INNATE IMMUNE ACTIVATION IN EXPERIMENTAL AUTOIMMUNE MYOCARDITIS

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Summary

Dilated cardiomyopathy (DCM) is a leading cause of heart failure and frequently results from postinfectious autoimmunity. Its pathophysiology is modeled by experimental autoimmune myocarditis (EAM), a $CD4^+$ T-cell mediated murine model of postinfectious heart disease. My thesis focuses on the role of innate mechanisms in inflammatory heart disease using the EAM model. In this context we specifically addressed the role of the Toll-like Receptor (TLR) signalling adaptor molecule MyD88 and the Interferon-alpha-beta Receptor (IFN$\alpha\beta$R).

First, we addressed the role of MyD88 in EAM induction. In contrast to wild-type (wt) control littermates, MyD88 deficient mice were protected from EAM after immunization with alpha-myosin heavy chain derived peptide (MyHC-alpha) and complete Freund`s adjuvant (CFA). Disease resistance of MyD88 deficient mice resulted from impaired expansion of heart-specific $CD4^+$ T-cells after immunization. We further showed that MyD88 deficient primary antigen presenting dendritic cells were defective in their capacity to prime $CD4^+$ T-cells. This defect mainly resulted from the inability of MyD88 deficient DCs to release TNF-alpha. However, repetitive injection of activated, MyHC-□lpha loaded wt bone marrow DCs (bmDCs) fully restored T-cell expansion and myocarditis in MyD88 deficient mice. We therefore conclude that autoimmune myocarditis induction depends on MyD88 signalling of self-antigen presenting cells in the peripheral compartments.

Second, we analyzed the role of MyD88 in the progression of heart failure. After wt bmDC immunization, MyD88 deficient and wt mice developed autoimmune myocarditis of the same severity and prevalence. Based on this phenotype we analyzed the role of MyD88 in the progression of autoimmune myocarditis to heart failure. We showed by echocardiographic heart function analysis, that wt mice but not MyD88 deficient mice have reduced heart function after initial bmDC induced autoimmune myocarditis. We further describe increased fibrosis in wild-type mice when compared to MyD88 deficient mice when immunized with a novel protocol combining bmDC and CFA immunization. We therefore hypothesize that

MyD88 mediated IL-1 receptor signalling or the production of MyD88 dependent proinflammatory cytokines contribute to the development of post myocardial inflammation induced heart failure. In addition, we provide new data to the ongoing debate about a heart specific innate stress program that contributes to the development of heart failure.

Third, we analyzed the role of type I interferon receptor signalling in EAM induction. MyD88 independent TLR signalling is mainly characterized by the induction of a type I interferon (IFN) response. We therefore decided to specifically address this alternative pathway of TLR signalling by analyzing EAM in IFN$\alpha\beta$R deficient mice. We found that IFN$\alpha\beta$R deficient mice are protected from bmDC induced autoimmune myocarditis. We provide evidence that the protection coincides with reduced $CD4^+$ T-cell priming. Further results from MyHC-specific wt and IFN$\alpha\beta$R T-cell transfer experiments indicate an additional role for type I IFN signalling in the recruitment of proinflammatory cells to the heart.

These studies characterize different pathways of TLR signalling and effector molecules in the pathogenesis of EAM. Our data clearly demonstrate that MyD88 and the IFN$\alpha\beta$R are essential modulators of the autoimmune process during myocarditis induction and heart failure development.

Aim of the thesis

The aim of this thesis was to assess the role of TLR signalling in Experimental Autoimmune Myocarditis.

In preliminary experiments we found that single TLR deficiencies for TLR2, TLR4 and TLR9 are redundant in the development of EAM. However, it was already shown that activation of IL-1 type 1 Receptor (IL-1R) is required for disease induction (1). TLRs and The IL-1R belong to the same receptor super-family and share intracellular signalling pathways including the Toll-IL-1 Receptor adaptor molecule MyD88 (2). Furthermore, MyD88 has been shown to be essential for the production of proinflammatory cytokines upon TLR stimulation (3). Interestingly, several proinflammatory cytokines, including IL-1, IL-6 and IL-12 have been described to be essential for EAM induction (2, 4, 5). We therefore decided to further address the role of MyD88 in autoimmune myocarditis. This was accomplished by examining the susceptibility of MyD88 deficient mice to EAM. In determining the function of MyD88 in this disease model, I aimed to open insights into the mechanisms involved in EAM induction and the development of autoimmunity in general.

Proinflammatory cytokines have also been associated with the pathogenesis of dilated cardiomyopathy and heart failure in general (6-11). However, the mechanisms and signals underlying the induction of proinflammatory cytokine production in the heart or increased systemic proinflammatory cytokine levels in patients with heart failure remain unknown. During the last years it was suggested that the heart possesses an innate stress program that is activated upon TLR stimulation in the heart (12). Interestingly, TLRs are upregulated in the heart in patients with idiopathic dilated cardiomyopathy (13). Hence, we decided to further address the role of MyD88 in the progression from autoimmune myocarditis to heart failure.

TLR signalling is generally divided into two signalling pathways, MyD88 dependent and MyD88 independent signalling (14). MyD88 independent signalling is mainly promoted by the TLR adaptor proteins TRIF and TRAM,

which induce amongst others the secretion of type I IFN (14). Members of the type I IFN family are major immune regulators and are already in use as therapeutic agents in autoimmune diseases, for example in Multiple Sclerosis or against chronic viral infection (15-17). On the other hand, type I IFN levels have been correlated with clinical manifestations of Systemic Lupus Erythematosus (SLE) (18) and Sjogren's syndrome (19). These studies highlight the complex role for type I IFN in the induction of autoimmune diseases with either protective or deteriorative function. Hence, the third part of my thesis was to determine the function of type I IFN in the induction of EAM. We therefore took advantage of IFN$\alpha\beta$R deficient mice, which are unable to signal both IFN-alpha and IFN-beta, to address the role of type I IFN signalling in EAM induction. We aim to better characterize the conditions necessary for the induction of autoimmunity. Understanding of these mechanisms is a prerequisite for the prevention of autoimmune diseases and the development of novel causal treatment strategies.

GENERAL INTRODUCTION

Myocarditis

Human disease

Myocarditis is clinically defined as inflammation of the heart muscle (20). Epidemiological studies suggest that myocarditis is a major cause of sudden death in adults less than 40 years of age (21). Inflammatory lesions are often focal in nature, which complicates diagnosis of myocarditis. To resolve the problem of differences in methods of diagnostic evaluation, the “Dallas Criteria” for the histological diagnosis of myocarditis were introduced (22).

In Europe and North America, myocarditis most often results from infections with enteroviruses such as coxsackievirus B3 or adenoviruses (20, 23, 24). But also cardiotropic bacteria such as *Borrelia* and *Chlamydia* can induce myocarditis and heart failure (25). A growing body of evidence suggests that myocarditis often results in dilated cardiomyopathy (DCM), which is the most common cause of heart failure in young patients (20, 26, 27).

Notably, many of the affected patients with DCM develop heart-specific autoantibody responses suggesting a role for autoimmunity in disease pathogenesis (28, 29). Evidence for autoimmunity in postviral cardiomyopathy also results from the observations of abnormal expression of HLA class II on endothelial cells and from the weak but significant association of dilated cardiomyopathy with HLA-DR4 (30). Further, immunosuppressive therapy can improve heart function in some patients, particularly in individuals without evidence for persistence of viral or bacterial genomes in heart biopsies (26, 31, 32). These observations suggest that post-infectious autoimmunity might play an important role in disease development (33, 34).

Mouse models of autoimmune heart disease

The idea that autoimmune mechanisms contribute to the development of myocarditis is additionally supported by experimental findings based on animal models. In several susceptible mouse strains such as BALB/c, A/J or SJL mice, enteroviral infections result in chronic myocarditis progressing to heart failure, even after clearance of the pathogen (35, 36). This chronic myocarditis following enterovirus infection is T-cell mediated because adoptive transfer of T-cells, but not serum from diseased mice, induces myocarditis in severe combined immunodeficiency (SCID) mice. Interestingly, peripheral blood lymphocytes from patients with dilated cardiomyopathy could adoptively transfer disease to SCID mice lacking B- and T-cells (37).

In autoimmune myocarditis, several key questions remain unanswered. What promotes the generation of self-reactive T-cells in the context of infections with cardiotropic microorganisms? Why are some individuals more susceptible than others? What are the risk factors that define the possibility of an individual to develop chronic myocarditis progressing to dilated cardiomyopathy? In order to answer these questions and to design novel therapeutic strategies against postinflammatory cardiomyopathy, researchers take advantage of murine disease models. In fact, the genetic tractability of the mouse immune system has made the study of mouse models of cardiac autoimmunity an emerging area of interest.

If chronic myocarditis resulting from infections is indeed autoimmune mediated, the question arises whether myocarditis can be induced experimentally by immunization with heart-specific self-antigens only. Immunization models offer the advantage of studying disease pathogenesis *in vivo* in the absence of infection. Neu and co-workers indeed showed that immunization of susceptible mice with heart specific alpha-myosin together with strong immunostimulants induces heart specific inflammation (Experimental Autoimmune Myocarditis = EAM) (38). EAM is a $CD4^+$ T-cell mediated disease (33, 34, 39) and the

pathogenic cardiac alpha-myosin heavy chain epitopes mediating myocarditis have been mapped for BALB/c and A/J mouse strains (40, 41). In contrast to whole myosin, alpha-myosin-peptide immunization results in higher disease scores in BALB/c mice. Histological inflammation scores usually peak 3 weeks after the first alpha-myosin immunization and many animals develop ventricular dilation and heart failure. Of note, impaired cardiac contractility in immunized A/J and BALB/c strains correlates with the extent of $CD4^+$ T-cell infiltrations in the heart (42).

Antigen presenting cells, especially dendritic cells, play a crucial role in induction of autoimmune diseases (43, 44). Urs Eriksson and co-workers developed a method where activated bone marrow dendritic cells loaded with a heart muscle-specific self peptide induces $CD4^+$ T-cell mediated autoimmune myocarditis (45).

Despite the fact that heart specific autoantibody responses parallel experimental autoimmune myocarditis, B-cell deficient mice on the BALB/c background are not protected from autoimmune myocarditis (4, 46). This latter finding clearly shows that autoantibodies and B-cells are not required for autoimmune myocarditis induction in most mouse strains. Autoantibodies against cardiac troponin I, however, contribute to the development of spontaneous heart failure in mice lacking the programmed cell death-1 (PD-1) immunoinhibitory co-receptor (47).

So far, several transgenic mouse models for autoimmune myocarditis have been developed. Immunization with ***human*** □lpha-myosin peptides induces $CD4^+$ mediated myocarditis in transgenic mice co-expressing human hCD4 and HLA-DQ6 (48). This finding fits the observation that HLA-DQ6 is epidemiologically linked to enhanced susceptibility to dilated cardiomyopathy. Other transgenic mouse models are based on the expression of ovalbumin or beta-galactosidase in cardiomyocytes and/or smooth muscle cells (49, 50). These rather artificial models are useful studying $CD8^+$ T-cell mediated myocyte specific inflammation but do not reflect the crucial contribution of $CD4^+$ T-cells in the induction of heart-specific autoimmunity.

Proinflammatory cytokines are key players in the induction of myocarditis and DCM. Amongst others, TNF-alpha is also involved in the pathogenesis of

autoimmune myocarditis and heart failure. Cardiac-specific overexpression of tumour necrosis factor-alpha causes lethal myocarditis in transgenic mice (51).

This section will review parts of the innate immune system with focus on the Toll-like Receptors (TLRs), including its adaptor protein MyD88 and the induction of type I IFN. For more comprehensive overview of the innate immune system I recommend the lecture of “ImmunoBiology, the immune system in health and disease” (52).

Toll-like Receptors

In 1993, Charles A. Janeway, Jr. wrote an article called “How the immune system recognizes invaders” (53). He predicted the existence of receptors of the innate immune system that would recognize pathogen-associated molecular patterns and would signal activation of the adaptive immune system. Now, 14 years later, we know he was right.

Toll, the founding member of the TLR family was first described in *Drosophila* and plays a critical role in the antifungal response (54). To date, 12 members of the TLR family have been identified in mammals (55) (Table 1). TLRs are type I integral membrane glycoproteins characterized by the extracellular domains containing leucine-rich-repeat motifs and a cytoplasmic signalling domain homologous to that of the interleukin 1 receptor, termed the Toll/IL-1R homology (TIR) domain (56). TLRs are germline-encoded pattern-recognition receptors (PRRs) recognizing pathogen-associated molecular patterns (PAMPs). TLRs are evolutionarily conserved from the worm *Caenorhabditis elegans* to mammals. The main function of TLRs is the detection of invading microorganisms.

Individual TLRs recognize distinct PAMPs that have been evolutionary conserved in specific classes of microbes. TLRs sense lipopolysacharide (LPS) (detected by TLR4), bacterial lipoproteins and lipoteichoic acids (detected by TLR2), flagellin (detected by TLR5), the unmethylated CpG DNA of bacteria and viruses (detected by TLR9), double stranded RNA (detected by TLR3) and single-stranded viral RNA (detected by TLR7) (Table 1) (57).

Table 1: TLR Recognition of microbial Components

Microbial components	*Species*	*TLR*
Bacteria		
LPS	Gram-negative bacteria	TLR4
Diacyl lipopeptides	Mycoplasma	TLR6/TLR2
Triacyl lipopeptides	Bacteria and mycobacteria	TLR1/TLR2
LTA	Group B Streptococcus	TLR6/TLR2
PG	Gram-positive bacteria	TLR2
Porins	Neisseria	TLR2
Lipoarabinomannan	Mycobacteria	TLR2
Flagellin	Flagellated bacteria	TLR5
CpG-DNA	Bacteria and mycobacteria	TLR9
ND	Uropathogenic bacteria	TLR11
Fungus		
Zymosan	Saccharomyces cerevisiae	TLR6/TLR2
Phospholipomannan	Candida albicans	TLR2
Mannan	Candida albicans	TLR4
Glucuronoxylomannan	Cryptococcus neoformans	TLR2 and TLR4
Parasites		
tGPI-mutin	Trypanosoma	TLR2
Glycoinositolphospholipids	Trypanosoma	TLR4
Hemozoin	Plasmodium	TLR9
Profilin-like molecule	Toxoplasma gondii	TLR11
Viruses		
DNA	Viruses	TLR9
dsRNA	Viruses	TLR3
ssRNA	RNA viruses	TLR7 and TLR8
Envelope proteins	RSV, MMTV	TLR4
Hemagglutinin protein	Measles virus	TLR2
ND	HCMV, HSV1	TLR2
Host		
Heat-shock protein 60, 70		TLR4
Fibrinogen		TLR4
ND = not determined	adapted from (14)	

The Toll-like Receptor family can be further divided into subfamilies, each of with recognizes related PAMPs: The subfamily of TLR 3, 7, 8, and 9 are specialized on the detection of viruses and nucleic acids, that are unique to the microbial world. These TLRs are localized to intracellular compartments. TLR1, 2, 4, 5 and 6 seem to mainly specialize in the recognition of bacterial products and are expressed on the cell surface.

In addition to ligand specificity, the functions of individual TLRs differ in their expression patterns and the signal transduction pathways they activate. The expression of TLR is dependent on the cell type and is modulated during an ongoing immune response. TLR expression has been described for various immune cells, including macrophages, dendritic cells, B-cells and T-cells, as well as non-immune cells like fibroblast, epithelial cells or cardiomyocytes express TLRs (56, 58, 59).

TLR signalling

Stimulation of TLRs by microbial components triggers expression of several genes that are involved in immune responses. After ligand binding, TLRs dimerize and undergo conformational changes. It was shown that TLR2 forms heterodimers with TLR1 or 6, but in other cases TLRs are believed to form homodimers (58, 60).

All TLR family members share homology in the cytoplasmic domains. In particular, a high degree of similarity exists within the Toll/interleukin-1 receptor (TIR)-domain. Signalling from TLRs involves the TIR domain of the receptor that recruits TIR-domain-containing adaptor molecules to the TIR domain of the TLR. So far, five TLR adaptor molecules have been described: myeloid differentiation primary-response protein 88 (MyD88), TIR-domain-containing adaptor protein (TIRAP) also named MAL, TIR-domain-containing adaptor protein inducing IFN-beta (TIRF) also named TICAM1, TRIF-related adaptor molecule (TRAM) and sterile alpha- and armadillo-motif-containing protein (SARM) (61). MyD88 is critical for the signalling of all TLRs except TLR3. A MyD88 dependent pathway

is analogous to IL-1R signalling (Figure 1). Upon stimulation, MyD88 associates with the cytoplasmic portion of TLRs and then recruits IL-1R-associated kinase 4 (IRAK-4) and IRAK-1 through homophilic interaction of death domains. Subsequently, IRAK-4 associates with TNFR-associated factor 6 (TRAF6) and initiates downstream activation of the transcription factor NF-□B and MAP kinases JNK and p38, resulting in induction of genes like proinflammatory cytokines involved in inflammatory responses and the induction of adaptive immunity (Figure 1).

TLR stimulation induces Type I IFN

Among the key requirements for the induction of adaptive immunity is the upregulation of costimulatory molecules (including but not limited to CD80 and CD86) on antigen-presenting cells (APCs) to stimulate T-cells. It is now clear, that the key event in upregulation of costimulatory molecules is the activation of a type I IFN response (62, 63). Interferon's, first discovered by Isaacs (1957), are a family of cytokines which act early in the innate immune response (64). Within the TLR family, TLR3, 4, 7 and 9 stimulation, but not TLR2 ligand stimulation, induces type I IFN production.

In general, type I IFN induction is shown to be MyD88 independent. MyD88 independent TLR signalling is initiated by the TLR-adaptor molecules TRIF and TRAM (65, 66). TRAM is specifically involved in TLR4 signalling whereas TRIF interacts with several TLRs. It is suggested that TRAM acts as a bridging adaptor between TLR4 and TRIF (Figure 1). TRIF activation leads to the induction of a signalling cascade activating IRF-3 and IRF-7. These transcription factors form homodimers, resulting in the expression of a set of IFN-inducible genes. IRF-3 and IRF-7 are essential for the production of type I IFN. The activity of these products leads to the maturation of APCs, expression of costimulatory molecules and immune activation in general (65). Interestingly, IFN type I induction is not completely MyD88 independent. TLR9-mediated IFN-alpha secretion occurs in a

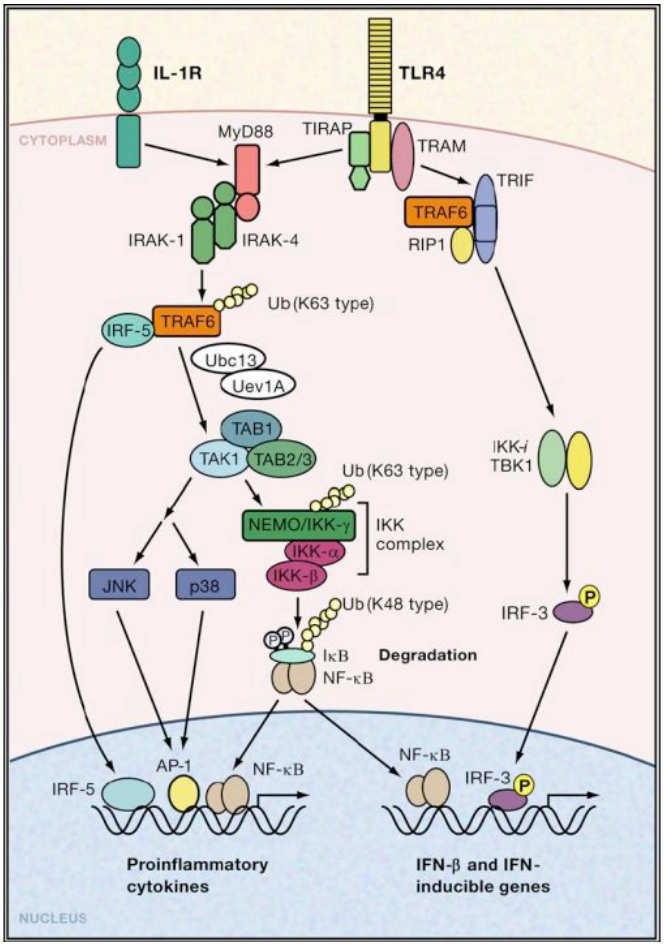

Figure1: TLR Signalling Pathway

TLRs and IL-1R share common signalling pathways in general. Ligand stimulation recruits TIR-domain-containing adaptors including MyD88 and TIRAP to the receptor, and subsequently induces the formation of a complex of IRAKs, TRAF6, and IRF-5. TRAF6 acts as an E3 ubiquitin ligase and catalyzes the K63-linked polyubiquitin chain on TRAF6 itself and NEMO with E2 ubiquitin ligase complex of UBC13 and UEV1A. This ubiquitination activates the TAK1 complex, resulting in the phosphorylation of NEMO and activation of the IKK complex. Phosphorylated IκB undergoes K48-linked ubiquitination and degradation by the proteasome. Once freed, NF-κB translocates into the nucleus and initiates the expression of proinflammatory cytokine genes. Simultaneously, TAK1 activates the MAP kinase cascades, leading to the activation of AP-1, which is also critical for the induction of cytokine genes. TLR4 triggers the MyD88-independent, TRIF-dependent signalling pathway via TRAM to induce type I IFNs. TRIF activates NF-κB and IRF-3, resulting in the induction of proinflammatory cytokine genes and type I IFNs. TRAF6 and RIP1 induce NF-κB activation and TBK1/IKK-i phosphorylate IRF-3, which induces the translocation of IRF-3. Adapted from (55)

MyD88-dependent manner. Also in TLR7 and 8 signalling, MyD88 dependent pathways contribute at least partially to the activation of IRF-3 and subsequently of type I IFN (14).

TLRs and autoimmunity

The main function of TLRs is the induction of inflammation and adaptive immunity. It is well established that the co-administration of antigens together with TLR ligands induces not only local inflammation, but also an adaptive immune response against the antigen. This process has been named the adjuvant effect. The most potent adjuvant currently used in experimental immunology is the complete Freund`s adjuvant (CFA). CFA consist of heat inactivated *Mycobacterium tuberculosis H37Ra* suspended in paraffin oil. CFA induces a strong T-cell immune response that can, if co-administered with a potent auto-antigen, break tolerance and induce autoimmunity. Several murine models for human autoimmune diseases are based upon these properties including Experimental Autoimmune Encephalomyelitis (EAE), Experimental Autoimmune Uveitis (EAU) and Experimental Autoimmune Myocarditis (EAM).

In EAE and EAU, the role of several TLR members has been investigated. EAE induction has been shown to be completely dependent on the TLR adaptor molecule MyD88 whereas TLR9 has at least a modulatory potential (67). In EAU, single TLR deficiencies for TLR2, 4 or 9 have no effect on disease induction whereas MyD88 or IL-1R deficiency completely prevents disease induction (68).

However, the role of TLRs in autoimmune diseases is not limited to the process of disease induction. TLR signalling has immunomodulatory potential and can directly influence the progression and outcome of autoimmune diseases. For example, TLR3-ligand administration has been shown to induce endogenous IFN-beta production that protects from EAE (69). In additional, Lang *et al.* demonstrated that TLR ligands could act directly on beta islet cells to up-regulate MHC I and enhance autoimmunity (70). Very recently it was shown in the model

of EAM that systemic TLR9-dependent CpG administration can restore T-cell proliferation and overcome disease resistance in PKC-theta deficient mice (71).
Thus far, diseases discussed are all T-cell mediated, however TLRs also play a major role in the pathogenesis of B-cell mediated autoimmune disease, namely Systemic Lupus Erythematosus (SLE) or Sjogren's syndrome (72). Both, DNA- and RNA-containing immune complexes have been shown to stimulate the production of autoantibodies through TLR7 and TLR9 in SLE or Sjogren's syndrome (73).

TLRs in the heart

Myeloid cells express TLRs but TLRs are also expressed in tissues without a recognized immune function, notably the heart and vasculature (59). Remarkably, TLR4 expression is increased in the heart of patients with dilated cardiomyopathy (13). In general, TLR2, 3, 4, 5 and 6 expression can be detected on cardiomyocytes and ligand activation of TLR2, TLR4 and TLR5 in cultured cardiomyocytes resulted in NF-κB activation and the expression of the inflammatory cytokine IL-6, the chemokine MIP-2 and the cell surface adhesion molecule ICAM-1 (59, 74). Furthermore, in a model of CVB3 induced myocarditis, it has been shown that MyD88 expression is upregulated during disease course in the heart (75).
Recent findings from mouse models of myocardial infarction show a role for TLR4 and TLR2 in the remodelling process after myocardial injury. Remodelling is a very complex process that induces changes in cardiac shape, size and composition in response to myocardial injury. It was shown, that TLR2 deficient and TLR4 deficient mice show higher survival, reduced inflammation, reduced myocardial fibrosis and improved heart function after myocardial infarction compared to control mice (76, 77). The exact mechanism that describes how TLR signalling influences cardiac remodelling and survival is not yet resolved. Due to the absence of infections, it is suggested that endogenous TLR ligands in

the heart might contribute to the remodelling and the pathogenic process of heart failure (77).

TLR stimulation could also boost heart failure by induction of proinflammatory cytokine secretion in the heart (12, 78). Proinflammatory cytokines are believed to be key players in the induction and progression of heart failure (79). Patients with chronic heart failure are characterized by systemic inflammation, as evident by raised circulating levels of inflammatory cytokines and chemokines (78). Furthermore, several studies have reported that the increased plasma levels of inflammatory cytokines correlate with deterioration of cardiac function (9, 11). The “cytokine hypothesis” for heart failure suggests that heart failure progresses as a result of the toxic effects exerted by endogenous cytokine cascades on the heart and peripheral circulation (80). Importantly, the myocardium itself may represent an important source of proinflammatory cytokines (81, 82). Amongst others, cytokines identified to contribute to heart failure also include TNF-alpha, IL-6 and IL-1, which are all members of the innate immune system (6).

Materials and Methods

Mice

MyD88ko (83) mice backcrossed for more than 9 generations on a H-2^d (BALB/c) background were kindly provided by Professor Shizuo Akira, Osaka, Japan.

IFN$\alpha\beta$Rko mice backcrossed for > 6 generations on a H-2^d (BALB/c) background were generated as described (84) and kindly provided by Professor Christian Bogdan, Freiburg, Germany. Depending on the experiment, wild-type BALB/c mice or heterozygous littermates from in-house breeding were used as controls. Experiments were in accordance with Swiss federal legislation and had been approved by the local authorities.

Generation of bone marrow chimeric mice

6- to 8-week-old recipient mice were reconstituted with bone marrow (bm) cells derived from tibiae and femurs from the respective donors. Bm cells (15 x 10^6 cells) were injected into the tail veins of recipients 24 hours after whole-body irradiation (2 x 250rad).

Induction of Experimental Autoimmune Myocarditis

Mice, older than 8 weeks were used for EAM experiments. For each mouse, 150µg murine alpha-myosin-heavy chain peptide (MyHC-alpha Ac-RSLKLMATLFSTYASADR-OH) (ANAWA Trading SA) was dissolved in 100µl complete Freund`s adjuvant (CFA) (Difco, 231131) and emulsified 1:1 with 100µl sterile PBS. For the preparation of the emulsion, a system with two 2ml luer-lock syringes (Braun Omnifix, 4617029V) was used. One syringe filled with CFA-MyHC the other syringe with the appropriate volume of PBS. The emulsion process was started pressing the PBS over a three-way stopcock (BD Connecta Plus 3, 394601) into the MyHC-CFA. After several minutes of intense mixing, an increase of resistance occurs indicating the successful completion of the MyHC-CFA emulsion. Each mouse was injected with a final volume of 200µl MyHC-CFA

emulsion on days 0 and 7. Depending on the experiment, mice were sacrificed 7, 14, 21 or 28 days after the first immunization.

Immunization with bone marrow derived dendritic cells

For the generation of bone marrow dendritic cells (bmDCs), whole bone marrow isolated from femur and tibiae was plated on several bacterial quality petri dish (Falcon, 100x15mm style, 351029) at a density of two-million cells per dish in 10ml bmDC medium (bmDC medium: RPMI 1640 (Bio-Whittaker, BE12-115F, containing 25mM HEPES and L-Glutamine) supplemented with 10% FCS, Pen/Strept (1/100, Gibco 15140-122), β–Mercaptoethanol (1/1000, Gibco 31350-010), Sodium Pyruvate (1/100, Gibco 11360-039), Non-Essential Amino Acids (1/100, Gibco 11140-035) and 200U/ml rmGM-CSF (Peprotech)).

After 8 days culturing, bmDCs were loaded with MyHC-alpha 10µg/ml for 1 hour and activated for additional 2 hours with 0.1µg/ml LPS (List Biological Laboratories INC Cat: 421) and 5□g/ml anti-CD40 (BD Pharmingen Cat: 553787). BmDCs were then harvested in ice cold PBS and 500'000 bmDCs per mouse were i.p. injected. BmDC immunization was performed two times on day 0 and 2. Mice were sacrificed 10, 15, 20, or 120 days after immunization depending on the goal of the experiment.

bmDC/CFA combined "double immunization"

For induction of myocarditis associated with strong fibrosis and heart dilation, mice were immunized with bmDCs on day 0 and 2 as described above and were additionally immunized with MyHC-CFA on day 10 and 17. Mice were sacrificed on day 31 and analyzed for myocarditis and fibrosis in the heart.

MyHC-alpha specific $CD4^+$ T-cell transfer.

$CD4^+$ T-cells were isolated with magnetic beads ($CD4^+$ T-cell isolation kit; Miltenyi Biotech GmbH) from diseased wt BALB/c mice d14 after MyHC-CFA immunization and cultured on irradiated (2000rad) wt BALB/c splenocytes at a 1:4 ratio in the presence of 2 µg/ml MyHC-alpha in RPMI 1640 complete medium

(RPMI 1640 (Bio-Whittaker, BE12-115F, containing 25mM HEPES and L-Glutamine), Pen/Strept (1/100, Gibco 15140-122), β–Mercaptoethanol (1/1000, Gibco 31350-010), Sodium Pyruvate (1/100, Gibco 11360-039), Non-Essential Amino Acids (1/100, Gibco 11140-035)), 10% FCS containing 10ng/ml rmIL-23 (provided by Burkhard Becher, Zürich, Switzerland) for 7 days. Cells were then washed and cultured in the presence of 20 U/ml of recombinant mouse IL-2 (Peprotech) and 10ng/ml rmIL-23 without MyHC-alpha peptide for an additional 7 days. This pulse/rest cycle was repeated at least three times. Finally, $CD4^+$ T-cells were restimulated for 4 days before i.p. injection of 3-6 x 10^6 $CD4^+$ T-cells per syngeneic recipient. Mice were sacrificed 10 days after adoptive transfer.

Histology

Histological analysis was performed in collaboration with Professor Stephan Dirnhofer, University Hospital Basel and PD Michael Kurrer, University Hospital Zurich.

After 24h fixation in 2% Paraformaldehyde-PBS, hearts were cut transversally into three pieces and embedded into paraffin. 5 µm thick sections were cut at various depths in the tissue section and stained with hematoxylin and eosin (H&E) to determine the level of inflammation. Myocarditis was scored using grades from 0 to 4 (0 - no inflammatory infiltrates; 1 - small foci of inflammatory cells between myocytes; 2 - larger foci of more than 100 inflammatory cells; 3 - more than 10% of a cross-section involved; 4 - more than 30% of a cross-section involved).

Fibrosis was detected with Chromotrope-Anilin Blue (CAB) staining detecting Collagen deposition. Fibrosis was scored according to the same criteria as Myocarditis which are the following: from 0 to 4 (0, no collagen deposition; 1, small collagen deposition between myocytes; 2, larger collagen deposition of > 5% of cross-section involved; 3, >10% of a cross-section involved; 4, >30% of a cross-section involved

FACS analysis

Cells were stained using fluorochrome-conjugated mouse-specific antibodies purchased from BD Pharmingen. Samples were acquired on a FACS Calibur flow cytometer (BD Biosciences) or on a FACS Cyan (DakoCytomation). Data was analyzed using FlowJo (TreeStar) software.

***Surface molecules staining*:** Fc-receptors were blocked in staining buffer (PBS, 1% FCS, 2mM EDTA) supplemented with 1% normal mouse serum for 15 minutes at 4°C with an additional antibody staining at 4°C for 20 minutes. Cells were washed and resuspended in staining buffer and immediately analyzed.

Intracellular staining: Before intracellular staining, cells were activated for 6 hours with 20ng/ml phorbol myristate acetate (PMA) and 1 µM ionomycin in the presence of 10 µg/ml Brefeldin A. Surface molecules staining was performed as described above. Cells were then fixed in freshly prepared PBS containing 4% Paraformaldehyde (PFA) for 30 minutes at 4°C and then permeabilized and stained for intracellular components in PBS, 1% FCS, 2mM EDTA, 0.5% Saponin for 30 minutes at room temperature. Cells were then washed with staining buffer and immediately analyzed.

bmDC migration assay

Bone marrow Dendritic Cells (bmDCs) were harvested after 10 days in culture with bmDC medium as described above. bmDCs were resuspended in PBS 5% FCS at 10 x 10^6 cells/ml. bmDCs were stained for 5 minutes at room temperature in PBS 5% FCS 5µM CFSE. The presence of FCS is essential to buffer the toxic effect of CFSE. bmDCs are then washed in PBS 10% FCS. 20 million bmDCs are injected intraperitoneal in wt BALB/c mice or IFN$\alpha\beta$Rko mice. bmDC migration to the spleen and mesenteric lymph nodes was assessed 2 days after injection.

Autoantibody ELISA

96-well plates (Nunc 430341) were coated over-night with porcine myosin (sigma 0-0531) 10 µg/ml in 0.1M $NaHCO_3$ buffer pH = 9.6. Wells were then washed with

PBS pH = 7.4 Tween-20 0.05% and further blocked with PBS 2% BSA 0.05% Tween-20 for 2h at room temperature. Wells were then washed with PBS 0.05% Tween-20 and mouse sera was added in dilutions ranging from 1:160 to 1:20480 in PBS, 2% BSA, 1% FCS, 0.05% Tween-20 for 2 h at room temperature.
Wells were then washed with Tris Buffer (Tris 50 mM, NaCl 37 mM, KCl 2.7 mM). The following detection antibodies were added 1:2000 in Tris Buffer 2% BSA 0.05% Tween-20): Goat anti-mouse total IgG AP (Southern Biotech 1030-04), Goat anti-mouse IgG 2b AP (Southern Biotech 1090-04), Goat anti-mouse IgG 2a AP (Southern Biotech 1080-04), Goat anti-mouse IgG1 AP (Southern Biotech 1070-04). Wells were washed with Tris buffer before Fluorescein diphosphate (FDP) substrate 10µM (Molecular Probes F2999) was applied for 1hour at room temperature. Fluorescence was measured at the following wavelengths: absorption 485 nm, emission 538 nm, cut off 530 nm.

Whole splenocytes restimulation *in vitro*

Whole splenocytes were isolated using 70µm cell strainers (BD Falcon Cat: 352350). After red blood cell lysis in ACK-buffer (For 250ml: 2g NH_4CL, 0.25g $KHCO_3$, 0.005g EDTA), whole splenocytes were cultured in 96-well plates for 72 hours with 0 - 10 µg/ml of MyHC-alpha peptide in RPMI 1640 complete medium (RPMI 1640 (Bio-Whittaker, BE12-115F, containing 25mM HEPES and L-Glutamine), Pen/Strept (1/100, Gibco 15140-122), β–Mercaptoethanol (1/1000, Gibco 31350-010), Sodium Pyruvate (1/100, Gibco 11360-039), Non-Essential Amino Acids (1/100, Gibco 11140-035)) supplemented with 1% of normal mouse serum. 2 x 10^{-5} mmol 3H-Thymidine (5 x 10^{-4} mCi) (Amersham TRK120) was added per well for additional 10 hours. 3H-Thymidine incorporation was measured using Top Count NXT (Packard).

$CD4^+$ T-cell-proliferation

$CD4^+$ T-cells were isolated with magnetic beads ($CD4^+$ T-cell isolation kit; Miltenyi Biotech GmbH) and cultured in 96-well plates for 72 hours on irradiated

(2000 rad) syngenic splenocytes, with 0-10 µg/ml of MyHC-alpha peptide in RPMI 1640 (Gibco) medium supplemented with 1% of normal mouse serum.
Naïve $CD4^+CD62L^+$ T-cells were stimulated in 96-well plates with either 5 µg/ml soluble anti-CD3□ antibody, 50 ng/ml PMA and 500 ng/ml Ionomycin, 1 µg/ml Concanavalin A (Con A) in the presence of irradiated wild-type DCs (1). Alternatively, naïve $CD4^+CD62L^+$ T-cells were stimulated with 1 µg/ml Con A on either $MyD88^{+/+}$ or $MyD88^{-/-}$ DCs in the presence or absence of recombinant murine TNF-alpha (PeproTech) or a TNF-alpha blocking antibody. Proliferation was assessed by measuring 3H-thymidine incorporation. All reagents and media were endotoxin free.

ReverseTranscription-PCR (RT-PCR)
Hearts were isolated and homogenized with a Polytron PT 1200 CL (Kinematica) in 2ml Tri Reagent (Molecular Research Center, Inc.). mRNA was extracted with Bromochloropropane (Molecular Research Center, Inc.), precipitated in Isopropanol and washed in 70% Ethanol. Following resuspension, mRNA samples were treated with Deoxyribonuclease I (Fermentas, Cat: EN0521). cDNA was subsequently generated using RevertAid M-MuLV Reverse Transcriptase (Fermentas, Cat:EP0441). PCR from cDNA was performed using TaqPCR Master Mix Kit (Qiagen Cat:201445). PCR products were analyzed on a 2% Agarose Gel (Invitrogen Cat:15510-027) containing 3µg Ethidiumbromide per 100ml Gel in TAE-Buffer. Gels were run for 45 minutes at 100 Volts in a PowerPac Universal (Biorad) system. Levels of gene expression were analyzed with the ChemImager 5500 (Alpha Innotech).
Primer sequences:

IL-1beta forward:	5’-CAG GAT GAG GAC ATG AGC ACC-3’
IL-1beta reverse:	5’-CTC TGC AGA CTC AAA CTC CAC-3’
IL-18 forward:	5’-ACT GTA CAA CCG CAG TAA TAC GC-3’
IL-18 reverse:	5’-TCC ATC TTG TTG TGT CCT GG-3’
TNF-alpha forward:	5’-GGC AGG TCT ACT TTG GAG TCA TTG C-3’
TNF-alpha reverse:	5’-ACA TTC GAG GCT CCA GTG AAT TCG T-3’

IL-6 forward:	5'-TTG CCT TCT TGG GAC TGA TGC-3'
IL-6 reverse:	5'-GTA TCT CTC TGA AGG ACT CTG G-3'
IFN-alpha forward:	5'-ATA ACC TCA GGA ACA ACA G-3'
IFN-alpha reverse:	5'-TCA TTG CAG AAT GAG TCT AGG AG-3'
IFN-beta forward:	5'-CCA CAG CCC TCT CCA TCA ACT ATA AGC-3'
IFN-beta reverse:	5'-AGC TCT TCA ACT GGA GAG CAG TTG AGG-3'
beta-Actin forward:	5'-TGT GAT GGT GGG AAT GGG TCA-3'
beta-Actin reverse:	5'-TTT GAT GTC ACG CAC GAT TTC C-3'

Echocardiography

For echocardiography, mice were anesthetized with Isofluran (5% initially, 1-2% for maintenance) resulting in a stable heart rate of 180 to 200 beats per minute. *In vivo* cardiac function was analyzed using a 15.0 mHz linear transducer attached to a Philips echocardiography system (Philips Medical System, Zurich, Switzerland). With the mouse in the left lateral decubitus position, the transducer was placed on the left hemithorax. Care was taken to avoid excessive pressure, which can induce bradycardia. End-diastolic left-ventricular diameter (EDD), end-systolic left-ventricular diameter (ESD) and ejection time were determined; end diastole being defined as the maximal left ventricle (LV) diastolic dimension and end systole as the most anterior systolic excursion of the LV posterior wall. The fractional shortening (FS) of the LV is expressed as a percentage as %FS=(EDD-ESD)/EDDx100. The velocity of circumferential fibre shortening (Vcf) is expressed in (circ/s) and calculated as Vcf=(EDD-ESD)/(ejection time x EDD).

Statistics

Dichotomous data were analyzed by Fisher`s exact test. The Mann-Whitney U test was used for the evaluation of severity scores. Proliferation responses and cytokine levels were compared using ANOVA and the t-test.

MyD88 Signalling Controls Autoimmune Myocarditis Induction

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Abstract

Background: Experimental autoimmune myocarditis (EAM) is a $CD4^+$ T-cell mediated mouse model of postviral cardiomyopathy. Activation of IL-1 type 1 and Toll-like receptors (TLR) sharing the common downstream adaptor molecule MyD88 is required for disease induction. The specific role of MyD88 in myocarditis, however, is not known.

Methods and Results: In contrast to control littermates, $MyD88^{-/-}$ mice were protected from myocarditis after immunization with □-myosin heavy chain derived peptide (MyHC-alpha) and complete Freund`s adjuvant (CFA). Disease resistance of MyD88ko mice resulted from impaired expansion of heart-specific $CD4^+$ T-cells after immunization. Intrinsic defects of MyD88ko $CD4^+$ T-cells were excluded. In contrast, MyD88ko but not wt primary antigen presenting dendritic cells (DCs) were defective in their capacity to prime $CD4^+$ T-cells. This defect mainly resulted from the inability of MyD88ko DCs to release TNF-alpha. The critical role of MyD88 signalling in DCs in the peripheral lymphatic compartments was finally proven by repetitive injection of activated, MyHC-alpha loaded wt DCs that fully restored T-cell expansion and myocarditis in MyD88ko mice.

Conclusions: Autoimmune myocarditis induction depends on MyD88 signalling in self-antigen presenting cells in the peripheral compartments. We conclude that MyD88 might become a target for prevention of heart-specific autoimmunity and cardiomyopathy.

Key words: myocarditis, cardiomyopathy, inflammation, autoimmunity, heart failure

Introduction

Dilated cardiomyopathy is the most common cause of heart failure in young patients and often results from enteroviral myocarditis (20, 28). Many patients show heart-specific autoantibodies (28, 29), and/or upregulation of activation markers on heart infiltrating cells (32). Interestingly, patients with heart-specific autoantibodies but no evidence for viral genome persistence in heart biopsies show improvement of their cardiac function upon immuno-suppression (31). Therefore, it is reasonable to conclude that autoimmunity is involved in the pathogenesis of postinflammatory cardiomyopathy (for review, see (33, 34)).

Animal models greatly advanced our knowledge on the pathogenesis of myocarditis and inflammatory cardiomyopathy. In susceptible mice, for example, infection with enteroviruses results in a biphasic myocarditis with an early acute stage 5-8 days after inoculation, followed by a chronic stage of low-grade inflammation (36). Interestingly, T-cells from mice with enteroviral myocarditis transfer disease in syngenic severe combined immunodeficiency (SCID) recipients lacking B- and T-cells, suggesting a crucial role for autoreactive T-cells in disease pathogenesis (85). Furthermore, immunization of susceptible mice with alpha-myosin derived peptides together with complete Freund`s adjuvant (CFA) results in $CD4^+$ T-cell mediated experimental autoimmune myocarditis (33, 34, 38).

TLRs belong to the Toll-interleukin 1 receptor superfamily of conserved surface molecules triggering innate mechanisms of immunity upon stimulation with microbial products or endogenous danger signals (86, 87). In the context of inflammatory heart disease, it has been shown that Toll-like receptor 4 is expressed together with enteroviral replication in hearts from patients with dilated cardiomyopathy (88). In addition, mice lacking Toll-like receptor 4 develop markedly reduced myocarditis after infection with enteroviruses such as Coxsackie B3 (CVB3) (89). In the experimental autoimmune myocarditis model activation of Toll-like receptors on selfantigen presenting dendritic cells (DCs) is essential for the induction of myocarditis and heart failure (45). Furthermore, IL-1

type 1 receptor signalling on DCs is critical for autoimmune myocarditis development (1). During myocarditis development, recruitment of activated, bone marrow derived dendritic cells precedes the accumulation of macrophages and T-cells in the heart (90-92). Accordingly, disease induction by adoptive transfer of heart-specific $CD4^+$ T-cells requires pretreatment of recipient mice with LPS and other strong Toll-like receptor stimulants upregulating MHC class II expression on heart resident DCs (91).

MyD88 is an essential adaptor molecule that mediates complex proinflammatory pathways involving a cascade of kinases integrating both Toll-like receptor 4 and IL-1 receptor type 1 activation (87). Its role in autoimmune heart disease is not known yet.

To assess the role of MyD88 in the expansion of heart-specific $CD4^+$ T-cells and the development of autoimmune heart disease, we assessed myocarditis susceptibility of mice genetically lacking MyD88. Here we describe that MyD88 is required for myocarditis induction after MyHC-CFA immunization. More specifically, we found that MyD88 signalling in dendritic cells is essential to prime heart specific $CD4^+$ T-cells *in vivo*.

Methods

Mice. MyD88ko mice were generated as described (83) and backcrossed for > 9 generations on a H-2^d (BALB/c) background. Wild-type or heterozygous littermates were used as controls. Experiments were in accordance with Swiss federal legislation or Austrian law, and had been approved by the local authorities.

Myocarditis induction. Mice were injected subcutaneously with 100□g/mouse of the murine □-myosin-heavy chain peptide (MyHC-alpha: Ac-RSLKLMATLFSTYASADR-OH) emulsified 1:1 with complete Freund`s adjuvant (CFA) on days 0 and 7 (4, 38). For myocarditis induction using dendritic cells (DCs), immature DCs were pulsed with MyHC-alpha and activated for 2 hours with 0.1 □g/ml of LPS and 5 □g/ml of anti-CD40 prior to intraperitoneal injection of 250000 DCs per mouse three times every second day. For adoptive transfer, we injected intraperitoneally 10^7 MyHC-alpha specific *in vitro* restimulated $CD4^+$ T-cells per mouse. Depending on the experiment, mice were sacrificed after 10, 14, 21, or 28 days.

Histopathology. Myocarditis was scored using grades from 0 to 4 (0 - no inflammatory infiltrates; 1 - small foci of inflammatory cells between myocytes; 2 - larger foci of more than 100 inflammatory cells; 3 - more than 10% of a cross-section involved; 4 - more than 30% of a cross-section involved) (1, 45).

Dendritic cells. For immunization experiments, bone marrow derived DCs were generated as described (45). Naïve, primary DCs were obtained from lymph nodes and spleens of non-immunized mice. Alternatively, we isolated DCs from draining lymph nodes 24 hours after MyHC-CFA immunization using magnetic beads (MACS DC isolation kit, Miltenyi Biotech GmbH) and cell sorting. For analysis of surface molecules, DCs were preincubated for 30 min at 4° with 1% normal mouse serum in staining buffer (Pharmingen) before staining with the

appropriate fluorochrome labelled antibodies from Pharmingen. Viable cells were assessed in FACS scatter plots by gating on Propidium Iodine negative populations. For cytokine analysis, primary DCs were plated at 1 x 10^6/ml in 96 well plates and activated with LPS at 0.1 □g/ml.

Cytokine analysis. Cytokine levels were measured in culture supernatants using commercially available Quantikine ELISA kits (R&D Biosystems, Minneapolis, U.S.A).

Autoantibodies. We assessed antibody responses against whole alpha-myosin with an ELISA as described (45), using AP-labelled goat anti-mouse IgG subclass antibodies (Southern Biotechnology Associates). Antibody titers were determined at half maximum OD_{405nm}.

$CD4^+$ T-cell-proliferation. $CD4^+$ T-cells were isolated with magnetic beads ($CD4^+$ T-cell isolation kit; Miltenyi Biotech GmbH) and cultured in 96-well plates for 72 hours on irradiated (2000 rad) syngenic splenocytes, with 0.01-10 □g/ml of MyHC-alpha peptide in RPMI 1640 (Gibco) medium supplemented with 1% of normal mouse serum.
Naïve $CD4^+CD62L^+$ T-cells were stimulated in 96-well plates with either 5 □g/ml soluble anti-CD3□ antibody, 50 ng/ml PMA and 500 ng/ml Ionomycin, 1 □g/ml Concanavalin A (Con A) in the presence of irradiated wild-type DCs (16). Alternatively, naïve $CD4^+CD62L^+$ T-cells were stimulated with 1 mg/ml Con A on either wt or MyD88ko DCs in the presence or absence of recombinant murine TNF-alpha (PeproTech) or a TNF-alpha blocking antibody. Proliferation was assessed by measuring (^{3}H)-methyl-thymidine incorporation. All reagents and media were endotoxin free.

Statistics. Dichotomous data were analyzed by Fisher`s exact test. The Mann-Whitney U test was used for the evaluation of severity scores. Proliferation responses and cytokine levels were compared using ANOVA and the t-test.

Results

Myocarditis induction requires MyD88 signalling

In order to define the role of MyD88 signalling in autoimmune myocarditis we first compared myocarditis susceptibility of mice lacking MyD88, heterozygous $MyD88^{+/-}$, or $MyD88^{+/+}$ (wt) littermates. As illustrated in figure 2A (right panel), figure 1C, and summarized in table 2, both wild-type littermates and $MyD88^{+/-}$ mice developed severe myocarditis with inflammatory infiltrates containing granulocytes, eosinophils, and mononuclear cells including macrophages and lymphocytes after two immunizations with MyHC-CFA. In contrast, MyD88ko mice were protected from disease, and developed minimal pericardial calcifications only (Figure 2A, left panels; Fig 2C; table). Differences in disease susceptibility between MyD88ko and $MyD88^{+/-}$ and wt mice were consistently observed at different time points, i.e. 14, 21 or 28 days after immunization (not shown). Therefore, the adaptor molecule MyD88 is crucial for the induction of autoimmune myocarditis after immunization with cardiac selfantigen together with CFA.

Impaired expansion of heart-specific $CD4^+$ T-cells in MyD88ko mice

Autoimmune myocarditis is a $CD4^+$ T-cell mediated disease (33, 45). In mice lacking MyD88, disease resistance paralleled impaired expansion of heart-specific T-cells, as suggested by the impaired *in vitro* proliferation of MyHC-alpha restimulated whole splenocytes (Figure 3A) and – more specifically - by the absence of *in vitro* proliferation of MyD88ko but not wt $CD4^+$ T-cells restimulated with MyHC-alpha pulsed irradiated splenocytes (Figure 3B). Accordingly, the production of IFN-gamma was reduced in supernatants of *in vitro* restimulated whole splenocytes from MyD88ko but not wt mice (Figure 3C). IL-4 levels, on the other hand, were uniformly low in supernatants from both, *in vitro* restimulated wt and MyD88ko splenocytes. Regarding humoral autoimmunity, MyD88ko mice still mounted myosin specific IgG autoantibodies upon immunization, albeit at significantly lower IgG1 titers. IgG2a and IgG2b anti-myosin responses, on the

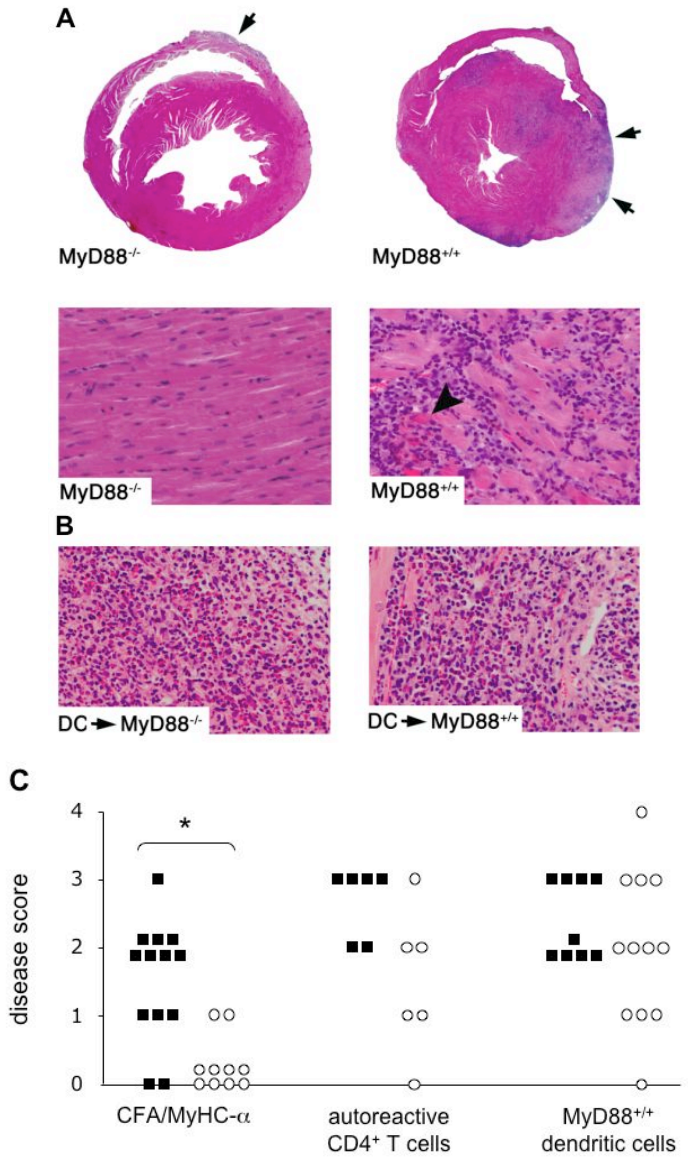


Figure 2: Myocarditis susceptibility of MyD88ko mice

(A) MyD88ko mice are protected from autoimmune myocarditis after MyHC-CFA immunization. Some MyD88ko mice develop minimal pericardial inflammation and calcifications (arrow) but no cardiac infiltrates (left panels). In contrast, wt mice develop severe myocarditis with inflammatory infiltrates surrounding necrotic cardiomyocytes (arrow).

(B) Immunization with activated, MyHC-alpha loaded DCs restores myocarditis in both, MyD88ko (left panel) and wt (right panel) recipient mice. All sections were stained with Hematoxylin and Eosin. 10 x, or 200 x original magnifications are shown.

(C) Disease severity scores of individual wt (filled squares) and MyD88ko (open circles) mice after either MyHC-CFA immunization, adoptive transfer of heart-specific wt $CD4^+$ T-cells, or after immunization with activated, MyHC-alpha loaded wt DCs.

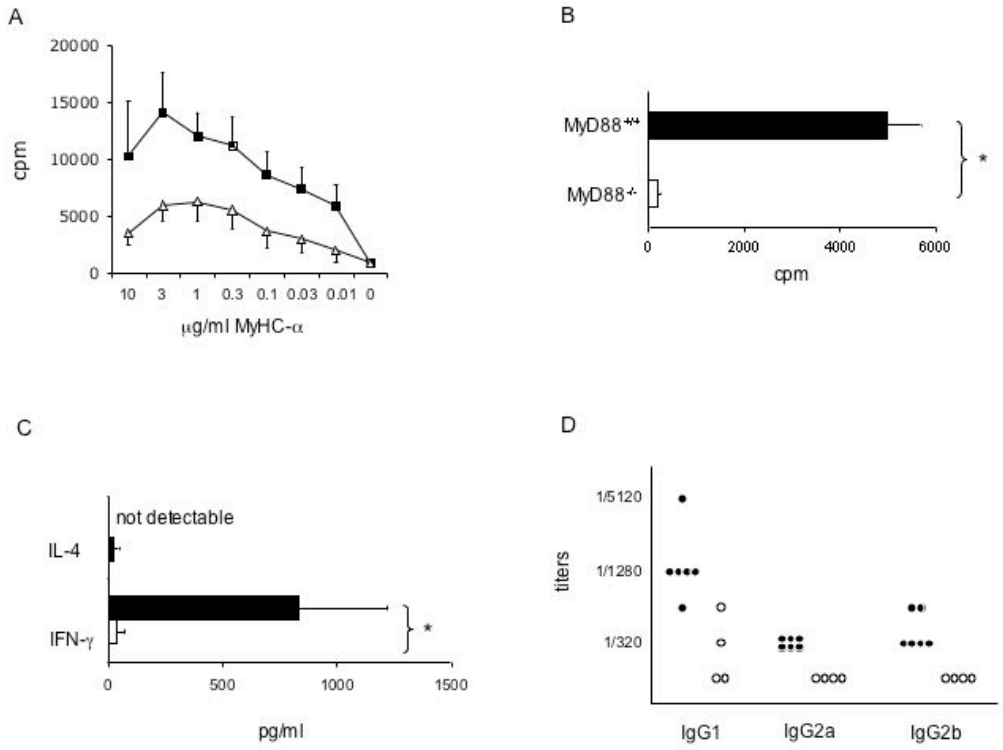


Figure 3

Impaired CD4$^+$ T-cell expansion and autoantibody production in MyD88ko mice

(A) Impaired proliferation of in vitro *MyHC-alpha restimulated splenocytes from MyD88ko mice (open triangles) compared to wt mice (filled squares), 21 days after immunization with MyHC-alpha and CFA. Proliferation was assessed by measurement of ^{3}H-Thymidine incorporation. Mean +/- SD of cpm values from 4-5 individual mice are shown.*

(B) Impaired proliferation of in vitro *MyHC-alphạrestimulated CD4$^+$ T-cells from MyD88ko and wt mice. CD4$^+$ T-cells were isolated 21 days after immunization and restimulated on irradiated syngenic splenocytes. Mean +/- SD of cpm values from 5 individual mice are shown.*

(C) Mean +/- SD of IFN-gamma and IL-4 in culture supernatants of MyHC-alpha restimulated whole splenocytes from MyHC-CFA immunized wt (black bars) vs. MyD88ko (white bars) mice. Each bar represents data from 5 individual mice.

(D) Impaired humoral IgG subclass responses to whole myosin of MyHC-/CFA immunized MyD88ko (open circles) vs. wt (filled circles) mice.

other hand, were markedly impaired in MyD8ko mice (Figure 3D). Taken together, the absence of MyD88 signalling impairs both, the expansion of heart-specific $CD4^+$ T-cells and the generation of heart-specific humoral autoimmunity. In conclusion, these findings strongly suggest impaired $CD4^+$ T helper cell function in MyD88ko mice after immunization with cardiac selfantigen.

MyD88 is not intrinsically required for $CD4^+$ T-cell activation

Next, we asked whether MyD88 signalling is intrinsically required for $CD4^+$ T-cell activation. We isolated naïve $CD62L^+CD4^+$ T-cells from wt and MyD88ko mice and compared their primary response upon various stimuli. As illustrated in figure 4, wt and MyD88ko $CD4^+$ T-cells did not differ in their capacity to proliferate upon stimulation with anti-CD3ε or PMA/Ionomycin. Furthermore, there was no difference in the primary responses between wt and MyD88ko $CD4^+$ T-cells upon stimulation with Concanavalin A (Con A) in the presence of irradiated wild-type antigen presenting cells. In conclusion, we found no *in vitro* evidence for impaired proliferation of MyD88ko $CD4^+$ T-cells upon T-cell receptor stimulation (anti-CD3ε), intrinsic activation (PMA/Inomycin), or antigen presenting cell dependent indirect activation (Con A and wild-type DCs) that would explain their impaired expansion in MyD88ko mice.

Heart-specific wt $CD4^+$ T-cells transfer myocarditis in MyD88ko recipients

Both, IL-1 receptor type 1 and TLR receptors sharing the common adaptor molecule MyD88 are expressed in various tissues including leukocytes, endothelial cells, and cardiomyocytes (88). Therefore, the absence of MyD88 might affect cardiac inflammation on many levels. Accordingly, we first asked whether heart-specific $CD4^+$ T-cells and other inflammatory cells get at all access to the heart in MyD88ko mice. To address this question we first created a highly autoreactive and heart-specific $CD4^+$ T-cell line. $CD4^+$ T-cells were isolated from immunized wt mice and restimulated for several times *in vitro* with MyHC-alpha pulsed irradiated splenocytes. A prolonged resting phase followed each restimulation. The resulting $CD4^+$ T-cell line was MyHC-alpha specific, and

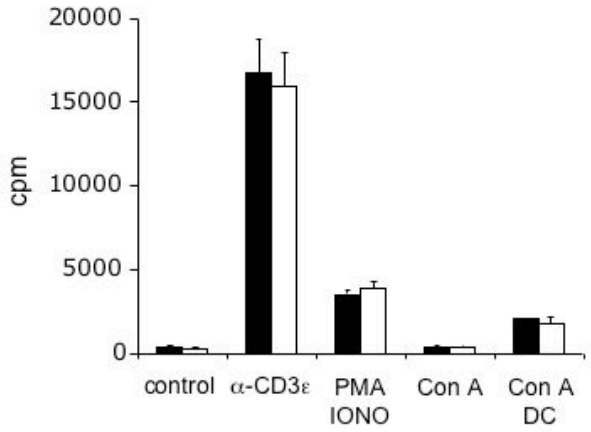


Figure 4

Primary responses of wt vs. MyD88ko CD4$^+$ T-cells

CD4$^+$CD62L$^+$ T-cells from naïve mice were stimulated with anti-CD3□, PMA/Ionomycin, or Con A in the presence of wild-type dendritic cells (DC). Proliferation was measured after 36 hours of culture. One out of several representative experiments is shown.

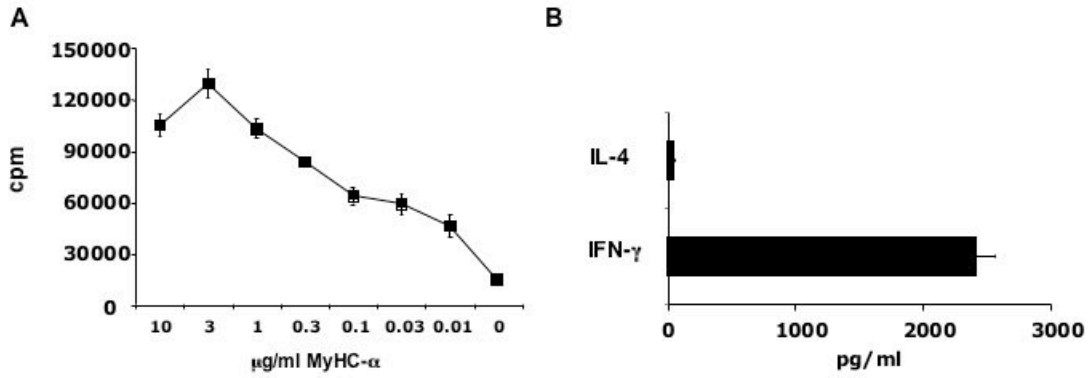


Figure 5

MyHC-alpha specific CD4$^+$ T-cell line

MyHC-alpha specific CD4$^+$ T-cells showed vigorous proliferation upon in vitro *MyHC-alpha restimulation on irradiated splenocytes (A), and produced high levels of IFN-gamma(B).*

produced high levels of IFN-□amma upon restimulation (Figure 5A,B). Adoptive transfer of 10^7 activated, heart specific CD4$^+$ T-cells per mouse induced myocarditis of similar prevalence albeit slightly reduced severity in both, wt and MyD88ko mice (Table 2). These findings suggest that MyD88 signalling is not decisive for the recruitment of autoreactive CD4$^+$ T-cells to the heart during myocarditis development.

Table 2: Myocarditis prevalence and disease severity in MyD88$^{-/-}$ mice and wild-type controls

Mice [genotype]	Treatments	Disease prevalence [# diseased/ # treated]	Severity grade [median (range)]
MyD88$^{-/-}$	MyHC-CFA (days 0,7)	2/ 8*	0 (0-1)
MyD88$^{+/+}$	MyHC-CFA (days 0,7)	11/13*	2 (0-3)
MyD88$^{-/-}$	(MyHC-alphą CD4^{+} T-cells	5/ 6	1.5 (0-3)†
MyD88$^{+/+}$	(MyHC-alphą CD4^{+} T-cells	6/ 6	3 (2-3)†
MyD88$^{-/-}$	MyHC-alpha pulsed dendritic cells	11/12	2 (0-4)
MyD88$^{+/+}$	MyHC-alpha pulsed dendritic cells	9/ 9	2 (2-3)

*P < 0.03 MyD88$^{-/-}$ vs. MyD88$^{+/+}$ for comparison of disease prevalence

†P= 0.08 MyD88$^{-/-}$ vs. MyD88$^{+/+}$ for comparison of severity (Mann Whithney)

MyD88ko DCs failed to prime wild-type CD4$^+$ T-cell responses

So far, our data suggest functionally intact CD4$^+$ T-cells in MyD88ko mice and exclude a relevant role for MyD88 signalling in the recruitment of CD4$^+$ T-cells to the heart. We, therefore, hypothesized that disease resistance of MyD88ko mice most likely results from the impaired capacity of antigen presenting cells to prime and expand autoreactive CD4$^+$ T-cells in the peripheral compartments *in vivo*. We therefore assessed the functionality of MyD88ko DCs to promote primary T-cell responses. To overcome the mechanisms of antigen processing and presentation we compared Con A induced proliferation of naïve wild-type CD4$^+$ T-cells in the presence of either wt or MyD88ko DCs. In fact, primary Con A mediated CD4$^+$ T-cell responses were significantly impaired in the presence of MyD88ko compared to wt DCs (Figure 6). The impaired functional capacity of MyD88ko DCs in peripheral lymph nodes, however, did not result from a defect in upregulation of essential costimulatory CD40, CD80, CD86, or MHC class II molecules because we found no difference in the expression of these surface molecules on dendritic cells isolated from draining lymph nodes after immunization (Figure 7A). In contrast, it is well established that MyD88ko DCs fail to produce relevant amounts of proinflammatory cytokines after maturation (3). Their impaired production in MyD88ko mice might contribute to the disease resistance on many levels including priming, expansion, and maintaining of autoreactive T-cells as well as suppression of regulatory T-cells. Given the fact, that a very short activation period with TLR stimulants is sufficient to render MyHC-alpha loaded DCs pathogenic (45), we assessed the production profiles of various cytokines and found that TNF-alpha, but not IL-6 or IL-12p40 production of primary wt DCs peak during the first 4 hours after TLR stimulation (not shown). As illustrated in Figure 7B, this early TNF-alpha production is markedly impaired in MyD88ko compared wt DCs. Given the central role of TNF-alpha in autoimmune myocarditis induction (34), we hypothesized that the absence of the early peak release of TNF-alpha from MyD88ko DCs might play an important role in the reduced capacity of MyD88ko DCs to prime naïve CD4$^+$ T-cells. Indeed, *in*

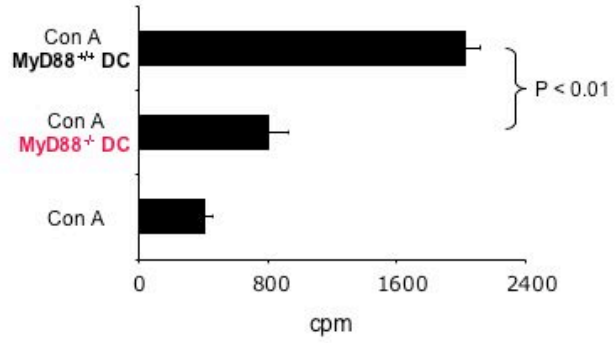


Figure 6

Primary responses of naïve CD4$^+$ T-cells in the presence of wt vs. MyD88ko DCs
(A) CD4$^+$CD62L$^+$ T-cells were purified from naïve mice and stimulated with 1 □g/ml Con A on either wt or MyD88ko DCs for 36 hours before measurement of ^{3}H-thymidine incorporation. Each value represents mean cpm values of four different culture wells. One out of several representative experiments is shown.

vitro blocking of TNF-alpha markedly reduced Con A mediated proliferative responses of naïve wild-type CD4$^+$ T-cells in the presence of wt DCs to the levels observed in the presence of MyD88ko DCs (Figure 7C). On the other hand, addition of recombinant mouse TNF-alpha restored Con A induced proliferative responses of naïve CD4$^+$ T-cells in the presence of MyD88ko DCs (Figure 7C). Taken together, MyD88ko primary DCs are defective in their capacity to prime naive T-cell responses. Impaired early TNF-alpha release by DCs lacking MyD88 might explain impaired T-cell priming.

MyHC-alpha loaded activated DCs restore myocarditis in MyD88ko mice

If myocarditis resistance of MyD88ko mice indeed results from the reduced priming capacity of MyD88ko antigen presenting DCs in the peripheral compartments, administration of activated MyHC-alpha loaded wt antigen presenting cells would restore CD4$^+$ T-cell expansion and myocarditis susceptibility of MyD88ko mice. To test this hypothesis, we injected groups of MyD88ko and wt mice with mature, CD11c$^+$CD11b$^+$CD8α^-, MyHC-alpha loaded wt DCs and compared disease susceptibility and the expansion of autoreactive

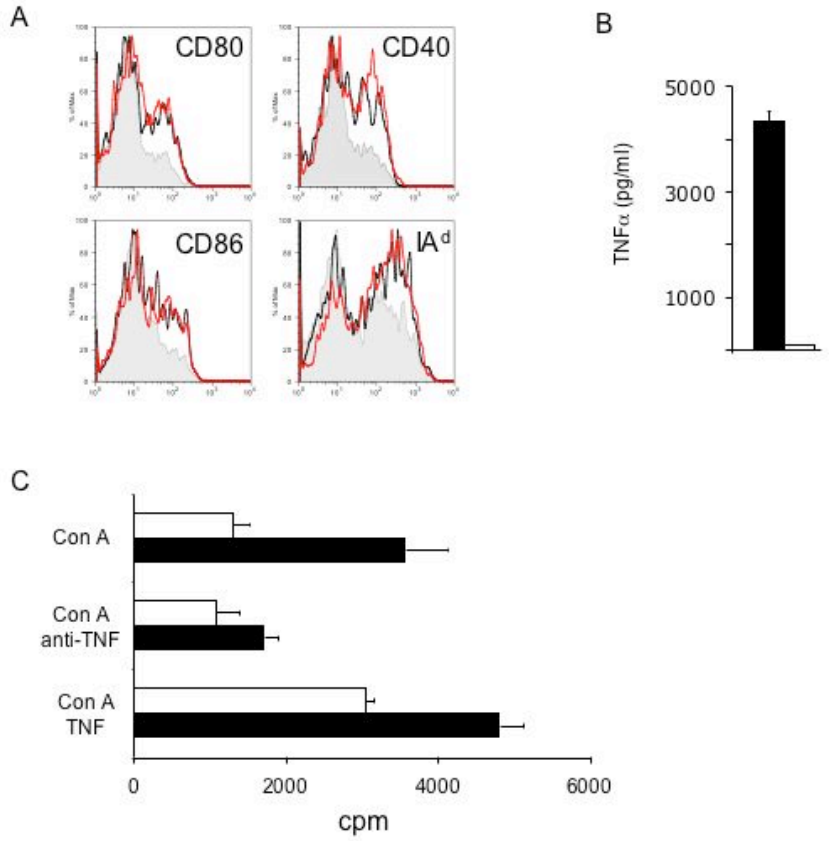


Figure 7

Functional capacity of MyD88ko vs. wt dendritic cells

(A) FACS analysis of MHC class II and costimulatory molecule upregulation on wt (black line) vs. MyD88ko (red line) DCs from draining lymph nodes after subcutaneous immunization with MyHC/CFA. Filled histograms represent control staining. Draining lymph nodes were removed 24 hours after immunization and cell suspensions were immediately stained with the appropriate antibodies. Histograms were gated on CD11c$^+$ live cells.

(B) TNF-alpha production of primary wt (black bars) vs. MyD88ko (white bars) DCs during 4 hours stimulation with 0.1 □g/ml LPS.

(C) Primary responses of CD4$^+$CD62L T-cells in the presence of wt (black bars) compared to MyD88ko (white bars) DCs after stimulation with 1 □g/ml Con A, in the presence of either 100 □g/ml TNF-alpha antagonist or 250 U/ml recombinant murine TNF-alpha. ^{3}H-thymidine incorporation reflecting proliferation was measured and is expressed as mean +/- SD of cpm values of 4 different culture wells. One out of several representative experiments is shown.

$CD4^+$ T-cells in wt vs. MyD88ko mice. Indeed, $CD4^+$ T-cells isolated from both, MyD88ko and wt mice after DC immunization, resulted in comparable albeit only mild myopericarditis after adoptive transfer (Figure 8). Most importantly, however, both, MyD88ko and wt mice developed severe myocarditis of the same prevalence and severity after injection of activated, self-antigen loaded wt DCs (Figure 2B, Figure 2C and Table 2). We therefore conclude that autoimmune myocarditis resistance of MyD88ko mice indeed results from a priming defect on the level of antigen presenting cells. Furthermore, our findings prove that in the presence of activated and competent wt DCs, MyD88 deficiency does not affect the functional capacity of the lymphatic system to provide an environment that allows the generation of autoreactive T-cells and autoimmune heart disease.

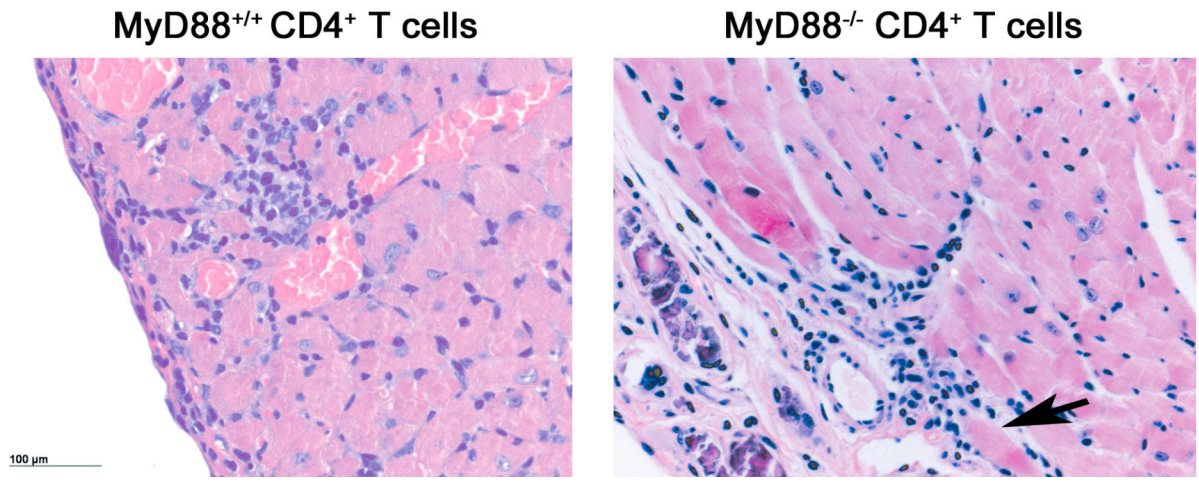


Figure 8

Myocarditis after adoptive transfer of myosin-specific wt and MyD88ko $CD4^+$ T-cells from dendritic cell immunized mice.

Wt and MyD88ko mice were immunized with in vitro *generated, activated and MyHC-alpha loaded wild-type dendritic cells. Then, $CD4^+$ T-cells were isolated from mice with myocarditis, expanded in FCS free medium, and injected into wt and MyD88ko recipients. Because of technical reasons both, wt and MyD88ko T-cells were much less pathogenic if isolated from DC immunized mice. Nevertheless, adoptive transfer still resulted in mild myopericarditis and there was no difference in the pathogenicity of wt and MyD88ko T-cells. Representative Hematoxylin and Eosin stained sections of grade 1 myopericarditis are shown. (200 x original magnifications)*

Discussion

In the present study we demonstrated that MyD88 signalling is essential for the stimulation of selfantigen presenting DCs to induce heart-specific $CD4^+$ T-cell responses in the peripheral compartments *in vivo*. In contrast, adoptive transfer of activated heart-specific autoreactive $CD4^+$ T-cells induced myocarditis in MyD88 deficient mice, suggesting that MyD88 signalling neither affects $CD4^+$ T-cell recruitment, nor accumulation of other inflammatory cells to the heart. More specifically, our findings provide a proof of principle that the lymphatic system of MyD88 deficient mice is fully competent to allow the development of autoimmune $CD4^+$ T-cell responses if it becomes substituted with appropriately activated self-antigen loaded antigen presenting cells.

In our experiments, we observed upregulation of costimulatory molecules on lymph node derived DCs after MyHC-CFA-immunization in both, MyD88ko and wt mice. These findings contrast the fact that the *in vivo* upregulation of costimulatory molecules is impaired in MyD88 deficient pulmonary DCs after Toll-like receptor activation (93). Obviously, depending on their mode of generation *in vitro* or resident location *in vivo*, DCs have different capacities to engage MyD88 dependent and MyD88 independent pathways in response to activation (93). On the other hand, lymph node derived DCs of MyD88ko mice showed markedly reduced production of proinflammatory cytokines, such as TNF-alpha or IL-12 after Toll-like receptor stimulation. However, even in the absence of exogenous Toll-like receptor stimulants we found an impaired capacity of MyD88ko DCs to promote Con A induced primary T-cell responses. This defect mainly resulted from a lack of TNF-alpha production in MyD88ko DCs and might reflect the fact that Con A directly mediates MyD88 dependent proinflammatory pathways. Otherwise the priming defect of MyD88ko DCs might result from impaired IL-1□eta mediated auto/paracrine activation of DCs because IL-1 type 1-receptor signalling involves MyD88 dependent proinflammatory cascades. Accordingly, it has recently been shown that IL-1 receptor type 1 deficient mice are protected from autoimmune myocarditis (1). The impaired capacity of MyD88 deficient DCs

to release specific cytokines certainly contributes to impaired T-cell priming and disease resistance in MyD88ko mice: TNF-alpha, IL-12p40, and IL-6 for example, are all essential for autoimmune myocarditis development (4, 5, 94).

Microbial products such as LPS acting on TLR4, CFA predominantly activating TLR2 and TLR4, or endogenous danger signals (95, 96) are critical for the capacity of antigen presenting cells to build up effective T-cell responses and to suppress regulatory T-cells (97, 98). Autoimmunity develops if TLR activation coincides release and uptake of self-antigen in lymphatic organs of genetically susceptible individuals (45). In the absence of TLR activation, uptake of selfantigen by dendritic cells is supposed to result in tolerogenic rather than autoaggressive T-cell responses (99-101). Based on our data, we cannot entirely exclude that the presence of tolerogenic T-cell populations in MyD88ko mice contributes to their myocarditis resistance. However, it was not possible to overcome disease resistance of MyD88ko mice by depletion of $CD25^+$ cells prior to immunization (Marty & Eriksson, unpublished).

Development of autoimmune myocarditis requires the recruitment of inflammatory cells to the heart (91, 92). Interestingly, systemic activation of the innate immune system with TLR stimuli, such as LPS results in upregulation of activation markers and MHC class II molecules on heart resident cells (91). Furthermore, LPS injection results in relapses of inflammatory infiltrates and more rapid progression of heart failure in immunized mice (34, 42, 45). In the context of CVB3 mediated myocarditis, treatments with both, LPS (102) and IL-1beta (103) enhances disease susceptibility of resistant mouse strains, most likely by activation of tissue resident dendritic cells. Based on these observations, one would expect that heart resident antigen presenting cells interact with autoreactive $CD4^+$ T-cells promoting their local expansion and the recruitment of other inflammatory cells such as macrophages, B cells, and granulocytes (34). Given the fact that MyD88 is a crucial common adaptor molecule mediating both, TLR and IL-1 type 1 receptor activation (86, 87), it was tempting to speculate that MyD88 signalling might also be essential for the recruitment and activation of heart infiltrating cells. Our data, however, clearly show that MyD88 signalling is

not required for the development of cardiac infiltrates in the presence of activated autoreactive T-cells. In fact, adoptive transfer of activated autoreactive T-cells induced myocarditis in both, wt and MyD88ko recipient mice. These findings argue for MyD88 independent mechanisms mediating the recruitment of inflammatory cells to the target organ in the presence of activated heart specific T-cells. Such mechanisms might include for example, MyD88 independent TLR signalling pathways (65).

Several studies suggested that the absence of MyD88 signalling on antigen presenting cells promotes default Th2 mediated immune responses in the presence of innate activation (104). Therefore, the question arises whether a possible Th1 to Th2 shift in MyD88ko mice might affect myocarditis susceptibility after MyHC-CFA immunization. In fact, our data show markedly impaired production of the Th1 cytokine IFN-gamma in MyD88ko compared to wt $CD4^+$ T-cells. Production of the Th2 cytokine IL-4, however, was uniformly low in both, wt and MyD88ko $CD4^+$ T-cells, suggesting that the failure to produce IFN-gamma rather reflects impaired expansion of heart-reactive $CD4^+$ T-cells than a relevant Th1 to Th2 shift in the MyD88ko mice.

In conclusion, we found a crucial role for MyD88 in rendering antigen-presenting cells capable of priming heart specific autoreactive T-cells. In addition, we provide first and direct evidence that the absence of MyD88 signalling in the lymphatic microenvironment of MyD88ko mice does not affect the generation of autoimmune heart disease if they become substituted with functional and activated wt DCs. From the clinical point of view, our findings suggest that treatment strategies targeting MyD88 signalling might contribute to the development of novel preventive and vaccination strategies that block the development of heart specific autoimmunity in the presence of a strong systemic inflammatory response and self-antigen release following cardiac injury.

Conflict of interest disclosure

None

Acknowledgments

The authors gratefully thank Regine Landmann and Stephanie Goulet for critical reading of the manuscript. Supported by the Novartis Foundation and the Swiss Society for Internal Medicine. U.E. holds a Swiss National Foundation professorship for Internal Medicine and Critical Care Medicine.

THE ROLE OF MYD88 IN THE PROGRESSION FROM AUTOIMMUNE MYOCARDITIS TO HEART FAILURE

Introduction

In the first part of my thesis I addressed the role of MyD88 in autoimmune myocarditis induction. I described that after bmDC immunization, MyD88 deficient and wild-type mice developed autoimmune myocarditis of the same severity and prevalence. Both, wt and MyD88 deficient mice showed comparable inflammation and damage in the heart. Based on this phenotype I will now analyze the role of MyD88 in the progression of inflammatory heart failure.

TLR signalling and heart failure

Toll-like Receptors recognize invariant patterns shared by groups of microorganisms (14). However, the “Danger-Model” of immunity suggested by Polly Matzinger proposes that cell damage, rather than foreignness, is what initiates an immune response (95). This concept implicates that stress responses and cell damage due to tissue injury of any cause might activate the innate immune system even in the absence of infections. TLRs and its signalling components are also expressed within the heart (74). It is therefore likely that endogenous ligands secreted by damaged or stressed cells within the heart activate innate immune reactions through TLRs.

Recent findings from myocardial infarction studies in mice show a role for TLR4 and TLR2 in the remodelling process after myocardial injury (76, 77). Remodelling is a very complex process that induces changes in cardiac shape, size and composition in response to myocardial injury (80). It was shown that TLR2 deficient and TLR4 deficient mice show higher survival, reduced inflammation, reduced myocardial fibrosis and improved heart function after myocardial infarction compared to control mice (76, 77). The exact mechanism of how TLR signalling influences cardiac remodelling and survival remain elusive. It is suggested that endogenous TLR ligands in the heart might contribute to the remodelling and the pathogenic process of heart failure (77).

TLR stimulation could also contribute to heart failure development by induction of proinflammatory cytokine secretion in the heart (12). Proinflammatory cytokines

are believed to be key players in the induction and progression of heart failure (79). The “cytokine hypothesis” for heart failure suggests that heart failure progresses as a result of the toxic effects exerted by endogenous cytokine cascades on the heart and peripheral circulation (80). Cytokines identified to contribute to heart failure are, amongst others, TNF-alpha, IL-6 and IL-1, namely all members of the innate immune system (78).

Interestingly, the IL-1 Receptor belongs to the same receptor superfamily as the Toll-like Receptors. TLRs and IL-1R have a conserved region in their cytoplasmic tails, which is known as the Toll/IL-1R (TIR) domain (2, 14) (Figure 9). Stimulation of TLRs triggers the association of MyD88 that further transmissions signalling. MyD88 has been shown to be essential for the production of proinflammatory cytokines upon TLR stimulation (62). We therefore identified MyD88 as an interesting player in the process of cardiac remodelling. To address the role of MyD88 in the development of autoimmune heart failure, we assessed heart function of mice that genetically lack the MyD88 signalling molecule after bmDC induced heart failure.

Here we describe for the first time, that MyD88 dependent proinflammatory cytokine induction and TLR signalling within the heart is involved in heart failure development.

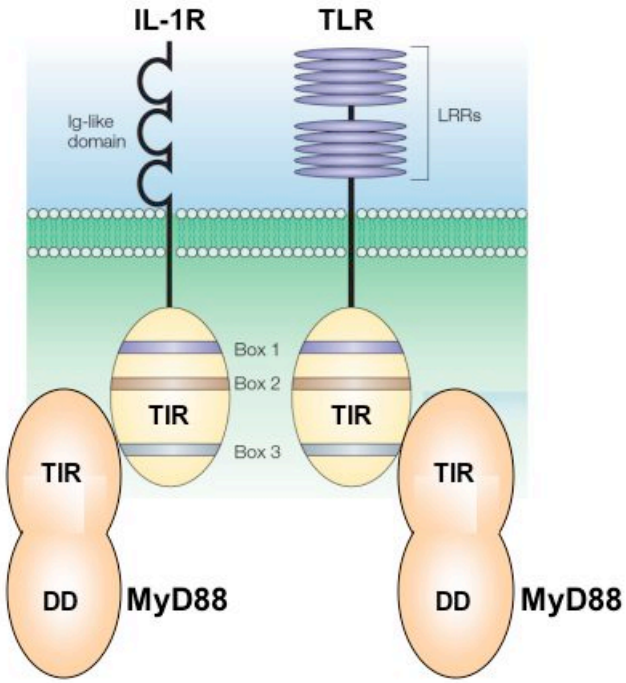


Figure 9: TLR/IL-1R structure homology

Toll-like receptors (TLRs) and interleukin-1 receptors (IL-1Rs) have a conserved cytoplasmic domain that is known as the Toll/IL-1R (TIR) domain. The TIR domain is characterized by the presence of three highly homologous regions (known as boxes 1, 2 and 3). Despite the similarity of the cytoplasmic domains of these molecules, their extracellular regions differ markedly: TLRs have tandem repeats of leucine-rich regions (known as leucine rich repeats, LRR), whereas IL-1Rs have three immunoglobulin (Ig)- like domains. In TLR/IL1R signalling, MyD88 couples receptor signals via TIR domain-TIR domain interactions and transmits down-stream signalling through its death-domain (DD). Adapted from (14)

Results

After wt bmDC immunization, wt mice and MyD88ko mice develop autoimmune myocarditis of the same severity and prevalence (Figure 10) (105). This characteristic phenotype allows us to further assess the role of MyD88 in the progression from autoimmune myocarditis to heart failure. In the model of bmDC induced autoimmune myocarditis, inflammation peaks at day 5 – 10 after immunization and starts to resolve at day 12. After resolution of inflammatory infiltrates in the heart, BALB/c mice develop heart failure (45).

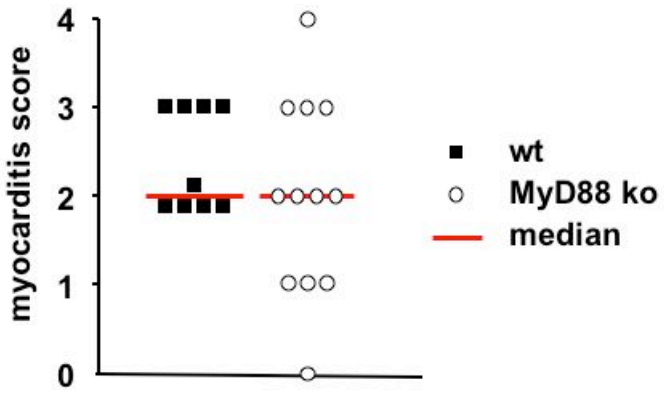


Figure 10: wt bmDC immunization induces comparable myocarditis severity in wt and MyD88ko mice

Myocarditis severity scores of individual wt and MyD88ko mice, immunized with MyHC-alpha loaded LPS/aCD40 activated wt bmDCs at day 0 and 2. Day 10 after immunization, hearts were removed and examined for myocarditis severity. MyD88ko and wt mice developed myocarditis of the same severity and prevalence.

Proinflammatory cytokine expression in the heart during autoimmune myocarditis

Cardiac remodelling is a very complex mechanism that occurs in different cardiac diseases including myocardial infarction and heart failure (79, 80).

Important parameters that contribute to the development of heart failure are proinflammatory cytokines. Among the cytokines described to be important in the

remodelling process are IL-1beta, IL-6 and TNF-alpha. Interestingly, all these cytokines have also been described to be essential for the induction of autoimmune myocarditis (1, 4, 5, 105). We therefore decided to measure proinflammatory cytokine levels in the heart at the peak of inflammation. Early differences in cytokine expression in the heart are likely to influence further development of heart failure (6).

Wild-type and MyD88ko mice were immunized with MyHC-alpha loaded LPS/aCD40 activated bmDCs at day 0 and day 2. Hearts were removed at day 10 after first immunization and mRNA was isolated. IL-1beta, TNF-alpha, IL-6 and IL-18 cytokine expression was analyzed by RT-PCR and expression was normalized to beta-Actin (Figure 11). We found significantly higher levels of IL-1beta expression in wt immunized mice when compared to MyD88ko immunized mice or wt control mice. There is a trend of elevated IL-6 and TNF-alpha expression in immunized wt mice, but this it is not statistically significant.

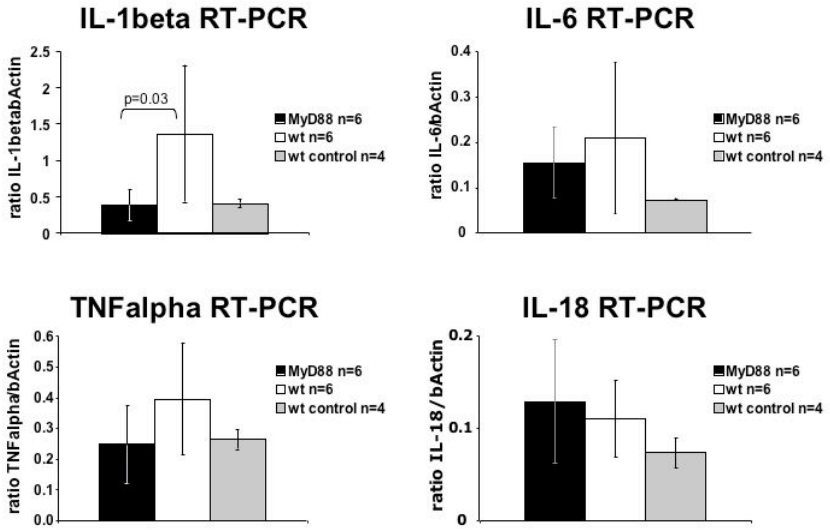


Figure 11: cardiac expression of IL-1beta, IL-6, TNF-alpha and IL-18 after bmDC immunization in wt and MyD88ko mice

RT-PCRs detecting IL-1beta, IL-6, TNF-alpha and IL-18 from heart mRNA. Wild-type and MyD88ko hearts were compared 10 days after bmDC immunization with healthy control hearts. A statistically significant increase of IL-1beta in wt immunized hearts could be detected. There is a trend of elevated IL-6 and TNF-alpha expression in immunized wt mice when compared to immunized MyD88ko mice and healthy control hearts, but not statistically significant. IL-18 expression was expressed at low levels only in all three groups.

MyD88 deficiency protects from progression of proinflammatory heart failure

To assess the development of heart function after autoimmune myocarditis we performed echocardiographic heart function measurements following post myocardial inflammation.

Heart function was measured using echocardiography of the left ventricle (Figure 12). Heart function is represented by two parameters named fractional shortening (FS) and velocity of circumferential fibre shortening (Vcf). Both parameters are derived from differences between end-diastolic left-ventricular diameter (EDD) and end-systolic left-ventricular diameter (ESD).

FS and Vcf were measured 120 days after bmDC immunization in wt and MyD88ko mice and compared to age matched untreated wt and MyD88ko control mice. As a consequence of autoimmune myocarditis, wild-type mice developed heart failure and showed reduced FS and Vcf compared to age matched control mice. Interestingly, MyD88ko mice are protected from heart failure development after autoimmune myocarditis (Figure 13A and 13B).

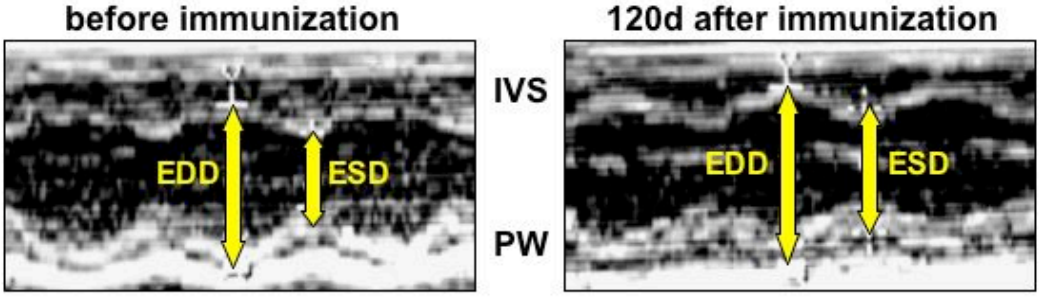


Figure 12: Echocardiographic analysis of heart function in mice before and after wt bmDC immunization in wt mice

*Exemplified pictures from echocardiographic heart function analysis before (left panel) and after bmDC immunization (right panel) in wt mice. Abbreviations**: EDD**: end-diastolic left-ventricular diameter; **ESD**: end-systolic left-ventricular diameter**; IVS**: intra-ventricular septum - **PW**: left-ventricular posterior wall.*

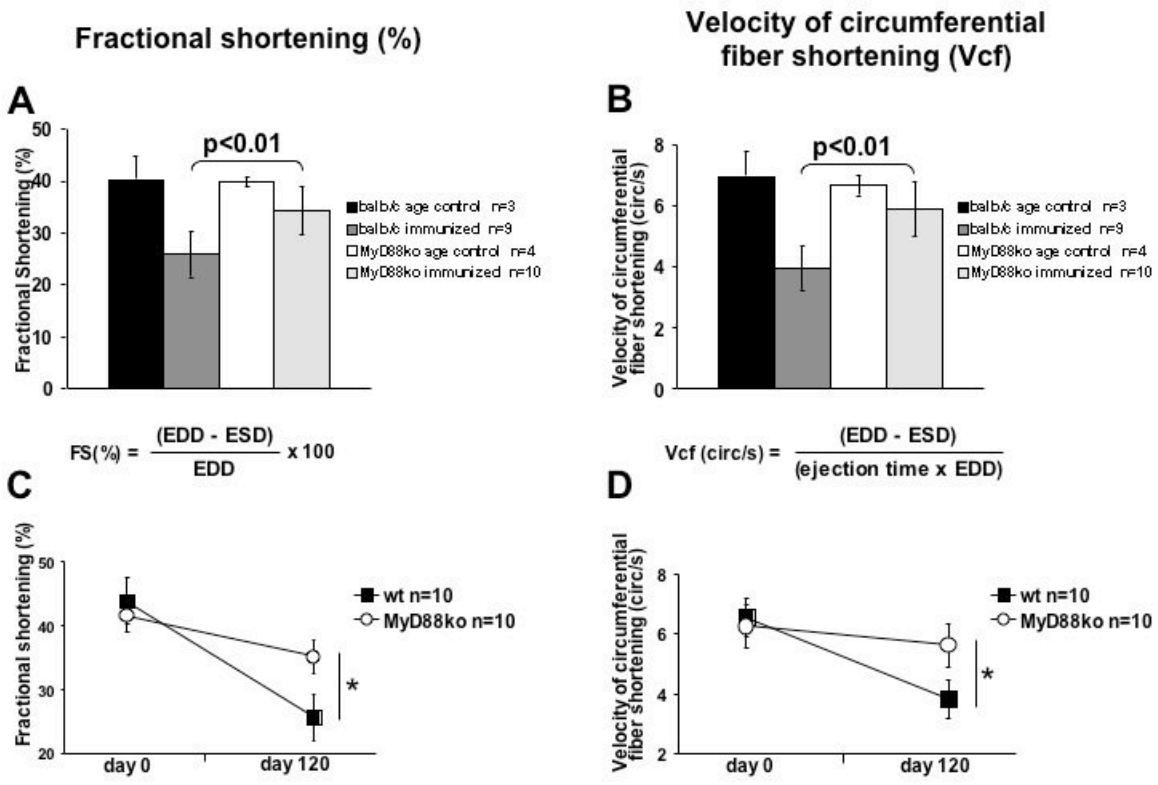


Figure 13: MyD88ko mice are protected from heart failure after autoimmune myocarditis

A) Analysis of fractional shortening (FS) 120 days after immunization in wt (dark grey bars) and MyD88ko (light grey bars) mice compared to untreated age-matched wt (black bars) and MyD88ko (white bars) control mice. MyD88ko immunized mice show a statistically significant increase of FS when compared to wt immunized mice.

B) Analysis of velocity of circumferential fiber shortening (Vcf) 120 days after immunization in wt and MyD88ko mice when compared to untreated age-matched wt and MyD88ko control mice. MyD88ko immunized mice show a statistically significant increase of Vcf when compared to wt immunized mice.

C) Analysis of FS in wt (filled squares) and MyD88ko (open circles) mice before and 120 days after wt bmDC immunization. No difference in FS was observed at day 0 before immunization between wt and MyD88ko mice. However, at day 120 after immunization, MyD88ko immunized mice show a statistically significant increased FS when compared to wt immunized mice.

*D) Analysis of Vcf in wt and MyD88ko mice before and 120 days after wt bmDC immunization. No difference in Vcf was observed at day 0 before immunization between wt and MyD88ko mice. However, at day 120 after immunization, MyD88ko immunized mice show a statistically significant increased Vcf when compared to wt immunized mice. * p<0.01*

To further exclude pre-existing intrinsic differences in heart function between wt and MyD88ko hearts, we measured heart function in wt and MyD88ko mice before and after bmDC immunization. At day 0, before immunization, wt and MyD88ko mice showed comparable heart function. We can therefore exclude intrinsic differences in heart function between wt and MyD88 mice. At day 120 after bmDC immunization, wt mice show reduced heart function when compared to MyD88ko mice (Figure 13C and 13D).

No differences in heart weight / body weight ratio or cardiomyocyte diameter between wt and MyD88ko mice after bmDC immunization

Heart failure is amongst others characterized by increased heart weight to body weight ratio (HW/BW) and increased diameter of cardiomyocytes that describes a dilated, hypertrophic and functionally impaired status of the heart. To further describe the protection of MyD88ko mice from heart failure we measured HW/BW ratio and cardiomyocytes diameter 120 days after bmDC immunization. To our surprise, the HW/BW ratio and cardiomyocyte diameter measurements do not reflect the differences observed between wt and MyD88 heart functions in echocardiographical measurements. There was no significant difference in HW/BW ratio or cardiomyocytes diameter between wt and MyD88ko mice (Figure 14A-C).

Autoantibodies and T-cell response in late stage myocarditis

In human myocarditis patients, autoantibodies against the heart are a key characteristic for an ongoing autoimmune response (34). We therefore measured autoantibodies against cardiac myosin 120 days after bmDC immunization in wt and MyD88ko mice. Only 2 out of 10 wt and 1 out of 10 MyD88ko mice showed high titers of IgG-total autoantibodies against myosin, most wt and MyD88ko mice show only low-level antibodies against the heart. Altogether, there is no significant difference in heart specific autoantibody response in the tested groups of 10 wt and 10 MyD88ko mice (Figure 15A and 15B). These results indicate that there is no pronounced ongoing humoral immune response against the heart 120

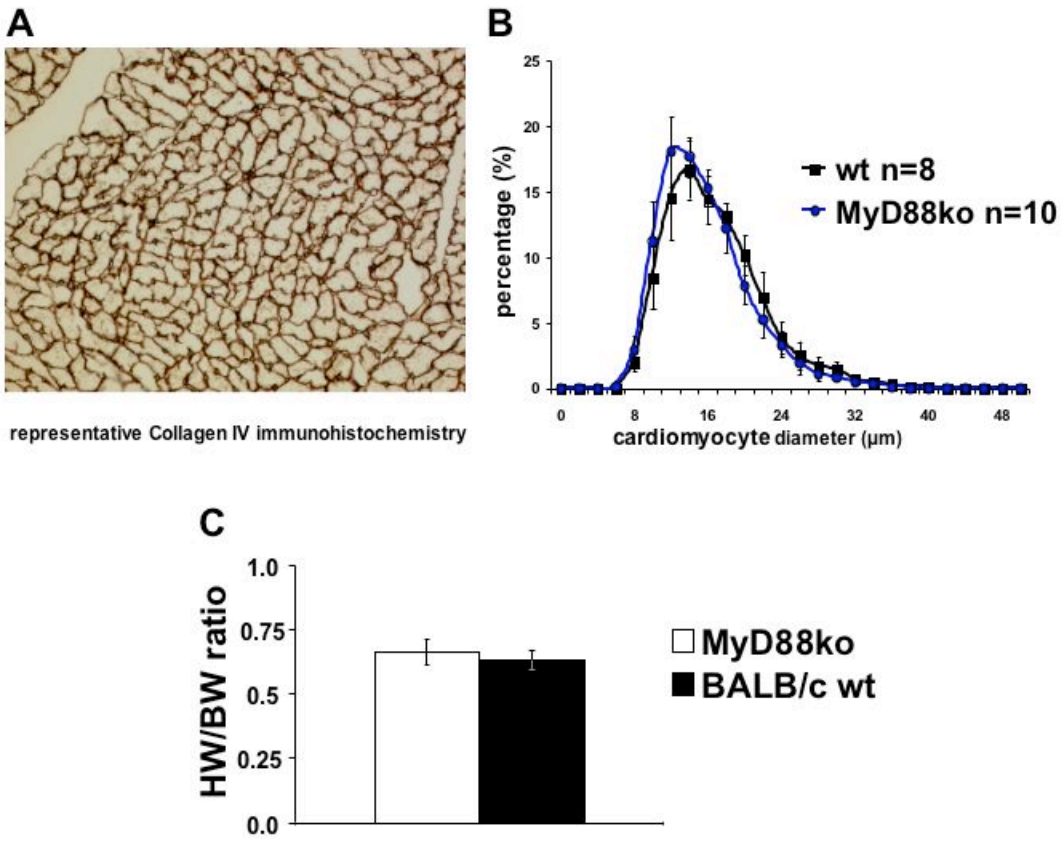


Figure 14: No differences in cardiomyocytes diameter or heart weight / body weight ratio between wt and MyD88ko mice after bmDC immunization

A) Representative picture from Collagen IV immunohistochemistry heart staining. wt and MyD88ko mice were immunized with MyHC-alpha loaded LPS/aCD40 activated wt bmDCs at day 0 and 2. 120 days after immunization, hearts were removed and stained for Collagen IV.

B) Hearts were analyzed for cardiomyocytes diameter: 10 pictures from Collagen IV immunohistochemistry were taken per heart and analyzed with "analySIS Image Processing" software for cardiomyocyte diameter. No significant difference was observed in cardiomyocyte diameter between wt and MyD88ko mice.

C) Heart weight / body weight (HW/BS) ratio was assessed 120 days after wt bmDC immunization in wt and MyD88ko mice. No difference was observed in HW/BW ration between wt and MyD88ko mice 120 days after bmDC immunization.

days after immunization. To further characterize ongoing T-cell immune response in wt and MyD88ko mice at different time points after bmDC immunization, we measured whole splenocytes proliferation response against MyHC-alpha peptide at day 10, 20 and 120 after bmDC immunization. Positive proliferation response was detected in both wt and MyD88ko mice at all measured time points (Figure 15C-E). We therefore conclude that the Myd88ko mice have comparable humoral and cellular immune response to MyHC-alpha peptide when immunized with wt bmDCs. These findings suggest that differences in the ongoing T-cell and B-cell response are not responsible for observed differences in heart function of MyD88 and wt mice.

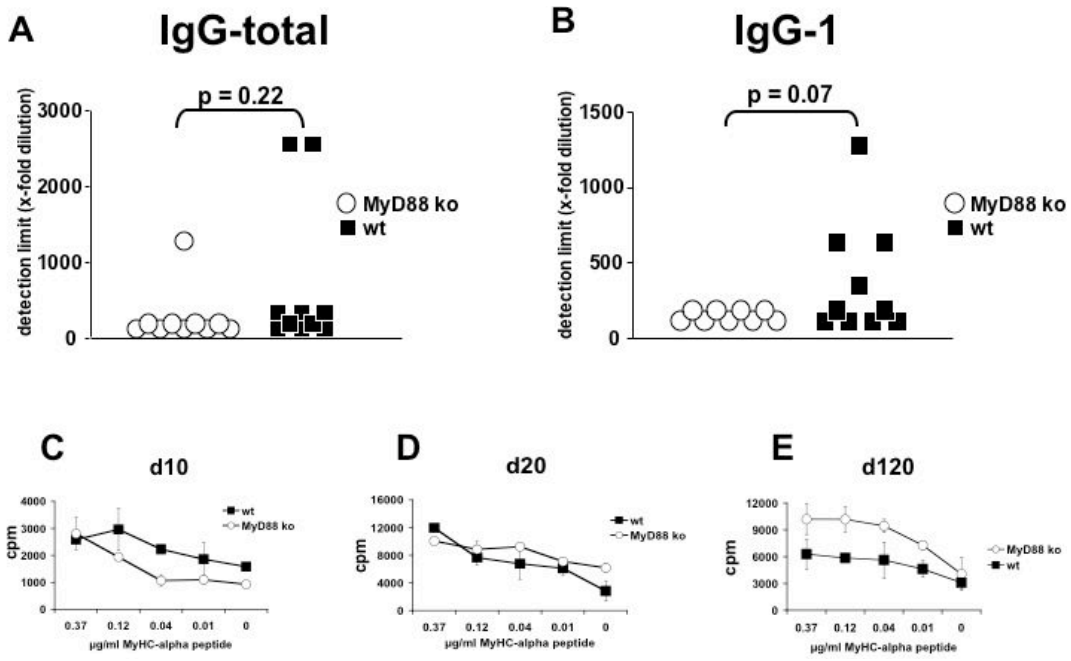


Figure 15: Humoral and cellular immune response characterization in wt and MyD88ko after bmDC immunization

A) Comparable humoral IgG-total response in wt (filled squares) and MyD88 (open circles) mice after bmDC immunization. Serum was collected 120 days after bmDC immunization from wt and MyD88ko mice and assessed for anti-myosin IgG-total antibodies.

B) Trend to increased IgG-1 subclass response in wt mice when compared to MyD88ko mice after bmDC immunization. Serum was collected 120 days after bmDC immunization from wt and MyD88ko mice and assessed for anti-myosin IgG-1 antibodies.

C - E) Whole splenocytes MyHC-alpha peptide restimulation in vitro from wt and MyD88ko mice day 10, day 20 and day 120 after bmDC immunization. Curves shown represent individual mice.

bmDC/CFA double immunization in wt and MyD88ko mice

Due to the time-consuming experiments of heart failure development, we recently created an immunization method combining bmDC and CFA immunization, called “double immunization”, where wild-type BALB/c mice develop severe myocarditis, strong fibrosis and heart dilation at day 30 after first immunization. In general, the double immunization dramatically shortens the experiment length and leads to a more pronounced heart failure phenotype when compared to bmDC immunization only. This shorter length of the experiment will allow us investigate the interplay between autoimmune inflammation and fibrosis that lead to the development of heart failure more efficiently.

We therefore decided to evaluate the myocarditis and fibrosis development in MyD88ko mice as a consequence of the novel bmDC/CFA double immunization protocol. We found that MyD88ko mice developed similar myocarditis severity at day 30 after first immunization when compared to wt mice (Figure 16A-D and 16I). These data support our previous findings that MyD88ko mice have similar myocarditis prevalence and severity day 10 after bmDC immunization (Figure 10) (105). However, when assessed for fibrosis development by CAB staining, MyD88ko mice showed significantly reduced fibrosis in the heart when compared to wt mice (Figure 16E-H and 16J). Also in the measured HW/BW ratio assessed at day 30 after first immunization, wt mice have increased HW/BW ratio when compared to MyD88ko mice, implying an impaired heart function and advanced progression of heart failure.

Figure 16A-D

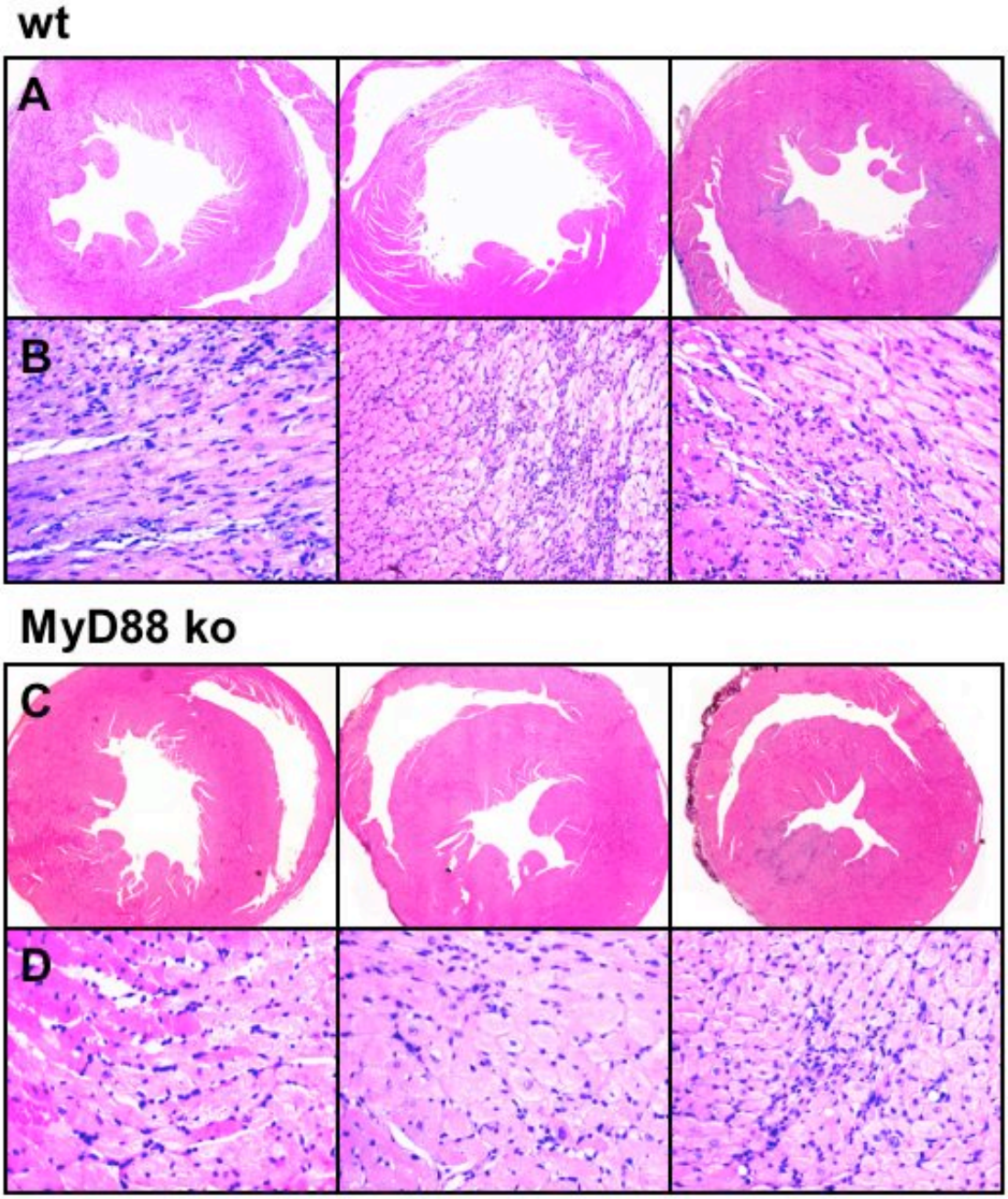
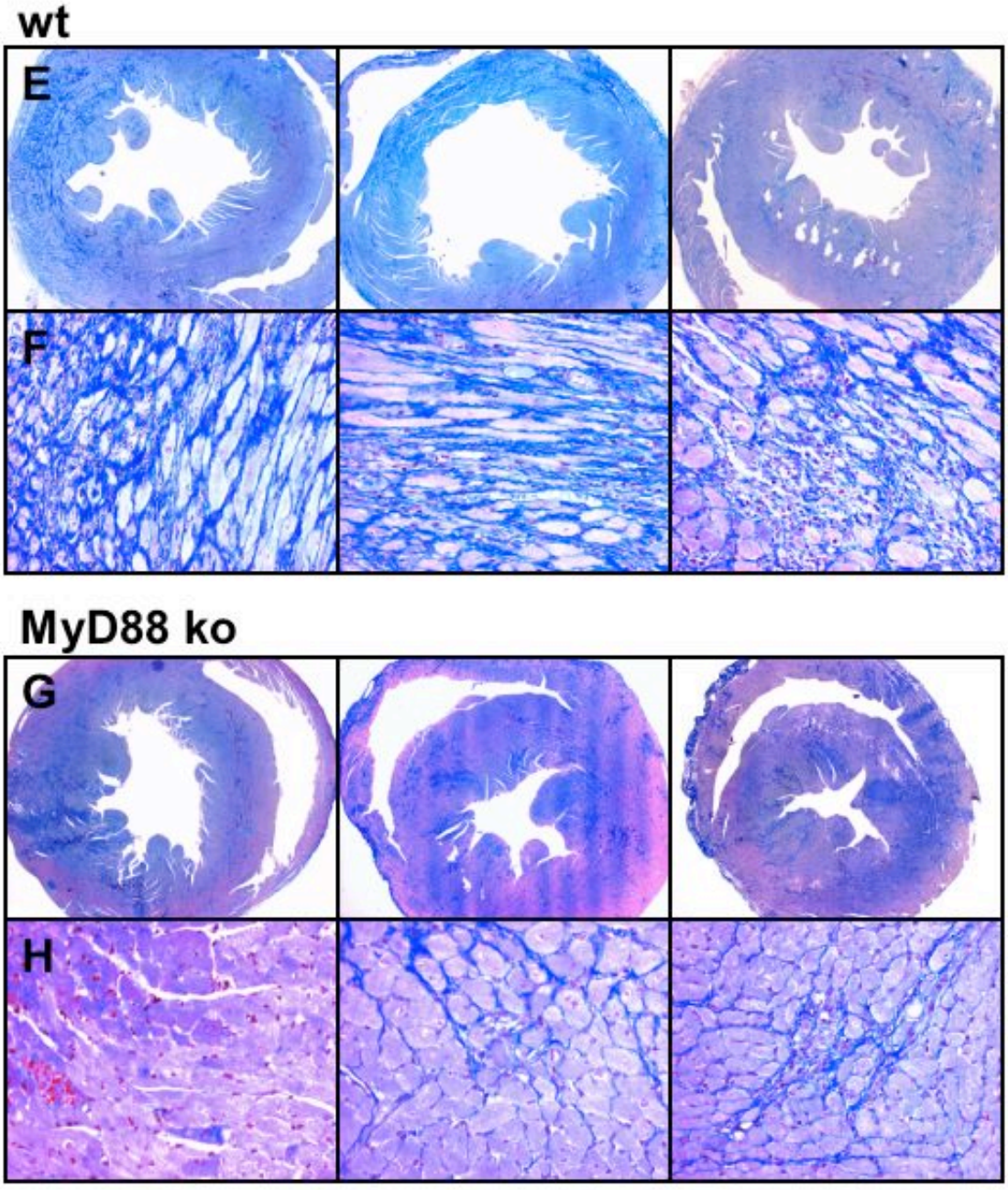


Figure 16E-H



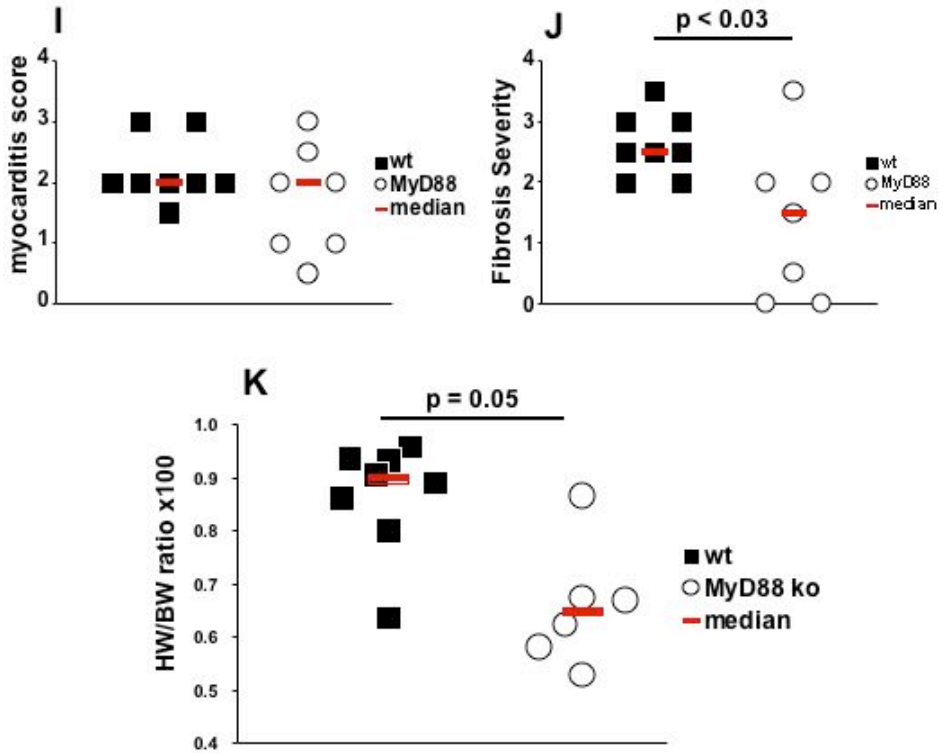


Figure 16: bmDC/CFA double immunization in wt and MyD88ko mice

wt and MyD88ko mice were immunized with MyHC-alpha loaded LPS/aCD40 activated bmDCs at day 0 and 2, followed by additional immunization with MyHC-CFA at day 10 and 17. Mice were sacrificed at day 30 and assessed for leukocyte infiltration by HE staining, Collagen deposition by CAB staining and heart weight-body weight ratio.

A and B) representative pictures from three individual HE-stained wt heart sections, 25x or 400x magnifications depticted.

C and D) representative pictures from three individual HE-stained MyD88ko heart sections, 25x or 400x magnifications depicted.

MyD88ko mice show decreased fibrosis at day 30 after first immunization (E-H and J).

E and F) representative pictures from three individual CAB-stained wt heart sections, 25x or 400x magnifications depicted.

G and H) representative pictures from three individual CAB-stained wt heart sections, 25x or 400x magnifications depicted.

I) wt and MyD88ko mice were assessed for myocarditis severity 30 days after bmDC/CFA double immunization. No significant differences in myocarditis severity observed.

J) wt and MyD88ko mice were assessed for fibrosis severity 30 days after bmDC/CFA double immunization. MyD88ko mice show significant decreased fibrosis when compared to wt mice.

K) MyD88 show decreased HW/BW ratio when compared to wt mice. HW/BW ratio was assessed 30 days after first immunization

Discussion

In the past few years it became apparent that Toll-like Receptor signalling may be involved in the development of heart failure (12). The “innate danger” model describes the induction of proinflammatory mediators in the heart due to TLR signalling. It is suggested that the heart possesses a germ-line encoded innate stress response. Ligands for TLRs in the heart without present infection might be derived from tissue injury including oxidative stress, stretch, ischemia/reperfusion, and neurohormonal activation (Figure 17) (12). In the present study we contribute to the ongoing debate about the role of TLR signalling in heart failure development (80). In previous studies we could show that mice deficient for the TLR adapter molecule MyD88 are protected from EAM when immunized with MyHC-CFA. However, when directly immunized with MyHC-loaded bmDCs, MyD88ko mice develop EAM with comparable prevalence and severity as wt mice (105).

We here show, for the first time, that mice deficient for MyD88 are protected from heart failure development after initial autoimmune myocarditis. In functional assessment of heart function via echocardiography, MyD88ko mice show increased heart function after initial autoimmune myocarditis when compared to wt mice. To address the mechanisms underlying the protection in MyD88ko mice, we analyzed the immune response against the heart at different time points after bmDC immunization. We describe that MyD88ko and wt mice have comparable autoaggressive cellular and humoral immune response against the heart after MyHC-loaded bmDC immunization. These findings suggest that the ongoing chronic immune response might not be responsible for the observed differences in heart function and development of heart failure. On the other hand, despite the comparable T-cell proliferation response, further investigations about the T_H-phenotype in late autoimmune myocarditis and heart failure in wt and MyD88ko mice is necessary. It was recently shown, that the newly described T_H17 phenotype is essential for the development of autoimmune myocarditis (106, 107). The role of IL-17 in the progression of heart failure, however, remains elusive.

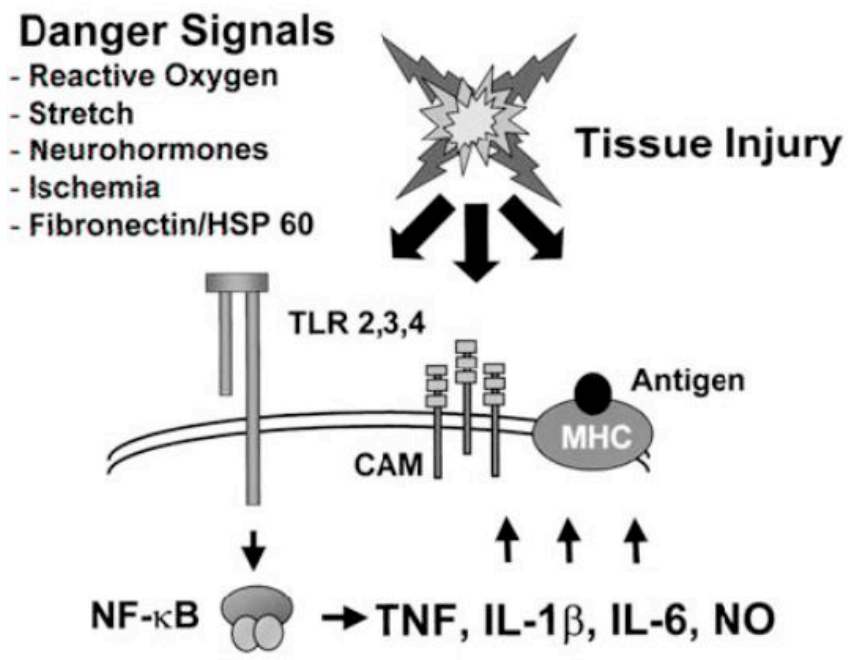


Figure 17: Proposed model for innate immune responses in the heart. *Inflammatory mediators such as tumour necrosis factor (TNF), interleukin 1 (IL-1), IL-6, and nitric oxide (NO) are expressed in the heart in response to "danger signals" that arise from diverse forms of tissue injury, including oxidative stress, stretch, ischemia/reperfusion, and neurohormonal activation. In addition, molecules released by stressed cells (e.g., heat shock protein 60 (HSP60) and fibronectin) are capable of eliciting inflammatory responses by binding to Toll-like receptor 4 (TLR4). Once expressed, the inflammatory mediators can exert direct effects on target cells by binding to their cognate receptors, or they can activate components of the adaptive immune system through antigen presentation or through upregulation of cell adhesion molecules (CAM) that attract neutrophils and monocytes. Adapted from (12)*

Therefore, it cannot completely be ruled out that memory immune responses contribute to the development of heart failure. More detailed investigations on memory immune responses during heart failure development are necessary. For example, TLR signalling has been described to be essential for the generation of $CD4^+$ T-cell memory, but not for the activation of memory $CD4^+$ T-cells (108). In contrast, memory B-cell express TLRs and can be directly activated through TLR stimulation (109).

We also investigated the expression of proinflammatory cytokines at the peak level of inflammation at day 10 after the first bmDC immunization. We

hypothesize according to the “cytokine hypothesis”, that the proinflammatory cytokine patterns expressed during acute inflammation might determine heart failure development. In the “cytokine hypothesis” of myocardial dysfunction, myocardial injury is associated with the elaboration of proinflammatory molecules by both immune effector cells, as well as cells intrinsic to the heart (6, 78). There is no single cytokine responsible for the development and progression of heart failure but rather a proinflammatory-cytokine cascade and their downstream effectors produce alterations in myocardial function. The induction of the proinflammatory cascade follows the production of three main cytokines, namely Interleukin-1, TNF-alpha and IL-6 (110).

The pathogenic role of IL-1 and IL-1R signalling in heart failure has been associated with increased IL-1beta expression in hearts of patients with idiopathic dilated cardiomyopathy (111). In addition, Il-1beta has also direct ionotrop/chronotrop effects on the heart (110, 112, 113). Furthermore, IL-1beta has been described to modulate adhesion molecule expression which is essential for the recruitment of proinflammatory cells to the heart (114).

We could show that wt mice significantly express higher amounts of IL-1beta in the heart during acute myocarditis. Interestingly, the IL-1 Receptor (IL-1R) uses the same signalling transduction pathways as the TLRs; both signal through the Toll/IL-1R adaptor molecule MyD88. Therefore, MyD88ko mice after autoimmune myocarditis induction not only have reduced IL-1 expression in the heart, but the remaining IL-1 levels are unnoticed due to the interrupted IL-1R signalling in MyD88ko mice. These findings lead to the hypothesis that IL-1R signalling plays an important role in the progression from autoimmune myocarditis to heart failure. Nevertheless, the ligands for TLR induced IL-1 expression in the heart remain unknown.

We further characterized myocarditis and fibrosis development in wt and MyD88ko mice with a novel immunization protocol combining bmDC- and CFA-immunization. This protocol leads to strong fibrosis development and dilation of wt hearts already at day 30 after first immunization. We describe that MyD88ko

mice have reduced cardiac fibrosis and improved HW/BW ratio when compared to wt mice.

In summary we could show that after initial autoimmune myocarditis, MyD88ko mice are protected from heart failure with increased heart function, reduced IL-1beta expression and reduced cardiac fibrosis. Our data strongly supports the theory that TLR signalling is involved in the development of heart failure. We believe that better understanding of TLR signalling pathways in the heart including the identification of endogenous TLR ligands may contribute to novel treatment strategies against autoimmune induced heart failure.

THE ROLE OF TYPE I INTERFERON RECEPTOR SIGNALLING IN EXPERIMENTAL AUTOIMMUNE MYOCARDITIS INDUCTION

Introduction

It is well-established that viral infections can cause autoimmunity (115). Diseases associated with viral induced autoimmunity are, amongst others, diabetes type I, myocarditis, thyroiditis and multiple sclerosis (20, 116, 117). However, the precise mechanisms of disease induction are not yet resolved (36, 118, 119).

The initiation of any host immune response against viral infections strongly depends on the expression of type I IFN (84). Type I IFN expression is initiated through pathogen interaction with toll-like receptors (TLR). Signal transduction mainly occurs via TLR3, TLR7, TLR8 and TLR9 signalling pathways (Figure 18) but TLR independent mechanisms can also lead to type I IFN expression (120). The type I IFN family consists of multiple members (α, β, ε, δ, κ, τ, ω and limitin), which are expressed by a variety of cell types and exert multiple immunomodulatory effects including stimulation of polyclonal T-cell responses, isotype switching, expression of class I major histocompatibility complex (MHC) molecules, and induction of dendritic cell differentiation (17, 121).

So far, there has been extensive research on the effect of type I IFN against viral infections in the heart (16, 23, 122-124). Kühl *et al.* showed in a phase II cohort study, that IFN-beta can be used to treat patients suffering from dilated cardiomyopathy with the presence of viral genomes in the heart (24). IFN-beta therapy has been shown to increase left ventricular function and to eliminate viral genomes in the heart (24). However, the role of type I IFN on the autoimmune properties of chronic myocarditis has not yet been investigated.

Type I IFN levels have been correlated with clinical manifestations of Systemic Lupus Erythematosus (SLE) (18) and Sjogren's syndrome (19). On the other hand, administration of IFN-beta1 has been shown to prevent progression of multiple sclerosis (125). In a recent publication addressing the role of TLRs in a transgenic mouse model of $CD8^+$ T-cell mediated autoimmune diabetes mellitus, it was shown that TLR activation induces IFN-alpha expression that further upregulates class I MHC expression initiating pancreas destruction (70). This

example highlights the importance of TLR induced type I IFN as immunomodulatory agents in the pathogenesis of autoimmune diseases.

For my thesis I am specifically interested in the role of the innate immune system in EAM development. In this context we further addressed the role of type I IFN signalling during EAM induction. Regarding the above-mentioned role of type I IFN in other inflammatory diseases, type I IFN signalling could both, protect from autoimmune myocarditis as well as aggravate the disease. In the context of human myocarditis, our research aims to contribute to the ongoing debate whether or not to treat chronic myocarditis patients with type I IFN.

To assess the role of type I IFN in the development of autoimmune heart disease, we examined the myocarditis susceptibility of mice that genetically lack the IFN type I receptor (IFN$\alpha\beta$Rko) (84). The multiple IFN-alpha/beta members share a ubiquitously expressed heterodimeric receptor composed of IFN-alpha-receptor subunit 1 (IFNAR1) and IFNAR2. Both receptor chains are required for signal transduction (121). The IFN$\alpha\beta$Rko mice genetically lack the IFNAR1 subunit and are therefore completely unresponsive to IFN-alpha and IFN-beta.

Here we describe for the first time that type I IFN signalling is critical in inducing heart-specific autoimmunity in the presence of autoreactive T-cell responses.

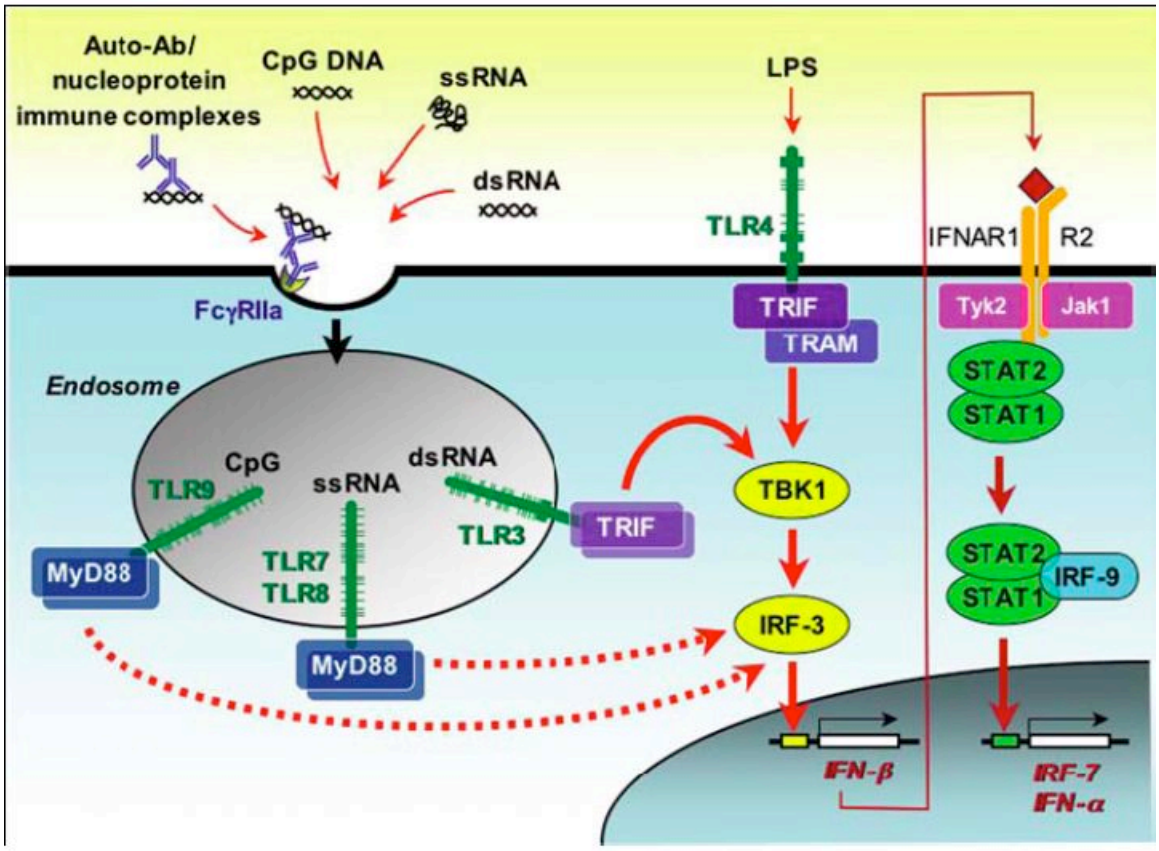


Figure 18:

TLRs engaged in IFN-alpha-beta production. Endogenous ligands, such as apoptotic/necrotic material and nucleoproteins complexed with autoantibodies, and exogenous ligands, such as bacterial lipopolysaccharide (LPS), bacterial hypomethylated CpG-DNA, and viral ssRNA or dsRNA, bind to the indicated TLRs on cell surfaces or in endosomal compartments. TLR signalling phosphorylates IRF-3 and initiates IFN-beta transcription. Subsequent signalling through IFN$\alpha\beta$R leads to IRF-7 and IFN-alpha expression. Adapted from (17)

Results

IFN-beta expression is upregulated in hearts with myocarditis

In a first experiment we induced autoimmune myocarditis in wt BALB/c mice using the dendritic cell immunization protocol (45). Dendritic cell immunization has certain advantages over the classical MyHC-CFA immunization (41). Dendritic cell immunization directly administers heart-specific peptide loaded antigen presenting cells that induce an organ specific autoimmune response against the heart without systemic inflammation or confounding TLR stimulation. It is therefore possible to measure changes in cytokine expression in the heart as a direct consequence of the autoimmune myocarditis response that is not falsified by a general inflammatory response to the *Mycobacterium tuberculosis H37Ra* present in the CFA.

We then measured IFN-alpha and IFN-beta expression in myocarditis hearts 9 days after wt MyHC-bmDC immunization. IFN-alpha expression could not be detected (not shown). IFN-beta expression is upregulated in five out of seven hearts from bmDC immunized wt mice compared to wt control hearts (Figure 19).

Interferon-alpha-beta receptor signalling is crucial for autoimmune myocarditis induction

To specifically address the role of type I IFN signalling in autoimmune myocarditis induction, we decided to assess the myocarditis phenotype in IFN$\alpha\beta$Rko mice. These mice do not express the IFN type I receptor and are therefore unresponsive to IFN-alpha as well as IFN-beta. To our surprise, we found that IFN$\alpha\beta$Rko mice are completely protected from wt bmDC immunization (Figure 20A & 20C). In the same experiment we further demonstrated that IFN$\alpha\beta$Rko splenocytes have a reduced *in vitro* proliferation response against MyHC-alpha peptide 10 days after bmDC immunization (Figure 20B).

In the model of bmDC induced autoimmune myocarditis in wt BALB/c mice, inflammation peaks at day 5 – 10 after immunization and starts to resolve at day

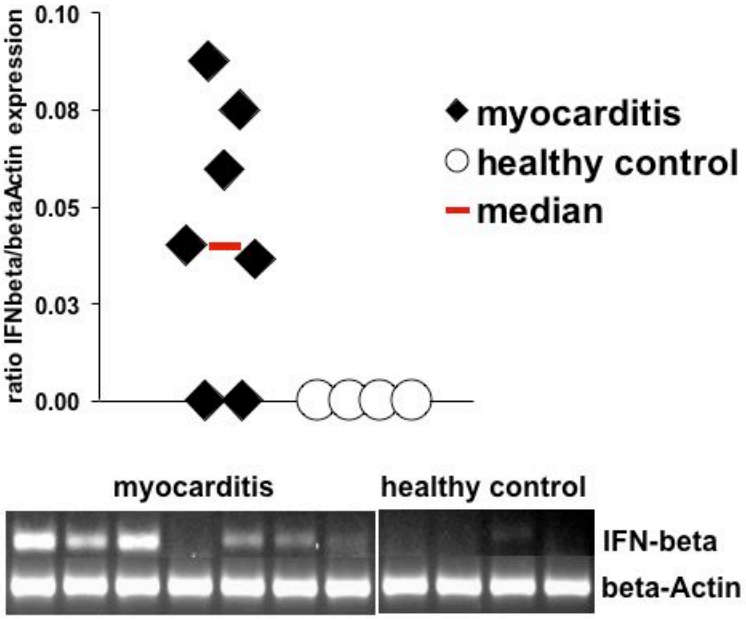


Figure 19: IFN-beta mRNA expression is upregulated in hearts with myocarditis

Myocarditis was induced by injecting MyHC-alpha loaded, LPS/aCD40 activated bmDCs at day 0 and 2. Hearts from immunized mice were isolated at day 9 and mRNA was extracted from immunized hearts (filled diamonds) as well as from naïve control hearts (open circles). IFN-beta expression was detected by RT-PCR. Myocarditis hearts express elevated levels of IFN-beta compared to healthy control hearts. IFN-beta expression levels were standardized against beta-Actin expression.

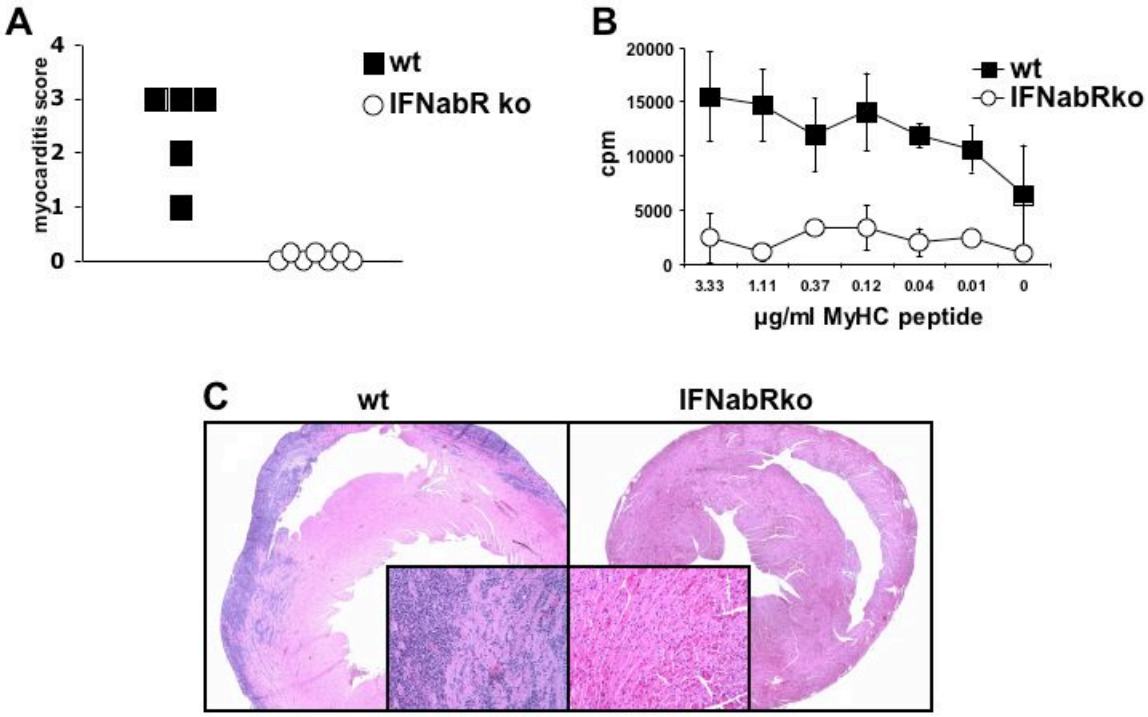


Figure 20: Protection from myocarditis in IFNαβRko mice after bmDC immunization

Myocarditis was induced by injecting MyHC-alpha loaded and LPS/aCD40 activated bmDCs at day 0 and 2. Wild-type bmDCs were injected into wild-type mice (filled squares) or in IFNαβRko mice (open circles).

A) After 10 days, hearts were scored histologically for inflammation. IFNαβRko mice were protected from bmDC induced myocarditis.

B) In the same experiment, spleens were removed and splenocytes were restimulated in vitro with MyHC-alpha at different concentrations and proliferation was assessed by 3H-Thymidine incorporation. IFNαβRko splenocytes showed reduced in vitro proliferation response against MyHC-alpha peptide 10 days after bmDC immunization when compared to wt mice.

C) Representative images of Hematoxilin & Eosin staining of wild-type and IFNαβRko heart sections. Original magnification 15x and 400x.

12 (45). Due to the reduced *in vitro* response of IFNαβRko splenocytes against MyHC-alpha peptide as shown in Figure 20B, we addressed the hypothesis that IFNαβRko mice might show delayed myocarditis development after wt bmDC immunization. We therefore tested myocarditis susceptibility of IFNαβRko mice 15 days after the first bmDC immunization. At day 15 post bmDC immunization, nine out of twelve IFNαβRko mice were completely protected from experimental autoimmune myocarditis (Figure 21A) and only two mice showed grade 1 myocarditis. We conclude that IFNαβRko mice are protected from bmDC induced myocarditis at every measured time point.

Interferon Type I Receptor deficient bmDCs induce myocarditis in wt mice

IFN type I signalling might influence priming of autoreactive T-cells not only on the level of T-cells but may also influence the antigen presenting cells directly. IFNαβR signalling modulates co-stimulation, cytokine secretion as well as migration and survival of dendritic cells *in vivo* (17, 126). To further address the role of type I IFN signalling on bmDCs during myocarditis induction we analyzed CD40, 80, 86 and IA-d surface molecules expression and TNF-alpha and IL-12p70 intracellular expression in IFNαβRko and wt bmDCs after *in vitro* LPS activation (Figure 21C-H). No major differences were found in surface molecule expression or cytokine production in INFabRko bmDCs compared to wt bmDCs. We then immunized wt mice with MyHC-alpha loaded IFNαβRko-bmDCs (Figure 21B). One out of five mice developed medium range myocarditis and three mice developed minimal myocarditis indicating that IFNαβRko bmDCs are able to induce autoimmune myocarditis although presumably at lower levels.

bmDC migration in wt and IFNαβRko mice

To further assess the mechanisms of protection in IFNαβRko mice after wt bmDC immunization, we tested bmDC migration capacity in wt and IFNαβRko mice. The reduction in T-cell priming in IFNαβRko mice could be explained by impaired migration of bmDCs in IFNαβRko mice to the site of T-cell priming. Our routine immunization pathway for bmDC immunization is intra-peritoneal

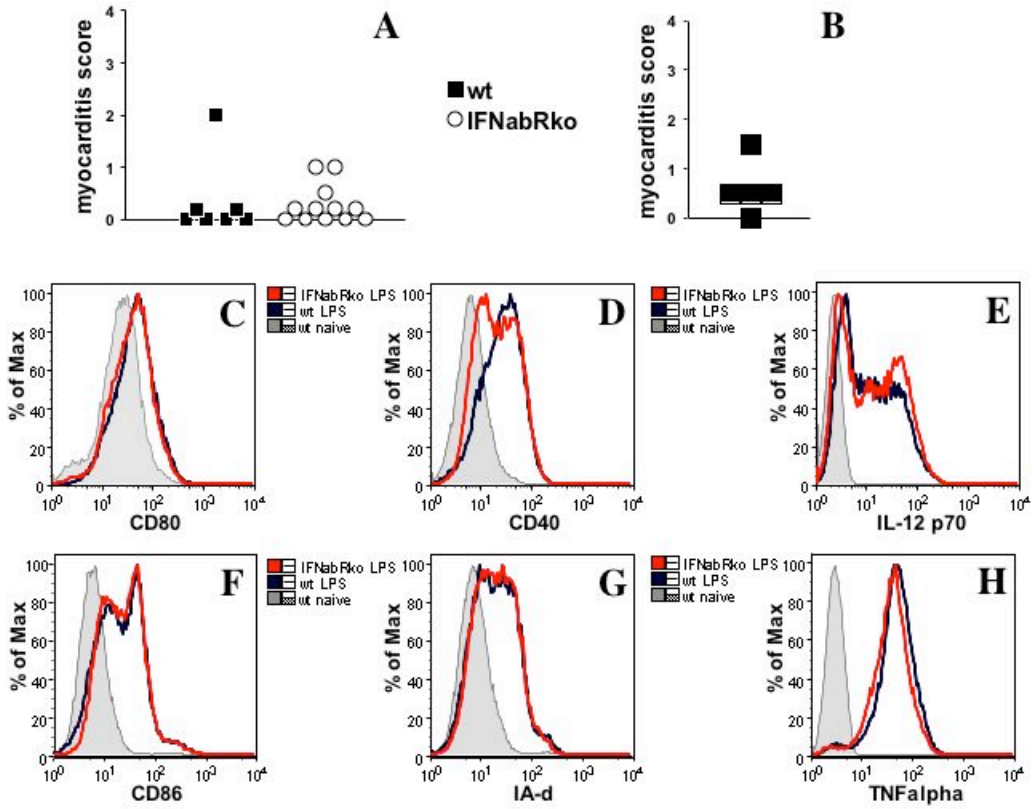


Figure 21:

A) Myocarditis score at late time point day 15 after bmDC immunization in wt and IFN$\alpha\beta$Rko mice. No significant differences observed between wt and IFN$\alpha\beta$Rko mice.

B) wt BALB/c mice were immunized at day 0 and 2 with 0.5 Mio MyHC-alpha loaded LPS/aCD40 activated IFN$\alpha\beta$Rko bmDC. Day 10 after first immunization, myocarditis severity was assessed. One out of five mice developed medium range myocarditis and three mice developed minimal myocarditis indicating that IFN$\alpha\beta$Rko bmDCs are able to induce autoimmune myocarditis although presumably at lower levels

C-H: FACS analysis of CD11positive in vitro generated bmDC before and after 4h LPS activation. Filled grey curve represents wt naïve bmDCs; black solid line represents LPS activated wt bmDCs; red solid line represents LPS activated IFN$\alpha\beta$Rko bmDCs.

C) Histogram of CD80 surface expression analysis on CD11c positive bmDCs

D) Histogram of CD40 surface expression analysis on CD11c positive bmDCs

E) Histogram of IL-12 p70 intracellular expression analysis on CD11c positive bmDCs

F) Histogram of CD86 surface expression analysis on CD11c positive bmDCs

G) Histogram of AI-d-MHC II surface expression analysis on CD11c positive bmDCs

H) Histogram of TNF-alpha intracellular expression analysis on CD11c positive bmDCs

injection. We know from previous experiments that bmDC migrate to the mesenteric lymph nodes and the spleen but not to the heart (45). Further experiments showed that the spleen is not necessary for autoimmune myocarditis development after bmDC immunization. Splenectomized wt BALB/c mice develop myocarditis after bmDC immunization (Figure 22A).

For migration assessment we transferred CFSE labelled wt CD11c positive bmDCs in wt and IFNαβRko mice intraperitoneal. Two days after transfer, we measured CFSE-positive CD11c-positive bmDCs in mesenteric lymph nodes (Figure 22B). Although reduced, there are comparable numbers of CFSE positive DCs in the lymph nodes of IFNαβRko mice and wt mice. Since priming of MyHC-specific T-cells in IFNαβRko mice is present but reduced and the fact that CFSE positive bmDCs migrate to mesenteric lymph nodes in IFNαβRko mice, we conclude that migration of bmDCs most likely is not the principle mechanism of protection in IFNαβRko mice. However, further experiments are required to address bmDC migration in IFNαβRko and wt mice in more detail.

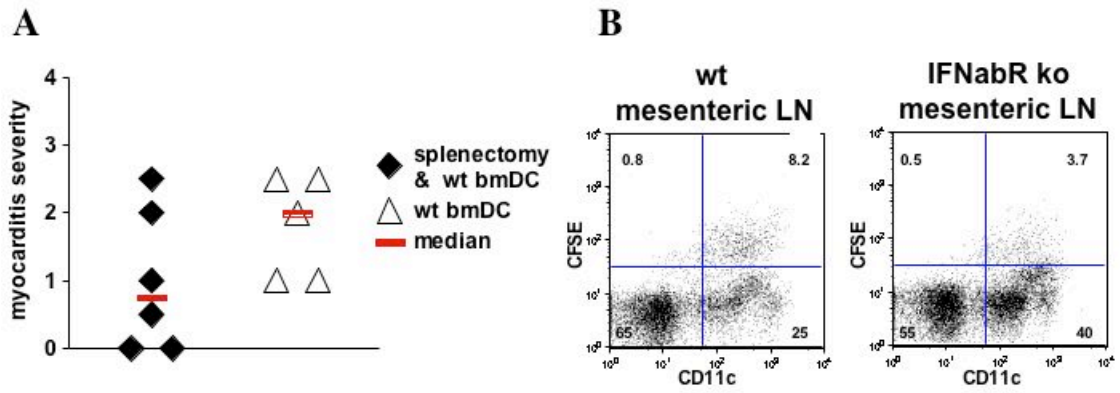


Figure 22: bmDC migration in wt and IFNαβRko mice

A) Splenectomyzed BALB/c mice develop myocarditis after bmDC immunization with slightly reduced severity and prevalence compared to control BALB/c mice after bmDC immunization. At day 10 and 12 after splenectomy, mice were immunized with MyHC-alpha loaded, LPS/aCD40 activated bmDCs and sacrificed at day 10 after first bmDC immunization.

B) Mesenteric lymph nodes were isolated 2 days after i.p. injection of CFSE labelled bmDCs. bmDC migration into mesenteric lymph nodes was analyzed by flowcytometry detecting CFSE and CD11c double positive bmDCs. IFNαβRko mice show slightly reduced migration of CFSE positive DCs to the mesenteric lymph nodes of IFNαβRko mice when compared to wt mice.

IFNαβRko mice are protected from autoimmune myocarditis after MyHC-alpha specific CD4$^+$ T-cell transfer

The IFN type I receptor is expressed in various cell types including APCs, B- and T-cells. Different members of the type I IFN family have pleiotropic effects on the induction and maintenance of immune response and are therefore key players in immune regulation (121). As shown in Figure 20B, IFNαβRko mice show reduced immune response to MyHC-alpha peptide after wt bmDC immunization. We further wished to assess whether expansion or migration mechanisms of autoreactive T-cells plays a role in myocarditis protection in IFNαβRko mice. We therefore generated a MyHC-alpha specific wt CD4$^+$ T-cell line. Adaptive transfer of MyHC-alpha specific wt CD4$^+$ T-cells circumvents *in vivo* priming and therefore we directly assessed the role of T-cell expansion and migration *in vivo*. The generated wt CD4$^+$ T-cells specific for MyHC-alpha peptide show high IFN-gamma (not shown) and intermediate IL-17 cytokine secretion levels (Figure 23A).

After adoptive transfer of heart specific T-cells, IFNαβRko mice were protected from autoimmune myocarditis (Figure 23B and 23C). Nevertheless, wt CD4$^+$ MyHC-specific T-cells could still be found in spleens of IFNαβRko mice after adoptive transfer. We isolated spleens 10 days after adoptive transfer and restimulated whole splenocytes *in vitro* with MyHC-alpha peptide. Both wt and IFNαβRko splenocytes responded to MyHC-alpha peptide, albeit with slightly different proliferation patterns depending on peptide concentration (Figure 23D). Reduced proliferation of heart-specific T-cells in IFNαβRko mice after adoptive transfer might indicate that the conditions for expansion of heart specific T-cells in secondary lymphoid organs in IFNαβRko mice are less favourable than in wt mice. This could indicate that APCs in the IFN receptor type I deficient mouse are less efficient in activating and expanding MyHC-alpha specific CD4$^+$ T-cell than wt APCs. However, protection of IFNαβRko mice from myocarditis could also arise from impaired recruitment of CD4$^+$ T-cells to the heart or impaired recruitment of inflammatory infiltrates to the heart.

These findings indicate that the IFNαβRko mice do not only have reduced adaptive immune response against MyHC-alpha peptide as shown in Figure 20C, but might also have further mechanisms that prevent autoimmune myocarditis.

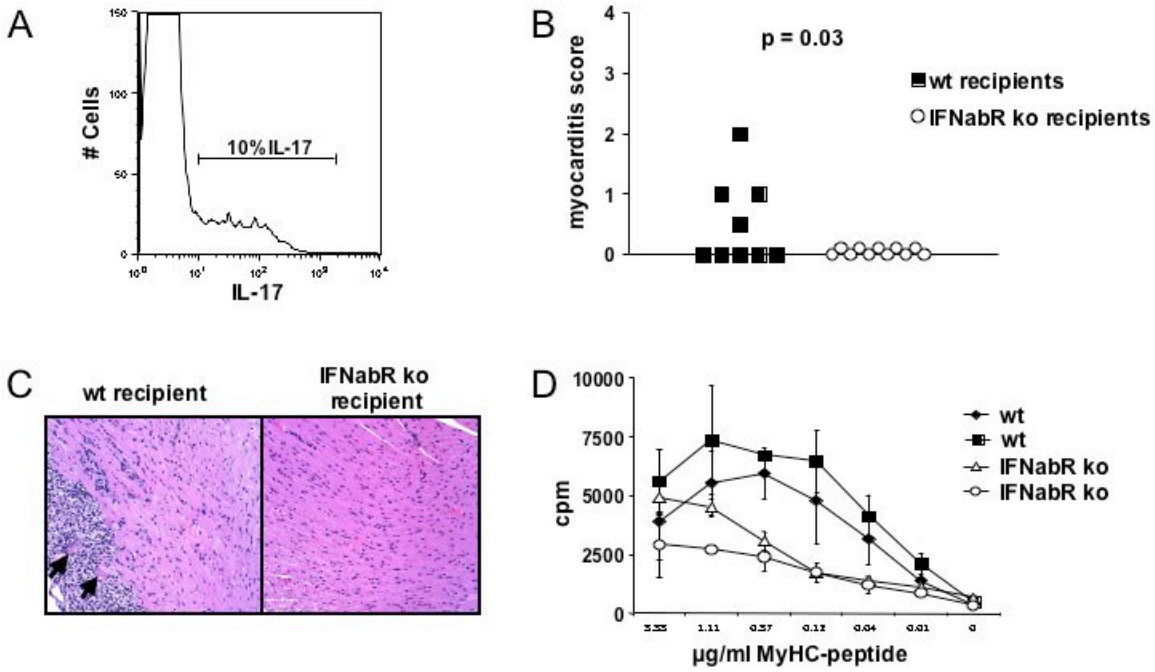


Figure 23: IFNαβRko mice are protected from myocarditis after MyHC-alpha specific adoptive wt T-cell transfer

A) FACS analysis of MyHC-alpha specific wt T-cells expressing intermediate amounts of IL-17.

B) 10 days after adoptive transfer, inflammation in hearts was scored by histology. IFNαβRko mice are completely protected from myocarditis after adoptive MyHC-alpha specific wt T-cell transfer.

C) Representative images of Hematoxilin & Eosin staining of wt and IFNαβRko heart sections. Original magnification 400x. Left figure panel: arrows pointing on cardiomyocytes (red) surrounded by infiltrating lymphocytes.

D) In the same experiment, spleens were removed and whole splenocytes were restimulated with different concentrations of MyHC-alpha peptide and proliferation was assessed by 3H-Thymidine incorporation. Each group represents a pool of two mice. Both wt and IFNαβRko mice respond to MyHC-alpha peptide, albeit with slightly different proliferation patterns depending on peptide concentration.

MyHC-alpha specific IFNαβR deficient CD4$^+$ T-cells transfer myocarditis in wt mice

To further investigate the protection mechanisms in IFNαβRko mice we addressed the potential of heart specific IFNαβRko T-cells in inducing myocarditis in wt mice.

If heart-specific IFNαβRko T-cells have intrinsic defects due to their inability to signal IFN type I cytokines, they would therefore fail to transfer myocarditis. On the other hand, if the protection mechanism in IFNαβRko mice after bmDC immunization is based on altered migration patterns of T-cells, then IFNαβRko MyHC-alpha specific T-cells should be able to induce myocarditis in wt mice. To address this hypothesis, we generated a MyHC-alpha specific IFNαβRko CD4$^+$ T-cell line (Figure 24A). After adoptive transfer of IFNαβRko T-cells, wt mice develop myocarditis (Figure 24B). Therefore we conclude, that IFNαβRko signalling is not essential in T-cells for the induction of autoimmune myocarditis. The protection mechanisms in IFNαβRko mice are more likely to be dependent on reduced expansion and impaired migration of MyHC-alpha specific T-cells from the secondary lymphoid tissue to the heart. Moreover, impaired recruitment of leukocytes to the heart would also be a possible mechanism of protection.

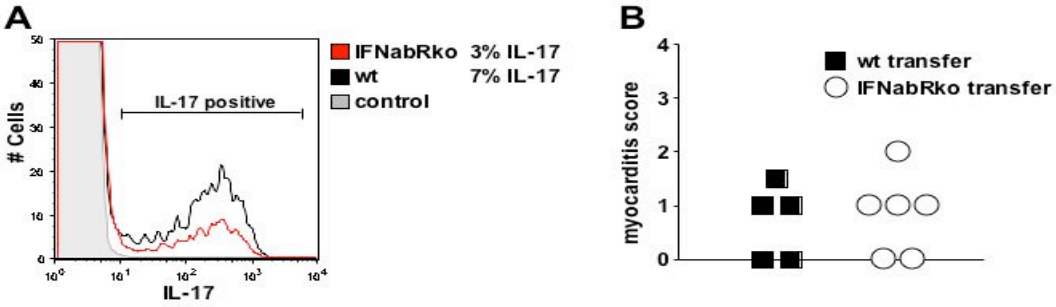


Figure 24: IFNαβRko MyHC-alpha specific T-cells induce myocarditis in wt mice

A) FACS analysis of wt and IFNαβRko MyHC-alpha specific CD4$^+$ T-cells expressing IL-17.

B) 10 days after adoptive transfer of MyHC-specific wt or IFNαβRko T-cells into wt BALB/c mice, inflammation in hearts was scored by histology. MyHC-specific IFNαβRko T-cells induced myocarditis in BALB/c mice with the same prevalence and severity as MyHC-specific wt T-cells.

bmDC induced myocarditis in chimeric wt and IFNαβRko mice

So far, we showed that MyHC-alpha specific IFNαβRko T-cells transfer myocarditis in wt mice but MyHC-alpha specific wt T-cells fail to transfer disease in IFNαβRko mice. It would hence be of interest to address the role of IFNαβR signalling in the vasculature and the heart for its function in the recruitment of leukocytes to the heart during autoimmune myocarditis induction after wt bmDC immunization. To better understand the importance of IFNαβRko signalling in the recruitment of autoreactive T-cells to the heart, we generated chimeric mice with either wt bone marrow in IFNαβRko mice or vice versa.

6 weeks after the generation of chimeras, we immunized them with wt bmDC. Myocarditis developed in both groups, however disease severity was low and no significant differences were observed between the two chimera groups at this time point after disease induction (Figure 25).

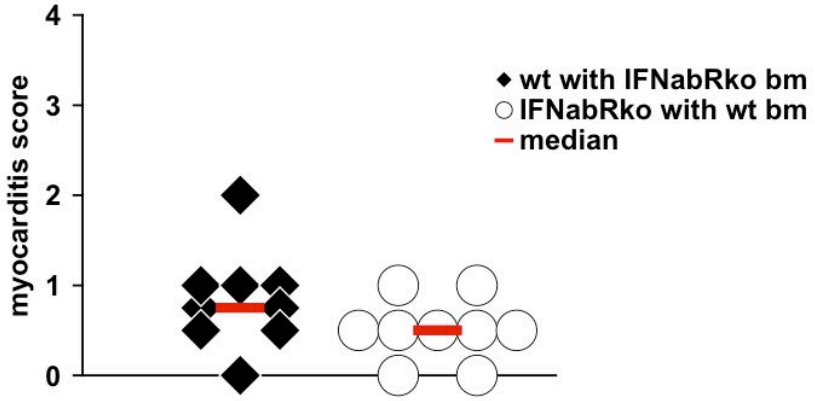


Figure 25: Myocarditis in wt and IFNαβRko bone marrow chimeras

Chimeric mice were generated with either wt bone marrow in IFNαβRko mice or vice versa. Myocarditis was induced by injecting MyHC-alpha loaded and LPS/aCD40 activated bmDCs at day 0 and 2. Wild type bmDCs were injected in wild type mice with IFNαβRko bone marrow (filled diamonds) or in IFNαβRko mice with wt bone marrow (open circles). After 10 days, hearts were scored histologically for inflammation. No significant difference was observed.

Discussion

In this study we examined the role of type I IFN receptor signalling in the development of autoimmune myocarditis. We could show for the first time that IFN-beta expression is upregulated in the heart during autoimmune myocarditis. Furthermore, taking advantage of the IFN type I receptor deficient mice, we report that IFN$\alpha\beta$R deficiency results in decreased autoimmune myocarditis. We showed that myocarditis incidence and severity was reduced in both bmDC-induced myocarditis as well as after adoptive transfer of MyHC-specific $CD4^+$ T-cells. Consistent with this observation was the reduced priming capacity of autoreactive $CD4^+$ T-cells after bmDC immunization in IFN$\alpha\beta$R deficient mice.

However, at the level of T-cells, *in vitro* expanded IFN$\alpha\beta$R deficient MyHC-specific T-cells transfer myocarditis into wt mice. We therefore conclude that IFN$\alpha\beta$R signalling at the level of T-cells is not required for T-cell infiltration in the heart and for the induction of autoimmune myocarditis. These findings lead to the question as to why IFN$\alpha\beta$Rko mice show reduced autoreactive T-cell priming when immunized with fully matured and activated wt bmDCs? And how does IFN$\alpha\beta$Rko deficiency in the host influence priming of autoreactive T-cells?

In the concept of the “adjuvant effect” (127), priming of autoreactive T-cell requires two preconditions. First, auto-antigen has to be released due to tissue damage or imitated by molecular mimicry of infectious agents. Second, infections acting as adjuvants induce an inflammatory milieu that favours priming of autoreactive T-cells and the development of autoimmunity (Figure 26). Noel R. Rose suggests that the inflammatory environment itself is important for the priming of autoreactive T-cells (127). We therefore hypothesize that type I IFN might be an important player in the induction of an inflammatory environment favouring priming of autoreactive T-cells.

In order to prime efficient T-cell responses, antigen-presenting cells, especially dendritic cells, need to be in an activated maturation status expressing high levels of MHC class I & II molecules, costimulatory molecules as well as proinflammatory cytokines. Type I IFN is essential for the maturation process of

antigen presenting cells to fully exert efficient T-cell priming. Recent studies have demonstrated that dendritic cells both produce type I IFN and undergo maturation in response to type I IFN (128). We hypothesize that mice lacking type I IFN signalling fail to induce a proinflammatory environment that favours the priming of autoreactive T-cell that subsequently induces autoimmunity. However, we could show that IFNαβRko bmDCs are able to induce myocarditis in wt mice. We therefore can exclude feedback mechanisms acting on the injected bmDCs. Therefore, the cell-types and signals contributing to an inflammatory milieu that enables efficient priming between wt bmDCs and IFNαβRko T-cells remain obscure.

The suggested “adjuvant effect” has similar characteristics as the described “bystander activation” of $CD4^+$ and $CD8^+$ T-cell during viral infection or innate activation (129). Bystander activation describes non-specific activation and proliferation of $CD4^+$ or $CD8^+$ T-cell upon infections. Infections lead to significant maturation and activation of APCs such as dendritic cells. Maturation can be induced directly through pathogen recognition receptors on DCs, in particular TLRs (130). In addition, DCs can respond indirectly to cytokines, secreted from other cells (131). These activated APCs could potentially activate preprimed autoreactive T-cells, which can then initiate autoimmune disease (bystander activation of autoreactive immune T-cells) (132).

So far we discussed the possible mechanisms that contribute to reduced priming of autoreactive T-cell in IFNαβRko mice. Further findings from T-cell transfer experiments suggest additional protection mechanisms in IFNαβRko mice. Adoptive transfer of MyHC-alpha specific wt T-cells directly induces autoimmune myocarditis in wt mice (105). In contrast to wt mice, IFNαβRko mice are protected from autoimmune myocarditis development after transfer of MyHC-specific wt $CD4^+$ T-cells. We found that MyHC-specific wt $CD4^+$ T-cells survive in IFNαβRko mice and remain antigen specific upon MyHC-alpha *in vitro* restimulation. However, restimulation capacity is reduced when compared to wt mice. Based on these results we suggest two possible mechanisms that could

protect IFNαβR deficient mice from autoimmune myocarditis. First, MyHC-alpha specific T-cells in IFNαβRko mice might be unable to infiltrate the myocardium. Second, once infiltrated in the heart, MyHC-specific $CD4^+$ T-cells might be unable to recruit further inflammatory cells such as DCs, macrophages or monocytes.

Autoimmune myocarditis infiltrates mainly consist of CD11b positive macrophage like cells, dendritic cells, eosinophils, neutrophils, B-cells, granulocytes, mast-cells as well as $CD4^+$ and $CD8^+$ T-cells (42). In the concept of disease induction, we believe that first, $CD4^+$ T-cells migrate to the heart and second, $CD4^+$ T-cells further recruit inflammatory cells to the myocardium. This process is amongst others orchestrated by proinflammatory cytokines secreted from heart specific $CD4^+$ T-cells that directly or indirectly induce chemokine expression and infiltrate recruitment (107). In one of our first experiments, we could show IFN-beta expression in the heart during active autoimmune myocarditis in wt mice. It is likely, that IFN type I receptor signalling in the heart is essential for the recruitment of additional infiltrating cells after the fist wave of $CD4^+$ T-cell infiltration. We hypothesize that type I IFN signalling influences migration and infiltration of leukocytes to the heart. However, further research has to be performed to address this specific characteristic of IFN-beta signalling within the heart.

The potential of type I IFN to modulate monocyte trafficking has been described in the context of other autoimmune diseases such as multiple sclerosis and its animal model of Experimental Autoimmune Encephalomyelitis (EAE) (133). The use of IFN-beta in treating MS is well known (15). Moreover, mice deficient in IFN-beta show augmented and chronic EAE (134). The mode of action of IFN-beta as a therapeutic agent for multiple sclerosis has multiple manifestations. Of the known immunomodulatory effects of IFN-beta are amongst others the inhibitory effect on the production of matrix metalloproteases (135), and modulatory effects on the expression of adhesion molecules and chemokines (133, 136, 137). We therefore further investigated the role of lymphocyte trafficking in the protection mechanism of IFNαβRko mice when immunized with

bmDCs. We generated chimeric IFNαβRko mice with wt bone marrow and chimeric wt mice with IFNαβRko bone marrow. After immunization with bmDCs, both groups develop autoimmune myocarditis. However, only low-level disease of myocarditis developed and no significant differences can be observed.

In summary, type I IFN receptor signalling affects autoimmune myocarditis induction on many levels. We could show that IFNαβRko mice are protected from disease induction, however, the precise protection mechanisms are still unknown. Our studies once more highlight the immunomodulatory potential of the type I IFN family in autoimmune diseases. Further work is required to determine the role of IFNαβR signalling in disease induction and progression of autoimmune myocarditis in more detail. For future research strategies, I believe that by narrowing down the IFNαβRko signalling pathway, (for example addressing EAM phenotype in IRF-7, or IFN-beta deficient mice), could greatly enhance our knowledge of type I IFN signalling in EAM. In addition, it would be of great interest to observe autoimmune myocarditis prevalence, severity and heart failure development during IFN-alpha or IFN-beta administration in wt mice.

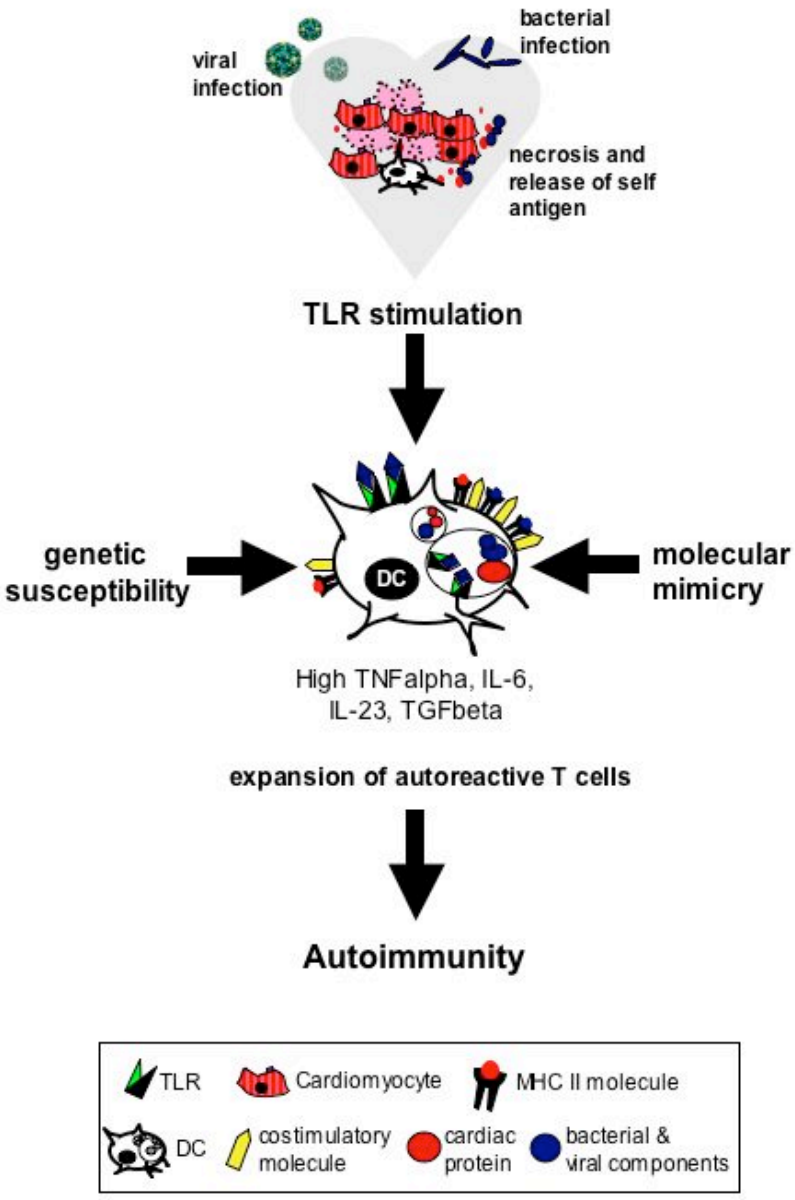


Figure 26

Microbial and viral infections result in tissue destruction, necrosis and release of cardiac selfantigens. In the presence of TLR stimuli – either microbial products or endogenous TLR ligands are released from dying cells – selfantigen presenting dendritic cells become activated in the draining lymphatic tissue. Excessive DC activation and release of high amounts of IL-23, IL-6, TNF-alpha and TGF-beta breaks peripheral tolerance and induces heart specific autoimmunity. Genetic predisposition and/or the presence of heart-specific T-cells from former infections with intruders mimicking cardiac selfantigens, both define the DC activation threshold required for the induction of autoimmunity. Adapted from (44)

GENERAL CONCLUSIONS AND DISCUSSION

This dissertation addresses the role of the innate immune system in EAM, a murine model of $CD4^+$ T-cell mediated autoimmune myocarditis. In this context I focussed my research on the contribution of the TLR adaptor molecule MyD88 and the IFN$\alpha\beta$R in disease induction.

We showed that MyD88 dependent TLR signalling is essential for the development of EAM upon MyHC-CFA immunization. We demonstrated that MyD88 is essential for the activation of self-antigen presenting DCs to induce heart-specific $CD4^+$ T-cell responses in the peripheral compartments *in vivo*. We further showed that MyD88 dependent proinflammatory cytokine secretion of APCs is essential for the priming of heart-specific autoreactive T-cells. Our findings provide a proof of concept that the lymphatic compartment of MyD88 deficient mice is fully competent to allow the development of autoimmune $CD4^+$ T-cell responses if it becomes substituted with appropriately activated self-antigen loaded antigen presenting cells. In addition, adoptive transfer of activated heart-specific autoreactive $CD4^+$ T-cells induced myocarditis in MyD88 deficient mice, suggesting that MyD88 signalling affects neither $CD4^+$ T-cell recruitment nor accumulation of other inflammatory cells in the heart.

Other experimental autoimmune murine disease models that use CFA as adjuvant confirmed our results of impaired $CD4^+$ T-cell priming in MyD88 deficient mice after MyHC-CFA immunization. MyD88 deficient mice are resistant to disease induction in Experimental Autoimmune Uveitis and Experimental Autoimmune Encephalomyelitis (67, 68). These studies highlight the role of APCs and DCs in particular as key players in the generation of autoimmune T-cell responses. Under normal physiological conditions, the most important function of DCs is the generation of innate and adaptive immunity to infections (131, 138). DCs are specialized to process antigens, presenting them as peptides bound to MHC products and initiating immunity. However, there is increasing evidence that DCs *in situ* induce antigen specific unsresponsiveness or tolerance in the periphery. Central tolerance is efficient, but not perfect.

Autoreactive T-cells may escape deletion, or self antigens may be expressed later in life, after the lymphocyte repertoire has been formed (139, 140). Peripheral tolerance is therefore necessary to support central tolerance (43). Immature dendritic cells continually capture and present self- and harmless environmental antigens. Naïve T-cells are deleted after recognizing ligands on these immature DCs and therefore maintain self-tolerance (98, 141). The concept of DC induced autoimmunity therefore implies that autoimmunity develops if TLR activation coincides with release and uptake of selfantigen in lymphatic organs of genetically susceptible individuals (45). In this case, DC maturation and presentation of antigens has to be tightly regulated especially at the site of infection where DCs not only capture the pathogen but also are likely to be taking up dying cells. How DCs avoid the risk of inducing autoimmunity to self-antigens and chronic reactivity to environmental proteins is a major topic of current research. Steinman and Nussenzweig suggest that immature DCs induce antigen-specific peripheral tolerance in the steady state, before DC maturation during inflammation and infection (98). During infection, DCs mature in response to pathogen signals. Two major receptor families play important roles in the maturation of dendritic cells: TLRs (142, 143) and TNF-receptors including CD40 (144, 145). This thesis therefore provides novel data that TLR induced maturation of DCs through the MyD88 signalling pathway is a prerequisite for the generation of autoimmune response and loss of peripheral tolerance.

We also addressed the role of MyD88 in the induction of heart failure. Heart failure is a complex multi-step disorder in which a number of physiologic systems participate in its pathogenesis (146). Chronic myocarditis is a leading cause of heart failure development (20). Numerous studies have demonstrated that heart failure patients have raised circulating levels of inflammatory cytokines such as TNF-alpha, IL-1, IL-6, as well as several chemokines, such as monocyte chemoattractant peptide (MCP-1), IL-8, and macrophage inflammatory protein (MIP-1alpha) (7-11). Chronic myocarditis patients only present with clinical symptoms weeks to months after the initial cardiac infection. The cause of heart

failure development is often unknown and complicates diagnosis and affects treatment strategy. Novel treatment strategies to stop the onset of heart failure development are therefore of great clinical interest.

Proinflammatory cytokines belong to the family of innate effector cytokines. We hypothesize that the innate immune system might contribute to the development of heart failure. Hence, we further addressed the role of MyD88 in the development of autoimmune myocarditis mediated heart failure. We could demonstrate by echocardiographic heart function assessment that MyD88 mice show increased heart function after autoimmune myocarditis when compared to wt mice. These results indicate an additional role for MyD88 and TLR signalling beyond immune system activation in the heart. We provide additional evidence that the heart possesses an innate danger program that reacts upon initial injury, which is involved in the development of heart failure. We hypothesize that MyD88 dependent cytokine signalling through the IL-1 Receptor or MyD88 dependent production of proinflammatory cytokines in the heart upon stress reaction or injury contribute to the development of heart failure. On the other hand, proinflammatory cytokines can also have protective properties in stress related responses in the heart (6). It is suggested that proinflammatory cytokines can induce short-term protective effects as for example the protection of cardiac myocytes against either hypoxic or ischemic injury (147). Knuefermann argues that due to the phyllogenetically ancient characteristics of the innate system, the innate stress response in the heart was developed to protect organisms with short live span. Otherwise, when activated over a prolonged time-period, maladaptive effects may abound. Thus, activation of the innate stress response system was never intended to provide long-term adaptive responses to the host organism (12).

During the course of assessing MyD88 induced heart failure we created a novel immunization protocol combining bmDC and CFA immunization. We here provide the first data that indicates a fast progression of fibrosis as well as dilation in hearts of wt BALB/c mice after double immunization. We believe that this specific protocol might be useful in future studies addressing innate stress responses in

heart failure development. So far, our research has focused on the contribution of MyD88 in the development of innate and adaptive immune responses against the heart and the induction of proinflammatory cytokine response within the heart. In the context of heart failure development it will be interesting to increase the area of investigation to further mechanisms that might contribute to disease onset. For example, preliminary data from Davide Germano in the Eriksson laboratory indicate that MyD88 might also play a role in the recruitment of bone marrow derived fibroblast and macrophage precursor cells in Bleomycin induced lung fibrosis. It will therefore be of great interest to further address the role of MyD88 in the recruitment of bone marrow derived precursor cells to the inflamed heart and its contribution to the development of fibrosis and heart failure.

The MyD88 independent TLR signalling pathways in EAM induction were also investigated. MyD88 independent signalling is mainly responsible for the induction of type I IFN. We therefore took advantage of IFN$\alpha\beta$R deficient mice to study the role of type I IFN signalling in EAM induction. We could show that IFN$\alpha\beta$R deficient mice are protected from bmDC induced autoimmune myocarditis. We provide evidence that the protection coincides with reduced T-cell priming and suggest an additional role for type I IFN signalling in the recruitment of proinflammatory cells to the heart. We showed for the first time that type I IFN is involved in the induction of autoimmune responses against the heart. However, the exact mechanisms contributing to reduced T-cell priming remain elusive. One possible mechanism might be the crosstalk of IFN$\alpha\beta$R signalling with other signalling pathways important for disease induction. For example, Mitani *et al.* recently reported a unique signalling crosstalk between IFN-alpha-beta and IL-6 signalling. It was shown that efficient IL-6 signalling requires constitutive subthreshold IFN$\alpha\beta$R signalling for the activation of IL-6 transcription factors (148). IL-6 is a critical factor for the induction of IL-17 expressing $CD4^+$ T-cells (149, 150). IL-17 has been shown to be essential for the development of EAM beyond the well established Th1, Th2 concept (106, 107).

In addition, also other disease models of autoimmunity are IL-17 depending such as EAE or collagen-induced arthritis (151-153).

Type I IFNs also act directly on activated T-cells. In the absence of inflammation, antigen injection into animals causes antigen-specific T-cells to become activated and, rapidly thereafter, die (154, 155). Marrack and co-workers identified type I IFN as a survival signal for activated T-cells (156). These results support our observed phenotype of reduced *in vitro* T-cell expansion after immunization with fully competent and activated wt bmDCs.

Taken together, type I IFN affects the induction of autoimmune myocarditis on many levels. Our research clearly demonstrates a role for type I IFN in disease induction. However, future investigations of type I IFN influencing pre-existing autoimmune responses will be of major interest to model IFN therapy of chronic myocarditis patients with autoimmune pathogenesis.

In summary, we could demonstrate that TLR signalling affects the induction of heart specific autoimmunity on many levels. We believe that further research on TLR signalling induced autoimmunity and the identification of endogenous TLR ligands may contribute to novel treatment strategies against autoimmune induced heart failure and also other autoimmune diseases.

Abbreviations

APC	Antigen-presenting cell
BALB/c	Mouse strain (IAd haplotype)
BmDCs	Bone marrow derived dendritic cells
CAB	Chromotrope-Anilin Blue Staining
CAM	Cell adhesion molecules
CD	Cluster of differentiation
CFA	Complete Freund`s adjuvant
CpG	Cytidine–phosphate–guanosine repeats
cpm	Counts per minute
CVB3	Coxsackievirus B3
DC	Dendritic cell
DCM	Dilated Cardiomyopathy
DD	Death-domain
EAE	Experimental Autoimmune Encephalomyelitis
EAM	Experimental Autoimmune Myocarditis
EAU	Experimental Autoimmune Uveitis
EDD	End-diastolic left-ventricular diameter
ELISA	Enzyme-linked immunosorbent assay
ESD	End-systolic left-ventricular diameter
FACS	Fluorescence-activated cell-sorting
FCS	Fetal calf serum
FS	Fractional shortening
HCMV	Human cytomegalovirus
HE	Hemotoxylin & Eosin
HSP	Heat-shock protein
HSV1	Herpes Simplex Virus I
HW/BW	Heart weight-body weight
i.p.	Intraperitoneal
i.v.	Intravenous

IFN	Interferon
IFN$\alpha\beta$Rko	Interferon-alpha-beta receptor knockout mice
Ig	Immunoglobulin
IL	Interleukin
IL-1R	Interleukin 1 receptor (IL-1R)
IRAK	IL-1R-associated kinase
IRF	Interferon-response factor
IVS	Intra-ventricular septum
KO	knock-out
LPS	Lipopolysacharide
LRR	Leucine rich repeats
LTA	Lipo-teichoic acid
MHC	Major histocompatibility complex
MMTV	Mouse mammary tumor virus
MyD88	Myeloid differentiation primary-response protein 88
MyHC-alpha	Myosin Heavy-Chain-alpha derived peptide
ND	Not determined
NF-kB,	Nuclear factor-kB
NO	Nitric oxide
PAMPs	Pathogen-associated molecular patterns
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PG	Peptidoglycan
PI	Propidium iodide
PKC	Protein Kinase C
PMA	Phorbol myristate acetate
PRRs	Pattern-recognition receptors
PW	Left-ventricular posterior wall
RSV	Respiratory syncytial virus
SARM	Sterile alpha- and armadillo-motif-containing protein
SCID	Severe combined immunodeficiency

SLE	Systemic Lupus Erythematosus
TIR	Toll/IL-1R homology domain
TIRAP	TIR-domain-containing adaptor protein
TLR	Toll-like Receptor
TNF-□lpha	Tumour necrosis factor alpha
TRAM	TRIF-related adaptor molecule
TRIF	TIR-domain-containing adaptor protein inducing interferon-beta
Vcf	Velocity of circumferential fibre shortening
wt	Wild-type

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Publications

Original articles

MyD88 signalling controls autoimmune myocarditis induction

Circulation. 2006 Jan 17;113(2):258-65

Marty RR, Dirnhofer S, Mauermann N, Schweikert S, Akira S, Hunziker L, Penninger JM, Eriksson U

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Review articles

Dendritic cells and autoimmune heart failure
Int J Cardiol. 2006 Sep 10;112(1):34-9
Marty RR and Eriksson U

Abstracts

Poster presentation at the keystone symposia "Tolerance, Autoimmunity and Immune Regulation"
March 21 - 26, 2006; Breckenridge, Colorado, USA
MyD88 but not TLR4 or TLR9 deficiency protects from Experimental Autoimmune Myocarditis
Marty RR, Dieterle T, Dirnhofer S, Mauermann N, Eriksson U

Poster presentation at the "11th Cardiovascular Biology and Clinical Implications Meeting"
October 6 - 8, 2005, Thun, Switzerland
MyD88 Signalling Controls Autoimmune Myocarditis Induction
Marty RR, Dirnhofer S, Mauermann N, Schweikert S, Eriksson U

Course Certificates

LTK2 / FELASA Cat. C: Course for persons directing animal experiments (Swiss Ordinance on the Education and Training of Persons Conducting Animal Experiments)
December 2006, Basel, Switzerland

“Key issues in drug discovery & development” organized by the universities of Basel and Zürich together with Novartis and Roche
November 2005, Basel & Zürich, Switzerland