es wurde postuliert, dass Fc γ RIIa der hoch affine Rezeptor für CRP ist (70), während Fc γ RI als niedrig affiner CRP-Rezeptor identifiziert wurde (72) – also genau im Gegensatz zur Affinität dieser Rezeptoren für IgG. In einer anderen Arbeit derselben Gruppe wurde die unterschiedliche Bindungsstärke von CRP an die verschiedenen Fc γ RIIa-131-Allele in Versuchen mit menschlichen Monozyten und Neutrophilen demonstriert (80).

Diese Befunde wurden der Kritik unterzogen. Die Anwendung von Fab-Fragmenten von anti-CRP Antikörpern erbrachte keinen Nachweis einer CRP-Bindung an Fc γ RIIa in Leukozyten und Fc γ RIIa-transfizierten IIA.6-Zellen (81). Da IgG₁ durch seinen Fc-Teil natürlicher Ligand für Fc γ RIIa ist, könnte die Anwendung von Antikörpern zu falsch positiven Resultaten führen. Darüber hinaus wurde postuliert, dass die beobachtete CRP-Bindung an Fc γ RIIa die Folge einer IgG-Kontamination des verwendeten CRP-Reagenzes ist (82).

Die Ergebnisse der vorliegenden Dissertationsschrift zeigen, dass es sich um substantielle Kritiken handelt. In dieser Arbeit werden CRP-Bindungsstudien an COS-7 Zellen, transfiziert mit verschiedenen Fc γ RIIa-131-Allelen, durch antikörper-abhängige Methoden und Bindungsstudien mit direkt markiertem CRP präsentiert.

Antikörper-abhängige Bindungsstudien deuteten zunächst darauf hin, dass Fc γ RIIa der wichtigste CRP-Rezeptor ist. Zellen transfiziert mit Fc γ RIIa (RR-131) und inkubiert mit CRP zeigten eine signifikant höhere Anfärbung als mock-transfizierte Zellen. Die Abwesenheit von CRP in diesem System zeigte eine wesentlich schwächere Färbung. Dies war ein Hinweis dafür, dass CRP die Ursache für das stark positive Ergebnis dieser Bindungsstudien sein könnte und bestätigte somit die publizierten Daten (70). Darüber hinaus hatte die IgG-Kontamination der CRP-Reagenzen keinen Einfluß auf die Anfärbung, da es keinen Unterschied zwischen Zellen, inkubiert mit teilweise-gereinigtem, hoch-

gereinigtem und rekombinantem CRP (ppCRP, hpCRP und rCRP) gab. Diese Experimente widerlegten die Aussage, dass die beobachtete CRP-Bindung an Fc γ RIIa in antikörperabhängigen Detektionsverfahren durch eine IgG-Kontamination der CRP-Reagenzien verursacht wird und sprachen damit zusätzlich für eine CRP-Bindung an Fc γ RIIa.

Die Ergebnisse weiterer Untersuchungen, sowohl durch Experimente mit den verschiedenen Fc γ RIIa-Allelen als auch mit direkt markiertem CRP, führten allerdings zu einer anderen Interpretation:

Obwohl berichtet wurde (80), dass große Unterschiede in der Affinität von CRP zu Fc γ RIIa-131-Allelen (RR : RH : HH = 290 : 134 : 1) auf Monozyten bestehen, ist es uns nicht gelungen, solche ausgeprägten Affinitätsunterschiede in der CRP-Bindung an COS-7 Zellen, transfiziert mit den verschiedenen Fc γ RIIa-Allelen, zu detektieren. In der **antikörperabhängigen Detektion** betrug das Verhältnis der Färbungsintensitäten für RR : RH : HH 1.6 : 1.2 : 1.0 in Anwesenheit von CRP, und die Unterschiede waren damit wesentlich kleiner als das erwartete Verhältnis aus den Experimenten mit Monozyten (80). Interessanterweise zeigten die Fc γ RIIa-Allele eine unterschiedliche Affinität sowohl zu den anti-CRP Antikörpern, als auch zu der IgG₁ Isotyp-Kontrolle. Dies entspricht der schon bekannten Affinität von Fc γ RIIa-RR-131 und Fc γ RIIa-HH-131 zu IgG₁, die aufgrund dieser Eigenschaft als „high“ und „low responder“ Formen beschrieben werden (74-77). Insgesamt deuten diese Befunde darauf hin, dass den positiven Ergebnisse in den antikörperabhängigen Verfahren eine Antikörper/Fc γ RIIa-Interaktion zugrunde liegt. Als ein weiterer Hinweis für diese Interaktion sind Experimente zu werten, in denen eine Behandlung der Fc γ RIIa-exprimierenden Zellen mit dem präformierten Antikörper/Antigen-Komplex (anti-CRP/CRP) durchgeführt wurde. Diese Experimente führten zu dem selben hoch positiven Ergebnis wie die Färbung der Zellen durch sequentielle Zugabe der einzelnen Komponenten (70, 80). Im Gegensatz dazu, ergab sich bei Inkubation der Zellen mit der IgG₁-Isotyp-Kontrolle in Anwesenheit von CRP keine Zunahme der Färbung. Da die Bindung von Antikörpern an ihre Liganden zu einer Erhöhung der Affinität der Antikörper an Fc-Rezeptoren führt (86), weisen diese Experimente ebenfalls darauf hin, dass die

hochpositive Färbung in **Abb.14** und **Abb. 18** Folge einer anti-CRP/Fc γ RIIa Interaktion ist.

Die Ergebnisse von **antikörper-unabhängigen Verfahren** unterstützen diese Interpretation. Weder die Behandlung mit dem fluoreszenz- noch mit dem radioaktiv markierten CRP führte zu einer signifikant erhöhten Bindung an Fc γ RIIa-transfizierte Zellen im Vergleich zu mock-transfizierten Zellen. Es ergaben sich keine statistisch signifikanten Unterschiede bei der F-CRP-Färbung zwischen Fc γ RIIa (RR-131)-transfizierten Zellen und mock-transfizierten Zellen. Diese wurden beide mit ansteigenden Konzentrationen von F-CRP behandelt. Des weiteren hatten die Fc γ RIIa-131-Allele keine Wirkung auf die Ergebnisse der F-CRP-Bindung. Auch für die ^{125}I -CRP-Bindung ergaben sich zwischen Fc γ RIIa (RR-131)-transfizierten und mock-transfizierten Zellen keine Unterschiede.

Zusammenfassend leiten sich aus den Experimenten der vorliegenden Dissertation folgende Schlussfolgerungen ab: 1) Antikörper-unabhängige Studien deuten daraufhin, dass CRP nicht spezifisch an Fc γ RIIa, exprimiert in COS-7 Zellen, bindet. 2) Das hochpositive Ergebnis der antikörper-abhängigen Methoden könnte durch eine hochaffine Bindung des Antikörper/Antigen-Komplexes an Fc-Rezeptoren bedingt sein (s. **Abb. 24**). Voraussetzung dieser Interpretation ist, dass CRP mit den COS-7 Zellmembranen Fc γ RIIa-unabhängig interagiert. Diese Interaktion könnte mit Lysophospholipiden der Zellmembran stattfinden. Zwei Beobachtungen legen diese Interpretation nahe: (a) Die Inkubation sowohl der mock-transfizierten als auch der Fc γ RIIa-transfizierten Zellen mit F-CRP und ^{125}I -CRP zeigte eine dosis-abhängige Zunahme des Bindungssignals. Diese Zunahme des Signals deutet auf eine Interaktion der Zellen mit markiertem CRP unabhängig von der Anwesenheit von Fc γ RIIa hin. (b) Die CRP-Bindung an mock-transfizierte Zellen steigt mit der Zellalterung. Diese Beobachtung ist vereinbar mit früheren Berichten über die CRP-Interaktion mit Zellmembranen (28-32, 87, 88).

Obwohl die Bindungsstudien an COS-7 Zellen keine direkte Interaktion zwischen CRP und Fc γ RIIa zeigen, gibt es einige funktionelle Hinweise, dass CRP in Monozyten/Makrophagen und promyelitische Zelllinie HL-60 biologische

Funktionen durch Fc γ RIIa vermitteln kann. Zum Beispiel wurde gezeigt, dass CRP die intrazelluläre Ca $^{2+}$ -Konzentration in Monozyten (80) und HL-60 Zellen (83) erhöht. In HL-60 Zellen induziert CRP durch Fc γ RIIa offenbar eine Erhöhung der Tyrosine-Phosphorylierung und eine Membranlokalisierung der Phospholipase-C γ 2. Sowohl CRP als auch aggregiertes IgG induzieren die gleichen Signalwege in HL-60 Zellen. Dies weist auf analoge Rezeptoren hin (83). Ferner wurde demonstriert, dass CRP ein sog. Fc γ RIIa-*capping* (Anhäufung) induziert und mit Fc γ RIIa auf Makrophagenmembranen nach einer Inkubation mit dem LDL/CRP Komplex kolokalisiert (55). Letztendlich zeigen γ -Ketten-defiziente Mäuse, im Vergleich zu Wildtypmäusen, einen Verlust der Fähigkeit, CRP-opsoniertes Zymosan (ein CRP-Ligand) zu phagozytieren (84).

Es ist daher denkbar, dass nur liganden-gebundenes CRP mit Fc γ RIIa interagiert (55, 83, 84). Es muss zudem im Betracht gezogen werden, dass im Gegensatz zu den COS-7 Zellen, die Monozyten/Makrophagen und die HL-60 Zellen Rezeptor-clusters exprimieren, welche die Fc-Rezeptoren beinhalten, und die CRP-Effekte vermitteln können.

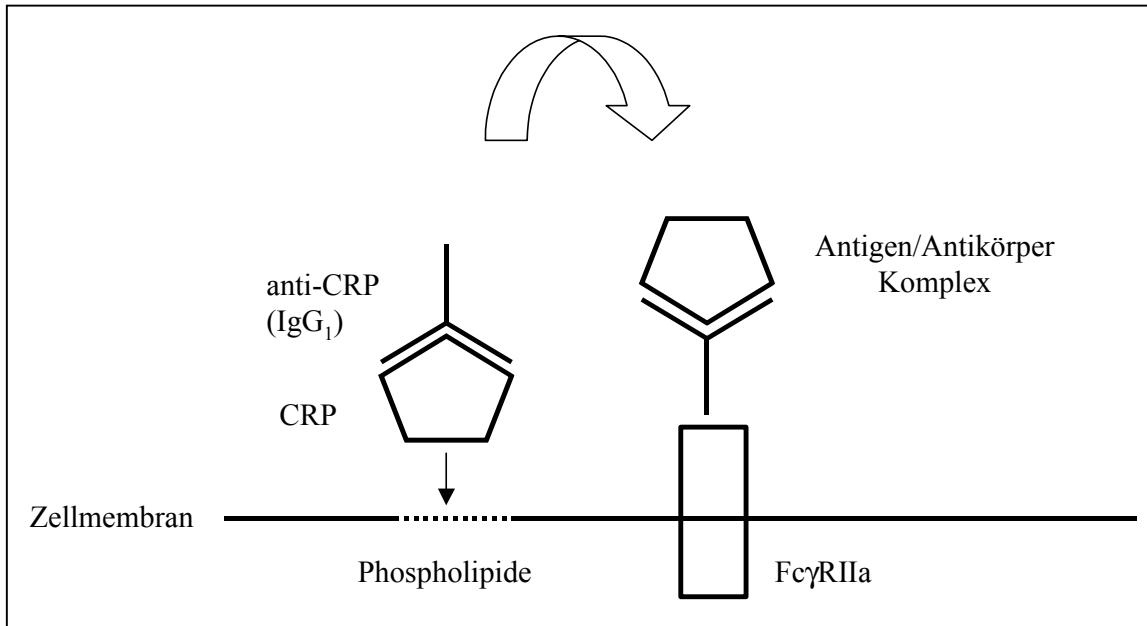


Abb. 24. Hypothetisches Modell der CRP/anti-CRP/Fc γ RIIa Interaktion. CRP bindet an Phospholipide von COS-7 Zellmembranen. Anti-CRP bindet CRP. Der Antigen/Antikörper-Komplex bindet mit verstärkter Affinität an Fc γ RIIa, im Gegensatz zu Antikörper ohne Ligand. Da das Epitop für anti-CRP, Klon 2C10, sich 5 mal wiederholt, kann ein mehrfacher IgG₁-Immunkomplex gebildet werden. Zur Vereinfachung ist nur ein einzelner Antikörper in der Abbildung gezeigt.

Insgesamt zeigt die vorliegende Dissertation, dass der direkte Beweis für Fc γ RIIa als wichtigster CRP-Rezeptor fehlt. Untersuchungen zur Natur der zellulären CRP-Rezeptoren müssen fortgesetzt werden. Im Kontext der Evidenzen zur pathogenetischen Rolle von CRP in Erkrankungen, insbesondere der Arteriosklerose, ist die Identifikation des zellulären CRP-Rezeptors von entscheidender Bedeutung.

2.5 Zusammenfassung

In den letzten Jahren wurde eine Bedeutung der angeborenen Immunität in der Genese kardiovaskulärer Erkrankungen diskutiert. In der vorliegenden Arbeit wird an zwei exemplarischen Beispielen eine potentielle pathophysiologische Bedeutung der angeborenen Immunität in kardiovaskulären Erkrankungen untersucht.

Es wurde durch kombinierte *ex vivo*- und *in vitro*-Untersuchungen demonstriert, dass der terminale Komplementkomplex C5b-9 möglicherweise zur Progression der Dilatativen Kardiomyopathie beiträgt, da C5b-9 im Myokard von Patienten mit Dilatativer Kardiomyopathie mit TNF- α kolokalisiert und *in vitro* eine Expression und Sekretion von TNF- α in Kardiomyozyten induziert.

Vor dem Hintergrund einer pathophysiologischen Bedeutung des C-reaktiven Proteins (CRP) im Rahmen der Arteriosklerose wurden Untersuchungen zur Frage des leukozytären CRP-Rezeptors durchgeführt. Es wurde gezeigt, dass CRP, im Gegensatz zu publizierten Daten, offenbar nicht an den Immunglobulinrezeptor Fc γ RIIa bindet und dass die antikörper-abhängige Detektion zur Definierung von Fc γ RIIa als CRP-Rezeptor von Antikörper/Fc-Rezeptor-Interaktionen beeinflusst waren. Die Suche nach dem zellulären CRP-Rezeptor sollte fortgesetzt werden.

2.6 Literaturverzeichnis

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2.7 Poster:

C-reactive protein-Fc γ RIIa-interactions – implications for
atherosclerosis

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C-reactive protein-Fc γ RIIa interactions – implications for atherosclerosis

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Introduction

C-reactive protein (CRP) is an acute phase protein in humans with an important role in innate immunity. During inflammation its concentration can be upregulated up to 1000-fold. CRP opsonizes biological particles, interacts with phagocytic cells and activates complement. CRP is involved in the pathogenesis of several diseases, including atherosclerosis, and is accepted as an important cardiovascular risk factor. The identification of the CRP-receptor on phagocytic cells is still a matter of debate.

Fc γ RIIa (CD32), the low affinity receptor for IgG, was recently described as the major receptor for CRP (1). Binding was demonstrated to be allele-specific with high affinity to Fc γ RIIa-RR-131, intermediate binding to Fc γ RIIa-RH-131 and no binding to Fc γ RIIa-HH-131 (2). As these observations were based on antibody-dependent assays the evidence was subjected to criticism (3).

In this study we investigate **CRP-binding to COS-7 cells transfected with different Fc γ RIIa-131 allelic forms using antibody-dependent and antibody-independent methods.** Our experiments suggest that Fc γ RIIa expressed on COS-7 cells does not bind CRP directly and the original observations were influenced by Fc γ RIIa-antibody interactions.

Materials and Methods

Cell culture: COS-7 cells – cell line that does not express Fc-receptors.

Cloning and Transfection: human Fc γ RIIa, coding for RR-131, and Fc γ RIIa, coding for HH-131, were cloned into pcDNA3.1. COS-7 cells were transfected with each of these vectors or with both together to express the heterozygous Fc γ RIIa-RH-131.

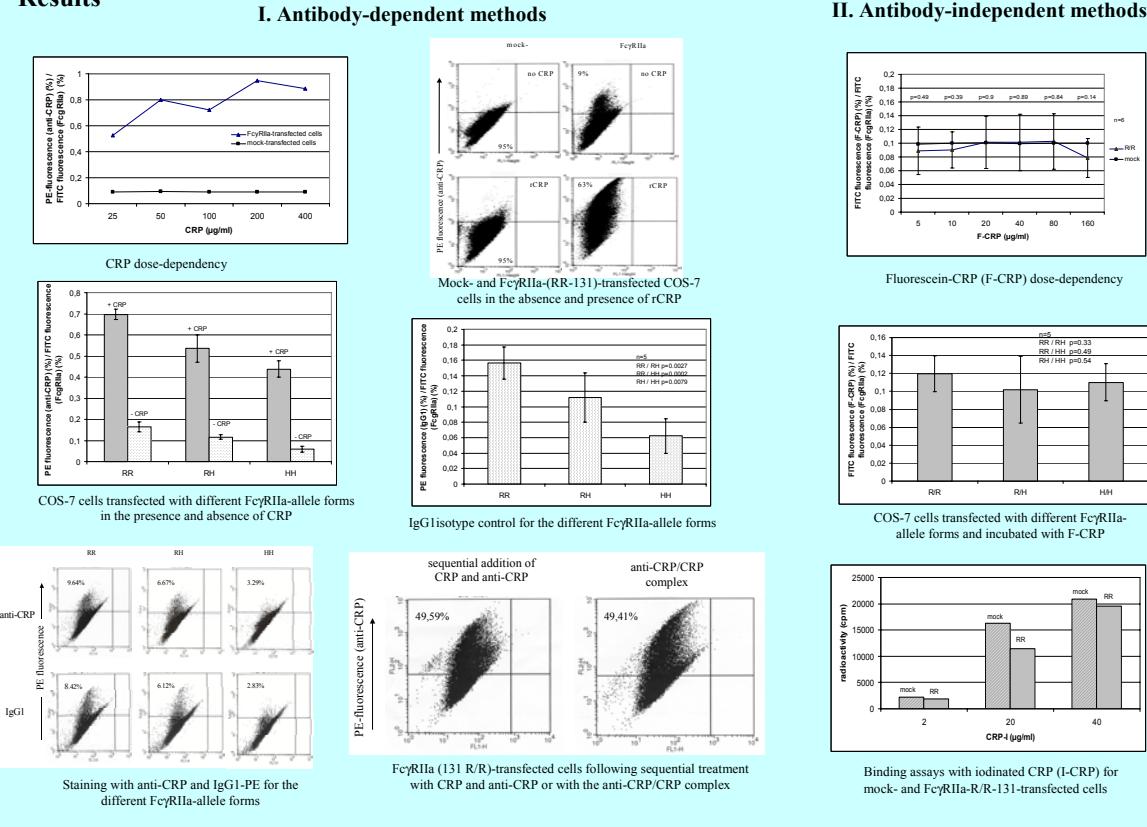
CRP Binding assay:

antibody dependent methods: cells transfected with different Fc γ RIIa-131 forms were incubated with CRP and further stained with anti-CRP, 2C10 and goat anti-mouse F(ab') $_2$ -PE.

antibody-independent methods: binding assays on transfected cells with Fluorescein-labelled or iodinated CRP (F-CRP and I-CRP).

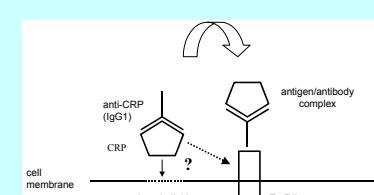
Detection: the anti-CRP and F-CRP staining of the transfected cells was tested through FACS analysis and the I-CRP binding through gamma counting.

Results



Discussion

- 1) antibody-independent assays suggest that CRP does not bind to Fc γ RIIa expressed in COS-7 cells
- 2) there seems to be non-specific interaction of CRP with COS-7 cell membranes, probably due to interaction with phospholipids
- 3) highly positive results in antibody-dependent assays may be influenced by high affinity binding of antigen/antibody complexes (CRP/anti-CRP) to Fc receptors (see Figure).



CRP binds to phospholipids in the COS-7 cell membrane. Anti-CRP binds CRP and the antigen/antibody complex binds to Fc γ RIIa with increased avidity as compared to the antibody without ligand.

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2.8 Manuscript:
C-reactive protein-Fc γ RIIa interactions
(eingereicht)

C-reactive protein-Fc γ RIIa interactions¹

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Abstract

Fc γ RIIa, the low affinity receptor for immunoglobulins, has recently been described to be a major receptor for C-reactive protein (CRP). CRP-binding to Fc γ RIIa on leukocytes was demonstrated to be allele-specific with high affinity binding to Fc γ RIIa (131 R/R), intermediate binding to Fc γ RIIa (131 R/H) and low affinity binding to Fc γ RIIa (131 H/H). As the original observations were based on antibody-dependent assays the evidence was subjected to criticism.

In this study we investigate CRP-binding to COS-7 cells transfected with different Fc γ RIIa-alleles using antibody-dependent methods and binding assays with directly labelled CRP. Whereas antibody-dependent methods using COS-7 cells expressing Fc γ RIIa (131 R/R) supported the conclusion of CRP binding, further experiments with Fc γ RIIa (131 R/H) and Fc γ RIIa (131 H/H)-expressing cells and use of directly labelled CRP led to the interpretation that, firstly, the original observations on CRP-binding to Fc γ RIIa were confounded by Fc γ RIIa-antibody-interactions and, secondly, CRP does not bind to Fc γ RIIa.
We conclude that investigations in cellular CRP receptors need to continue.

Introduction

C-reactive Protein (CRP)³ is the prototype acute-phase protein in humans and plays an important role in innate immunity (1, 2). In acute phase response CRP concentrations can raise up to 1000-fold compared to normal. The major biological functions identified up to now are binding to ligands (3), opsonization of biological particles (4, 5) and activation of the complement system (6). CRP is involved in the pathology of several diseases, and recently a contribution of CRP to atherogenesis has been proposed (7-13).

CRP displays binding to several ligands. For example, it binds to membrane phospholipids like lecithin and sphingomyelin (6, 14, 15), histones (16), to bacterial C-polysaccharide (3) and modified low density lipoprotein (LDL) (17, 18) via its Ca^{2+} -dependent phosphocholine (PCh) binding site. Ligand complexed CRP binds C1q and thereby initiates complement activation (6, 10).

CRP binding to cells has long been investigated. It was initially demonstrated that CRP binds to necrotic cells or damaged cell membranes only (19, 20), but not to normal cells. CRP was shown to bind to PCh in the membranes of damaged cells and this process requires partial hydrolysis of the membrane phospholipids (21). Also, CRP was reported to bind to apoptotic cells (22, 23).

Furthermore, CRP-binding to cellular receptors has been discussed. Whereas some reports provided evidence for specific CRP-receptors on leukocytes (24-28) other experiments demonstrated interaction with Fc receptors (29, 30). The high affinity IgG-receptor Fc γ RI was identified as a low affinity CRP-receptor (31), whereas, recently, the low affinity IgG-receptor Fc γ RIIa was described as the major receptor for CRP (32). There are two codominantly expressed allelic variants of Fc γ RIIa resulting in a change at amino acid position 131 displaying different affinity to IgG₁ and IgG₂ (33-37). Fc γ RIIa (131 R/R) has been defined as the “high responder” form of the receptor and Fc γ RIIa (131 H/H) as the “low responder” form for IgG binding. It has been reported that these two allelic variants confer distinct CRP-

binding to leukocytes (38). In monocytes, CRP-binding to cells expressing Fc γ RIIa (131 H/H) was hardly detectable whereas significant binding has been demonstrated for Fc γ RIIa (131 R/H) and Fc γ RIIa (131 R/R). However, as the initial reports (32, 38) used anti-CRP antibodies in order to demonstrate CRP-binding to Fc γ RIIa it was suggested that detection of CRP-binding to Fc γ RIIa results from interaction of the Fc-part of the anti-CRP-antibodies with Fc γ RIIa itself (39). In experiments using F(ab')₂ fragments of anti-CRP antibodies (Clone CRP 8, Sigma) no CRP-binding to Fc γ RIIa-R131 was found on polymorphonuclear leucocytes and Fc γ RIIa-transfected IIA.6 cells. Other authors proposed that the observation of CRP-binding to Fc γ RIIa might be due to IgG-contamination of the CRP reagent used in the binding studies (40). However, evidence for Fc γ RIIa being a CRP receptor is not confined to results from these direct binding assays, but there is some functional evidence for CRP-Fc γ RIIa interaction from other *in vitro*-experiments (13, 41) and from experiments with γ -chain-deficient mice (42).

In the light of substantial evidence for an intimate involvement of CRP in the pathology of several diseases the identification of the major CRP-receptor gains increasing interest. In this paper we describe antibody-dependent approaches as well as assays with directly labelled CRP in a system utilizing COS-7 cells transfected with the different Fc γ RIIa-alleles.

Materials and methods

Reagents and Antibodies.

CRP: Partially purified CRP (ppCRP) was obtained from Sigma (C-4063). Highly purified CRP (hpCRP) was kindly provided by Dr. Terry W. Du Clos (New Mexico University, Albuquerque, NM), it was purified from human pleural fluids as described (16). Recombinant CRP (rCRP) was obtained from Calbiochem (236608). Human IgG was obtained from Sigma, (I-2511). Monoclonal anti-CRP, clone 2C10 (mouse IgG₁), was generously provided by Dr. Terry W. Du Clos, with the kind permission of Dr. Larry Potempa (ImmTech, Evanston, IL). Anti-CD32-FITC, clone FLI8.26 (2003) and PE-mIgG₁ isotype control, was purchased from BD PharMingen, affinity-isolated F(ab')₂ PE-GAM from Caltag Labs and HRP-conjugated anti-human IgG (Fc specific) from Dianova.

Fluorescein-labelling of rCRP (F-CRP) was prepared using Alexa Fluor 488 protein labelling kit from Molecular Probes following the manufacturer's instructions.

Iodination of rCRP (125I-CRP) using Chloramin T Method was performed by the Central Laboratory of Radioisotope Application of Ulm University.

Cell Culture.

COS-7 cells were obtained from DSMZ - German Collection of Microorganisms and Cell Cultures. The cell line was maintained in DMEM with 10% FCS and 1% Penicillin/Streptomycin/L-Glutamine.

Fc γ RIIa-cloning and transfection.

pcDNA3.1-Fc γ RIIa was prepared as follows: Human Fc γ RIIa cDNA, containing G/G genotype, (coding for allele Fc γ RIIa R/R-131) was generated by RT-PCR and cloned into pcDNA3.1 using Directional TOPO Expression Kit (Invitrogen). cDNA Fc γ RIIa, containing

A/A genotype, (coding for allele Fc γ RIIa H/H-131) was generated from Fc γ RIIa G/G genotype vector using site-specific mutagenesis by overlap extension (43). Both vectors were sequenced. 6-well plates were seeded at 2.5×10^5 cells/well and grown to 70-80% confluence. COS-7 cells, a cell line that does not express Fc-receptors, were transfected with cDNA-Fc γ RIIa vectors using Polyfect Transfectant Reagent according to manufacturer's protocol (QIAGEN). COS-7 cells expressing the heterozygous Fc γ RIIa RH-131 (Fc γ RIIa G/A genotype) were obtained by cotransfection with both, the Fc γ RIIa G/G and A/A vector. Control cells (mock-transfected cells) were treated with transfectant reagent only.

CRP Binding Assays

CRP binding to Fc γ RIIa R/R-131-transfected COS 7-cells was investigated using antibody dependent assays as well as approaches with directly labelled CRP (F-CRP and 125 I-CRP). For antibody-dependent assays, cells were detached using PBS/EDTA 0.02% 48h after transfection and centrifuged at 300g for 5min. Three wells were pooled to a cell number of 7.5×10^5 to 10^6 cells to form one sample. Cells were further washed with calcium-free PBS and then with ice-cold PBS containing 0.05% sodium azide and 0.1% BSA (PAB). Cells were resuspended in 100 μ l of ice-cold PAB and incubated with different concentrations of CRP for 1h on ice. Cells were washed twice with ice-cold PAB. Cells were then stained with anti-CRP, 2C10, for 0.5h on ice. Cells were washed twice with PAB and stained with PE-GAM-F(ab')₂ for 0.5h on ice in dark. Finally, cells were washed twice, resuspended in 500 μ l PAB/sample and subjected to FACS analysis. Furthermore, cells transfected with different Fc γ RIIa alleles were investigated. Transfection efficiency (Fc γ RIIa-allele expression) was tested by staining cells with anti-Fc γ RIIa-FITC.

For binding assays with directly labelled CRP, detached cells (7.5×10^5 to 10^6 cells per 100 μ l PAB) were incubated for 1h on ice with increasing concentrations of F-CRP. CRP binding to

cells was investigated after two washing steps. Furthermore F-CRP binding to cells transfected with different Fc γ RIIa alleles was investigated. F-CRP binding was detected by FACS-analysis. For radioactive binding assays, detached cells (7.5×10^5 to 10^6 cells per 100 μ l PAB) were incubated for 1h on ice with increasing concentrations of ^{125}I -CRP. Competitive binding assays with cold CRP were performed as controls. ^{125}I -CRP binding to cells was detected using 1261 Multigamma counter (LKB Wallac).

Flow Cytometry.

Cells were analyzed using FACSCalibur® flow cytometer equipped with CellQuest software (Becton Dickinson). 30 000 cells were gated by fluorescence-1 and -2 (FL1-Height and FL2-Height).

Western blot.

Definitive amounts of CRP and IgG samples were boiled 1:1 with Laemmli's sample buffer and separated in 12% SDS-PAGE. Nitrocellulose membrane (Hybond-C extra, Amersham) was incubated with HRP-conjugated anti-human IgG (Fc specific), 1:10 000, in blocking solution (4% skim milk powder, 1% BSA in PBS) for 1h. After 6 washing steps the membrane was treated with ECL detection reagent and then a Hyperfilm (Amersham) was exposed on it overnight.

Statistics

Results are expressed as mean \pm SD. Scores were compared using Students paired t-test (Microsoft Excel 2000). A p<0.05 was considered statistically significant.

Results

Antibody-dependent methods:

Dose-response dependency.

Cells were incubated with various CRP concentrations and FACS analysis was performed using anti-CRP antibodies. For each CRP-concentration, 95% of mock-transfected cells treated in the same way, were assessed as background. In accordance with the original data (29, 32, 38) parallel staining of mock-transfected and Fc γ RIIa (131 R/R)-transfected cells in the presence of CRP showed a maximum increase in PE-staining for Fc γ RIIa (131 R/R)-transfected cells at a CRP-concentration of 200 μ g/ml (**Fig. 1**).

Effect of IgG-contamination of the CRP reagent.

IgG contamination of various CRP reagents was tested through Western immunoblot (**Fig. 2A**). Three different CRPs were tested: partially purified CRP (ppCRP), highly purified CRP (hpCRP) and recombinant CRP (rCRP). Decreasing amounts of IgG were used as positive controls. Samples were separated using SDS-PAGE and immunoblotted with HRP-conjugated anti-human IgG (Fc specific). The 50kDa (H chain) human IgG band was detected in partially purified CRP and evaluated as <0.1%. No IgG was detected in highly purified CRP and recombinant CRP.

In order to test whether the result of anti-CRP-antibody dependent binding assays depends on the presence of IgG in the CRP reagents, Fc γ RIIa (131 R/R)-transfected and parallel mock-transfected COS-7 cells were incubated with different CRP-preparations (ppCRP, hpCRP and rCRP, see *Materials and methods*) at 200 μ g/ml. Again, 95% of mock-transfected cells were assessed as background. A marked increase in fluorescence was observed in all cases (**Fig. 2B**).

Fc γ RIIa-alleles and involvement of anti-CRP-antibody/Fc γ RIIa-interactions.

CRP binding to mock-transfected and Fc γ RIIa-transfected cells was investigated in the presence and absence of CRP. Again, 95% of mock-transfected cells were assessed as background. **Fig. 3A** shows a weak increase in PE-staining (9%) in Fc γ RIIa (131 R/R)-transfected cells compared to mock-transfected cells in the absence of CRP. In contrast, staining of Fc γ RIIa (131 R/R)-transfected cells in the presence of recombinant CRP showed a remarkable increase in PE-staining.

In order to test the effect of allelic variants of Fc γ RIIa on the result of antibody-dependent binding assay cells were transfected with different Fc γ RIIa-alleles. Transfection efficiency as assessed by FACS analysis was similar for the different alleles (~50%). Binding assays were performed in the presence and absence of rCRP (**Fig. 3B**). In accordance to the results given in figure 2B and 3A incubation with CRP led to a marked increase in PE-staining compared to cells not incubated with CRP. Different staining was observed for the different alleles. The ratio RR : RH : HH in the presence of CRP was 1.6 : 1.2 : 1.0. In the absence of CRP, the ratio for anti-CRP-antibody staining was 2.8 : 2.0 : 1.0. The observed differences were statistically significant. The ratios for both, staining in the presence and absence of CRP, showed similar tendency to decrease in the order RR : RH : HH.

In analogy, the mouse IgG₁ isotype control revealed similar results for binding to Fc γ RIIa-forms (**Fig. 3C**) with a ratio of 2.5 : 1.8 : 1.0 and statistical significance (**Fig. 3D**). Addition of CRP to this system did not influence the results (not shown).

Lastly, preincubation of anti-CRP and CRP in order to generate the CRP/anti-CRP complex with consecutive treatment of cells with this complex led to the same highly positive results (Fig. 3E, right) as obtained by addition of CRP to the cells and later incubation with anti-CRP (Fig. 3E, left or figures 2B, 3A, respectively).

CRP binding to ageing cells

Comparison of early passages and later passages of mock-transfected COS-7 cells incubated with CRP showed a remarkable increase in anti-CRP/PE-staining with cell ageing (**Fig. 3F**).

Binding assays using directly labelled CRP:

Lack of detection of CRP binding to Fc γ RIIa

CRP binding to mock-transfected and Fc γ RIIa-transfected cells was investigated using Fluorescein-labelled CRP (F-CRP). 95% of cells incubated with the lowest CRP-concentration (5 μ g/ml) were assessed as background. **Fig. 4A** shows a dose-response dependent increase in F-CRP staining using F-CRP-concentrations ranging from 5 to 160 μ g/ml. F-CRP-staining signal increased for both, mock-transfected and Fc γ RIIa (131 R/R)-transfected cells in a dose-dependent manner. Fc γ RIIa (131 R/R)-transfected cells did not show more intense staining signals compared to mock-transfected cells. Furthermore, no differences were observed for F-CRP (80 μ g/ml) binding to cells transfected with different Fc γ RIIa-alleles (**Fig. 4B**).

Binding assays with increasing concentrations of 125 I-CRP showed dose-response dependent increase in radioactivity (**Fig. 4C**). Radioactivity (cpm) increased for both, mock-transfected and Fc γ RIIa (131 R/R)-transfected cells. Again, Fc γ RIIa (131 R/R)-transfected cells did not show more intense radioactive signals as compared to mock-transfected cells. Furthermore, competitive binding studies with cold ligand did not show any inhibition of 125 I-CRP-binding to the cells (**Fig. 4D**).

Discussion

In this study we investigate CRP-binding to COS-7 cells transfected with different Fc γ RIIa-alleles using antibody-dependent method and binding assays with directly labelled CRP.

Antibody-dependent binding assays indeed suggested that Fc γ RIIa is a major receptor for CRP as cells transfected with Fc γ RIIa (131 R/R) and incubated with CRP showed significantly higher staining than mock-transfected cells. Absence of CRP from this system revealed significantly less staining, thus indicating that CRP is the essential cause for the strongly positive result of these assays. Furthermore, IgG contamination of CRP-preparations did not influence the staining as there was no difference between Fc γ RIIa (131 R/R)-transfected cells incubated with partially purified, highly purified or recombinant CRP. Thus, these experiments supported recent findings that Fc γ RIIa is a major CRP-receptor (32, 38).

However, further experiments investigating CRP-binding to different allelic variants of Fc γ RIIa expressed in COS-7 cells led to a different interpretation. Although a previous report (38) described extensive differences in the affinity of CRP to monocytes expressing the various Fc γ RIIa-alleles (RR : RH : HH = 290 : 134 : 1) we were not able to detect such differences in our CRP binding assays on COS-7 cells transfected with the different alleles using anti-CRP-staining. The ratio between staining intensities for RR : RH : HH in the presence of CRP was 1.6 : 1.2 : 1 and thus, much smaller than expected from the experiments with monocytes (38). Interestingly, the Fc γ RIIa allelic variants showed different affinities to the anti-CRP-antibody as well as its isotype-matched control, and the ratios were of similar tendency as the ones observed in the presence of CRP. The latter results are in line with the known affinity of the Fc γ RIIa allelic variants to IgG₁ described as “high”- and “low responder” forms (33-37) and suggest an involvement of antibody/Fc γ RIIa interactions in the positive results obtained with CRP.

Notably, preincubation of anti-CRP and CRP in order to generate the antigen/antibody complex with consecutive treatment of cells with this complex led to the same highly positive results as those obtained by sequential addition of these components as performed in the original experiments (32, 38). In contrast, experiments with isotype-matched control antibody and CRP did not result in any increase in staining. As it is known (44) that binding of an antibody to its ligand increases the affinity of the Fc-part of the antibody to Fc receptors these experiments suggest that highly positive staining in figure 3A and 3E results from interaction of Fc γ RIIa with anti-CRP.

This interpretation is supported by use of antibody-independent assays. Neither use of fluorescein-nor radioactively labelled CRP led to detection of significantly increased binding to Fc γ RIIa-transfected cells as compared to mock-transfected cells. For F-CRP, there was no statistically significant difference in staining between Fc γ RIIa (131 R/R)-transfected cells treated with increasing F-CRP concentrations as compared to mock-transfected cells. Furthermore, the Fc γ RIIa allelic variants did not influence the results of F-CRP binding. Also, for 125I-CRP no differences in binding between Fc γ RIIa (131 R/R)-transfected and mock-transfected cells were observed.

Our experiments tend us to draw the following conclusion: Firstly, the antibody-independent assays suggest that CRP does not bind to Fc γ RIIa expressed in COS-7 cells. Secondly, highly positive results in antibody-dependent assays are influenced by high affinity binding of antigen/antibody complexes (CRP/anti-CRP) to Fc receptors (see **Fig. 5**). The requirement for this interpretation is that CRP interacts with COS-7 cell membranes in an Fc γ RIIa-independent manner, most likely due to interaction with lysophospholipids. The latter is supported by two observations: (a) Incubation of mock-transfected cells with F-CRP and 125I-CRP both shows dose-dependent increase in binding signal clearly suggesting an interaction of cells with labelled CRP independent from presence or absence of Fc γ RIIa. (b)

Cell ageing increases CRP binding to mock-transfected cells, and this observation is in line with previous reports on CRP interaction with cell membranes (19-21, 45, 46).

Although these binding assays in COS-7 cells clearly suggest that there is no direct interaction between CRP and Fc γ RIIa, there is some recent functional evidence that CRP, in monocytes/macrophages and promyelitic cell line HL60, might mediate biological functions through Fc γ RIIa. For example, CRP has been shown to induce transient intracellular calcium elevation in monocytes (38) and HL60 cells (41). In monocytes, CRP-induced transient Ca-influx was allele-dependent suggesting an involvement of Fc γ RIIa in CRP-mediated effects. In HL60 cells, CRP and aggregated IgG showed the same signalling pathways, thus indicating analogous receptors (41). Furthermore, it has been demonstrated that CRP induces Fc γ RIIa-capping and is colocalized with Fc γ RIIa on macrophage cell membranes after incubating these cells with native LDL and CRP (13). Lastly, experiments with γ -chain-deficient mice showed loss of ability to phagocytose CRP-opsonized zymosan as compared to wild-type mice (42). It is thus conceivable that only ligand bound CRP interacts with Fc γ RIIa (13, 41, 42) or it has to be considered, that, in contrast to COS-7 cells, monocytes/macrophages and HL60 cells may express receptor clusters involving Fc receptors that mediate CRP effects.

We conclude that direct proof of Fc γ RIIa as the major CRP-receptor is still a matter of debate. Investigations in the nature of cellular CRP receptors need to continue. In light of substantial evidence for a pathogenic role of CRP in disease this issue gains increasing importance.

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³Abbreviations used:

CRP, C-reactive protein; LDL, low density lipoprotein; PCh, phosphocholine; pp(CRP), partially purified; hp(CRP), highly purified; PE-GAM, PE-conjugated F(ab')₂ goat anti-mouse IgG₁; F-CRP, Fluorescein-labelled CRP; ¹²⁵I-CRP, iodinated CRP; PAB, PBS containing 0.1% BSA and 0.05% sodium azide

Figure legends

Figure 1

Antibody-dependent method: Dose-response curve.

Mock-transfected and Fc γ RIIa (131 R/R)-transfected cells were incubated with increasing concentrations of rCRP. Staining was performed using anti-CRP, 2C10, and F(ab')₂-PE-GAM following FACS analysis. For each CRP-concentration, 95% of mock-transfected cells treated in the same way, were assessed as background. Figure shows CRP dose-dependent increase in PE-fluorescence with a maximum at 200 μ g/ml rCRP.

Figure 2.

Antibody-dependent method: IgG contamination.

A. Western blot of different CRP reagents for IgG contamination.

ppCRP - partially purified CRP, hpCRP - highly purified CRP, rCRP - recombinant CRP.

Lane 1: 50 μ g ppCRP, lane 2: 50 μ g hpCRP, lane 3: 25 μ g ppCRP, lane 4: 25 μ g hpCRP, lane 5: 25 μ g rCRP, lane 6 to lane 11: IgG - 2 μ g, 1 μ g, 500ng, 250ng, 100ng, 50ng.

50 μ g of ppCRP contains less than 50ng (< 0.1%) IgG.

B. FACS analysis of anti-CRP/PE-staining after incubation of parallel mock- (left) and Fc γ RIIa (131 R/R)-transfected (right) COS-7 cells with ppCRP, hpCRP and rCRP. Results were independent from IgG-contamination.

Figure 3.

Antibody-dependent method: Antibody/Fc γ RIIa-interactions. For each CRP-concentration, 95% of mock-transfected cells treated in the same way, were assessed as background.

- A.** FACS analysis of mock-transfected (left) and Fc γ RIIa (131 R/R)-transfected (right) COS-7 cells in the absence and presence of rCRP (200 μ g/ml). Fc γ RIIa (131 R/R)-transfected cells show a 9% PE-staining in the absence and a 63% staining in the presence of CRP.
- B.** Results for COS-7 cells transfected with different Fc γ RIIa alleles in the presence and absence of CRP. Anti-CRP/PE-staining was related to Fc γ RIIa-allele expression as assessed by staining with anti-CD32-FITC (see methods). RR : RH : HH - staining ratio in the presence of CRP is 1.6 : 1.2 : 1.0 and in the absence of CRP is 2.8 : 2.0 : 1.0.
- C.** FACS analysis of Fc γ RIIa (131 R/R-), Fc γ RIIa (131 R/H-), Fc γ RIIa (131 H/H)-transfected COS-7 cells incubated with anti-CRP/ (upper row) and mouse IgG₁-PE isotype control (lower row) in the absence of CRP. Figure shows representative experiment.
- D.** IgG₁ isotype control: Results for COS-7 cells transfected with different Fc γ RIIa alleles in the absence of CRP. Again, IgG₁-PE-staining was related to Fc γ RIIa-allele expression as assessed by staining with anti-CD32-FITC. RR : RH : HH - staining ratio is 2.5 : 1.8 : 1.0.
- E.** FACS analysis of Fc γ RIIa (131 R/R)-transfected COS-7 cells following either sequential treatment first with CRP and later with anti-CRP (left) or, alternatively, treatment with the preincubated anti-CRP/CRP complex (right). No differences were observed.
- F.** Effect of cell ageing: Comparison of mock-transfected COS-7 cell passages 5 and 17 incubated with CRP showed a remarkable increase in anti-CRP/PE-staining at passage 17 (low right).

Figure 4

Binding assays with directly labelled CRP:

A. Mock-transfected and Fc γ RIIa (131 R/R)-transfected cells were incubated with increasing concentrations of F-CRP. 95% of cells incubated with the lowest CRP-concentration (5 μ g/ml) were assessed as background. Figure shows dose-dependent increase in F-CRP-staining using F-CRP-concentrations ranging from 5 to 160 μ g/ml. F-CRP-staining signal increased for both, mock-transfected and Fc γ RIIa (131 R/R)-transfected cells in a similar way.

B. F-CRP binding to COS-7 cells transfected with different Fc γ RIIa-alleles. F-CRP-staining was related to Fc γ RIIa-allele expression as assessed by staining with anti-CD32-FITC. No differences were observed for F-CRP (80 μ g/ml) binding to cells transfected with different Fc γ RIIa-alleles.

C. Binding assays with different concentrations of 125I-CRP for mock- and Fc γ RIIa (131 R/R)-transfected cells. A similar dose-dependency of 125I-CRP-binding was observed for both, Fc γ RIIa (131 R/R)- and mock-transfected cells.

D. Competitive binding assays with cold ligand. Fc γ RIIa (131R/R)-transfected cells were treated with different concentrations of rCRP and constant concentration of 125I-CRP (1 μ g/ml). No competitive inhibition of 125I-CRP binding was observed.

Figure 5

Hypothetical model of CRP/anti-CRP/Fc γ RIIa interactions. CRP binds to phospholipids in the COS-7 cell membrane. Anti-CRP binds CRP and the antigen/antibody complex binds to Fc γ RIIa with increased avidity as compared to the antibody without ligand. As the epitope for the 2C10 mAb is repeated 5 times a multimeric IgG₁-immune complex with CRP may be formed. For reasons of simplification, only one antibody is delineated in the figure.

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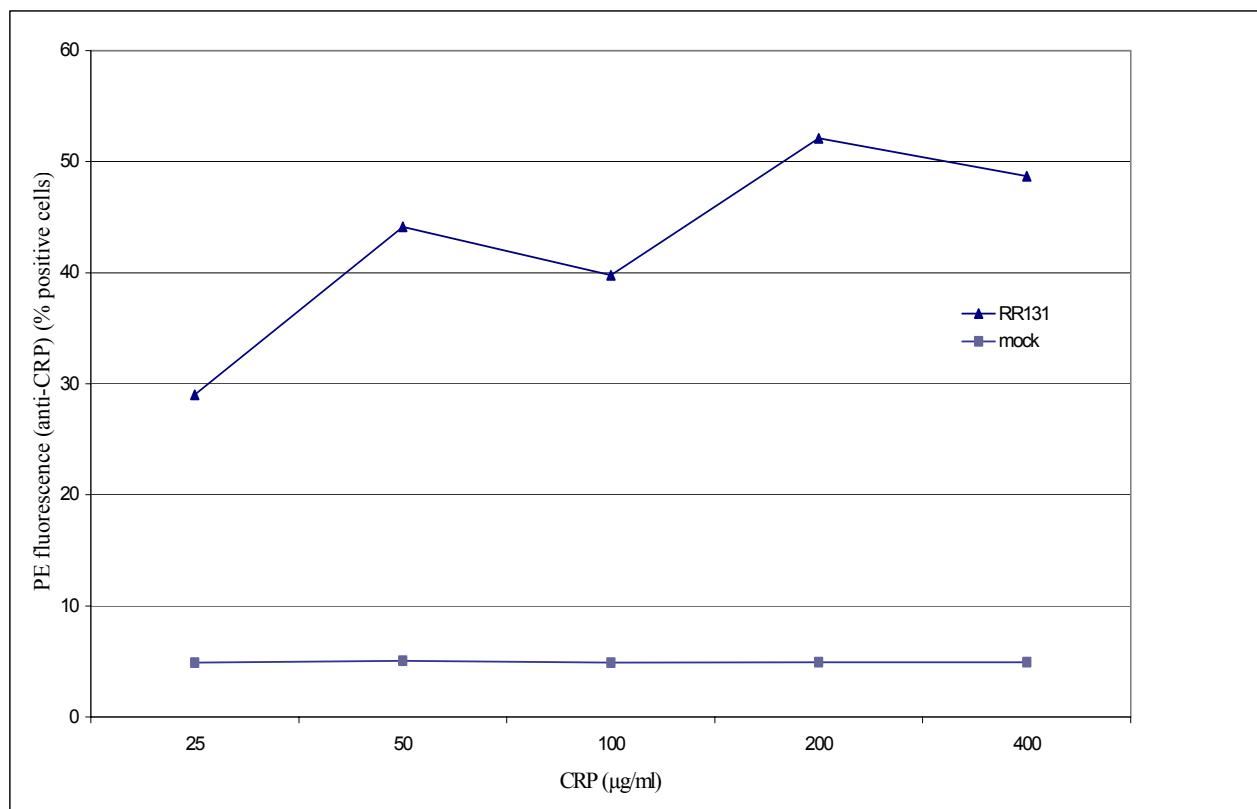


Fig. 1

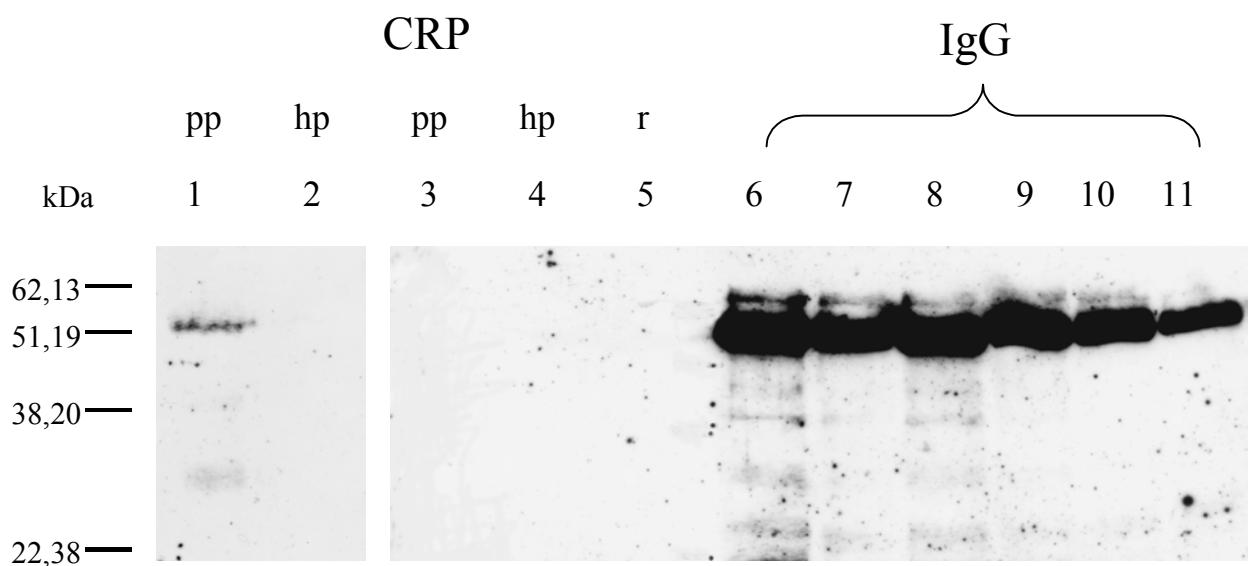


Fig. 2 A

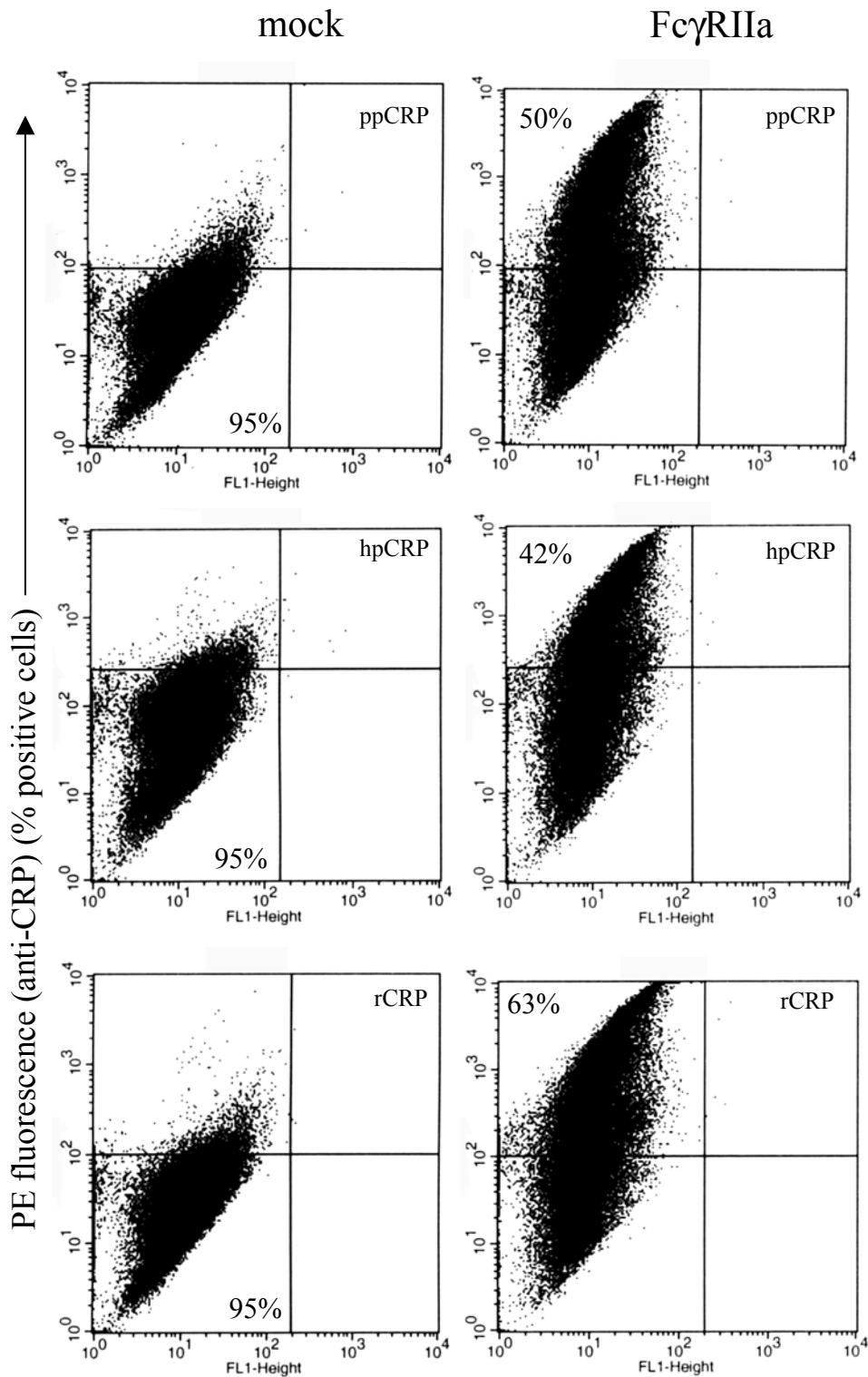


Fig. 2 B

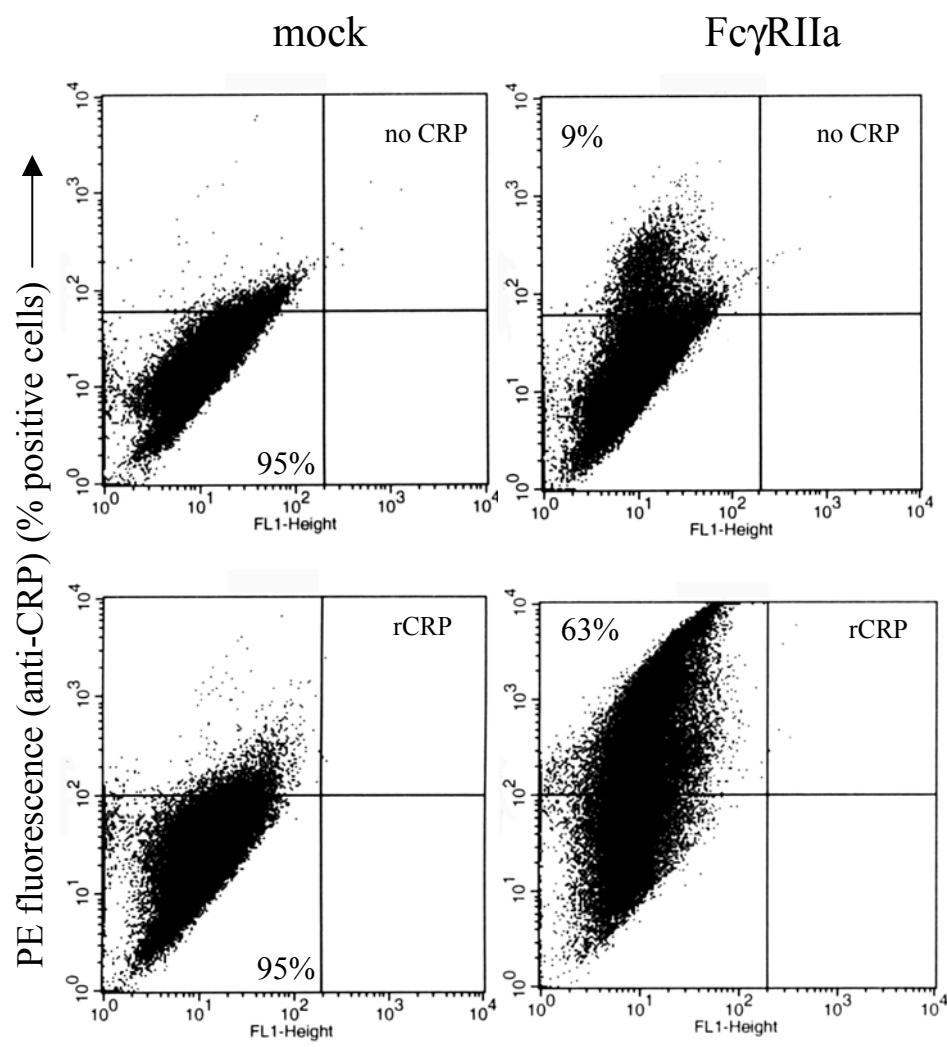


Fig. 3 A

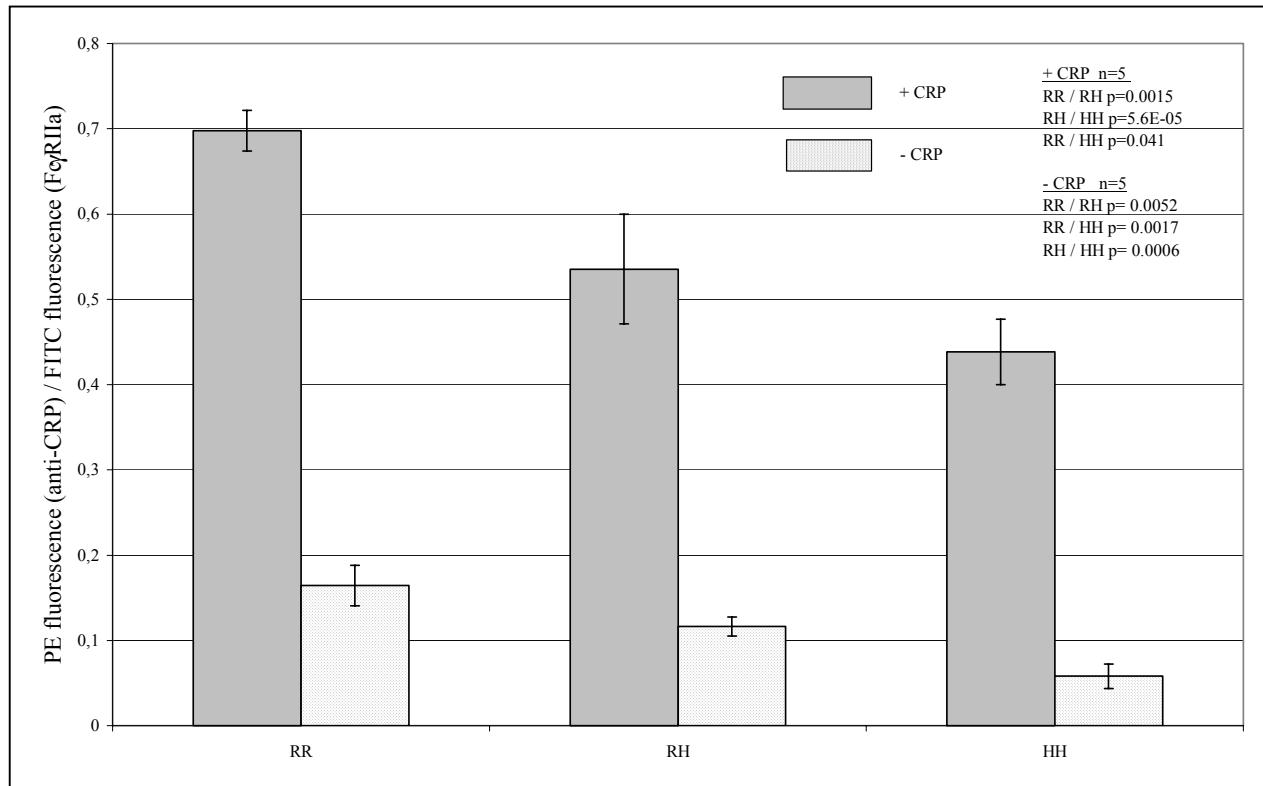


Fig. 3 B

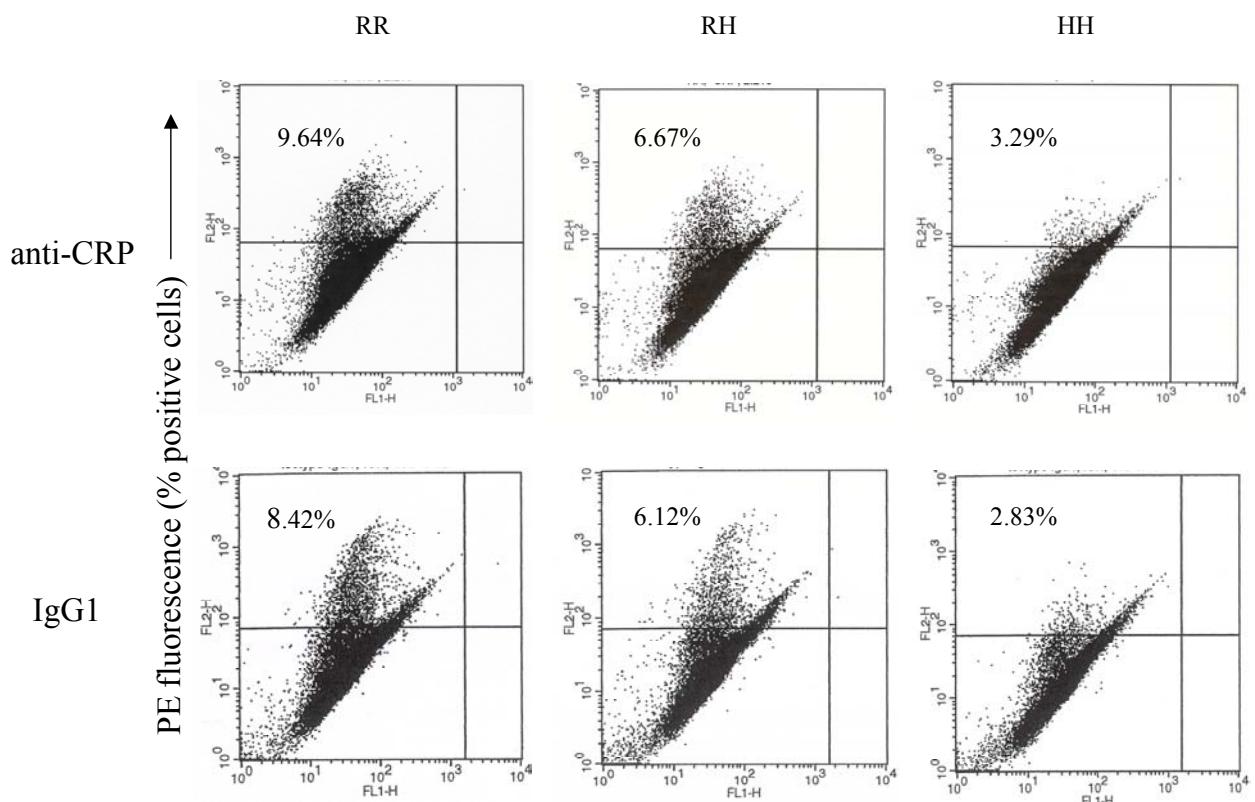


Fig. 3 C

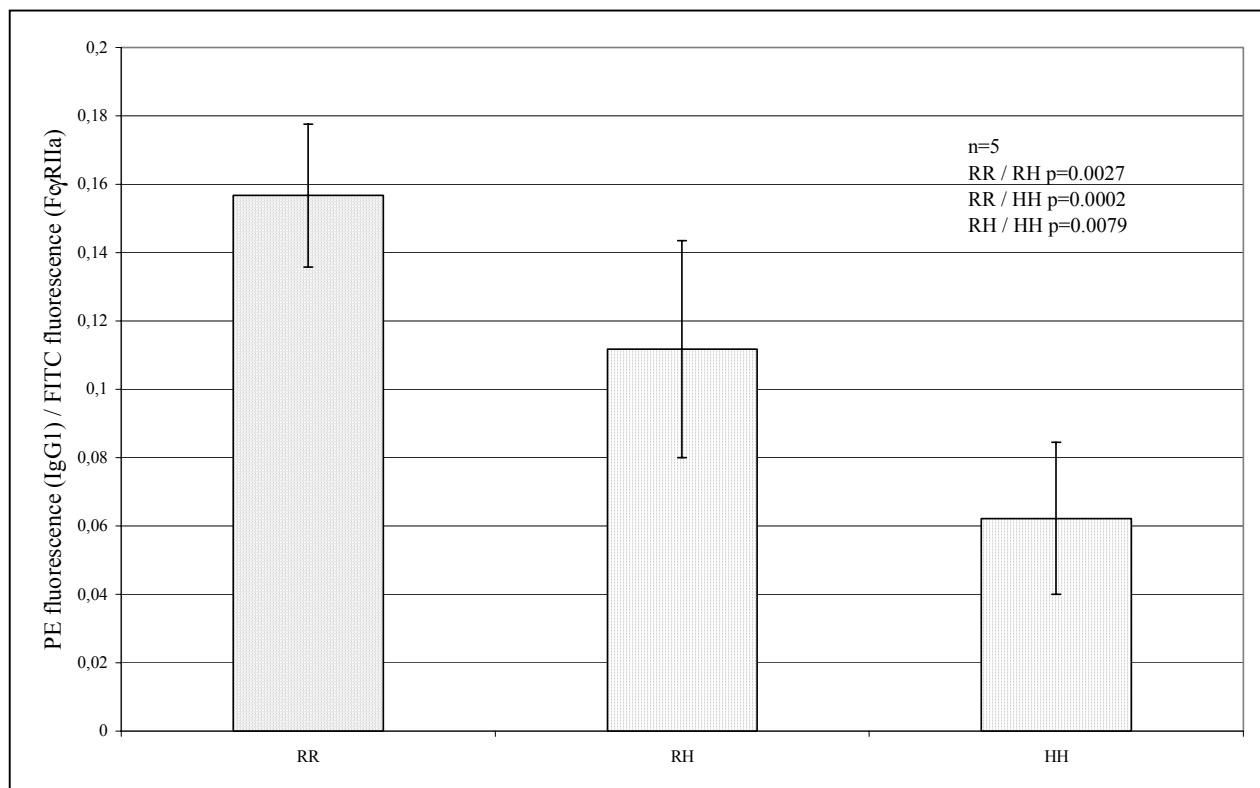


Fig. 3 D

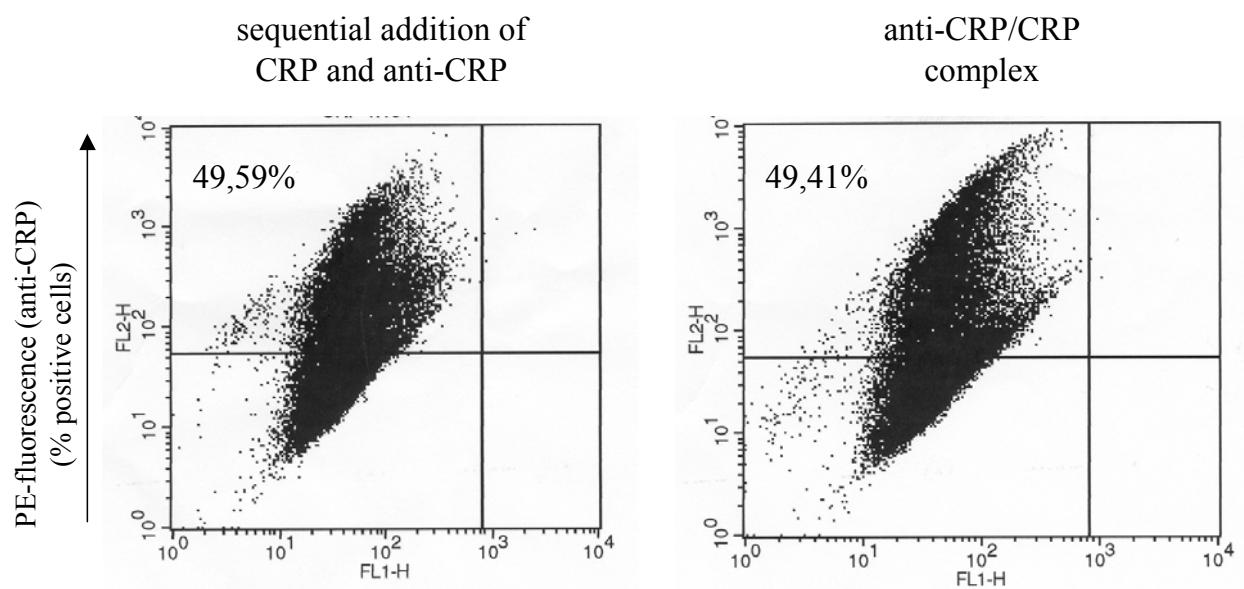


Fig. 3 E

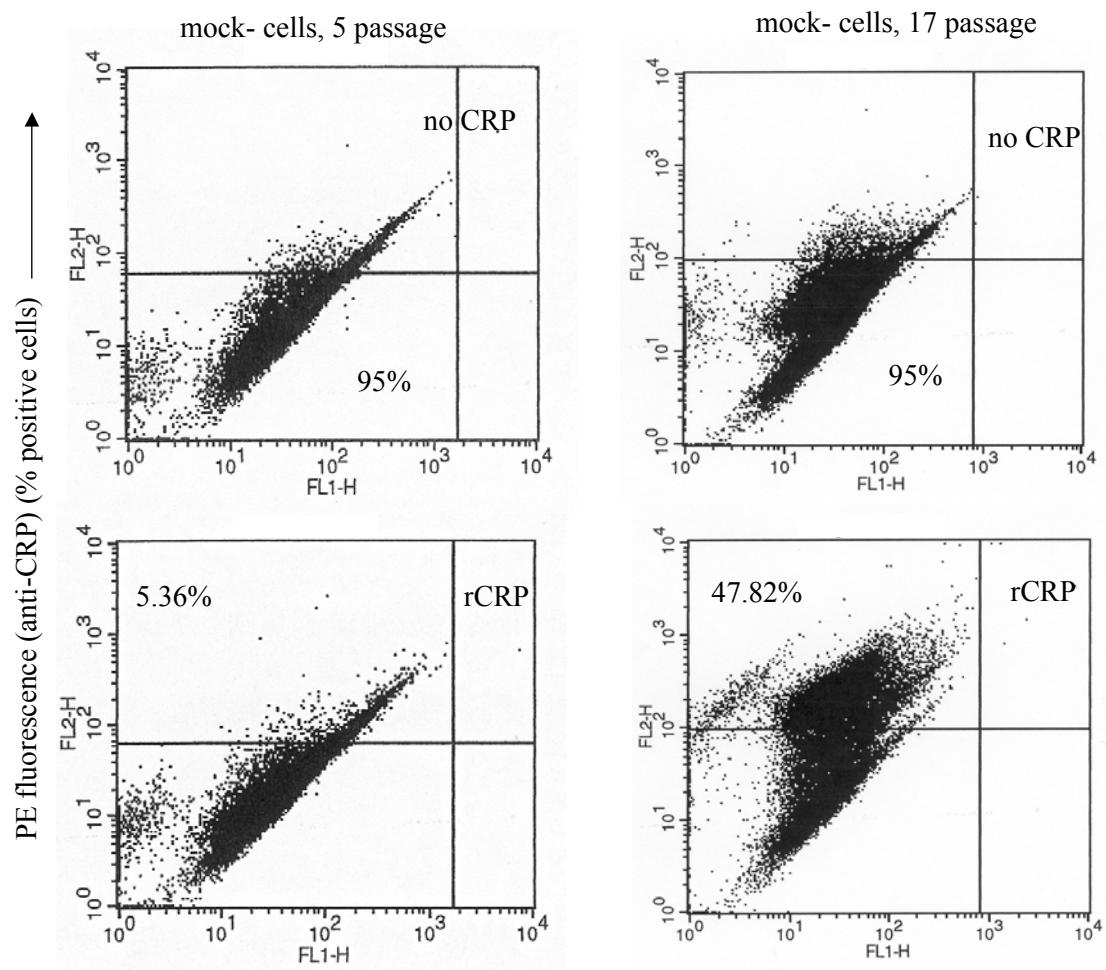


Fig 3 F

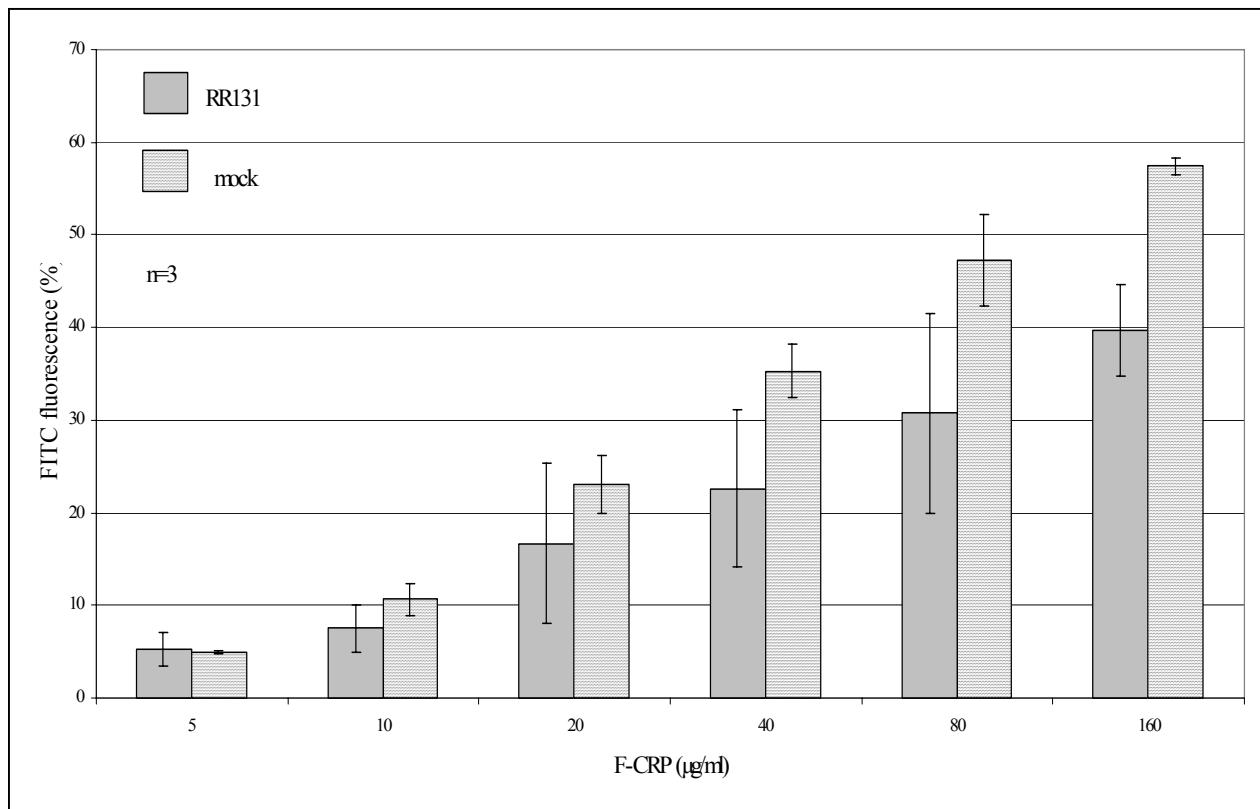


Fig 4 A

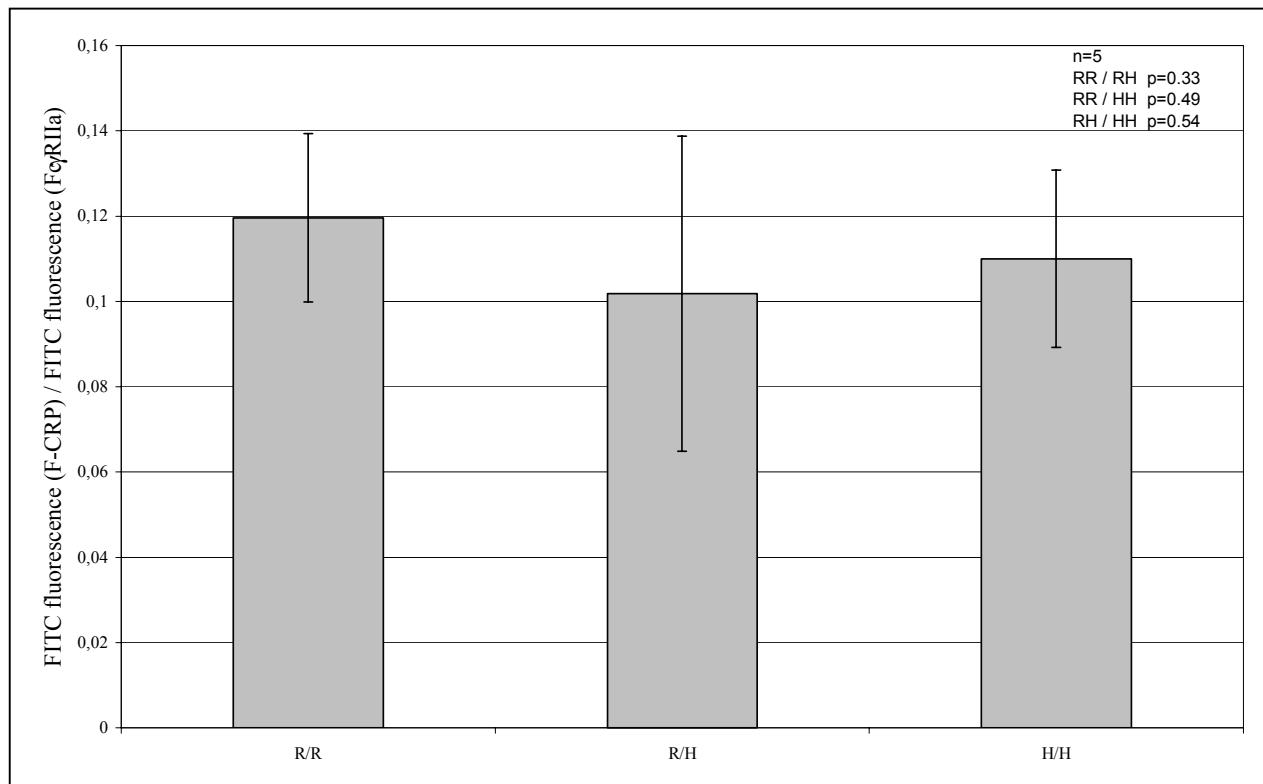


Fig 4 B

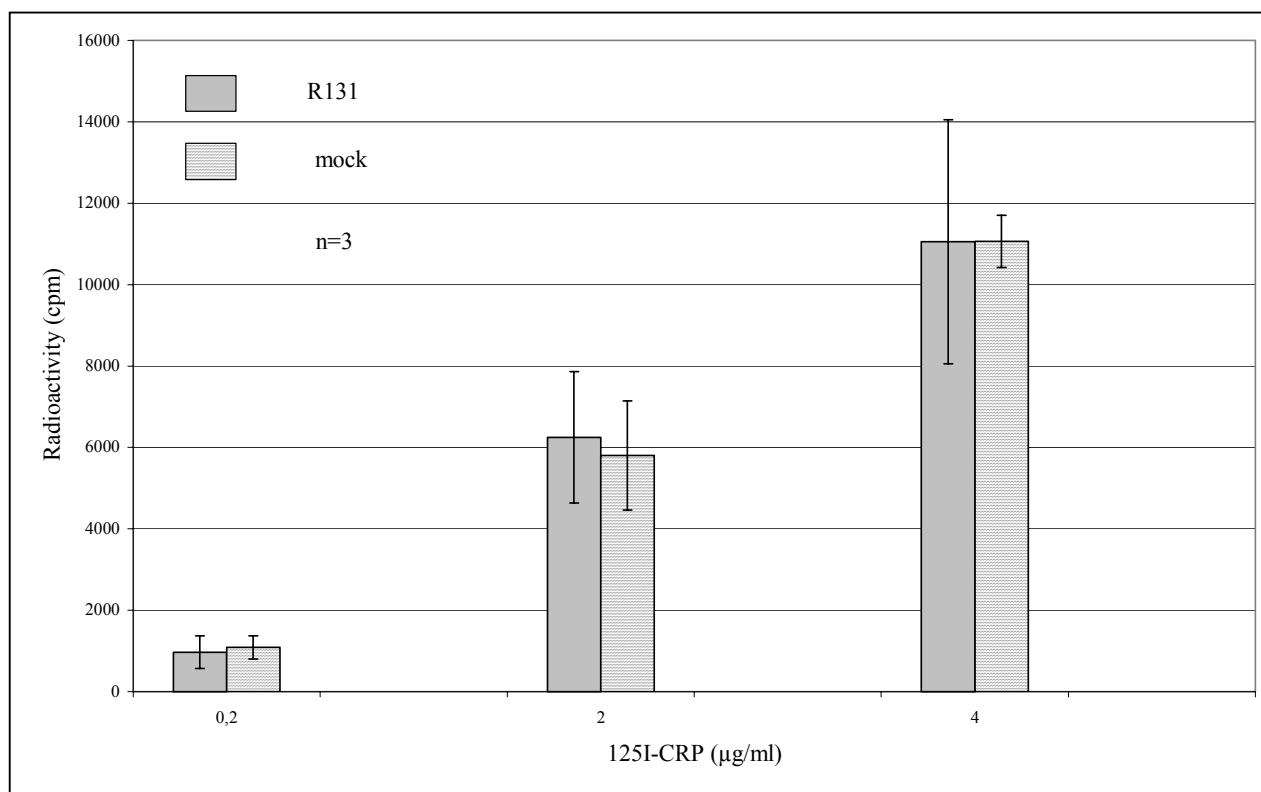


Fig. 4 C

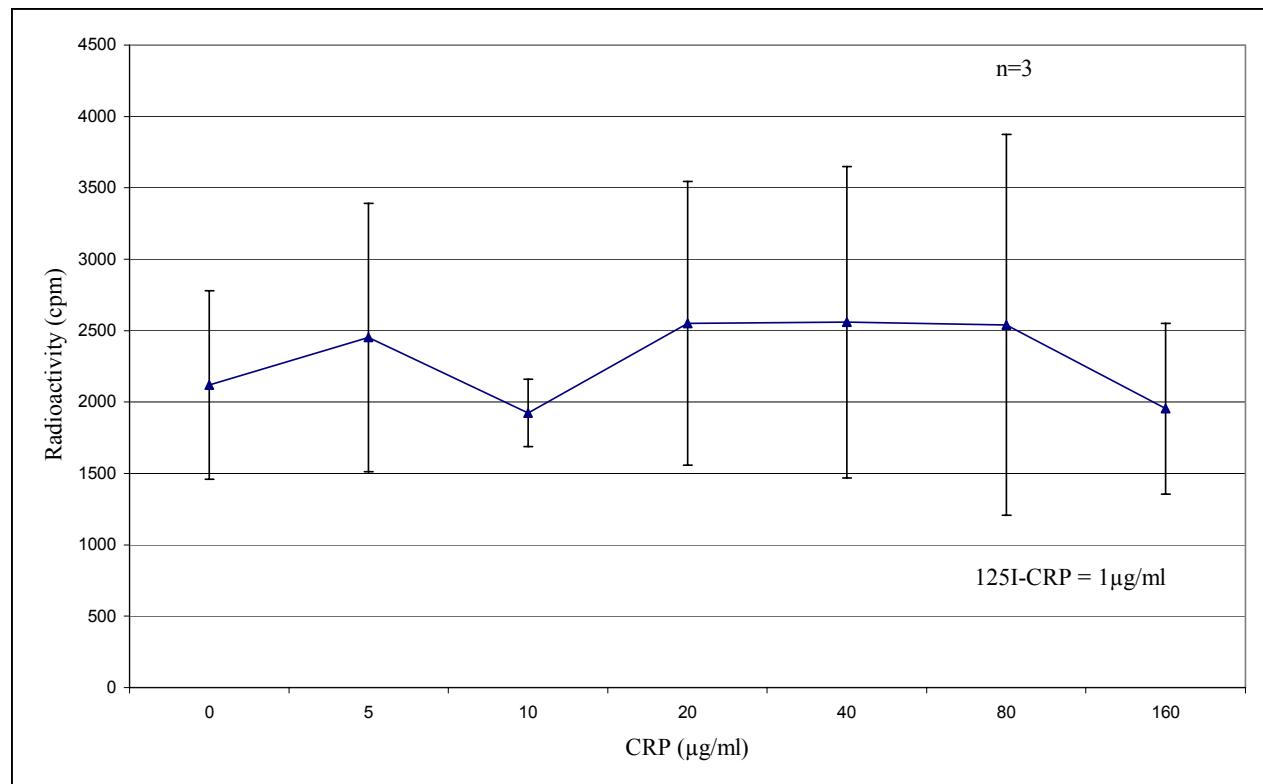


Fig 4 D

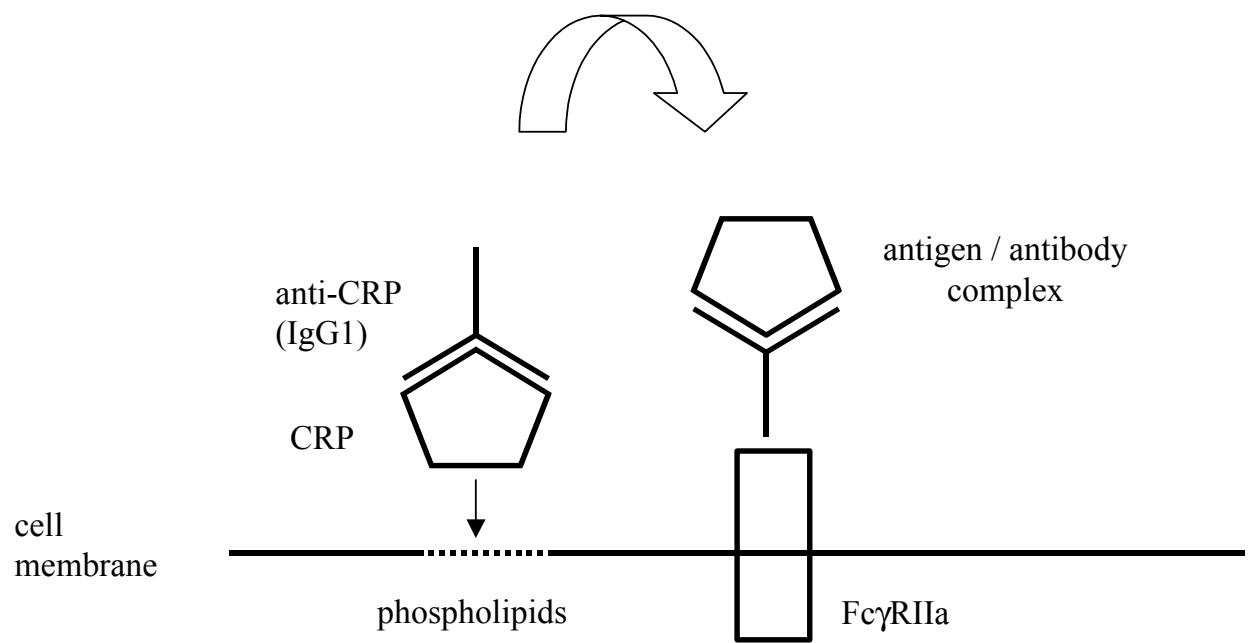


Fig. 5

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