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LNA oligonucleotide mediates an anti-inflammatory effect in autoimmune myocarditis via targeting lactate dehydrogenase B

Mariella Bockstahler[1,2] | Christian Salbach[1,2] | Anna-Maria Müller[1,2] | Andrea Kübler[1,2] | Oliver J. Müller[3] | Hugo A. Katus[1,2] | Norbert Frey[1,2] | Ziya Kaya[1,2] 🔟

[1]Department of Internal Medicine III, University of Heidelberg, Heidelberg, Germany

[2]DZHK (German Centre for Cardiovascular Research), Partner Site Heidelberg/Mannheim, University of Heidelberg, Heidelberg, Germany

[3]Department of Internal Medicine III, University of Kiel, Kiel, Germany

Correspondence
Prof. Dr. med. Ziya Kaya, Department of Internal Medicine III, University of Heidelberg, Im Neuenheimer Feld 410, 69120 Heidelberg, Germany.
Email: ziya.kaya@med.uni-heidelberg.de

Senior author: Prof. Dr. med. Ziya Kaya, Department of Internal Medicine III, University of Heidelberg, Im Neuenheimer Feld 410, 69120 Heidelberg, Germany.

Abstract

Treatment of myocarditis is often limited to symptomatic treatment due to unknown pathomechanisms. In order to identify new therapeutic approaches, the contribution of locked nucleic acid antisense oligonucleotides (LNA ASOs) in autoimmune myocarditis was investigated. Hence, A/J mice were immunized with cardiac troponin I (TnI) to induce experimental autoimmune myocarditis (EAM) and treated with LNA ASOs. The results showed an unexpected anti-inflammatory effect for one administered LNA ASO MB_1114 by reducing cardiac inflammation and fibrosis. The target sequence of MB_1114 was identified as lactate dehydrogenase B (mLDHB). For further analysis, mice received *mLdhb*-specific GapmeR during induction of EAM. Here, mice receiving the *mLdhb*-specific GapmeR showed increased protein levels of cardiac mLDHB and a reduced cardiac inflammation and fibrosis. The effect of increased cardiac mLDHB protein level was associated with a downregulation of genes of reactive oxygen species (ROS)-associated proteins, indicating a reduction in ROS. Here, the suppression of murine pro-apoptotic Bcl-2-associated X protein (mBax) was also observed. In our study, an unexpected anti-inflammatory effect of LNA ASO MB_1114 and *mLdhb*-specific GapmeR during induction of EAM could be demonstrated *in vivo*. This effect was associated with increased protein levels of cardiac mLDHB, mBax suppression and reduced ROS activation. Thus, LDHB and LNA

Abbreviations: 5'UTR, 5'-untranslated region; Afog, Acid fuchsin orange G-stain; ASO, Antisense oligonucleotide; CFA and PBS control, Control; CFA, Complete Freund's adjuvant; DCM, Dilated cardiomyopathy; EAM, Experimental autoimmune myocarditis; GAPDH, Glyceraldehyde 3-phosphate dehydrogenase; HE, Haematoxylin and eosin; hsTnT, High-sensitive troponin T; IL-17, Interleukin-17; IL-1α, Interleukin-1 alpha; IL-1β, Interleukin-1 beta; IL-2, Interleukin-2; IL-6, Interleukin-6; LDH, Lactate dehydrogenase; LNA Ctrl., LNA ASO control; LNA ASOs, Locked nucleic acid antisense oligonucleotides; mBax, Murine Bcl-2-associated X protein; mLDHA, Murine lactate dehydrogenase A; mLDHB, Murine lactate dehydrogenase B; mNox2, Murine NADPH oxidase 2; mp47 phox, Murine NADPH oxidase cytosolic protein p47phox; mp67 phox, Murine NADPH oxidase cytosolic protein p67phox; mSod2, Murine superoxide dismutase 2; mtDNA, Mitochondrial DNA; mUcp2, Murine uncoupling protein 2; NADPH, Nicotinamide adenine dinucleotide phosphate; ROS, Reactive oxygen species; SMARTer RACE, Switching Mechanism At 5'-end of RNA Transcript Rapid Amplification of cDNA Ends; TLR, Toll-like receptor; TNF-α, Tumour necrosis factor alpha; TnI, Troponin I.

The first authors Mariella Bockstahler and Christian Salbach contributed equally to the study (joint first authorship).

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ASOs may be considered as a promising target for directed therapy of myocarditis. Nevertheless, further investigations are necessary to clarify the mechanism of action of anti-inflammatory LDHB-triggered effects.

INTRODUCTION

Particularly in young patients, myocarditis represents a leading cause of heart failure and sudden cardiac death [1,2]. Myocarditis is characterized by inflammatory infiltration of the myocardium and subsequent necrosis of cardiomyocytes [3,4]. In 10–20%, myocarditis may progress to dilated cardiomyopathy (DCM) [3,4]. Due to unknown pathomechanism leading from myocarditis to DCM, therapeutic regimes for myocarditis patients and subsequent DCM are generally restricted to heart failure treatment [5,6]. Mechanistically, cell damage induced by cardiac inflammation in terms of myocarditis may trigger autoimmune reactions leading to an unregulated chronic autoimmune response and subsequently heart failure [7,8].

In order to study mechanisms leading from myocarditis to DCM and to investigate underlying principles of an autoimmune response in myocarditis, our group developed a mouse model of troponin I (TnI)-induced experimental autoimmune myocarditis (EAM) [9].

Antisense oligonucleotides (ASOs) are short synthetic single-stranded DNA/RNA-like oligonucleotides, which are designed to selectively bind RNA (Watson–Crick pairing) to regulate protein expression [10]. Here, protein expression is regulated by affecting processing or translation of target RNA or by activation of RNase H and subsequent degradation of target RNA [10,11]. Due to their potential to modify protein expression, ASOs are currently used to treat diseases such as hereditary transthyretin amyloidosis [12]. In order to use ASOs as therapeutic agents, they are modified to achieve better bioavailability and higher target RNA specificity [13]. For this purpose, DNA nucleotides of ASOs are replaced with locked nucleic acid (LNA) nucleotides [14]. Here, GapmeRs, made of LNA bases flanking a central DNA sequence, provide a potent target mRNA inhibition. Mechanistically, GapmeRs form DNA-RNA hybrids after binding their target RNA. These DNA-RNA hybrids are recognized by RNase H catalysing RNA cleavage [13].

Lactate dehydrogenase (LDH) is an important tetrameric metabolic enzyme that catalyses the interconversion of lactate and pyruvate. LDH is composed of non-identical subunits (H-(LDHB) and M-(LDHA)) encoded by the LDHB and LDHA gene, respectively [15,16]. Through combination of these subunits, five isoenzymes (A4,

A3B1, A2B2, A1B3 and B4) with tissue-specific distribution are formed [17]. Isoenzyme combinations containing LDHB are predominantly found in heart tissue, whereas combinations containing LDHA are particularly located in anaerobic tissues [18].

In our study, we were able to demonstrate an anti-inflammatory effect of LNA ASOs during EAM induction *in vivo* mediated via increased protein levels of cardiac mLDHB and subsequent pro-apoptotic Bcl-2-associated X protein (mBax) suppression, as well as downregulation of genes of reactive oxygen species (ROS)-associated proteins, indicating a reduction in ROS. Thus, LNA ASOs and LDHB may be a promising therapeutic approach for targeted treatment in autoimmune myocarditis.

METHODS

Mice

A/J mice were obtained from Envigo (Huntingdon, Cambridgeshire, UK). For all experiments, 5-to 7-week-old female mice were used. Mice were maintained at the animal facility unit of the University of Heidelberg, Germany. This study was performed in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the German Animal Welfare Act, which is based on the Directive of the European Parliament and of the council on the protection of animals used for scientific purposes (Directive 2010/63/EU). The local Animal Care and Use Committee Karlsruhe approved all procedures involving the use and care of animals.

Experimental autoimmune myocarditis

To induce EAM, mice were subcutaneously immunized with an emulsion of murine cardiac TnI and complete Freund's adjuvant (CFA), which was supplemented with 5mg/mL of *Mycobacterium tuberculosis* H37Ra (Sigma, St Louis, MO, USA). For animal experiments, 120μg TnI was used. For the experiments with LNA ASOs, A/J mice were immunized with TnI on days 0, 7 and 14 and received 5mg/kg of the LNA ASO mmu-miR-21a-5p, LNA ASO mmu-miR-146b-5p, MB_1114 ASO or LNA ASO control (Exiqon)

intraperitoneally (i.p.) 5 times in total (one injection per week). Furthermore, for analysis of the effect of MB_1114, A/J mice were immunized with Control (CFA+PBS) (for a detailed experimental setting, see Methods S1, Figure S1 and Figure S2 and Table S1). For the *mLdhb*-specific GapmeR experiment, mice were immunized on days 0 and 7 with TnI or Control and received 10mg/kg of the ASOs (Exiqon) i.p. 6 times in total (for a detailed experimental setting, see Methods S1, Figure S3 and Table S1). Murine cardiac TnI was provided and prepared as already described [19–21]. On day 28, mice were anaesthetized by i.p. injection of 120mg/kg ketamine and 16mg/kg xylazine and blood was taken from facial vein for measurement of high-sensitive troponin T (hsTnT) serum level. Afterwards, mice were killed by cervical dislocation. Hearts were explanted, cut longitudinal vertical to septum and snap-frozen or fixed in 10% formalin for further analyses.

Western blotting

For Western blot analysis, proteins of frozen heart tissues were extracted as described and separated on 12% NuPage Bis-Tris gels [21,22]. Afterwards, proteins were transferred, and membranes were blocked as previously described [22]. Membranes were incubated with primary antibody at 4°C overnight and corresponding secondary antibody (Table S2 and Table S3). Protein bands were detected using chemiluminescence. Quantitative analysis was performed using ImageJ or FluorChem Q software. GAPDH served as a loading control. The mean of at least 3 technical replicates per sample is depicted in the graphs.

Measurement of hsTnT

Measurement of hsTnT serum level was performed as previously described [22]. For the measurement, serum was diluted 1:10. Measured hsTnT serum levels were normalized against the mean hsTnT level of the Ctrl. B./Control group.

Measurement of cardiac ROS level

For determination of cardiac ROS levels, DCFH-DiOxyQ assay was used (described in Methods S1, see also Figure S4).

Histopathology

Explanted hearts were fixed in formalin and subsequently embedded in paraffin. Heart sections (3–5μm) were cut and stained with haematoxylin and eosin (HE) or with acid fuchsin orange G-stain (Afog) to assess the grade of inflammation and fibrosis, using standard staining protocols and reagents. HE- and Afog-stained sections were analysed using light microscopy. Histopathological evaluation was carried out by two independent examiners, who were blinded to the treatment and immunization status of the respective groups. Inflammation and fibrosis were assessed in percentage of the examined heart section as previously described [22].

Quantitative real-time polymerase chain reaction (qPCR)

Measurement of qPCR was performed as previously described using 25ng of cDNA [21,22]. *L32* was used as reference gene. The relative expression represents the mean of 3 technical replicates. Primer sequences used for qPCR analysis are shown in Table S4.

Target identification and verification of LNA ASO MB_1114

To identify the mRNA target of LNA ASO MB_1114, Switching Mechanism At 5′-end of RNA Transcript (SMARTer) Rapid Amplification of cDNA Ends (RACE) was performed (detailed methods in the Methods S1 and in Table S5). Sequence of SMARTer RACE PCR product was determined using TOPO-TA cloning and sequencing (detailed methods in the Methods S1).

Cytokines

For analysis of cytokine levels in splenocytes, the spleen was explanted. Cytokine levels from spleen homogenate after TnI stimulation were measured for murine IL-1β, IL-6, IL-2, IL-17, and TNF-α as previously described [23].

Statistical analysis

Results are shown as mean ±standard error of the mean (SEM) and plotted as individual points. To determine whether samples within one group are normally distributed, the D'Agostino–Pearson test was used. For the comparison of more than two normally distributed groups, two-way ANOVA with a conservative Bonferroni post hoc test was used to control the type I error rate. To compare groups with a single control group, one-way ANOVA with Dunnett's post hoc test was used. Treatment (MB_1114 vs.

LNA control vs. solvent Ctrl. or GapmeR vs. Ctrl. B) and the immunization (TnI vs. Control) were tested as independent variables. Differences between two parametric groups were analysed using the unpaired t-test. To compare two non-parametric groups, the Mann–Whitney test was performed. Values of $P < 0.05$ were considered statistically significant and marked by *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, and ****$P < 0.0001$. Statistical analysis was performed using GraphPad Prism 7 (GraphPad Software, La Jolla, California, USA).

RESULTS

LNA ASO MB_1114 showed an anti-inflammatory effect in TnI-induced EAM

In order to identify new therapeutic approaches in EAM, the contribution of miRNAs in EAM was examined. For that purpose, A/J mice immunized with TnI for EAM induction received LNA ASOs directed against mmu-miR-21a-5p and mmu-miR-146b-5p, as well as scrambled LNA ASO control (MB_1114) or solvent Ctrl. 5 times in total

(once a week). However, not the miR-specific LNA ASOs, but the treatment with MB_1114, showed an unexpected anti-inflammatory effect on EAM (Figure 1a, Figure S5). In order to examine the sequence-specific effect of the discovered anti-inflammatory LNA ASO MB_1114, the initial experiment was repeated using MB_1114, solvent Ctrl. and another scrambled LNA ASO control (LNA Ctrl.). Furthermore, some mice were immunized with CFA+PBS (control) in addition to the i.p. injections with LNA ASOs and solvent Ctrl. This repetition of the initial experiment showed the same results. Accordingly, the consolidated results of the two experiments showed a significant reduction in inflammation and fibrosis for MB_1114-treated and TnI-immunized mice compared to TnI-immunized mice treated with LNA Ctrl. or solvent Ctrl., respectively (Figure 1b,c,d). These changes could not be observed for Control-immunized mice (Figure 1b,c,d). To characterize cardiac damage, hsTnT serum level was measured on day 28. The results showed no significant difference in hsTnT serum level in TnI-immunized and MB_1114-treated mice compared with LNA Ctrl.-treated mice (Figure 1e). Taken together, the results showed a significant anti-inflammatory effect of MB_1114-treated mice.
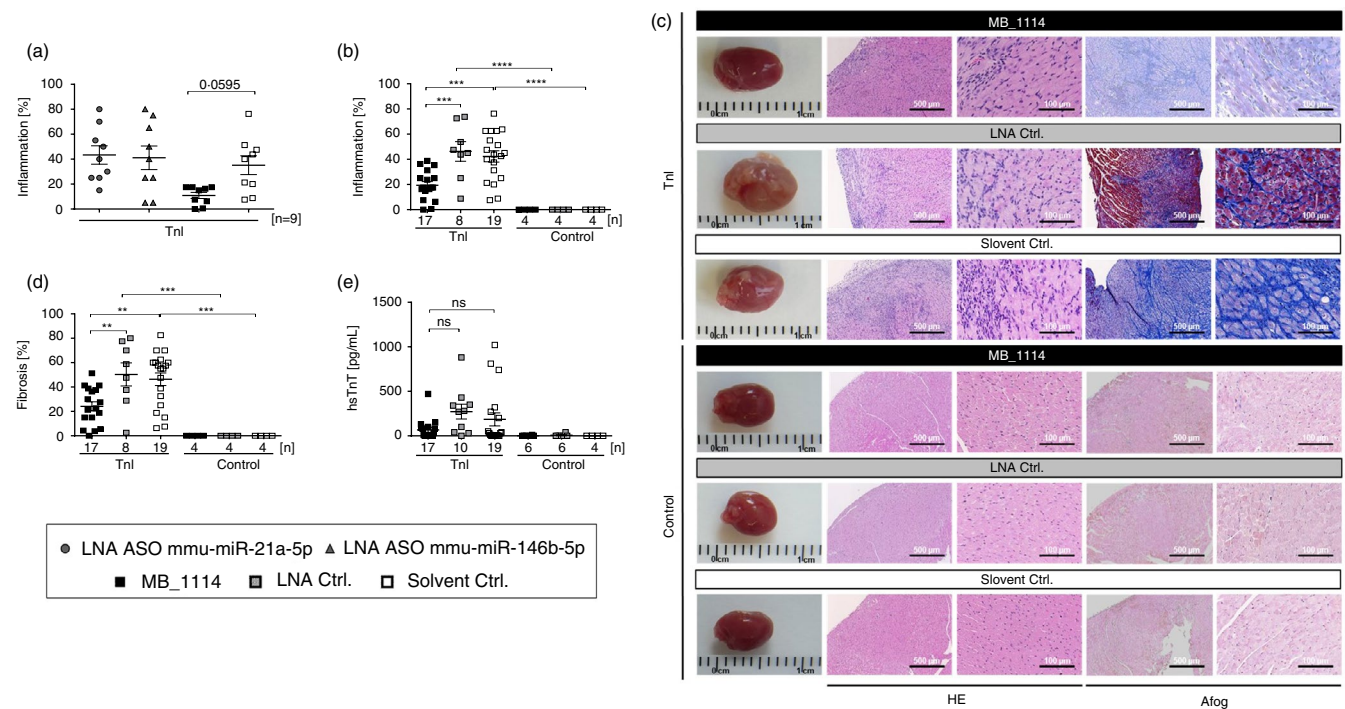
FIGURE 1 LNA ASO MB_1114 showed an anti-inflammatory effect in TnI-induced EAM. (a) Myocardial inflammation of TnI-immunized mice treated with LNA ASO mmu-miR-21a-5p and LNA ASO mmu-miR-146b-5p, as well as MB_1114 and solvent Ctrl. The following panels show the consolidated results of the initial and repeated experiment. (b, d) Myocardial inflammation and fibrosis of TnI-immunized mice treated with MB_1114 compared to LNA Ctrl.- or solvent Ctrl.-treated mice. (c) Representative macroscopic pictures (column 1) and histopathological exterminations of HE-stained (columns 2, 3) and Afog-stained (columns 4, 5) heart sections. (e) hsTnT serum level of TnI-immunized mice treated with LNA ASO MB_1114 compared to LNA Ctrl.- and solvent Ctrl.-treated mice. Data are displayed as mean ±SEM. Statistical analysis was performed using one-way or two-way ANOVA with the Bonferroni post hoc test. Values of $P < 0.05$ were considered statistically significant and marked by *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ and ****$P < 0.0001$

LNA ASO MB_1114 triggered an increase in mLDHB protein level potentially mediating anti-inflammatory *in vivo* effects

Due to the optimized design of LNA ASOs leading to improved hybridization of modified ASOs to complementary RNA [24], we assumed that the target of the LNA ASO MB_1114 was a RNA. To identify the RNA target sequence of LNA ASO MB_1114, a 5' SMARTer RACE PCR was performed. The BLAST search of the generated PCR product revealed m*Ldhb* as the RNA target sequence of MB_1114 (Figure S6). Due to the substance class of MB_1114 as LNA ASO, we expected an inhibition of m*Ldhb* mRNA translation mediated via steric hindrance of the translation machinery [25]. Hence, the influence of MB_1114 on mLDHB protein level was analysed and correlated to inflammation and fibrosis. Contrary to our expectation, the Western blot analysis showed a significantly increased mLDHB protein level in TnI-immunized mice treated with MB_1114 compared to LNA Ctrl.- and solvent Ctrl.-treated mice (Figure 2a). These effects could not be observed in the Control-immunized mice. To exclude potential effects of MB_1114 on m*Ldha* through high sequence homology of 74%, Western blot analysis was performed. The results showed no effect of MB_1114 on mLDHA protein level (Figure 2b). However, in general, mice immunized with TnI showed a significantly lower mLDHA protein level compared to Control-immunized mice. These results indicated that LNA ASO MB_1114 could mediate its anti-inflammatory effect via increased protein levels of mLDHB.

mLdhb-specific GapmeR showed an anti-inflammatory effect in EAM mice

As MB_1114, primarily designed as an LNA ASO miRNA inhibitor, stimulated the expression of its identified target mLDHB, we investigated whether the administration of a specific mLDHB ASO was also able to cause the same anti-inflammatory effect. Thus, a specific anti-*mLdhb* LNA GapmeR (GapmeR) was designed, validated (Figure 7) and administered to A/J mice during EAM. An unspecific GapmeR control (Ctrl. B) was used as inhibitor control. Mice were immunized with TnI to induce an EAM or with CFA+PBS (Control), and effects of *mLdhb*-specific GapmeR in EAM mice were analysed. Here, we observed a significant lower inflammation and fibrosis in cardiac tissue of GapmeR-treated mice compared with Ctrl. B-treated mice, both groups immunized with TnI (Figure S3A,B,C, Figure S8). Furthermore, our results showed a significantly lower hsTnT serum level between GapmeR- and Ctrl. B-treated mice both immunized with TnI

(Figure 3d). Heart weight/body weight ratios were significantly lower in GapmeR-treated and TnI-immunized mice than in Ctrl. B-treated and TnI-immunized mice (Figure 3e). Furthermore, a successful delivery of LNA ASOs in heart tissue was detected (Figure S9). Our results showed an *mLdhb*-specific GapmeR-mediated anti-inflammatory *in vivo* effect.

mLdhb-specific GapmeR triggered increased mLDHB protein levels during anti-inflammatory effects *in vivo*

To determine whether the *mLdhb*-specific GapmeR increased the expression of cardiac mLDHB levels, the cardiac protein levels were analysed. Similar to the experiment with MB_1114, we were able to show a significant increase in mLDHB protein level in EAM mice treated with GapmeR compared to Ctrl. B (Figure 4a). In contrast to that, no significant difference in the *mLdhb* mRNA levels could be observed for these two groups (Figure S10). Furthermore, no difference in cardiac mLDHA protein expression was observed between the various groups (Figure 4b). These results supported our hypothesis of a regulatory mechanism, which is induced by the LNA ASO-mediated inhibition of m*Ldhb* mRNA translation leading to enhanced translation efficiency.

mLdhb-specific GapmeR administration reduced pro-inflammatory cytokines in EAM mice

To study the effect of *mLdhb*-specific GapmeR administration on pro- and anti-inflammatory stimuli, we analysed the expression of various cytokines of supernatant of stimulated splenocytes and in cardiac tissue. The supernatant of TnI-stimulated spleen cell homogenate explanted from TnI/ASO treated mice showed a significant reduction in pro-inflammatory cytokines mIL-1β, mIL-6 and mIL-2, as well as a reduction in mIL-17 and mTNF-α in GapmeR-treated and TnI-immunized mice compared with Ctrl. B-treated and TnI-immunized animals (Figure 5a–e). Additionally, cardiac levels of *mIL-1α* mRNA showed a downregulation of cardiac *mIl-1α* mRNA levels in TnI-immunized and GapmeR-treated mice compared with Ctrl. B-treated and TnI-immunized mice (Figure 5f). Furthermore, a successful delivery of LNA ASOs in spleen tissue was detected (Figure S9). These results indicated a *mLdhb*-specific GapmeR caused reduction in pro-inflammatory cytokines in EAM mice.
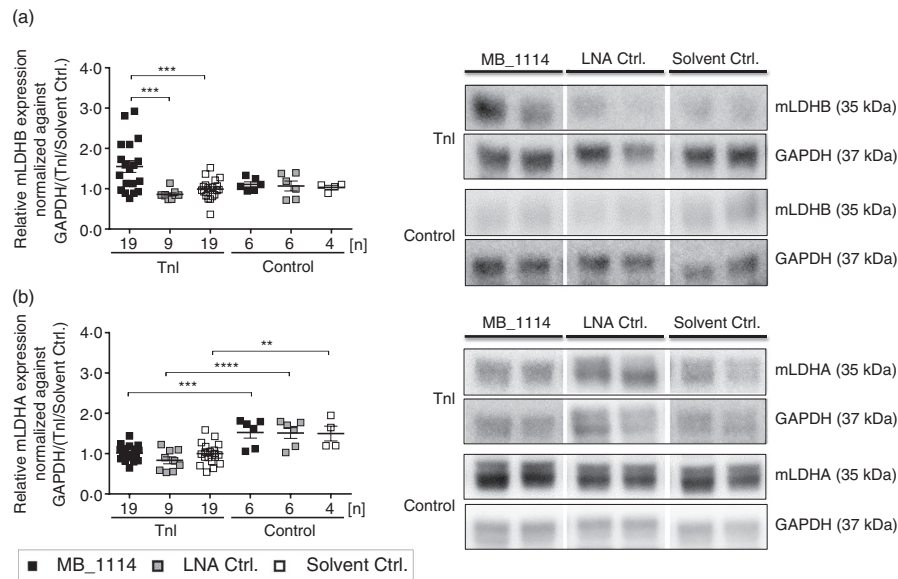
FIGURE 2 LNA ASO MB_1114 triggered an increased mLDHB protein level potentially mediating anti-inflammatory *in vivo* effects. (a) Heart tissue was analysed by Western blot and fold increase in cardiac murine LDHB (mLDHB) of mice immunized with TnI or Control and treated with LNA ASO MB_1114 (n = 19), LNA Ctrl. (n = 10) or solvent Ctrl. (n = 19) normalized against GAPDH expression of TnI-immunized mice treated with solvent Ctrl. Representative Western blot images of mLDHB protein expression. (b) Western blot analysis of cardiac murine LDHA (mLDHA) protein of different groups normalized against GAPDH expression of TnI-immunized mice. Representative Western blot images of mLDHA protein expression. Data are displayed as mean ±SEM. Statistical analysis of comparison between groups was performed using two-way ANOVA with the Bonferroni post hoc test. Values of $P < 0.05$ were considered statistically significant and marked by ** $P < 0.01$, *** $P < 0.001$ and **** $P < 0.0001$

mLdhb-specific GapmeR-triggered anti-inflammatory effect in A/J mice led to a reduced activation of ROS and mBax suppression

To elucidate potential mechanisms involved in anti-inflammatory effects observed in GapmeR-treated and TnI-immunized animals, we first examined the role of cardiac ROS levels, as well as mRNA levels of ROS-associated proteins such as *mp47 phox*, *mp67 phox*, *mNox2*, *mUcp2* and *mSod2* in TnI-induced EAM. Here, an upregulation of cardiac ROS levels, as well as an upregulation of cardiac mRNA levels of ROS-associated proteins such as *mp47 phox*, *mp67 phox*, *mNox2* and *mUcp2* in TnI-induced EAM mice, was observed, whereas cardiac *mSod2* mRNA levels were downregulated (Figure S11). These results revealed the importance of ROS-associated proteins in TnI-induced EAM model.

In a next step, we investigated the mRNA levels of ROS-associated proteins, which we previously identified to be important in our EAM model, in GapmeR- or Ctrl. B-treated and TnI- or Control-immunized A/J mice. Here, a significant downregulation of cardiac mRNA levels from ROS-associated proteins *mp47 phox*, *mp67 phox*, *mNox2* and *mUcp2* in GapmeR/TnI-treated compared with Ctrl. B/TnI-treated mice was observed, indicating a GapmeR-mediated reduction in ROS (Figure 6a–d). These results suggested that GapmeR-treated mice seemed to show less ROS

activation despite TnI immunization. In accordance with these results, significantly higher cardiac m*Sod2* mRNA levels in GapmeR-treated and TnI-immunized mice compared with Ctrl. B-treated and TnI-immunized mice were observed (Figure 6e). Taken together, these results indicated a downregulation of ROS in *mLdhb*-specific GapmeR-treated and TnI-immunized mice.

To gain better insights into mechanisms involved in *mLdhb*-specific GapmeR-mediated upregulation of mLDHB protein expression and subsequent anti-inflammatory mechanisms observed in our study, we examined the expression of pro-apoptotic protein mBax, as mBax was described by Sheibani et al. to be suppressed by LDHB protein and by Wu et al. to be linked with TnI auto-antibodies [26,27]. Here, we observed a significant downregulation of mBax protein levels in GapmeR-treated and TnI-immunized mice compared with Ctrl. B-treated and TnI-immunized mice (Figure 7). These results indicated a potential mechanism of mLDHB, which may be mediated via mBax protein suppression followed by potential ROS reduction in GapmeR-treated and TnI-immunized mice.

DISCUSSION

Underlying mechanisms leading from myocarditis to DCM are not fully understood so far. Therapies to reduce the
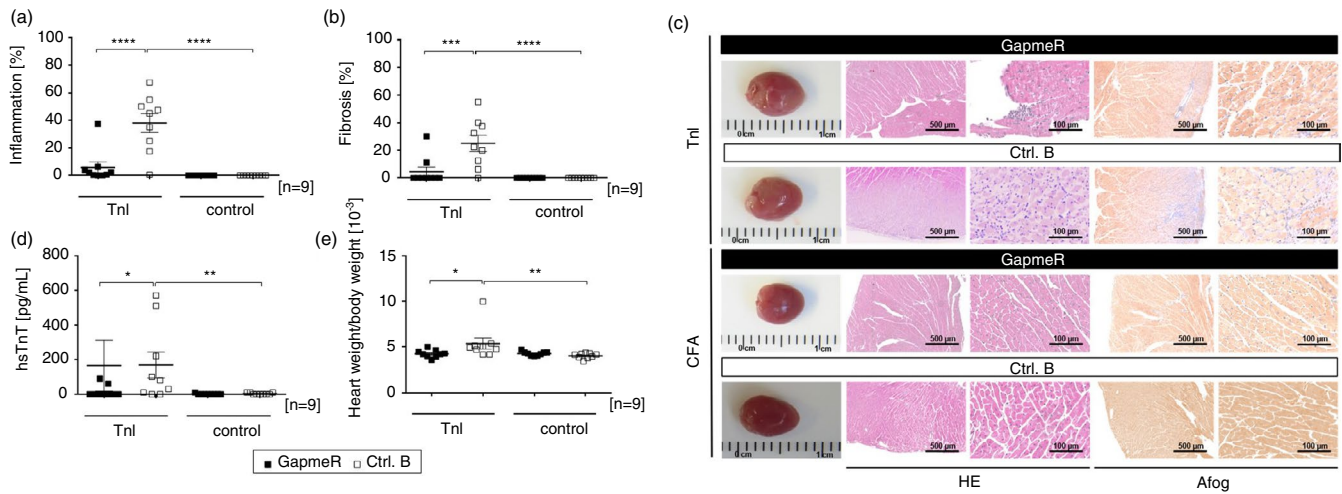
FIGURE 3 *mLdhb*-specific GapmeR showed an anti-inflammatory effect in EAM mice. (a, b) Inflammation and fibrosis of A/J mice, immunized with TnI or Control and treated with GapmeR (n = 9/group) or Ctrl. B (n = 9/group). (c) Representative macroscopic pictures (column 1) and histopathological exterminations of HE-stained (columns 2, 3) and Afog-stained (columns 4, 5) heart sections. (d) hsTnT levels in serum of immunized mice treated with GapmeR and Ctrl. B. (e) Heart weight/body weight ratio of examined groups. Data are displayed as mean ±SEM. Statistical analysis of comparison between groups was performed using two-way ANOVA with the Bonferroni post hoc test. Values of $P < 0.05$ were considered statistically significant and marked by *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ and ****$P < 0.0001$
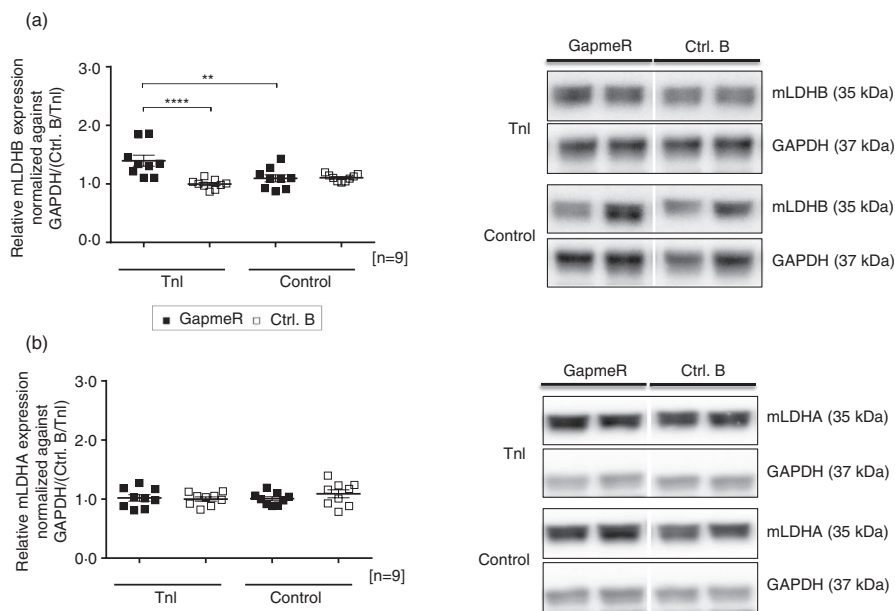
FIGURE 4 *mLdhb*-specific GapmeR-triggered increased levels of cardiac mLDHB protein expression. (a) Western blot analysis of cardiac murine LDHB (mLDHB) protein expression of A/J mice immunized with TnI or Control and treated with GapmeR (n = 9/group) or Ctrl. B (n = 9/group). mLDHB protein expression was normalized against GAPDH in TnI-immunized mice treated with Ctrl. B. Representative Western blot images of mLDHB. (b) Western Blot analysis of cardiac murine LDHA (mLDHA) protein level. Protein expression was normalized against GAPDH of Control-immunized mice treated with Ctrl. B. Representative Western blot images of mLDHA. Data are displayed as mean ±SEM. Statistical analysis of comparison between groups was performed using two-way ANOVA with the Bonferroni post hoc test. Values of $P < 0.05$ were considered statistically significant and marked by ***$P < 0.001$ and ****$P < 0.0001$

progress from myocarditis to DCM are currently limited to heart failure treatment [5,6]. Thus, our team focused on identifying potential therapeutic approaches for new targeted therapies in myocarditis using a TnI-based EAM mouse model [9]. One substance class, which is already

under investigation for the treatment of inflammatory diseases, is ASOs [28]. In our study, we demonstrated for the first time that LNA ASOs have an anti-inflammatory effect during EAM induction *in vivo*. This is potentially induced by increased mLDHB protein levels and probably subsequent
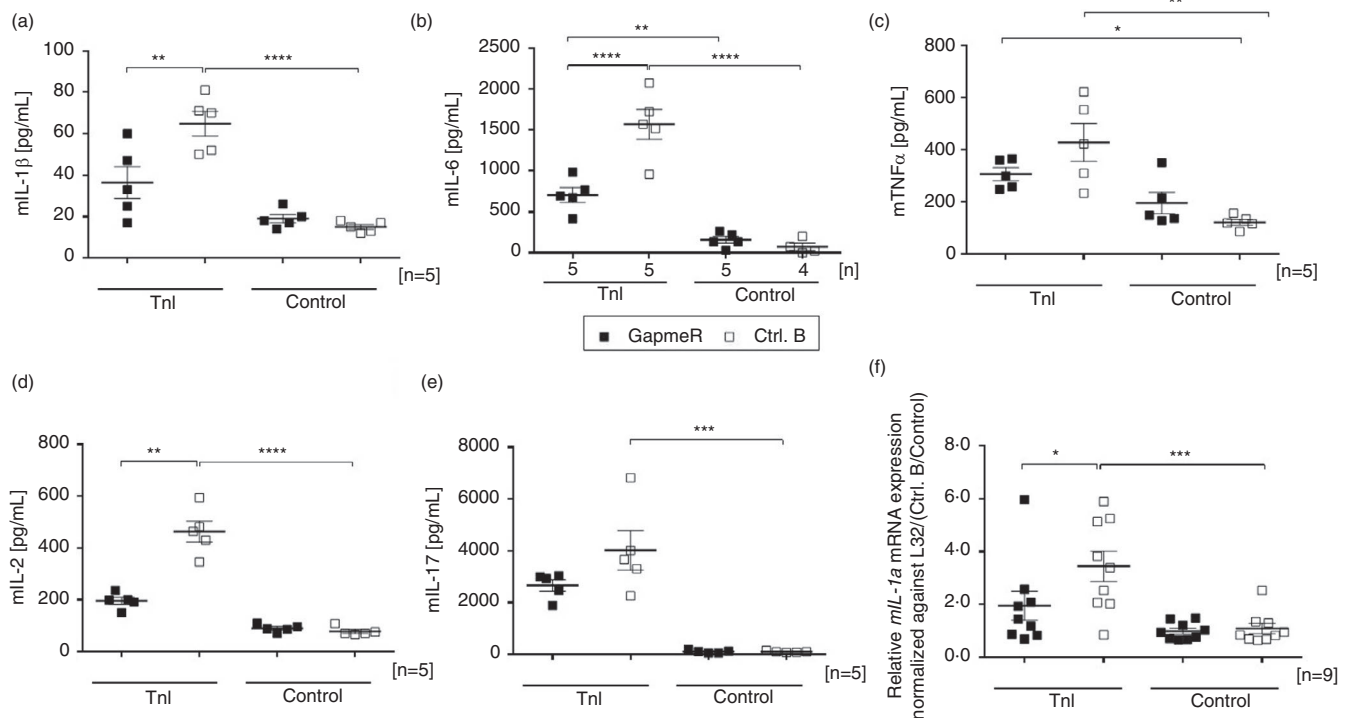
FIGURE 5 *mLdhb*-specific GapmeR administration reduced pro-inflammatory cytokines and led to reduced cardiac *mIL-1α* levels in EAM mice. Cytokine levels from supernatant of TnI-stimulated spleen cell homogenate. (a) mIL-1β, (b) mIL-6, (c) mTNF-α, (d) mIL-2 and (e) mIL-17 were analysed using ELISA. Myocardial mRNA expression of (f) *mIl-1α* was analysed by qPCR from mice, immunized with TnI or Control and treated with GapmeR (n = 9/group) or Ctrl. B (n = 9/group). qPCR data were normalized against m*L32* in Control-immunized mice treated with Ctrl. B. Data are displayed as mean ±SEM. Statistical analysis of comparison between groups was performed using two-way ANOVA with the Bonferroni post hoc test. Values of $P < 0.05$ were considered statistically significant and marked by *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ and ****$P < 0.0001$

reduced ROS activation mediated via a suppression of protein levels of pro-apoptotic mBax during TnI-induced EAM. Hence, LNA ASOs directed against *Ldhb* seem to be a promising substance class for targeted treatment of myocarditis. Nevertheless, exact evidence for the mechanism of action of LDHB-mediated anti-inflammatory activity is still lacking. Furthermore, off-target effects mediated by LNA ASOs might also contribute to the anti-inflammatory effect observed in our study.

## LNA ASO MB_1114 showed an anti-inflammatory effect in EAM mice

In this study, we observed an anti-inflammatory effect in EAM mice treated with LNA ASO MB_1114, which was primarily designed as a scrambled LNA ASO control. The results showed reduced signs of inflammation, fibrosis and cardiac damage. Due to the sequence-specific anti-inflammatory off-target effect of MB_1114, we hypothesized the anti-inflammatory potential of this LNA ASO not to be ascribed to unspecific phosphorothioate backbone interactions. However, it was already described

that the phosphorothioate backbone directly contributes to sequence-dependent off-target effects of LNA ASOs. These effects showed higher potency with phosphorothioate backbone compared with a phosphodiesterase one [29]. Thus, the phosphorothioate backbone seems to support but not mediate the sequence-dependent effect of MB_1114.

LNA ASO MB_1114 triggered an increase in mLDHB protein level potentially mediating anti-inflammatory *in vivo* effects

Our results point to a MB_1114-induced post-transcriptional mechanism regulating the cardiac mLDHB protein expression. Hence, we postulate that binding of LNA ASO MB_1114 to the m*Ldhb* mRNA indeed could sterically inhibit the mRNA translation. However, the inhibition may be counteracted via enhanced translation efficiency of the accessible m*Ldhb* mRNA. Schwanhäusser et al. demonstrated that the cellular abundance of proteins is predominantly controlled at the level of translation [30]. Another mechanism potentially involved in LNA-triggered
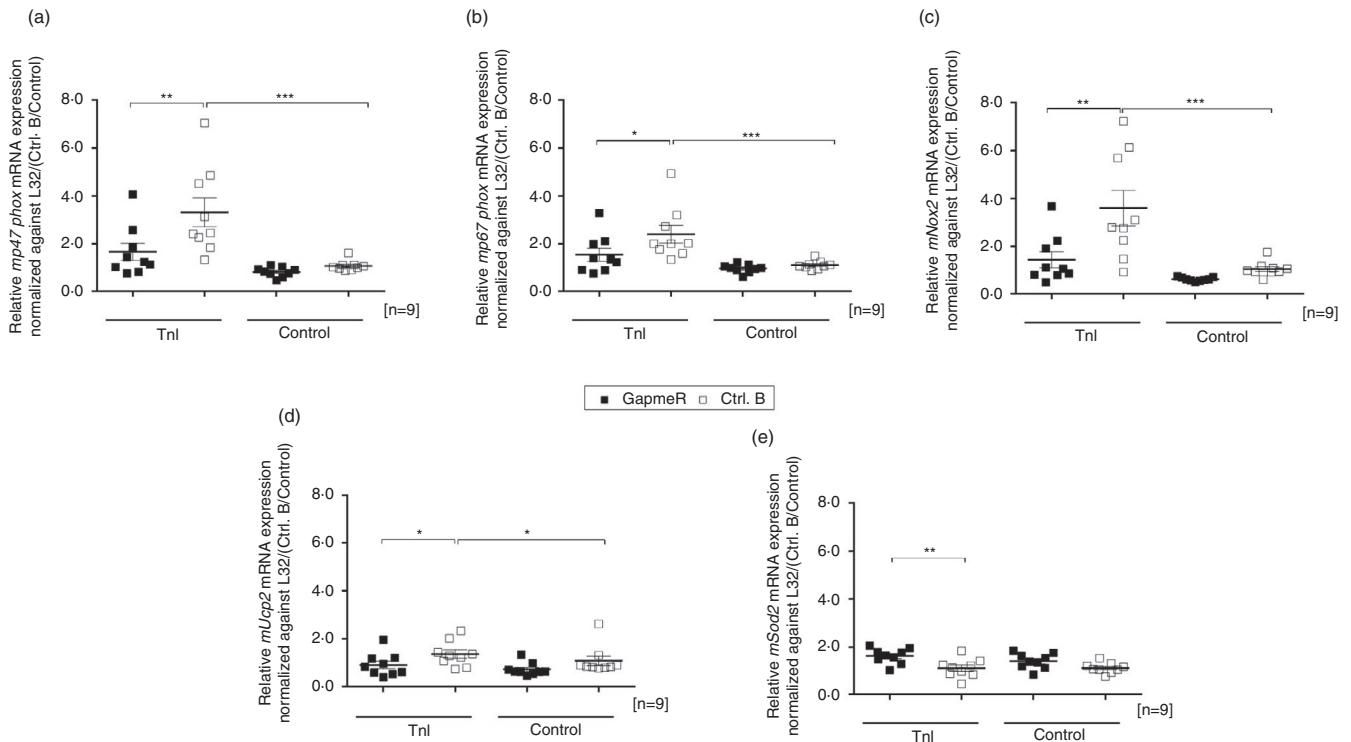
FIGURE 6 *mLdhb*-specific GapmeR-treated mice showed reduced cardiac levels of ROS-associated proteins. qPCR analysis of ROS-associated proteins. (a) *mp47 phox*, (b) *mp67 phox*, (c) *mNox2*, (d) *mUcp2* and (e) *mSod2* in hearts of mice immunized with TnI or Control and treated with GapmeR (n = 9/group) or Ctrl. B (n = 9/group). mRNA levels were normalized against m*L32* and the corresponding mRNA expression level of Control-immunized mice treated with Ctrl. B. Data are displayed as mean ±SEM. Statistical analysis of comparison between groups was performed using two-way ANOVA with the Bonferroni post hoc test. Values of $P < 0.05$ were considered statistically significant and marked by *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$
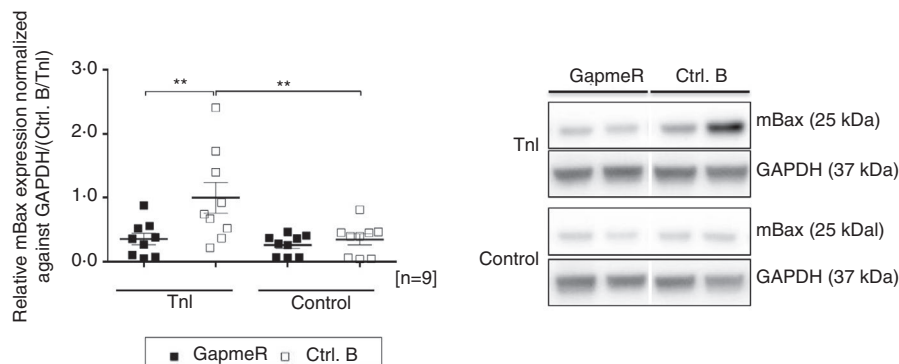
FIGURE 7 *mLdhb*-specific GapmeR-treated mice showed reduced protein levels of mBax. Western blot analysis of cardiac mBax protein normalized against GAPDH and the mBax protein level of Control-immunized mice treated with Ctrl. B. Representative Western blot images of mBax. Data are displayed as mean ±SEM. Statistical analysis of comparison between groups was performed using two-way ANOVA with the Bonferroni post hoc test. Values of $P < 0.05$ were considered statistically significant and marked by *$P < 0.05$ and **$P < 0.01$

upregulation of mLDHB protein levels might be the phenomenon of genetic compensation. This mechanism, which might be responsible for observed unintended upregulation, is caused by a development of tolerance against LNA ASOs and subsequent upregulation of the target mRNA. Liang et al. described a mechanism where LNA ASOs increase the pre-mRNA levels of their target mRNA [31].

These pre-mRNAs might afterwards travel back to nucleus and trigger the proposed genetic compensation mechanism proposed by El-Brolosy et al. [32]. However, in our study we could not observe an upregulation of *mLdhb* mRNA levels in GapmeR-treated compared to Ctrl. B-treated mice. Hence, such a feedback mechanism might be an interesting hypothesis, but lacks evidence in our study. Therefore,

we hypothesized an enhanced translation efficiency to be responsible for the observed boost in mLDHB protein levels. Moreover, this regulatory mechanism seems to play a role only in EAM mice. Accordingly, no effect of LNA ASO could be observed in Control-immunized and MB_1114-treated mice. Therefore, MB_1114 seems to show only an effect in diseased mice predestinating the LNA ASO as an ideal therapeutic agent. However, further investigations will be necessary to reveal the precise regulatory mechanism by which MB_1114 mediates its effect.

mLdhb-specific GapmeR showed an anti-inflammatory effect on EAM

Similar to the experiments with MB_1114, mice treated with the *mLdhb*-specific GapmeR in addition to TnI immunization showed reduced signs of inflammation, fibrosis, cardiac damage and better heart weight/body weight ratio. These observations confirmed our hypothesis that LNA ASO MB_1114 seems to mediate its anti-inflammatory effect via mLDHB. A correlation between mLDHB level and immune reaction was already described by Ratter et al. They showed that the change in LDHB expression upon stimulation of peripheral blood mononuclear cells *in vitro* correlated significantly with immune cell function [33]. This observation in addition to our results refers to a yet unknown role of mLDHB in inflammatory processes.

mLdhb-specific GapmeR-triggered increased mLDHB protein levels during anti-inflammatory effects *in vivo*

To determine whether the *mLdhb*-specific GapmeR similar to LNA ASO MB_1114 led to an enhanced cardiac mLDHB expression, the cardiac protein expression was analysed. Similar to the experiment with MB_1114, we were able to show a significant increase in cardiac mLDHB protein levels in EAM mice treated with GapmeR compared with Ctrl. B. To explain why the mLDHB protein increased by GapmeR was only seen in EAM mice but not in control mice, we determined the cardiac concentration of GapmeR and Ctrl. B in TnI- and Control-immunized mice. The analyses showed that the cardiac GapmeR concentration of GapmeR-treated and TnI-immunized mice seems to be higher than the cardiac GapmeR concentration of GapmeR-treated and Control-immunized mice. Thus, the mLDHB protein increase in the GapmeR/TnI group could be based on the higher cardiac GapmeR concentration. The enhanced cardiac uptake of GapmeR might be explained by an inflammation-induced enhanced permeability of endothelial cells. Accordingly, TNF-α, which

was more highly expressed in TnI-immunized compared with Control-immunized mice, seems to increase the permeability of endothelial monolayers to lower weight solutes and macromolecules [34]. Considering this hypothesis, the increased cardiac concentration of ASOs in TnI-immunized mice should also be detectable for Ctrl. B. However, for Ctrl. B, no difference in ASO concentration was observed between TnI- and Control-immunized mice. These inconsistent results could be based on the small number of mice in each group. To prove the hypothesis of increased uptake mediated via inflammation and leading to enhanced mLDHB expression, a further study with a larger group size would be required.

In contrast to mLDHB, no difference in cardiac mLDHA protein level was observed between the various groups. These results support our hypothesis of a regulatory mechanism, which is induced by the LNA ASO-mediated inhibition of m*Ldhb* mRNA translation leading to enhanced translation efficiency. A regulated negative feedback mechanism was already verified for the transcription of LDHA. Zhang et al. could demonstrate that an inhibition of LDHA led to a negative feedback overexpressing c-Myc, which in turn induced LDHA expression [35]. This observation suggests that there might exist yet unknown feedback loops for LDHB expression as well. Taken together, these results seem to support our hypothesis of an anti-inflammatory role of mLDHB in the TnI-induced EAM. Despite evidence collected and discussed, it is still unexpected that the m*Ldhb* GapmeR did not decrease m*Ldhb* mRNA but increased mLDHB protein levels. As discussed, we collected data supporting the hypothesis of an enhancing translation efficiency mediated by m*Ldhb*-specific LNA ASO. However, the possibility of unspecific off-target effects mediated by LNA GapmeR could not definitely be ruled out and thus might also be an explanation for our results [36,37]. In particular, it is not clear whether the mLDHB protein increase in heart tissue is a direct result of GapmeR in the heart or mediated via downstream events of the GapmeR in other tissues.

mLdhb-specific GapmeR administration caused reduction in pro-inflammatory cytokines in EAM mice

To study the effect of mLDHB on pro-inflammatory stimuli, we analysed the expression of various cytokines in TnI-stimulated spleen cell homogenate and in heart tissue. Here, we could observe a reduction in pro-inflammatory cytokines mIL-1β, mIL-6, mTNF-α, mIL-2 and mIL-17 in the GapmeR-treated and TnI-immunized group compared to the group immunized with TnI and treated with Ctrl. B. IL-1β and TNF-α are early pro-inflammatory cytokines,

which seem to be necessary to induce the transition from infectious to autoimmune myocarditis. Additionally, IL-6 and IL-17, late pro-inflammatory cytokines, are crucial in the progression from autoimmune myocarditis to fibrotic dilated cardiomyopathy [38]. Moreover, the protection against EAM in Lewis rats can be ascribed to splenocytes secreting reduced levels of IL-2 [39]. The cardiac expression of *mIl-1α* was also decreased in the TnI-immunized GapmeR group compared with the Ctrl. B-treated and TnI-immunized mice. Due to their importance for the induction and progression of an EAM, the decreased expression of all these pro-inflammatory cytokines could explain reduced inflammation, fibrosis and cardiac damage observed for the GapmeR group. Therefore, the decreased levels of these cytokines seem to be responsible for the better disease outcome in the GapmeR-treated and TnI-immunized group. However, the possibility cannot be ruled out that other organs such as kidney, liver or spleen, which also showed successful ASO delivery, are also involved in the downstream anti-inflammatory effects. Thus, further studies are necessary to analyse the involvement of these organs.

mLdhb-specific GapmeR-triggered anti-inflammatory effect in A/J mice led to reduced activation of ROS and mBax suppression

The expression of the analysed pro-inflammatory cytokines during myosin-mediated EAM seems to be induced via Toll-like receptor (TLR) 4 activation and enhanced via ROS formation. Hence, Wu et al. demonstrated in their study that TLR4 activation mediates inflammation and cytokine expression via ROS production. ROS in turn led to subsequent mitochondrial DNA (mtDNA) damage and release, which promotes apoptosis of cardiomyocytes [40]. Additionally, a connection between ROS generation and LDHB could be observed in cancer cells [41]. Due to these discoveries and to elucidate the influence of LDHB on ROS generation, we first analysed the contribution of ROS and the role of mLDHB in TnI-induced EAM. Here, a significant increase in ROS and significantly elevated transcriptional levels of ROS-associated proteins could be observed in TnI-immunized mice. These proteins are NADPH oxidase *Nox2* and components of Nox (*mp47 phox*, *mp67 phox*), which produces ROS, as well as *Ucp2*, a negative regulator of mitochondrial ROS production induced by ROS [42–44]. Furthermore, the transcription of *Sod2*, which catalyses the conversion of superoxide into oxygen and hydrogen peroxide, was significantly reduced in EAM mice [45]. These observations confirmed the results of Wu et al. Thus, ROS generation seems to play an important role in the induction and progression of TnI-induced EAM.

Contrary to that, our results did not show any significant difference in mLDHB expression between TnI- and Control-immunized mice. These results suggest that mLDHB does not negatively affect disease progression and therefore contradict the findings of Ratter et al., who postulated an interaction between LDHB and immune cell function [33].

To analyse the influence of *mLdhb*-specific GapmeR administration and subsequent elevated mLDHB protein levels on ROS, the transcriptional levels of ROS-associated proteins in GapmeR- and Ctrl. B-treated mice were determined. The studies showed a significant reduction of *mp47 phox*, *mp67 phox*, *Nox2* and *Ucp2* transcription level in GapmeR-treated EAM mice compared with Ctrl. B-treated animals. Furthermore, significantly increased transcription could be observed for *Sod2*. According to these results, the GapmeR-mediated mLDHB protein levels might lead to a reduction in cardiac ROS in EAM. First indications on how mLDHB could mediate its positive effect on ROS are provided by Sheibani et al. They identified LDHB as a novel Bax suppressor preventing apoptosis in yeast [27]. Bax, which is a pro-apoptotic effector protein, promotes mitochondrial outer membrane permeabilization by pore formation leading to the release of pro-apoptotic factors. Furthermore, Bax triggers the release of mtDNA causing a pro-inflammatory type of cell death [46]. According to the results of Wu et al., the release of mtDNA could be responsible for the manifestation of an EAM by binding to TLR 9 and triggering the production of further pro-inflammatory cytokines such as IL-1β, TNF-α and IL-6, as well as ROS [40]. Additionally, Oka et al. also demonstrated that released mitochondrial DNA leads to TLR 9-mediated inflammatory responses in cardiomyocytes and is able to induce myocarditis and dilated cardiomyopathy *in vivo* [47]. To investigate whether increased mLDHB expression in GapmeR-treated and TnI-immunized mice could lead to a mBax suppression and therefore potentially resulting in lower ROS activation, we analysed the cardiac mBax expression in GapmeR and Ctrl. B-treated mice. The results showed a significantly reduced cardiac mBax protein expression in GapmeR-treated and TnI-immunized mice compared with Ctrl. B-treated animals. These results point to an anti-inflammatory effect of the *mLdhb*-specific GapmeR mediating a mBax suppression potentially via mLDHB overexpression. However, further investigations are necessary to confirm the mLDHB-Bax-ROS axis in the EAM model.

CONFLICTS OF INTERESTS

AUTHOR CONTRIBUTIONS

Mariella Bockstahler, Christian Salbach, Anna-Maria Müller and Andrea Kübler performed the experiments; Mariella Bockstahler and Christian Salbach analysed the data; Mariella Bockstahler, Christian Salbach, Anna-Maria Müller and Ziya Kaya designed the study; and Mariella Bockstahler, Christian Salbach and Anna-Maria Müller wrote the paper.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request. The corresponding author had full access to all data in the study and took responsibility for its integrity and data analysis.

ORCID

Ziya Kaya https://orcid.org/0000-0002-3745-0836

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