Figure 10. Representative immunohistochemical stainings of CD11b+ (a to d) and CD68+ (e to h) macrophages in the left ventricle. 200×magnification. Specific epitopes are stained red.

3.4.2 Cytokines

Our further investigations were focused on analyzing the expression of pro-(IL-1, TNF-α)/anti-(IL-10) inflammatory cytokines, which are associated with the etiology of viral myocarditis and expressed under the control of the TLR systems and effector molecules. In order to evaluate the expression of cardiac cytokines during the immune response to viral infection seven days after CVB3 infection, we used the real-time PCR method. As expected, low expression levels of pro- and anti-inflammatory cytokines were detected in the left ventricle of the healthy control groups. Seven days after CVB3 infection, a generalized exacerbation of pro-inflammatory cytokines in the heart tissue of infected mice was indicated by the significantly increased mRNA expression levels of TNF-α and IL-1β , (P <0.05, Figure 11). The expression of both pro-inflammatory cytokines was significantly higher in WTCVB3 mice than in NOD2$^{-/-}$CVB3 mice (P <0.05, Figure 11). For IL-1β, there was no significant difference between the NOD2$^{-/-}$ and NOD2$^{-/-}$CVB3 groups. IL-10 expression was significantly higher in WTCVB3 mice compared to NOD2$^{-/-}$CVB3 mice (P <0.05, Figure 12).

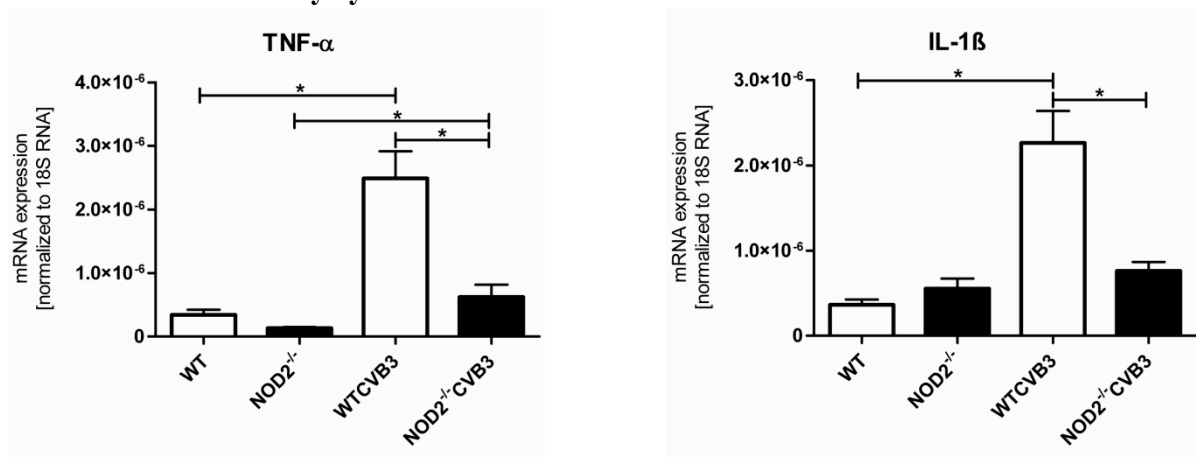### 3.4.2.1 Pro-inflammatory cytokines

Figure 11. The mRNA expression of TNF-α and IL-1β in the left ventricle seven days after viral infection measured by real-time PCR. The mRNA expression of TNF-α and IL-1β in the CVB3-infected groups is significantly higher than in the respective healthy groups. A generalized exacerbated expression of both pro-inflammatory cytokines was detected in WTCVB3 mice in comparison to NOD2$^{-/-}$CVB3mice (*= p<0.05). All data are reported as the mean value (MV) ± the standard error of the mean (SEM). WT = wild type; CVB3= coxsackie virus B3.
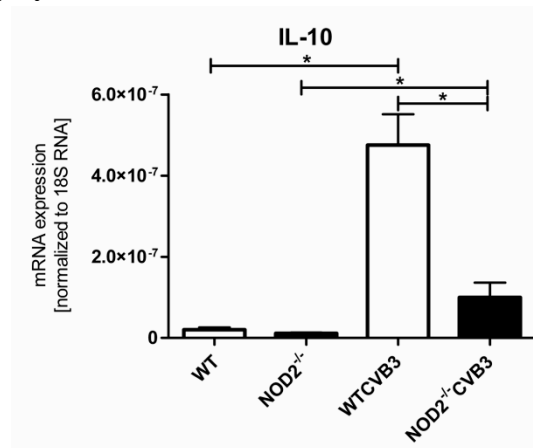
3.4.2.2 Anti-inflammatory cytokines

Figure 12. The mRNA expression of IL-10 in the left ventricle seven days after viral infection measured by real-time PCR. The mRNA expression of IL-10 in the CVB3-infected groups is significantly higher than in the respective healthy groups. A significant difference in IL-10 expression between WTCVB3 and NOD2$^{-/-}$CVB3 was also detected (*= $p<0.05$). All data are reported as the mean value (MV) ± the standard error of the mean (SEM). WT = wild type; CVB3= coxsackie virus B3.

3.4.3 Monocyte chemotactic protein-1

Chemokines also play a crucial role in the inflammation process of CVB3-induced myocarditis by attracting immune cells into the heart. Immunohistochemistry and real-time PCR method were both used to evaluate the expression of the chemokine MCP-1 (CCL2) seven days after infection. The immunohistochemical results of MCP-1, measured by using digital image analysis, were matched to the mRNA expression results ($p <0.05$, Figure 13).
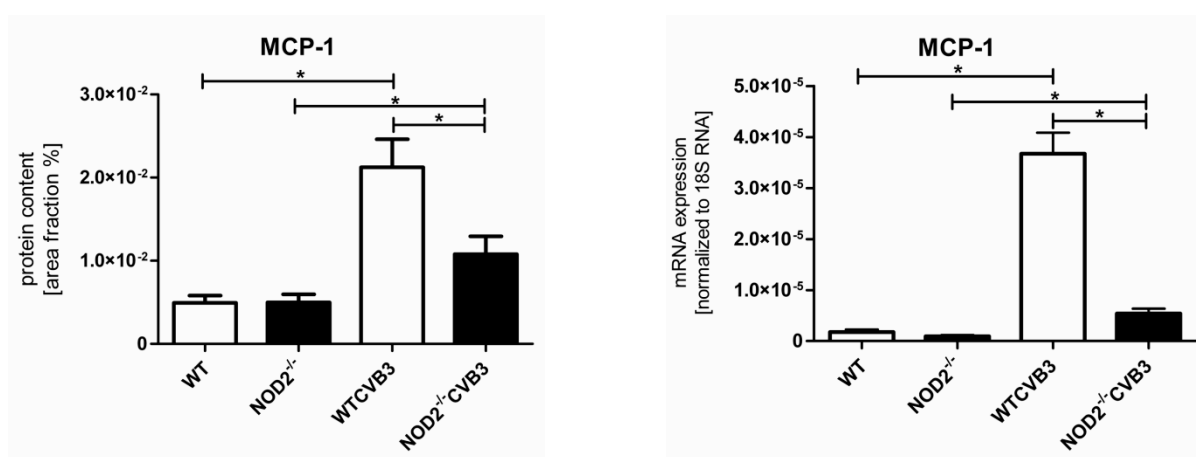

Figure 13. Immunohistochemically determined levels of MCP-1 and MCP-1 mRNA expression in the left ventricle seven days after CVB3 infection. MCP-1 expression is higher in CVB3-infected groups compared to respective healthy groups. WTCVB3 mice express significantly higher MCP-1 than NOD2$^{-/-}$CVB3 mice (*=$p <0.05$). Data are shown as the mean value (MV) ± standard error of the mean (SEM). WT = wild-type, CVB3 = coxsackie virus B3.
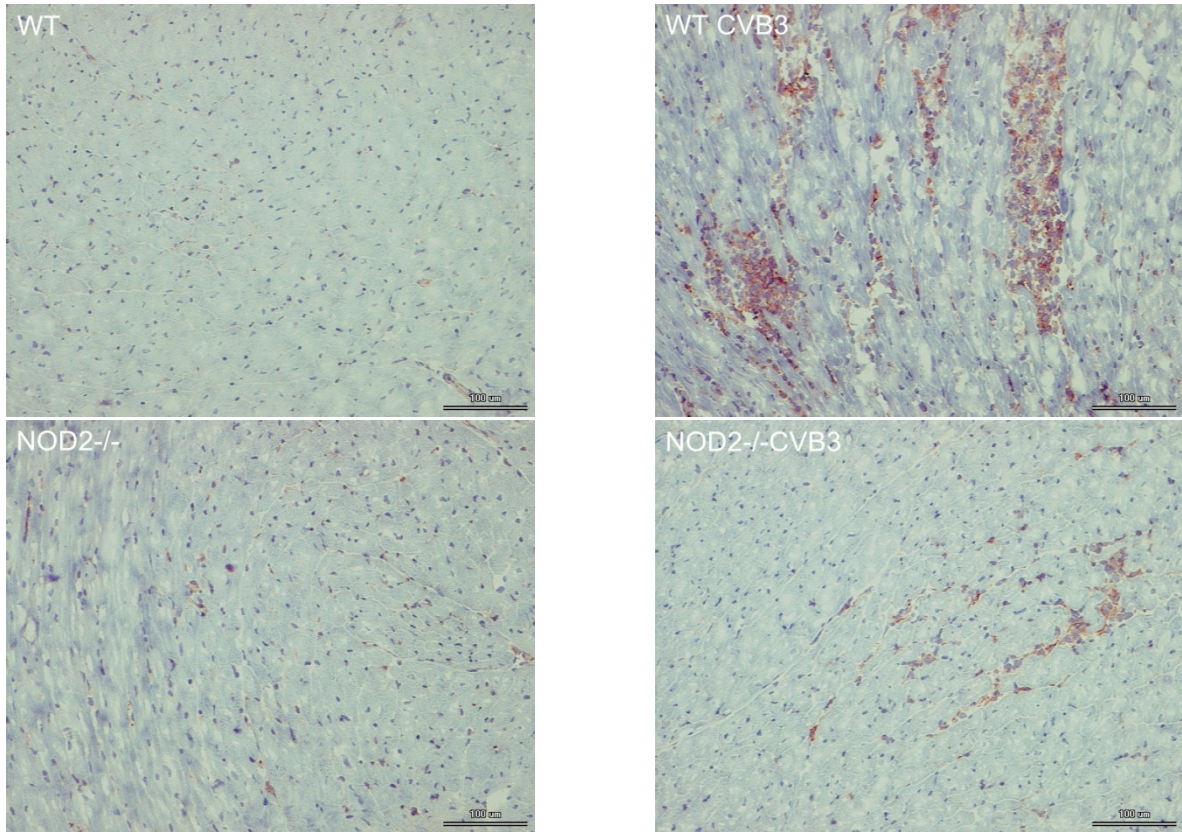
Figure 14. Representative immunohistochemical stainings for images of MCP-1 in the left ventricle. 200×magnification. MCP-1 specific epitopes are stained red.

3.5 Myocardial fibrosis

After heart tissue injury and inflammation, the healing process takes place with a significant deposition of collagen [125]. We examined the mRNA expression of collagen type I and III as a marker of fibrosis in the myocardium by real-time PCR and the protein content of type I and III collagen by immunohistochemistry.

No significant differences in *collagen I* and *III* mRNA expression were found between the two control groups. In contrast, *collagen I* and *III* mRNA expression was significantly higher in WTCVB3 mice compared to NOD2$^{-/-}$CVB3 mice. Immunohistochemical results revealed a distinctly higher collagen I / III ratio in WTCVB3 mice compared to the other three groups. The expression of collagen I and III in the four groups can be seen in Figure 15.

In parallel, mRNA expression of the pro-fibrotic factor TGF-β was significantly elevated in WTCVB3 compared to WT control mice and to NOD2$^{-/-}$CVB3 mice. Furthermore, mRNA expression of α-SMA, a marker of myofibroblast transdifferentiation, was significantly higher in

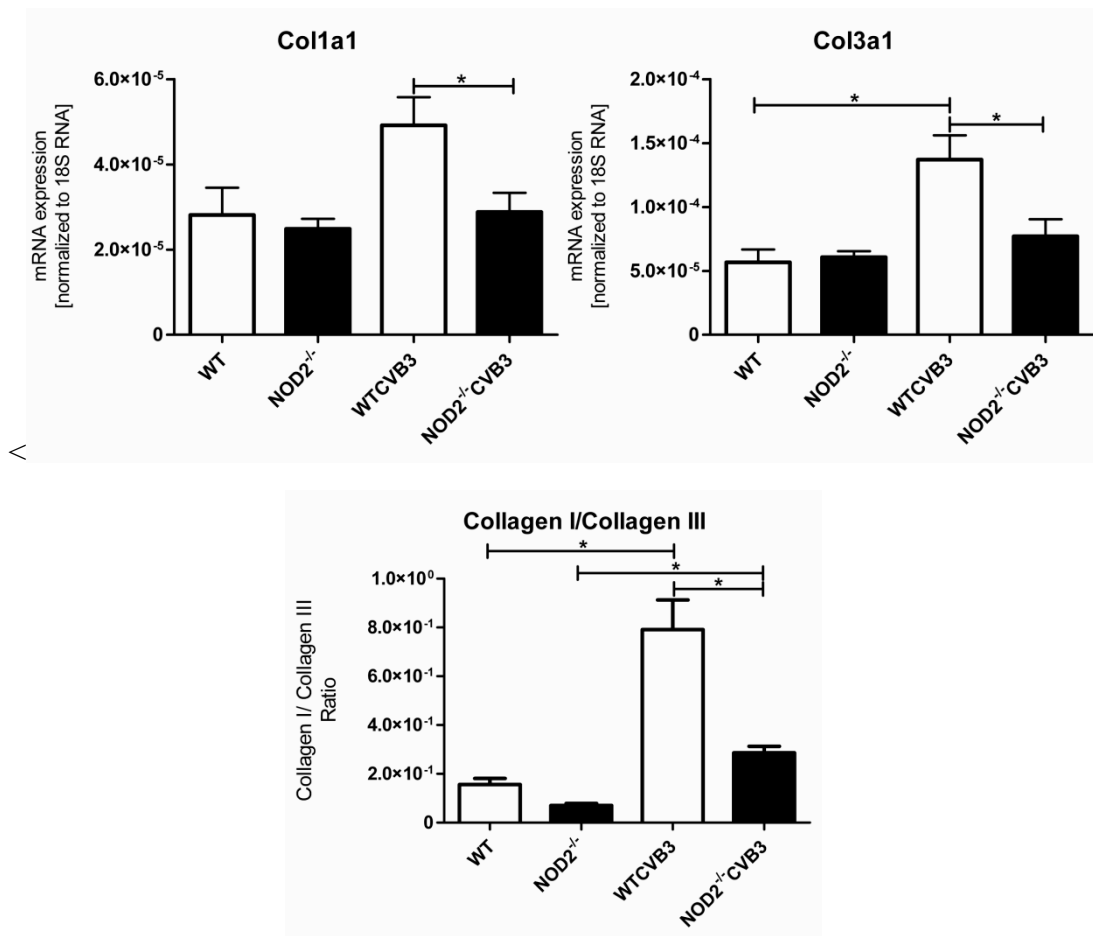WTCVB3 compared to to NOD2^{-/-}CVB3 mice (Figure 17).

Figure 15. The left ventricular mRNA expression of collagen I and III and the ratio of left ventricular collagen I / III content. The mRNA expression of collagen I and III was significantly lower in NOD^{-/-}CVB3 mice compared to WTCVB3 mice seven days after CVB3 infection (*= $p<0.05$). A significantly lower collagen I/III ratio was detected by immunohistological staining in NOD^{-/-}CVB3 mice compared to the WT CVB3 mice seven days after CVB3 infection (*= $p<0.05$). All data are reported as the mean value (MV) ± the standard error of the mean (SEM). WT = wild type; CVB3= coxsackie virus B3.
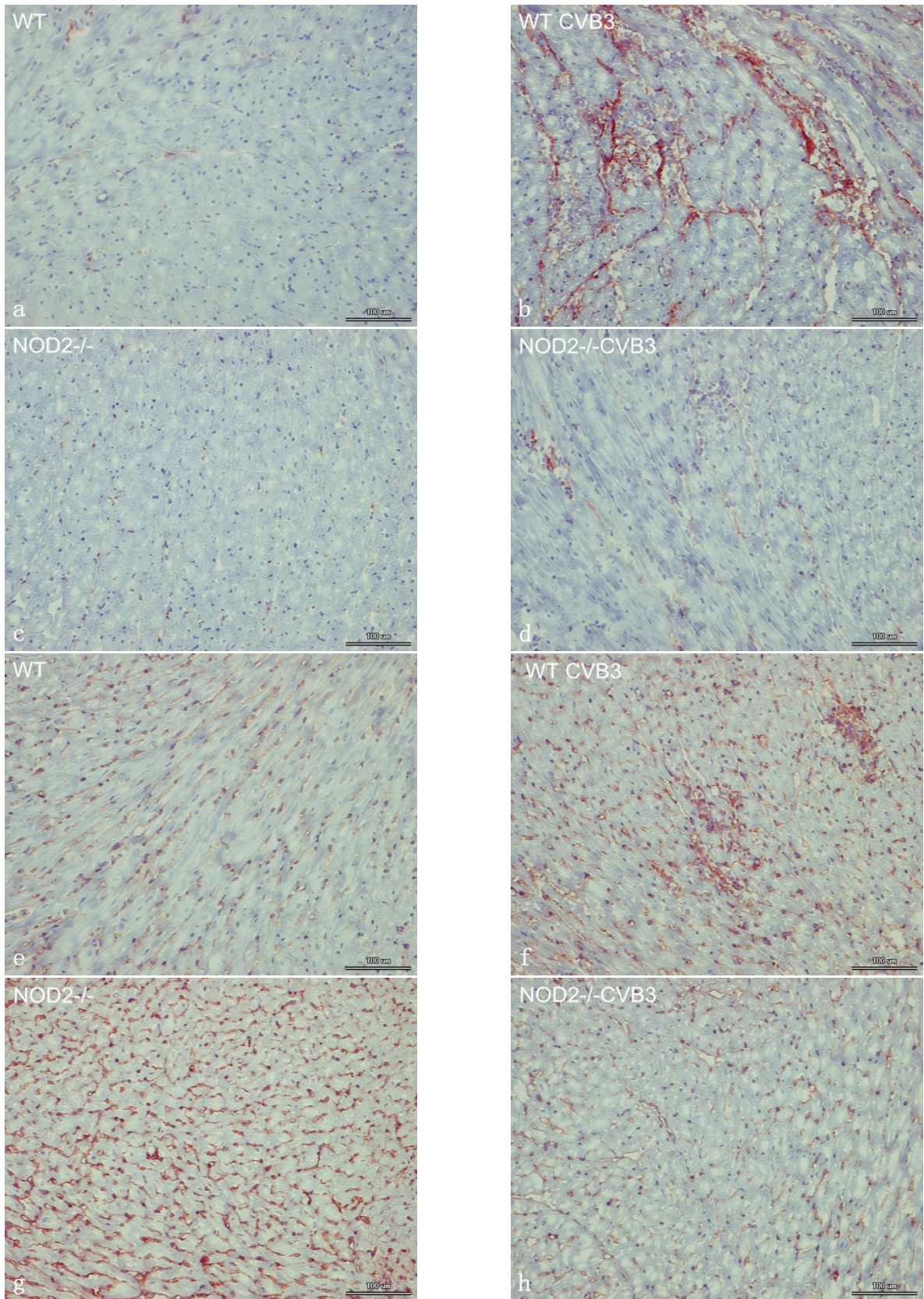
Figure 16. Representative immunohistochemical stainings of collagen I (a to d) and collagen III (e to h) in the left ventricle. 200×magnification. Collagen-specific epitopes are stained red.
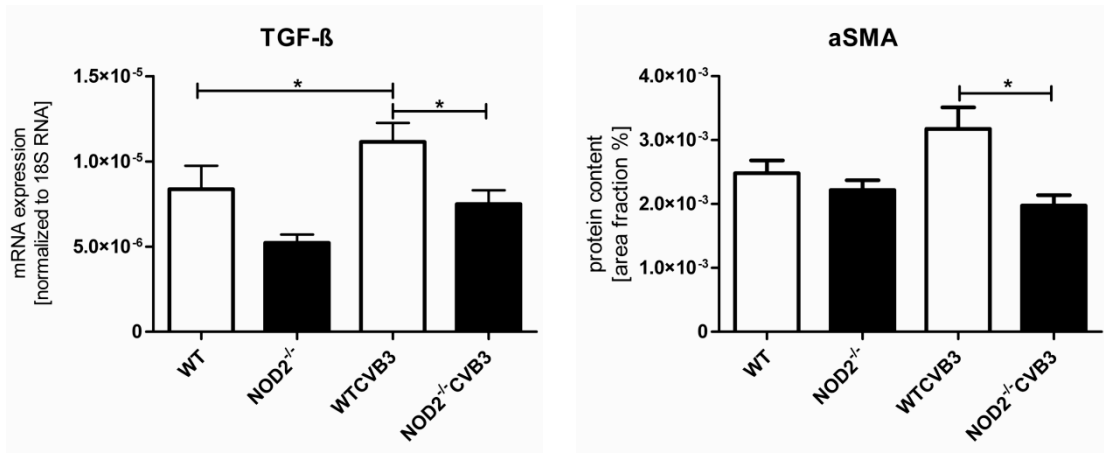
Figure 17. The mRNA expression of TGF-β and α-SMA in the left ventricle measured by real-time PCR was shown seven days after viral infection. Generalized exacerbation of TGF-β and α-SMA was detected in WTCVB3 mice seven days after CVB3 infection in comparison to NOD2$^{-/-}$CVB3mice (*= $p<0.05$). All data are reported as mean value (MV) ± standard error of the mean (SEM). WT = wild type; CVB3= coxsackie virus B3.
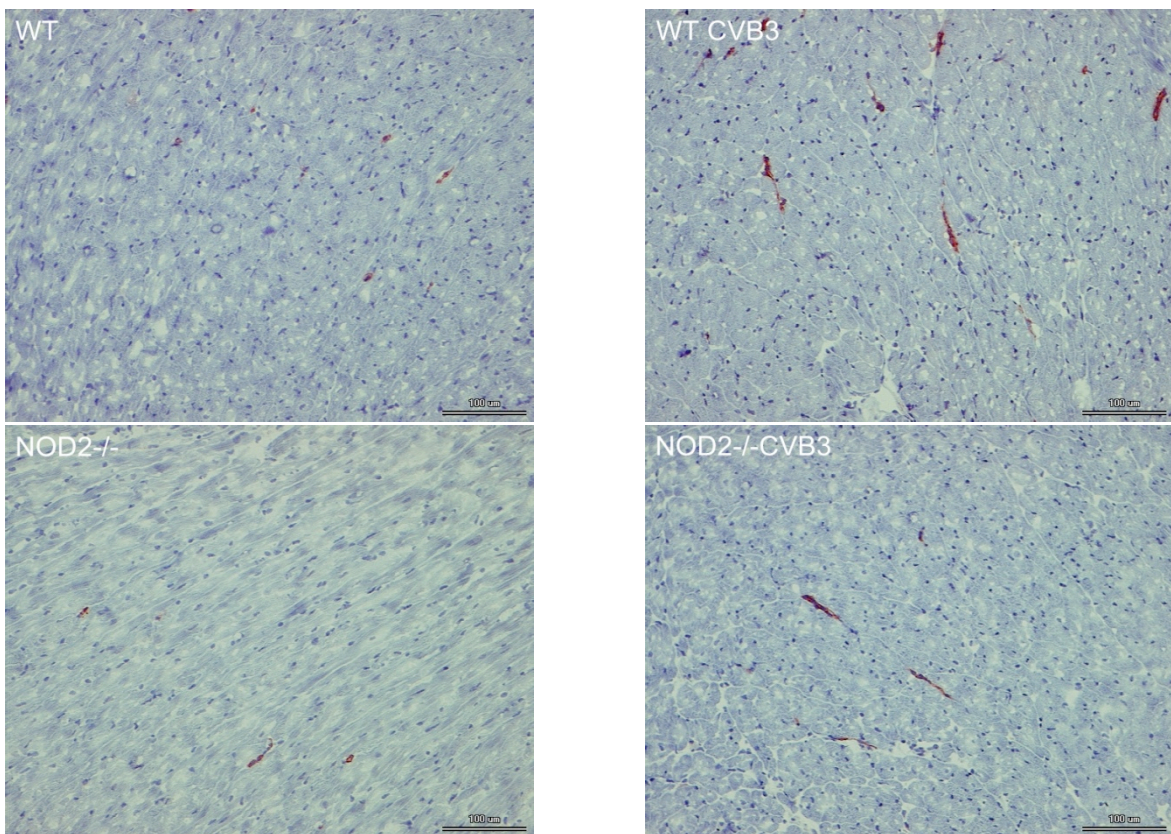

Figure 18. Representative immunohistochemical stainings of α-SMA in the left ventricle. 200×magnification. α-SMA-specific epitopes are stained red.

3.6 Anti-virus response

3.6.1 Virus load

In order to detect the effect of NOD2 on   viral replication, we investigated the number of viral

RNA copies, as a marker of viral load in the myocardium, using real-time PCR. The number of CVB3 RNA copies was significantly higher in WTCVB3 than in NOD2$^{-/-}$CVB3 (10.75-fold), which indicated a sign of lower viral replication in the myocardium of NOD2$^{-/-}$CVB3 animals (Figure 19).
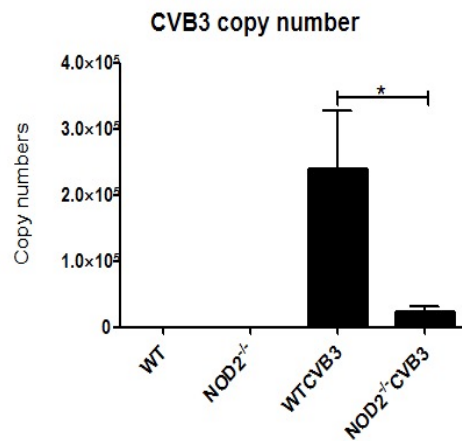
Figure 19. CVB3 copy number in the myocardium seven days after CVB3 infection. The number of virus RNA copies in WTCVB3 mice was significantly higher compared to NOD2$^{-/-}$CVB3 mice (*= $p<0.05$). All data are reported as the mean value (MV) ± the standard error of the mean (SEM). WT = wild type; CVB3= coxsackie virus B3.

3.6.2 Receptor

Coxsackie adenovirus receptor (CAR) is the main receptor for CVB3 viral entry**.** To investigate whether the lower viral copy number in NOD2-/-CVB3 compared to WTCVB3 was due to a reduction in viral uptake, CAR mRNA expression was evaluated via real-time PCR. No difference in CAR expression was detected between the four groups ($p>0.05$).

Figure 20. Cardiac CAR mRNA expression. CAR mRNA expression is not significantly different among the different groups. All data are reported as the mean value (MV) ± the standard error of the mean (SEM). WT = wild type; CVB3= coxsackie virus B3.

3.6.3 Anti-viral cytokines

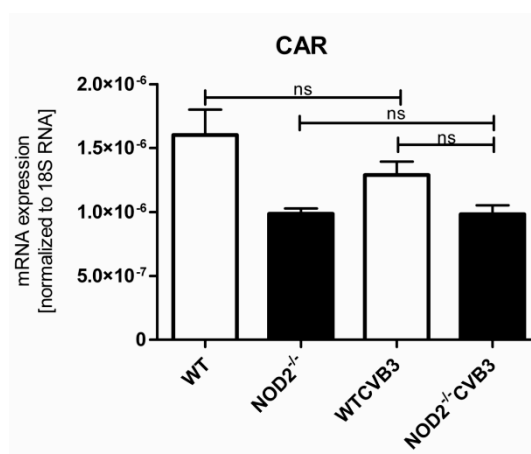IFNs are believed to contribute to the antiviral immune response by limiting viral replication. We measured the mRNA expression of IFN-β and –γ seven days after infection by real-time PCR. The expression of both IFNs was significantly higher in CVB3-infected mice compared to the respective control groups. In addition, IFN-β and – γ mRNA expression was significantly higher in WTCVB3 mice compared to NOD2$^{-/-}$CVB3 mice ($p < 0.05$).
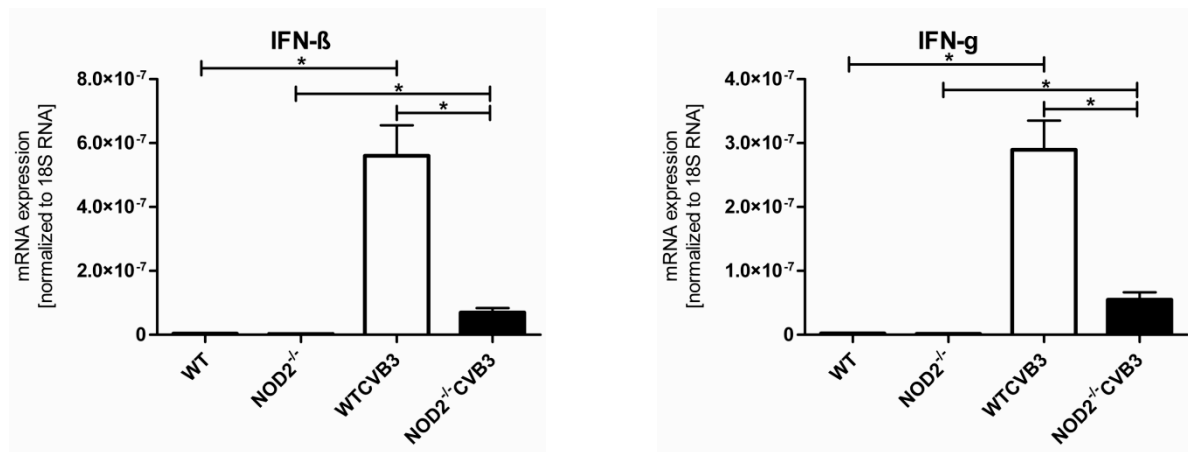
Figure 21. Left ventricular mRNA expression of IFN-β and IFN-γ measured by real-time PCR. Interferon expression is significantly higher in CVB3-infected mice compared with respective controls (*= $p < 0.05$). All data are reported as the mean value (MV) ± the standard error of the mean (SEM). WT = wild type; CVB3= coxsackie virus B3.

3.7 Apoptosis

In addition, we investigated whether the LV dysfunction seven days after CVB3 infection was associated with cardiac apoptosis. The amount of apoptotic cells in the heart muscle didn't show any difference between the NOD2$^{-/-}$ and NOD2$^{-/-}$ CVB3 mice. However, in the WTCVB3 group, a significantly increased level of apoptotic cells was found compared to WT control mice (10.9-fold, $p < 0.05$) and NOD2$^{-/-}$CVB3 mice (18.8-fold, $p < 0.05$) (Figure 22).

Figure 22. The content of apoptotic cardiac cells was verified by the TUNEL method. All data are reported as the mean value (MV) ± the standard error of the mean (SEM). (*= p<0.05) WT = wild type; CVB3= coxsackievirus B3.

Figure 23. Representative immunohistochemical stainings of TUNEL+ cells in the left ventricle. 200×magnification. TUNEL specific epitopes are stained red.

4. Discussion

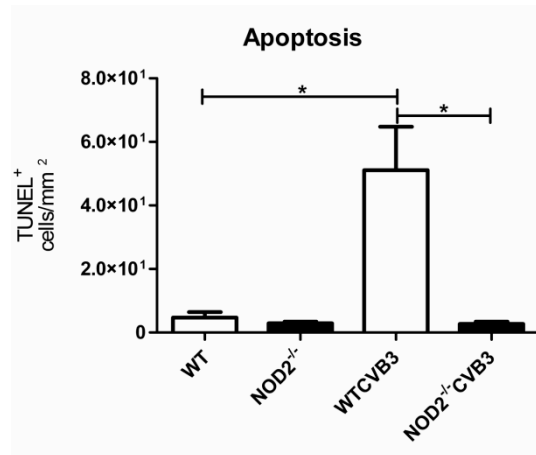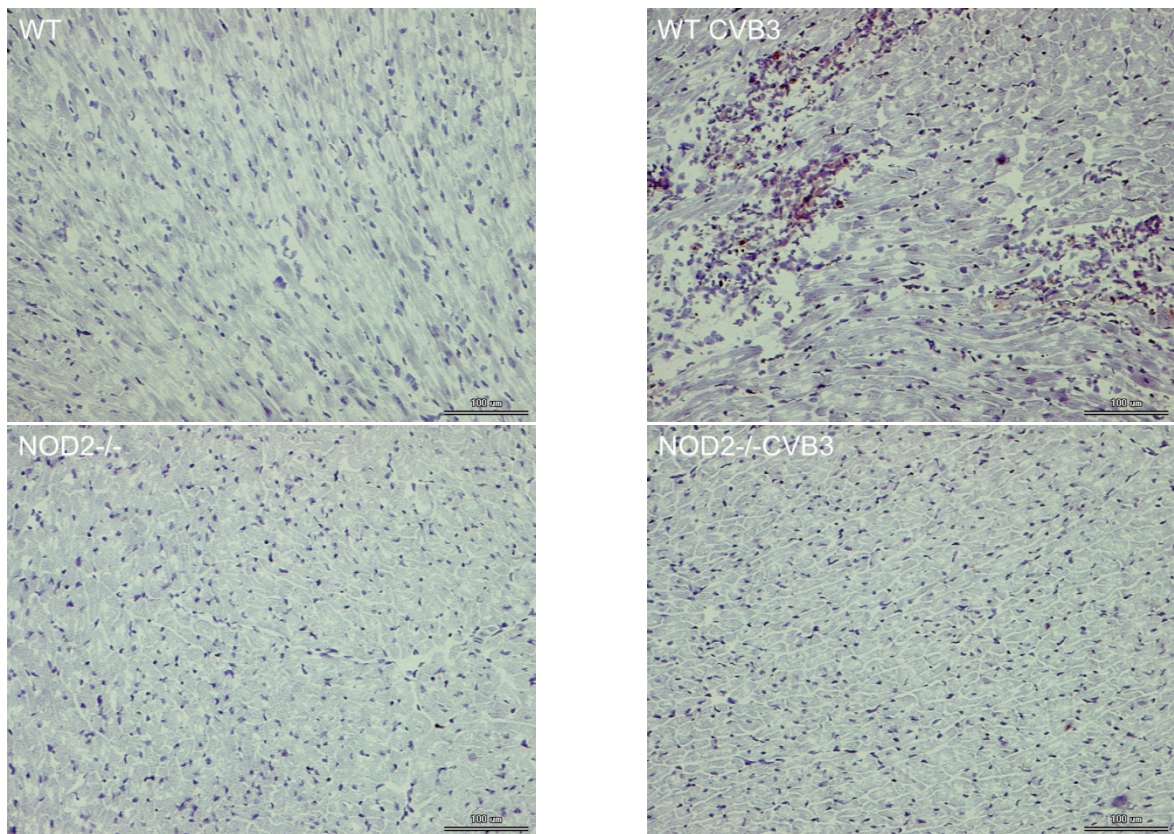NOD2 has been considered a new PRR that recognizes conserved microbial structures such as viruses containing an ssRNA genome. Previous results have demonstrated *in vitro* that NOD2 facilitate type I IFN production after infection with viruses such as RSV, VSV and influenza virus [123]. So far, the role of NOD2 in CVB3-induced myocarditis remains unclear. The present study, as far as we know, examines for the first time the effects of NOD2 depletion in an animal model of CVB3-induced myocarditis. We could show that: 1) left ventricular dysfunction was less pronounced in NOD2$^{-/-}$CVB3 mice compared to WT CVB3 mice; 2) deficiency of NOD2 led to a decreased cardiac inflammatory cell infiltration and myocardial fibrosis seven days after CVB3 infection; 3) lower cytokine and chemokine expression was detected in NOD2$^{-/-}$CVB3 compared to WT CVB3 mice ; 4) CAR expression was not different between NOD2$^{-/-}$ mice and wild type mice; 5) less cardiac apoptosis was detected in the myocardium of NOD2$^{-/-}$CVB3 mice compared to WT CVB3 mice.

4.1 Body and heart weights

The body weight is one of the main clinical criteria for the assessment of morbidity in acute myocarditis. According to our measurements, both NOD2$^{-/-}$CVB3 and WT CVB3 mice had a significantly reduced body weight in contrast to the respective healthy mice seven days after viral infection. Between the two infected groups, NOD2$^{-/-}$CVB3 mice showed a higher body weight compared to WT CVB3 mice, but there was no significant difference.

4.2 Hemodynamic function

In the present study, the global heart function was characterized by means of the heart rate (HR), LV volumes (LVESV, LVEDV), stroke volume (SV), ejection fraction (EF) and cardiac output (CO). The systolic function was described in detail by the maximum and end-systolic LV pressure (LVP$_{max}$, LVESP), and the maximal LV pressure rise rate (dP/dt$_{max}$). The diastolic function was evaluated by the maximum rate of pressure drop (dP/dt$_{min}$), the Tau value, the minimum LV pressure (LVP$_{min}$), and the end-diastolic volume (LVEDP).

4.2.1 Hemodynamic function under basal conditions

Under basal conditions, the two non-infected groups of WT and NOD2$^{-/-}$ mice showed no significant difference in either the systolic or diastolic function, indicating that deficiency of NOD2 in mice without virus infection does not influence cardiac function.

4.2.2 Hemodynamic function during acute myocarditis

4.2.2.1 Global cardiac function

The WT CVB3 group in our study developed reduced global cardiac function compared to the non-infected groups as shown by reduced EF value seven days after CVB3 infection compared to respective control group. However, NOD2$^{-/-}$ CVB3 mice showed a milder LV dysfunction, which benefited from higher EF value. There was no significant difference in the LV Volume (LVESV, LVEDV and SV) between WT mice and WT CVB3 mice. Similar results have been observed in previous studies of acute CVB3 myocarditis. During acute CVB3-induced myocarditis, the volumes remain approximately constant, since there is no cardiac dilation in infected wild type mice [126].

The heart rates of all groups shown in the hemodynamic measurements were not statistically different. Therefore, the possibility, that the variance of heart rate influences the measurement results, could be excluded.

4.2.2.2 Systolic cardiac function

The LVP$_{max}$ and dP/dt$_{max}$ are the earliest parameters which indicate the development of systolic dysfunction in myocarditis [127]. In the present study, we also evaluated these parameters to evaluate the systolic heart function seven days after CVB3 infection. A significantly lower LVP$_{max}$, dP/dt$_{max}$, and LVESP was detected in WT CVB3 mice compared to control mice, indicating the deterioration of systolic pump function in this group seven days after CVB3 infection. In contrast, NOD2$^{-/-}$ CVB3 mice showed no significant differences in any of these parameters compared to healthy mice. In addition, the LVP$_{max}$ of NOD2$^{-/-}$ CVB3 mice was significantly higher in contrast to WT CVB3 mice. Overall, the evaluated parameters impressively showed significantly impaired contractility and reduced pressure conditions in systolic cardiac function in WT CVB3 mice compared to NOD2$^{-/-}$ CVB3 mice.

4.2.2.3 Diastolic cardiac function

The emergence of diastolic dysfunction is described as an early indicator of cardiac dysfunction in viral myocarditis. dP/dt_{min} is the main parameter which indicates diastolic dysfunction. Seven days after virus infection the value of dP/dt_{min} was significantly reduced in the experimental group of WT CVB3 mice in contrast to NOD2$^{-/-}$CVB3 mice. A relevant lower value of dP/dt_{min} was found in the infected compared to the non-infected groups, but there were no statistical differences. Moreover, a significant lower LVEDP was detected in WT CVB3 mice compared to NOD2$^{-/-}$CVB3 mice. A possible explanation for this observation could be the more pronounced weight loss in the WT CVB3 compared to the NOD2$^{-/-}$CVB3 mice. The greater fluid reduction could in fact lead to lower intravascular volume and thereby reduced filling volumes and pressures in the left ventricle. Furthermore, the more severe fibrosis, which is associated with myocardial stiffness, in WT CVB3 compared to NOD2$^{-/-}$CVB3 mice can be the other reason. In summary, we believe that diastolic cardiac function is less impaired in NOD2$^{-/-}$CVB3 mice compared to WT CVB3 mice seven days after CVB3 infection.

The impaired cardiac function in CVB3-induced myocarditis, can be explained by several mechanisms.

First of all, the direct effect of the virus on cardiomyocytes is considered a crucial reason. After virus entry, the synthesis mechanism of the cell is switched off. Some cytoskeletal cellular proteins, such as dystrophin, will be degraded by the viral proteases [128]. Furthermore, the energy of the cells, which is necessary for the relaxation of the heart during the diastolic process, is consumed by the processes of viral replication [129]. Furthermore, cardiomyocytes are lysed by the virus released from the infected cells and adjacent cells. So, there is a reduction of cardiomyocytes and the function of the remaining cells is also impaired by infection. In our study, the higher viral load in WT CVB3 mice could explain the poorer heart function.

Second，during inflammation, the circulating inflammatory mediators (cytokines, chemokines, antibodies) will increase. This can also lead to a disturbance of systolic and diastolic function by several mechanisms including stimulation of hypertrophy and fibrosis through direct effects on cardiomyocytes and fibroblasts, and reduced myocardial contractile function through direct effects on intracellular calcium transport. Particularly, TNF-α, IL-1β and MCP-1 are involved in the pathways of heart failure [130, 131]. We could observe in our study that the left ventricular

expression of TNF-α, IL-1β and MCP-1 was significantly higher in the infected mice, especially in the WT CVB3 animals, compared to non-infected CVB3.

Furthermore, the stiffness of the heart, which is directly dependent on the collagen content and quality in the myocardium, plays an important role in diastolic dysfunction [132]. Significant myocardial fibrosis was found in the infected mice compared to the healthy animals, accompanied with a raised content of collagen expression. Recent studies demonstrate an important role for T cells in increasing the activity of the enzyme lysyl oxidase, which can induce increased collagen cross-linking and myocardial stiffness [133]. In our study, a significantly increased presence of T lymphocytes could be detected in infected mice, particularly in WT CVB3 mice, compared to healthy mice. This might also explain in part the more severe impaired diastolic cardiac function and fibrosis observed in WT CVB3 mice.

Overall, in the present study, CVB3-infected mice showed a significant cardiac dysfunction compared to healthy mice. The less pronounced cardiac dysfunction in NOD2$^{-/-}$CVB3 mice compared to WT CVB3 mice can be explained by the relatively low T lymphocyte infiltration and cardiac expression of cytokines, the lower fibrosis, apoptosis, and virus load in the myocardium.

4.3 Inflammation

4.3.1 Cell infiltration

The expression of NOD2 mRNA and protein was found in CD4+ T and CD8+ T cells at substantial levels [134]. This phenomenon suggests that those immune cells play a critical role in NOD 2 signal transduction. Furthermore, the influx of CD11b+ monocytes is reduced in the colon of NOD2$^{-/-}$ mice after *C. rodentium* infection, which indicates that NOD2 is involved in the regulation of the influx of circulating monocytes [135]. However, the determination of immune cells involved in the NOD2-mediated immune response to CVB3-induced myocarditis remains unclear. In this respect, our investigation was focused on the identification of possible immune cell populations in the heart that are responsible for mediating the excessive inflammatory response in the NOD2-mediated pathway after CVB3 infection.

In our experiments, all infected animals showed a stronger immune response with an increased influx of inflammatory cells into the heart muscle. This reaction in WT CVB3 mice was more

pronounced compared to the NOD2$^{-/-}$CVB3. Immunohistochemistry showed significantly higher presence of macrophages (CD68+), monocytes (CD11b+) and T cells (CD3+ and CD4+) in the WT CVB3 compared to NOD2$^{-/-}$CVB3 mice.

In several other studies, it has been shown that in CVB3-induced myocarditis the excessive migration of inflammatory cells induces an increased damage to the heart tissue. Opavsky *et al.* showed the potentially negative effect of CD4+ and CD8+ cells in the CVB3 myocarditis [136]. Mice lacking both CD4 (CD4$^{-/-}$) and CD8 (CD8$^{-/-}$) were protected from CVB3-induced myocarditis. They were associated with elevated IFN-γ and decreased TNF-α expression compared to CVB3-infected WT mice. Henke *et al.* showed less pronounced inflammation and diminished mortality in CD4$^{-/-}$ mice in which CD8+ T lymphocytes were depleted by CD8+-directed antibodies [137]. These findings indicate that the enhanced immune cell migration into the myocardium is one of the crucial mediators of CVB3-induced cardiomyopathy. Furthermore, expression patterns of the specific cytokines TNF-α, IL-1β, or IFN-γ, are regarded as one of the mechanisms by which CD4+ and CD8+ T-cells influence the pathogenic progress of myocarditis.

4.3.2 Cytokines

It has been proven in several human and animal studies that both viral infection and the development of heart failure are closely associated with a broad cytokine activation. The release of proinflammatory cytokines (such as TNF-α, IL-1β) by monocytes has been detected with the stimulation with NOD2-activating agonists [101], which indicates that activation of NOD2 is associated with release of these cytokines. TNF-α andIL-1β are both cytokines with exert harmful effects in CVB3-inducedmyocarditis. This follows from experiments with CVB3-infected mice, wherein administration of IL-1β and TNF-α resulted in an increased myocardial inflammation and necrosis, as well as poor prognosis [68, 138]. Furthermore, activation of TNF-α and IL-1β can amplify the development of LV dysfunction and cardiac remodeling [139]. In summary, it can be shown that the increased production of theses cytokines plays a critical role in the pathogenesis of CVB3-induced myocarditis and leads to an exacerbation of viral infection.

In our study, we were able to confirm these observations: the mRNA levels of TNF-α and IL-1β

were significantly increased in WT CVB3 mice compared to NOD2$^{-/-}$CVB3 mice in the myocardium seven days after CVB3 infection. These findings were consistent with the more severe cardiac dysfunction and myocardial fibrosis in WT CVB3 mice compared to NOD2$^{-/-}$CVB3 mice. We suggest that the slighter activation of pro-inflammatory cytokines in cardiac tissue NOD2$^{-/-}$CVB3 mice seven days after CVB3 infection might be due to a less pronounced induction of the NF-κB signaling pathway or to the lower induction of NF-κB independent pathways triggered by viral infection or cardiac injury.

Interestingly, an increased expression of IL-10, which is generally considered an anti-inflammatory cytokine, was detected in WT CVB3 mice compared to NOD2$^{-/-}$CVB3 mice. IL-10 mRNA levels of both infected groups were significantly higher than in the respective healthy groups. IL-10 has been shown to exert a protective role in experimental autoimmune myocarditis [140]. However, its exact role in myocarditis is not clear. In a human study, high serum IL-10 levels were accompanied with fulminant myocarditis [141]. Alternatively, it is possible that the induction of an IL-10 immunosuppressive effect could decrease the host defense against viral infection. This could partly explain the higher virus load in WT CVB3 mice compared to NOD2$^{-/-}$CVB3 mice, in which the CVB3-induced cardiac *IL-10* mRNA expression is less pronounced compared to that in WT CVB3 mice.

4.3.3 Chemokines

In the acute phase of myocarditis, local levels of chemokines are up-regulated in the myocardium [49]. The importance of chemokines in the induction of cardiac damage is demonstrated from homozygous MIP-1α mutant mice which are found to be resistant to CVB3-induced myocarditis in contrast to wild-type mice [72]. These findings suggest a crucial role of chemokines in virus myocarditis. These chemokines may control the chemotactic movement of vicinal immune cells and induce the immune response. NOD2$^{-/-}$ mice express lower MCP-1 (CCL2), a monocyte chemoattractant, in the serum and colon after *C. rodentium* infection compared to WT mice, which is associated with reduced inflammation during the early phase of the infection [135]. This indicates the possibility that NOD2 positively regulates the production of MCP-1 after infection. The expression of MCP-1 is associated with increased monocytes in the infected area, which may contribute to the increased inflammatory activity in the myocardium [75] and result in

exacerbation of DCM and development of congestive heart failure [76, 142]. MCP-1 may also directly induce dysfunction of the cardiac muscle by enhancing effects on spontaneous generation of reactive oxygen species in monocytes, which is involved in the increased apoptosis of cardiomyocytes [142].

Furthermore, it has been shown that MCP-1 is released by T lymphocytes and monocytes in peripheral blood. In our study, mRNA levels and immunohistochemical results of MCP-1 were significantly higher in infected groups accompanied with significantly increased cardiac presence of CD3+ T lymphocytes and CD11b+ monocytes. Specially, these results are higher in WT CVB3 mice compared to NOD2$^{-/-}$CVB3 mice, which is consistent with the more severe apoptosis and impaired hemodynamic function in WT CVB3 mice compared to NOD2$^{-/-}$CVB3 mice. Thus, the results of our study suggest that MCP-1 is also released by T lymphocytes and monocytes in the myocardium under the positive regulation of NOD2 and it might contribute to cardiac dysfunction through direct myocardial damage and immune response.

4.4 Fibrosis

Heart injury of myocarditis can end up in a final pathway of pathologic remodeling and fibrosis, promoting heart failure development. Our investigations were made seven days after infection, which was too early to observe any evidence of remodeling, since there were no changes in the SV and LV volume (LVEDV and LVESV) after infection. However, we detected the onset of fibrosis.

Myocardial fibrosis in the context of inflammation is a part of the normal healing process after virus infection. Enhanced fibrosis is always characterized by a disproportionate accumulation of collagen, with type I collagen being the main form, contributing to stiffness of ventricles and diastolic dysfunction [143]. In our study, significantly higher mRNA levels of collagen I and III were found in WT CVB3 mice compared to NOD2$^{-/-}$CVB3 mice. There was no significant difference between NOD2$^{-/-}$CVB3 and non-infected mice. Immunohistochemical results indicated a significantly higher collagen I/III ratio in infected groups, particularly in the WT CVB3 group, in comparison to the respective healthy groups. We also found a higher protein expression of α-SMA, an actin isoform expressed in myofibroblasts that contributes to fibrosis in fibrocontractive lesions, in WT CVB3 mice compared to NOD2$^{-/-}$CVB3 ones.

The progression of fibrosis in myocarditis is critically controlled by a number of cytokines. Among them, TGF-β, a profibrotic cytokine, was found to induce conversion of fibroblasts or progenitor cells into α-SMA-expressing myofibroblasts [144, 145] and to induce endothelial-to-mesenchymal transition which is associated with cardiac fibrosis [146]. Our study discovered a significantly increased expression of TGF-β in WT CVB3 mice compared to WT and NOD2$^{-/-}$CVB3 mice. These findings are in line with the higher expression of α-SMA and higher mRNA levels of collagen I and III in WT CVB3 mice.

TGF-β, which regulates the progression of fibrosis in myocarditis, is believed to be produced by inflammatory infiltrating cells, particularly monocytes and T cells. So the significantly increased accumulation of monocytes and T cells in WT CVB3 mice in our study can be regarded as the source of higher expression of TGF-β in WT CVB3 mice.

All these results indicate an exacerbation of fibrosis in WT CVB3 mice compared to NOD2$^{-/-}$CVB3 mice and healthy mice seven days after CVB3 infection. This suggests that NOD2 contributes to fibrosis in CVB3-induced myocarditis by inducing the infiltration of inflammatory cells and subsequent production of TGF-β.

4.5 Antiviral response

4.5.1 Viral load and CAR

A relatively surprising observation in our study was the significantly higher viral load in WT CVB3 animals compared to the NOD2$^{-/-}$CVB3 animals. It would be expected that the virus could be eliminated effectively by the strong immune response. The expression of CAR showed no significant difference between any groups, indicating that the lower viral load found in NOD2$^{-/-}$CVB3 mice compared to WT CVB3 mice cannot be explained due to a lower viral uptake.

The Coxsackievirus has the property of infecting not only cardiomyocytes but also cells of the immune system, which even constitutes one of the main ways via which the virus reaches its target organs [40, 147]. A possible explanation for the observed viral load could be a strong infiltration of infected immune cells in the myocardium. Previous studies indicated that viral RNA was also detected in CD4+ T cells and Mac-1+ macrophages [148]. Consistently, increased transmigration of CD4+ T cells and Mac-1+ macrophages (CD11+ cells) in the myocardium of

WT CVB3 mice compared to NOD2$^{-/-}$CVB3 was observed in our study. These findings would support the hypothesis that immune cells in the myocardium could contribute to viral persistence in WT CVB3 mice and serve as a major non-cardiac virus reservoir [147].

DeBiasi *et al*. demonstrated that the reovirus titer in the heart was significantly decreased in caspase 3 knockout mice accompanied with the decrease in apoptotic response 48h after infection [149]. Moreover, gp130 receptor signaling, a well-known anti-apoptosis pathway in cardiomyocytes, also has a protective role against CVB3 infection of the heart [63]. This suggests that apoptosis in the myocardium may not be beneficial in inhibiting virus replication and spread in the acute phase of infection. In our study, a significantly increased apoptotic response was found in WT CVB3 mice compared to NOD2$^{-/-}$CVB3 mice (mentioned below). This could be another reason for the higher virus load in the cardiomyocytes of WT CVB3 mice. The probable hypothesis is that activation of apoptosis may increase virus propagation by disrupting the sarcolemmal membrane, which is regarded as the barrier to inhibit virus propagation to adjacent cardiomyocytes.

4.5.2 Antivirus cytokines

Type I IFN, particularly IFN-β, is found to play an important role during innate antiviral response by activating the JAK-STAT system [65-67]. It can reduce the number of myocardial lesions in CVB3-infected mice [150]. Sabbah *et al*. indicated the role of NOD2 to mediate IFN-β production in cells stimulated *in vitro* with ssRNA. In the same study, NOD2$^{-/-}$mice were more susceptible to respiratory syncytial virus (RSV) *in vivo* accompanied with lower IFN-β production. This highlights the possibility that NOD2 participates in the induction of the type I IFN pathway to induce the antiviral response. Furthermore, a very recent study demonstrated that IFN-β also upregulates NOD2 transcription 24h after RSV infection [151]. These results suggest that NOD2 would not only mediate IFN-β production after infection by ssRNA virus, but it could also be positively regulated by IFN-β. In our present study, a significant increased expression of IFN-β was detected in infected mice compared to the healthy mice, indicating that IFN-β was involved in the immune response against CVB3 infection. In agreement with the role of NOD2 in IFN-β production, IFN-β mRNA expression seven days after CVB3 infection was less pronounced in NOD2$^{-/-}$CVB3 compared to that of WT CVB3 mice. .

Unlike IFN-β, the function of IFN-γ, as the only known type II interferon, remains controversial in view of the conflicting data as to whether IFN-γ is pathological or protective in the heart. Most of the conflicting studies focus on its function in cardiac remodeling. IFN-γ is believed to play an antiviral role in the situation where a virus is involved, such as its role and effectiveness against encephalomyocarditis virus-induced myocarditis and its protective role against CVB3-induced myocarditis in mice [152, 153]. In our study, all infected groups showed a significantly increased mRNA level of IFN-γ compared to non-infected groups. As for IFN-β, IFN-γ mRNA expression was significantly higher in WT CVB3 mice than $NOD2^{-/-}$CVB3 mice. Our results suggest that NOD2 may induce IFN-γ as well as IFN-β in the myocardium seven days after CVB3 infection.

4.6 Cardiac apoptosis

Coxsackieviruses are found to induce activation of caspase enzymes [154], which are the key effector molecules in apoptosis. The induction of myocardial apoptosis is present in the early stage of CVB3-induced myocarditis [54]. At this time, the virus from the infected apoptotic cell is released, and there is a re-infection of adjacent cells in the myocardium. However, the content of apoptotic cells varies between the different mouse strains [54]. Furthermore, the induction of apoptosis appears to be mediated directly by viral mechanisms and closely associated with the extent of the virus replication in cardiomyocytes, since the virus proteins are found in TUNEL-positive cells.

In our study, the rate of apoptosis in WT CVB3 animals was significantly higher than in the $NOD2^{-/-}$CVB3 animals by TUNEL staining of apoptotic cells. This can be explained firstly by the considerable persistence of the virus and the increased inflammation in the heart. In addition the direct effect of virus, the cytotoxic productions of leukocyte action, such as perforin and oxidative stress, are found to induce apoptosis as well [155]. Furthermore, apoptotic cells and pro-apoptotic proteins lead to cytokine production, which triggers an excessive inflammatory response. It can be speculated that a vicious circle exists in the WT CVB3 animals where the inflammatory reaction is most pronounced. The viral replication leads to increased apoptosis in cardiomyocytes as well as to an increase in immigrant infiltration of immune cells. As a result, this is a secondary release of more viruses that could infect other adjacent cells in the heart.

Consequently, the expression of cytokines and chemokines increases, which in turn increase the migration of immune cells. Many infected immune cells then lead to an increase in the total viral load in the myocardium and thus to further infection and apoptosis of cardiac cells.

Moreover, inhibition of NF-κB, the main factor of the NOD2-mediated pathway, is found to inhibit virus-induced apoptosis following experiments with stable overexpression of an IκB suppressor or with an proteasome inhibitor which blocks IκB degradation [156]. This suggests the possibility that NOD2 exerts a pro-apoptotic influence through the NF-κB pathway after virus infection. It also can explain the significant increased apoptosis in WT CVB3 mice compared to the NOD2$^{-/-}$CVB3 ones.

Since apoptosis is also required for optimal viral replication and spread, inhibition of apoptosis results in a reduction of virus titer [149]. Therefore, the lower viral load in NOD2$^{-/-}$CVB3 mice compared to WT CVB3 ones might be explained by the decreased cardiac apoptosis, despite the lower expression of the antiviral cytokine IFN-β in NOD2$^{-/-}$CVB3 group compared to the WT CVB3 group.

4.7 Study limitations and perspectives

To investigate the effect of the NOD2 on experimental CVB3-induced myocarditis, we used "knock-out" mice in which the gene of the NOD2 was deleted. Mice are used as experimental models for many diseases due to their low cost and size, which makes their attitude and treatment easier, and especially due to the ability to create transgenic or knockout mice, which makes it possible to reproduce many human diseases, and to study the role of specific factors in this disease.

Although the heart and the circulatory physiology of the mouse shows many similarities to that of humans, there are also differences: for instance the heart rate in mice is approximately 400-600 beats per minute [157]. Nevertheless, important conclusions concerning human physiology and pathology can be drawn. One limitation of knockout mice is that the elimination of a gene can have direct and indirect effects on the phenotype of the animal under basal conditions. In our hemodynamic evaluation of cardiac function by conductance catheter, we did not detect any differences between NOD2$^{-/-}$and WT control mice, indicating that NOD2$^{-/-}$mice can be a good model for studying cardiovascular diseases. Species such as dogs and pigs are

indeed closer related to the human physiology, but they are difficult to manipulate genetically, because transgenically altered offspring are rarely available and they are not sufficiently suitable for our experiments.

The hemodynamic study of the cardiac function by conductance catheter has proven to be an accurate method comparable with other methods, such as echocardiography [158]. The specific advantages of the conductance catheter are the simultaneous recording of the pressure and volume in the heart, and thus of pressure-volume curves in real time. With their help, preload- and afterload-independent data can be reliably obtained. However, the animals must be anesthetized for the application of the method. Most anesthetics, including thiopental, show a cardiodepressive effect, which is dependent on the mouse strain and sex [159].

Therefore, we particularly paid attention to achieve an optimal balance between inadequate anesthesia and less pronounced cardiomyopathy in our study. Protocols for the application of conductance catheter in conscious mice have been described, but they are not yet sufficiently established and show several limitations [160]. Another limitation is the injection of 10% saline into the jugular vein to determine the parallel conductance. The method assumes that the injection has no effect on the hemodynamic function, but both the volume and the hypertonicity of the salt can affect heart function [127].

Additionally, various restrictions could be applied to the molecular biological methods. Our immunohistochemical staining was performed with frozen sections, which were previously frozen at -80∘C. Therefore, the possibility of a change in antigen cannot be entirely excluded. The TUNEL method is relatively specific for the determination of the rate of apoptosis, but in some cases, necrotic cells whose DNA show the same changes as the apoptotic cells may be involved incorrectly [161].

In experiments, various proteins of different gene expression were investigated using real-time PCR. The specific mRNA levels can give insights into protein levels in cardiac tissue. The exact concentration of protein levels, however, can vary physiologically by their modification or their degradation, so that the actual amounts of protein are not always in agreement with the measured mRNA levels. Nevertheless, the investigation of gene expressions provides important information on the regulatory mechanisms in cells and tissues.

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Curriculum Vitae

Mein Lebenslauf wird aus datenschutzrechtlichen Gründen in der elektronischen

Version meiner Arbeit nicht veröffentlicht.

STATEMENT IN LIEU OF OATH

I declare that the experiments described in the thesis were carried out by me.

Eidesstattliche Versicherung

„Ich, [Yu Xia], versichere an Eides statt durch meine eigenhändige Unterschrift, dass ich die vorgelegte Dissertation mit dem Thema: [The role of nucleotide-binding oligomerization domain containing protein 2 (Nod 2) in coxsackievirus B3 induced myocarditis in mice model] selbstständig und ohne nicht offengelegte Hilfe Dritter verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel genutzt habe.

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Datum 17.07.2013   Unterschrift   Yu Xia

Anteilserklärung an etwaigen erfolgten Publikationen

No publication.

Acknowledgements