Priming with synthetic oligonucleotides attenuates pressure overload-induced inflammation and cardiac hypertrophy in mice

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Received 1 September 2011; revised 30 July 2012; accepted 24 August 2012; online publish-ahead-of-print 12 September 2012

Time for primary review: 35 days

Aims

Inflammation and Toll-like receptor (TLR) signalling have been linked to the development of cardiac hypertrophy following transverse aortic constriction (TAC). In the present study, we investigated whether pre-treatment with the synthetic TLR9 ligands 1668-thioate or 1612-thioate modulates the progression of TAC-induced cardiac inflammation and hypertrophy.

Methods and results

C57BL/6N-mice were pre-treated with 1668-thioate, 1612-thioate (0.25 nmol/g, i.p.), or phosphate-buffered saline 16 h prior to TAC or sham surgery. Heart-weight/body-weight ratio (HW/BW), cardiomyocyte cell size, cellular macrophage accumulation, myofibroblast differentiation, and collagen deposition were investigated for up to 28 days. Cardiac function was monitored using a pressure–volume catheter and M-mode echocardiography. Inflammatory gene expression in the heart was analysed via gene array, while the time course of mRNA expression of key inflammatory mediators was assessed via RT-qPCR. TAC increased the HW/BW ratio and cardiomyocyte cell size and induced macrophage accumulation, myofibroblast differentiation, and collagen deposition. These changes were accompanied by cardiac inflammation and a significant loss of left ventricular function. Pre-treatment with cytosine-phosphate-guanine (CpG)-containing 1668-thioate attenuated the inflammatory response, the progression of cardiac hypertrophy, and cardiac remodelling, which resulted in a prolonged preservation of left ventricular function. These changes were induced to a smaller extent by the use of the non-CG-containing oligodeoxynucleotide 1612-thioate.

Conclusion

Pre-treatment with 1668-thioate attenuated cardiac hypertrophy following pressure overload, possibly by modifying the hypertrophy-induced inflammatory response, thereby reducing cardiac growth and fibrosis as well as delaying loss of cardiac function.

Keywords

Inflammation • Cytosine-phosphate-guanine oligodeoxynucleotide • Innate immunity • Cardiac hypertrophy • Cytokine • Transverse aortic constriction

1. Introduction

Chronic hypertension can initiate the development of cardiac hypertrophy and, ultimately, congestive heart failure.⁵ While the relationship between hypertension and congestive heart failure is firmly established, the molecular mechanisms by which hypertension mediates these effects have not been completely defined. It is widely accepted that pro- and anti-inflammatory mediators are expressed within the heart in response to mechanical pressure overload and ischaemic injury.⁶ This inflammatory state has been shown to induce...
deleterious changes in the structure of the cardiac extracellular matrix characterized by macrophage invasion and fibrosis driven by differ-
entiation myofibroblasts. Inflammatory- and fibrosis-associated genes are commonly involved in this process. Cardiac fibrosis can impair the electrical and mechanical function, inducing arrhythmia, increased myocardial stiffness and, subsequently, reduced ventricular compliance. Furthermore, cardiac fibrosis is associated with diffuse chronic interstitial inflammation. Recent studies have demonstrated that increasing fibrosis during the development of cardiac hypertrophy can be modulated by inflammatory sig-
nalling of innate immunity.

Cardiac fibrosis can be induced by pressure overload from the development of cardiovascular diseases. TLR expression is ele-
vated in the human myocardium during heart failure and cardiac ischae-
mia and is of central importance in animal models of cardiac dysfunction, including ischaemia/reperfusion injury. Furthermore, TLR ligands are involved in myocardial tolerance to cardiac injury. In a model of ischaemia/reperfusion injury, preconditioning by TLR4 stimulation with lipopolysaccharide resulted in a modified induction of inflamma-
tory molecules, thereby reducing cardiac necrosis and improving aortic flow. This effect was not restricted to lipopolysaccharide, because other bacterial components, such as bacterial DNA, have recently been shown to attenuate ischaemia/reperfusion injury in the brain. In contrast to mammalian DNA, the bacterial DNA is non-methylated and contains a higher number of cytosine-phosphate-guanine oligodeoxynucleotides (CpG-ODNs). Synthetic CpG-ODNs, such as 1668-thioate, can activate an innate immune response via TLR9, poten-
tially leading to septic shock and cardiac dysfunction. In contrast to 1668-thioate, 1612-thioate is a non-cytosin-guanine (CG)-containing ODN; hence, 1612-thioate is often used as a control ODN to monitor unspecific immune modulation. Furthermore, TLR4 signalling has been shown to interfere with the development of cardiac hypertrophy in a murine model of transverse aortic constriction (TAC). Given that TLR4 and TLR9 signalling converge to common end-points, e.g. nuclear factor-κB (NF-κB) acti-
vation, the protective potential of TLR9 signalling on TAC-related cardiac injury is worth elucidating.

In analogy to a recent study on CpG-ODNs in brain ischaemia/reper-
fusion injury, we applied the synthetic TLR9 ligands 1668-thioate, con-
taining a CG-motif, and the non-CG-containing 1612-thioate, prior to transverse aortic constriction. Thereby, we tested the hypothesis that pre-treatment with ODNs alters TAC-induced cardiac hypertrophy in mice and that the CG motif is essential.

2. Methods

All mice were handled in accordance with the Guide for Use and Care of Laboratory Animals published by the US National Institutes of Health (NIH publication no. 85-23, revised 1996). Protocols were approved by the local authority (LANUV, Recklinghausen, Germany).

2.1 Experimental groups and protocols

To evaluate whether ODN pre-treatment attenuates the development of afterload-dependent cardiac hypertrophy, 10- to 12-week-old male C57BL/6N mice (Charles River Laboratories, Sulzfeld, Germany; n = 8 per group) were randomized to receive an intraperitoneal (i.p.) injection of a CpG-ODN (1668-thioate; 5′-TCC-ATG-TTC-CTG-ATG-C; TibMolBiol, Berlin Germany; 0.25 nmol/g), a non-CG containing ODN (1612-thioate; 5′-GCT-AGA-TGT-TAG-CGT; TibMolBiol; 0.25 nmol/g) or phosphate-buffered saline (PBS) injection 16 h prior to TAC surgery. PBS was used as the control. The effects of ODN are dose dependent and can be disruptive; hence, the amount of synthetic ODNs adminis-
tered in our model was considerably below the amount used in other in vivo models to induce severe inflammation.

2.2 Transverse aortic constriction

The TAC model was used to generate pressure overload-induced heart failure. Mice were anaesthetized with isoflurane (2 vol%). A 27-gauge needle was used to standardize the degree of aortic constriction. For an-
glesia, buprenorphine was administered (0.1 mg/kg subcutaneously). Sham
control animals underwent surgery without aortic restriction. Mice were killed 4 and 24 h after CpG-ODN priming, and 3, 7, 14, and 28 days following surgery the hearts were removed and prepared for further analyses.

2.3 Collagen content and cardiomyocyte size

Excised hearts were formalin fixed (Anatech Ltd, Battle Creek, MI, USA) and embedded in paraffin. The heart was cut from the base to the apex, and a block of 10 μm-thick sections was mounted on glass slides at 250 μm inter-
vals. The sections below the papillary muscles were identified, and 5-μm-thick sections were stained with haematoxylin and eosin (HE) or picrosirius red, or were used for immunohistochemistry. Quantitative planimetric analysis was performed on five pictures per animal and averaged. The interstitial picrosirius red-positive stained area was quantified as a percentage of the total myocardial area. Also, cardiomyocyte cross-sections (>250 cells per slide) were measured to evaluate hypertrophy data.

2.4 Immunohistochemistry

Sections from excised hearts were stained with the following primary antibodie:
MAC-2 clone 3/38 rat anti-mouse antibody for macrophages (Cedarlane, Burlington, Ontario, Canada); and α-smooth muscle actin (α-SMA) anti-mouse monoclonal antibody (clone 1A4; Sigma, Taufkirchen, Germany). For further immunohistochemical staining, Vectastain Elite ABC kits and diaminobenzidine (AXXORA, Lörrach, Germany) were utilized. An M.O.M immunoedetection kit (AXXORA) was used for mouse-derived antibodie. Macrophase density was assessed by an investigator blinded to group assignment and expressed as cells per square millimetre.

2.5 Microarray analysis

2.5.1 Microarray hybridization

Cyanine (Cy)3-labelled reference and Cy5-labelled sample complemen-
tary DNAs (cDNAs; 10 μL each) were combined, denatured by heating for 2 min at 98 °C, and mixed with 36 μL of hybridization solution at 42 °C (Ambion, Austin, TX, USA). Murine microarrays ( NimbleGen; Roche, Basel, Switzerland) were overlaid with this solution and hybridized for 18 h at 42 °C using an actively mixing MAUI hybridization system (BioMicro Systems, Salt Lake City, UT, USA). Post-hybridization, the arrays were washed in 1× saline-sodium citrate (SSC)/0.05% SDS and 0.1× SSC, centrifuged to remove remaining liquid with unbound cDNA, and dried. Arrays were scanned and intensity values generated using an array scanner (NimbleGen; Roche). Data were up-loaded to the mAdb database (Microarray Database, a collaboration of CIT/BIMAS and NCI/CCR at the NIH: http://nciarray.nci.nih.gov/) and formatted via the export function for use with BRB ArrayTools (Biometric Research Branch, NCI, Frederick, MD, USA).

2.5.2 Analysis of gene expression

Data from three independent experiments and three PBS controls were used for all statistical analyses. Expression analyses were performed using BRB ArrayTools. Data were background corrected, flagged values were removed, spots in which both signals were <100 were filtered out,
ratios were log base 2 transformed, and Lowess intensity-dependent normalization was used to adjust for differences in labelling intensities of the cyanine (Cy)3-labelled and cyanine (Cy)5-labelled dyes. Analysis was restricted to genes present on >50% of the arrays after filtering. The gene expression profile of all treatment groups was compared with that of the control groups. A P-value cut-off of 0.0001 was used to identify genes whose expression was significantly up-regulated after CpG-ODN stimulation when compared with controls. Data were evaluated using Ingenuity Pathway Analysis (IPA; Ingenuity Systems Inc., Redwood City, CA, USA). IPA maps each gene within a global molecular network, pathways, and functional groups developed from information contained in the Ingenuity Pathways Knowledge Base.

2.5.3 Statistical analysis
Genes that were differentially expressed in the treatment groups were identified using a random-variance t-test. The random-variance t-test is an improvement over the standard separate t-test because it permits sharing of information among genes about within-class variation without assuming that all genes have the same variance. Genes were considered statistically significant if their P-value was <0.0001.

2.6 RNA extraction and Taqman® real-time quantitative RT-qPCR
Upon excision of the hearts, total RNA was isolated (TRizol; Applied Biosystems, Carlsbad, CA, USA), and first-strand cDNA was synthesized using the High-Capacity cDNA transcription kit (Applied Biosystems) with random hexameric primers as described in the manufacturer’s protocol. RT-qPCR was performed and analysed with 1:10 diluted cDNA on an ABI Prism 7900 Sequence Detection System with SDS2.2 Software (Applied Biosystems). Target gene expression was normalized to an internal control and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as non-inducible house-keeping gene, and results from TAC-operated mice were further normalized to their respective sham group. All primers were measured using fluoresceine-marked reporter dye and tetramethylrhodamine as quencher dye (FAM-TAMRA chemistry) and dissociation curve analysis was performed to determine the amplification of a single PCR product.

Hereupon, mRNA expression levels of the cytokines tumour necrosis factor-α (TNF-α), interleukin (IL)-1β, IL-6, IL-10, CC-chemokines CCL2 and CCL4, transforming growth factor-β (TGF-β1), the antioxidative enzyme haem oxygenase-1 (HO-1), the remodelling marker tenasin-C (TNC), the macrophage activation factor osteopontin (OPN), Toll-like receptors (TLR1, TLR2, TLR4, TLR6 and TLR9), and CD14 were determined using Taqman® real-time quantitative PCR (RT-qPCR; Applied Biosystems).

2.7 Invasive pressure–volume measurements
The haemodynamic parameters heart rate (HR), left ventricular systolic pressure (LVSP), left ventricular end-diastolic pressure (LVEDP), stroke volume (SV), cardiac output (CO), and ejection fraction (EF) were recorded using a pressure–volume catheter according to the manufacturer’s manual (Millar® Instruments, Houston TX, USA). Mice injected with PBS, 1668-thioate, or 1612-thioate were investigated 16 h after application and served as controls. All other mice underwent TAC surgery 16 h after priming and were monitored 14 or 28 days later. Following induction of anaesthesia and intubation, a 1.4-French pressure-conductance catheter was retrogradely inserted through the right carotid artery into the left ventricle (LV). After stabilization, haemodynamic parameters were recorded while anaesthesia was maintained at 0.8 vol% isoflurane.

2.8 Echocardiography
Following 3 and 28 days of TAC, mice (n = 6 per group) underwent two-dimensional guided short-axis M-mode echocardiography (HDl-5000; ATL, Phillips, Oceanside, CA, USA) using a linear array transducer (CL15-7) working at 15 MHz and providing frame rates up to 284 Hz. General anaesthesia was maintained with isoflurane (0.8 vol%). Heart rate was monitored during examination to minimize cardio depression-related effects. The heart was examined from base to apex using a parasternal short-axis view, and echocardiographic data were acquired at the level of the papillary muscle in order to assure reproducibility. Images were analysed by an investigator blinded to treatment. We calculated fractional shortening (FS) as a parameter of global LV function, and anterior wall thickening (AWT) as a parameter of regional LV function using the following formula: FS (%) = [(LVd – LVs)/LVd] × 100 and AWT (%) = [(AWs – AWd)/AWs] × 100 (LVd = diastolic left ventricular diameter; LVS = systolic left ventricular diameter; AWs = diastolic anterior wall diameter; AWd = systolic anterior wall diameter). Furthermore, left ventricular end-systolic diameter (LVESd) and left ventricular end-diastolic diameter (LVEDd) were calculated.

2.9 Statistics
Data were normally distributed and presented as means ± SEM. Statistical analysis was performed by one-way ANOVA and Newman–Keuls post hoc testing using GraphPad PRISMS (Graphpad Inc., La Jolla, CA, USA); P < 0.05 was considered statistically significant. Statistics for microarray analysis are described in detail in the Supplementary material online.

3. Results
3.1 1668-Thioate pre-treatment attenuated cardiac hypertrophy
We calculated the HW/BW ratio (Figure 1A) and HW/tibia length ratio (supplement) on days 7, 14, and 28 following TAC or sham. TAC induced significant increases in HW/BW and HW/tibia length ratios in PBS-injected mice, reaching a stable maximum by 7 days. Pre-treatment with 1668-thioate prevented TAC-dependent increases of both parameters over 28 days. Pre-treatment with 1612-thioate delayed the TAC-induced HW increases. However, the HW equalled that of PBS-injected animals after 28 days and was thus significantly greater than that of 1668-thioate pre-treated animals.

To evaluate whether changes of HW were partly caused by cardiomyocyte growth, we measured the area of cardiomyocyte cross-sections 28 days after TAC (Figure 1B). This analysis revealed a significant increase of cell size in all pre-treatment groups as a result of TAC. However, cells from the TAC-PBS group had a significantly higher cross-sectional area than those of 1668-thioate pre-treated mice. TAC initiated intermediate cardiomyocyte growth in 1612-thioate-primed mice, because the cross-sectional area of these cells did not differ significantly from those of either of the two other groups.

3.2 1668-Thioate modulated cardiac gene expression during the priming phase
To elucidate CpG-ODN-induced changes mRNA expression levels were monitored in the heart by microarray 4 h after priming, i.e. 12 h before TAC surgery. Ingenuity Pathway Analysis was used to identify the pattern of regulatory interactions underlying CpG-ODN-dependent gene activation in vivo. 1668-Thioate modulated several inflammatory pathways. The most significantly up-regulated pathways were the ‘interferon
pathway’ (P < 1.2 × 10⁻¹¹), ‘activation of interferon (IRF) by cytosolic pattern recognition receptors’ (P < 2.8 × 10⁻¹¹), and ‘acute phase response signalling’ (P < 5.3 × 10⁻⁸; see Supplementary material online, Table S1A). Furthermore, functional groups of activated genes were identified. The most significantly up-regulated functional groups were ‘cellular growth and proliferation’ (P < 1.4 × 10⁻¹⁶), ‘cellular development’ (P < 1.7 × 10⁻¹⁶), and ‘organism survival’ (P < 7.1 × 10⁻¹⁶; see Supplementary material online, Table S1B). While 1668-thioate induced 649 genes, only three genes were found to be up-regulated in the 1612-thioate group 4 h after priming (Table 1).

### 3.3 1668-Thioate decreased CCL2 and CCL4 induction and modulated macrophage activation and infiltration

Basic histological evaluations (haematoxylin and eosin) revealed strong cellular invasion into the LV myocardium until 3 days in all TAC groups, while these TAC-induced changes were not detectable in animals pre-treated with 1668-thioate or 1612-thioate at later time points (data not shown).

In order to characterize this cellular invasion further, sections taken on days 3, 7, and 14 were stained with anti-macrophage antibody MAC-2. In accordance with total cellular invasion, MAC-2 staining revealed a large macrophage accumulation after 3 days in all TAC groups. Later, macrophages could be detected only in hearts from the TAC-PBS group, while the number of MAC-2-positive cells decreased in both of the ODN pre-treated groups until 14 days (Figure 2A–D).

Compared with sham animals, TAC induced a transient, more than two-fold increase of expression of the mRNA for the macrophage-attracting chemokine CCL2 in the PBS- and 1612-thioate-treated mice. Furthermore, CCL2 mRNA expression was elevated in mice treated with TAC-PBS and 1612-thioate compared with the 1668-thioate pre-treated groups (Figure 2E). Interestingly, TAC induced a high, transient CCL4 mRNA expression, which was significantly above that of the 1668- and 1612-thioate pre-treated animals over the whole observation period, with a peak induction after 3 days (Figure 2F). In accordance, OPN-1, a marker for macrophage activation,²⁹,³⁰ was significantly increased in the TAC-PBS group after 3 days compared with all other groups. 1668-Thioate completely prevented OPN induction (Figure 2G).

### 3.4 1668-Thioate modulated the cardiac inflammatory response following TAC

The murine myocardium exhibited a marked, but transient, mRNA up-regulation of TLRs (also see Supplementary material online, Figure S1B–E), chemokines, cytokines, and other mediators following TAC compared with sham (Figure 3A–G). The TAC-dependent up-regulation of most genes measured in this study was maximal 3 days after surgery in the TAC-PBS group. At this time point, expression of all genes was significantly lower due to 1668-thioate pretreatment. In comparison to 1668-thioate, 1612-thioate priming was less effective, i.e. IL-6 and HO-1 mRNA expression were the same as in TAC-PBS-treated mice.

### Table 1 Microarray analysis

<table>
<thead>
<tr>
<th>Pathway</th>
<th>P-value</th>
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<tr>
<td>(A) Ingenuity Pathway Analysis of 1668-thioate-dependent gene activation</td>
<td></td>
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<tr>
<td>in vivo 4 h after priming</td>
<td></td>
</tr>
<tr>
<td>Interferon signalling</td>
<td>1.2 × 10⁻¹¹</td>
</tr>
<tr>
<td>Activation of interferon regulatory factor (IRF) by cytosolic pattern</td>
<td>2.8 × 10⁻¹¹</td>
</tr>
<tr>
<td>recognition receptors</td>
<td></td>
</tr>
<tr>
<td>Acute-phase response signalling</td>
<td>5.3 × 10⁻⁹</td>
</tr>
<tr>
<td>Antigen presentation</td>
<td>7.6 × 10⁻⁷</td>
</tr>
<tr>
<td>Dendritic cell maturation</td>
<td>1.3 × 10⁻⁶</td>
</tr>
<tr>
<td>Interleukin-17 signalling in fibroblasts</td>
<td>5.6 × 10⁻⁶</td>
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<tr>
<td>Functional groups</td>
<td></td>
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<tr>
<td>(B) Ingenuity Pathway Analysis to identify functional groups of</td>
<td></td>
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<tr>
<td>1668-thioate-activated genes in vivo 4 h after 1668-thioate stimulation</td>
<td></td>
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<tr>
<td>Cellular growth and proliferation</td>
<td>1.4 × 10⁻¹⁶</td>
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<tr>
<td>Cellular development</td>
<td>1.7 × 10⁻¹⁶</td>
</tr>
<tr>
<td>Organism survival</td>
<td>7.1 × 10⁻¹⁶</td>
</tr>
<tr>
<td>Cellular movement</td>
<td>1.4 × 10⁻¹⁵</td>
</tr>
<tr>
<td>Haematological system development and function</td>
<td>1.4 × 10⁻¹⁵</td>
</tr>
<tr>
<td>Immune cell trafficking</td>
<td>1.4 × 10⁻¹⁵</td>
</tr>
<tr>
<td>Cell-to-cell signalling and interaction</td>
<td>2.7 × 10⁻¹⁴</td>
</tr>
<tr>
<td>Tissue morphology</td>
<td>1.9 × 10⁻¹²</td>
</tr>
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</table>

A minimum of three animals per group were independently analysed (stringency cut-off: P < 0.0001 above controls).
3.5 ODN pre-treatment attenuated TAC-induced collagen deposition

Transforming growth factor-β1 is involved in fibroblast stimulation and differentiation to myofibroblasts that release collagen precursors. Tenascin-C is also a marker for reorganization of the extracellular matrix. Three days after TAC, the mRNA expression of these mediators was significantly elevated in TAC-PBS-treated hearts above all other groups. Pre-treatment with 1668- or 1612-thioate prevented the TAC-induced mRNA up-regulation of both mediators (Figure 4A and B).

To verify myofibroblast development further, sections of hearts taken 7 days after TAC surgery were stained for α-SMAC. The TAC-PBS-treated hearts showed a marked accumulation of myofibroblasts (Figure 4C). This was attenuated by pre-treatment with either of the ODNs (Figure 4D and E).

Cardiac fibrosis was evaluated on picrosirius red-stained sections at the papillary muscle level (Figure 4F–H). Planimetric analysis of the collagen area provided a significantly increased and continuously progressing collagen deposition in the TAC-PBS and the 1612-thioate pre-treated hearts compared with sham after 3, 7 and 14 days (Figure 4I), with 1612-thioate being less effective at 14 days.
Pre-treatment with 1668-thioate abolished collagen deposition at all time points.

### 3.6 1668-Thioate pre-treatment preserved cardiac function in LV pressure–volume catherization

In order to evaluate whether ODN pre-treatment attenuates pressure overload-induced cardiac dysfunction following TAC, we examined pressure–volume parameters of LV function 14 and 28 days after surgery.

Heart rate progressively increased during the observation period in all groups (Figure 5A). Left ventricular systolic pressure significantly increased in all TAC groups compared with the respective controls. However, after 14 days the LVSP in the 1668-thioate group was significantly lower than that in the mice pre-treated with PBS or 1612-thioate. After 28 days, the LVSP of 1668-thioate pre-treated mice reached the same levels as in both other groups (Figure 5B).

TAC-dependent changes of SV, CO, and EF followed the same pattern; in PBS- and 1612-thioate pre-treated mice, all three parameters decreased steadily over time. In the case of the 1668-thioate pre-treated mice, cardiac functional parameters stayed at the control level for the first 14 days, thus being significantly higher than both other groups at this time point. Fourteen days later, all three parameters had also significantly decreased in the 1668-thioate group but were still above those of the PBS group. Interestingly, the value of SV, CO, and EF in the 1668-thioate group equalled that of the 1612-thioate group after 28 days (Figure 5C–E).

### 3.7 1668-Thioate pre-treatment delayed pressure overload-induced cardiac decompensation, as revealed by echocardiographic analysis

In order to specify early and late changes (3 and 28 days) in LV function further, ventricular performance was assessed by short-axis...
Figure 4 Cardiac fibrosis. (A and B) mRNA expression of myofibroblast stimulation- and differentiation-related transforming growth factor-β1 (TGF-β1) and remodelling-related marker tenascin C (TNC) 6 h, 24 h, 3 days, and 7 days after TAC surgery. Animals were pre-treated with PBS, 1668-thioate, or 1612-thioate 16 h prior to TAC. Results are normalized to sham (dashed line). (C–E) Left ventricular (LV) sections stained for myofibroblast marker α-SMAC 7 days after TAC. Arrows indicate myofibroblast accumulation. Scale bars represent 100 μm. (F–H) Picrosirius red histology of LV sections 7 days after TAC surgery. Arrows indicate collagen deposit. Scale bars represent 200 μm. (I) Planimetry of the LV collagen deposition as a percentage of LV wall. *p < 0.05, #p < 0.05 vs. respective sham; n = 8 per group.
M-mode echocardiography. Fractional shortening as a parameter of global LV function and anterior wall thickness as a parameter of regional LV function were recorded, and left ventricular end-systolic (LVESd) as well as left ventricular end-diastolic diameter (LVEDd) were calculated (Figure 6A–D). Following TAC, both FS and AWT decreased significantly in all groups at both time points. 1668-Thioate pre-treatment attenuated the decrease of FS compared to other groups 3 days after TAC. TAC induced an increase of LVESd and LVEDd at both time points, reaching the level of significance only in the case of LVEDd. 1668-Thioate pre-treatment prevented this increase at least for the first 3 days.

4. Discussion
This study shows, for the first time, that priming with synthetic ODNs attenuates pressure overload-induced development of cardiac hypertrophy in mice. The beneficial effect of ODNs seems to depend on modulation of the inflammatory response initiated by pressure overload. Two different synthetic ODNs were chosen for the experiments; 1668-thioate contains a CpG motif typical of bacterial DNA and was chosen for its TLR9-specific stimulating properties, whereas 1612-thioate, which is a non-CG ODN, was applied as a control ODN to monitor unspecific immune modulation.32

Figure 5 Haemodynamic parameters. (A–E) Catheter measurement in controls, TAC-PBS-, TAC-1668-thioate-, and TAC-1612-thioate-primed animals 14 and 28 days after TAC surgery, as follows: heart rate (A), left ventricular systolic pressure (LVSP; B), stroke volume (SV; C), cardiac output (CO; D), and ejection fraction (EF; E). *P < 0.05, #P < 0.05 vs. respective control; n = 8 per group.
In the present study, TAC surgery induced a significant increase in HW/BW and HW/tibia length ratios after 7 days. Further increase was not achieved during the observation time until day 28. This is consistent with a study by Nakamura et al., who observed a maximal increment of about 60% in HW/BW and HW/tibia length ratios in TAC-operated mice after 10 days.\textsuperscript{33} 1668-Thioate pre-treatment significantly attenuated the TAC-induced increase in HW/BW ratio. In contrast, 1612-thioate did not attenuate, but only delayed this increase compared with non-pre-treated animals, suggesting that priming with ODNs effectively modulates the development of cardiac hypertrophy in a ligand-specific manner. These findings are corroborated by evaluations of cardiomyocyte cross-sectional area.

Figure 6 Short-axis M-mode echocardiography. (A–D) Evaluation of functional and morphological parameters 3 and 28 days after TAC in PBS-, 1668-thioate-, and 1612-thioate-primed mice, as follows: fractional shortening (FS; A), anterior wall thickness (AWT; B), left ventricular end-systolic diameter (LVES\textsubscript{d}; C), and left ventricular end-diastolic diameter (LVED\textsubscript{d}; D). *\(p < 0.05\); \(n = 8\) per group.

Table 2 Quantification of gene activation 4 h after priming

<table>
<thead>
<tr>
<th>P-value</th>
<th>1612 ODN-treated mice vs. PBS-treated mice</th>
<th>1612 ODN-treated mice vs. untreated mice</th>
<th>1668 CpG-ODN-treated mice vs. untreated mice</th>
</tr>
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<tbody>
<tr>
<td>(p &lt; 0.0001)</td>
<td>1 gene</td>
<td>3 genes</td>
<td>649 genes</td>
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TAC increases intracardiac pressure and, thereby, mechanical force to the ventricular wall. The response to this mechanical force is a multi-step procedure. One part is the transition of force to the myocardial cells via interaction of the extracellular matrix with cardiomyocytes, leading to myocyte hypertrophy.\textsuperscript{34}

Recently, it has been demonstrated that receptor activator of NF-\(\kappa\)B ligand can be activated by TAC and thus induce cytokine expression of cardiomyocytes, thereby activating and attracting inflammatory cells.\textsuperscript{35} Accordingly, the observed strong mRNA up-regulation of the macrophage chemoattractants CCL2 and CCL4\textsuperscript{36} was followed by sustained infiltration of MAC-2-positive cells. Furthermore, pre-treatment with synthetic ODNs reduced or
even permanently abolished CCL2 and CCL4 mRNA induction and, consequently, macrophage accumulation. On day 3 after TAC, the presence of MAC-2-positive cells was surprisingly high in all three groups; later on, the number of MAC-2-positive cells decreased in the two ODN-primed groups. Thus, the behaviour of macrophages does not mirror the mRNA expression of the recorded chemokines. An explanation for this divergence cannot be deduced from our data.

Another important and new finding was the marked alteration in OPN, a marker of macrophage activation.\textsuperscript{35} 1668-Thioate pre-treatment abolished OPN mRNA expression at day 3 most effectively, indicating that macrophages in the 1668-TAC group are less active and, thereby, provide a lower inflammatory stimulus. These data suggest a specific role for 1668-thioate in the attenuation of the inflammatory response to TAC.

Even though CpG-ODN-induced effects have been investigated by several authors, little is known about the CpG-ODN-induced functional groups of genes in the heart. Therefore, we examined whole-genome expression during the priming phase induced by 1668-thioate. Several identified acute-phase cytokines and interferon-driven pathways (Table\textsuperscript{A} and\textsuperscript{B}) may be responsible for dampening the inflammatory response during the subsequent injury induced by TAC, given that they have been shown to be associated with cell survival and immune suppression, e.g. IRF7 and interferon.\textsuperscript{38} The priming with 1612-thioate served as a control for unspecific immune induction, because the charged backbone of phosphorothioate ODNs can induce a great magnitude of non-specific immune reactions.\textsuperscript{21} 1612-Thioate up-regulated only three genes, compared with 649 genes in the case of 1668-thioate, thus the effect of 1668-thioate seems to depend mainly on the CpG motif.

According to the results of the gene array analysis, we examined the expression of TLR9-dependent inflammatory parameters, such as PRRs and cytokines, after TAC. There was a significant increase in the mRNA of all examined PRRs in the TAC-PBS group. In contrast, 1668-thioate pre-treatment delayed and partly abolished up-regulation of these receptors (see Supplementary material online, Figure S1B–E). As CD14 acts as a co-receptor for various TLRs, including TLR9,\textsuperscript{39} CD14 up-regulation might be regarded as the organism’s attempt to sensitize a key regulatory molecule for inflammatory responses. TAC induced an up-regulation of all investigated inflammatory mediators in PBS-pretreated mice, while 1668-thioate pre-treatment prevented pressure overload-induced up-regulation of inflammatory mediators at all time points. These findings further support the idea of an attenuated TAC-induced inflammatory response after 1668-thioate priming. Interestingly, there was a concomitant up-regulation of anti-inflammatory IL-10\textsuperscript{40} and the anti-oxidant enzyme HO-1, which has known anti-hypertrophic effects.\textsuperscript{41} in mice treated with TAC-PBS or TAC 1612-thioate, indicating the necessity to limit excessive inflammation in these groups.

It is well known that the development of an increased HW/BW ratio due to pressure overload depends on hypertrophy of cardiomyocytes and increasing fibrosis.\textsuperscript{42} Fibrosis is linked to the maturation/differentiation of myofibroblasts.\textsuperscript{36} In our study, each ODN pre-treatment inhibited cardiac myofibroblast differentiation, as seen by a down-regulation of the myofibroblast differentiation marker TGF-β1. In consequence, pre-treatment with ODNs led to less myocardial α-SMAC-positive cells, implicating a smaller number of collagen-producing myofibroblasts. Our study revealed striking collagen deposition, shown by picrosirius red staining in TAC-PBS-treated hearts. In contrast, 1668-thioate pre-treatment most effectively abolished TAC-induced collagen deposition, whereas 1612-thioate pre-treatment only delayed the progression of fibrosis after day 7. These findings suggest that synthetic 1668-thioate pre-treatment specifically attenuates both components of increased heart weight, i.e. collagen deposition and the afore-mentioned cardiomyocyte hypertrophy.

The observed reduction of cardiac mass might be explained by the following suggested mechanism: priming the animals with 1668-thioate leads to an activation of IRF pathways prior to TAC, as indicated by the presented microarray evaluations. It has been shown that the activation of IRF7 is an essential common pathway activated by TLR4, TLR9, and ischaemic pre-conditioning in neuroprotection.\textsuperscript{19} IRF7 has been demonstrated to re-route TLR-dependent signal transduction away from NF-κB and towards activation of interferon genes.\textsuperscript{38} Inhibition of NF-κB resulted in a reduction of cardiac hypertrophy in the context of transverse aortic banding.\textsuperscript{43} The 1668-thioate induced activation of IRF7 in our experimental setting might have a comparable effect, because it re-routes the signal from NF-κB.\textsuperscript{44}

Furthermore, TLR4 signalling supports the development of cardiac hypertrophy.\textsuperscript{7} This phenomenon seems to rely on the binding of endogenous TLR4 ligands and the subsequent activation of NF-κB.\textsuperscript{45} Inhibition of TLR4 by Eritoran\textsuperscript{46} has recently been shown to diminish TAC-induced cardiac growth and inflammation.\textsuperscript{45} It is important to note that the microarray data after 1612-thioate stimulation showed almost no induction of gene expression, while there were notable effects seen in the PCR. This observation can be explained by a lower sensitivity of the microarray analysis compared with PCR; in the microarray analysis, the probability level is set to relatively high values to avoid false-positive findings in some cases, resulting in a loss of information compared with the PCR analysis. This phenomenon has been termed ‘fold-compression’.\textsuperscript{46}

Finally, the question of whether the observed attenuation in cardiac hypertrophy resulted in improved cardiac function was addressed by LV pressure–volume measurements (days 14 and 28) and short-axis M-mode echocardiography (days 3 and 28). Pre-treatment with 1668-thioate sustained cardiac function on days 3 and 14, whereas 1612-thioate injection had no such effects at these time points. On day 28, cardiac function was impaired in all groups; however, the 1668-thioate pre-treated group, as well as the 1612-thioate pre-treated group, was less severely affected than TAC-PBS mice. Echocardiography revealed an overall reduction of FS due to TAC, but this was significantly attenuated in the TAC-1668 group after 3 days. This beneficial influence of priming with 1668-thioate seems to persist until day 14, because LVSP, SV, CO, and EF were also superior relative to these parameters in the PBS- and 1612-thioate primed mice. The preceding attenuation of inflammation in 1668-thioate primed hearts may explain this improved performance. At day 28, haemodynamic function was impaired in all groups; however, the 1668-thioate pre-treated group, as well as the 1612-thioate pre-treated group, was less severely affected than TAC-PBS mice, as demonstrated by catheter recordings. This difference did not appear in echocardiographic recordings at this time point.

Our findings support the hypothesis that modulation of the inflammatory response by pre-treatment with TLR9 ligands attenuates subsequent cardiac hypertrophy and delays consecutive loss of LV function during pressure overload. In conclusion, our data suggest that modulation of the inflammatory response may constitute a promising therapeutic option to counteract maladaptive cardiac adaptation.
Supplementary material

Supplementary material is available at Cardiovascular Research online.

Conflict of interest: none declared.

Funding

R.L. was supported by a scholarship from BONFOR.

References