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DISSERTATION

Sex differences in macrophage polarization in experimental autoimmune myocarditis (EAM)

Geschlechterunterschiede in der Makrophagen Polarisation bei experimenteller autoimmuner Myokarditis (EAM)

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Preamble

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1 List of Abbreviations

(v/v)	(volume/volume)
(w/v)	(weight/volume)
Abl	Abelson murine leukemia viral oncogene homolog 1
ACE	angiotensin converting enzyme
Amp	ampicillin
Alpha-SMA	alpha-smooth muscle actin
ANOVA	analysis of variance
B-Cell	b lymphocyte
BCF	blood clotting factor
BMM	bone marrow-derived macrophage
BNP	brain natriuretic peptide
Bp	base pairs
BSA	bovine serum albumin
Cat.	catalogue
C/EBP	CCAAT-enhancer-binding proteins
cAMP	cyclic adenosine monophosphate
CAR	coxsackievirus adenovirus receptor
CCL	CC chemokine ligand
CD	cluster of differentiation
cDNA	complementary deoxyribonucleic acid
CFA	Freund's Complete Adjuvant
COL3A1	collagen3 A1
CREB	CAMP-responsive element binding protein
c.s.	charcoal stripped
CTLA4	cytotoxic t-lymphocyte-associated protein 4
CVB3	coxsackie virus b3
CX3CR	C-X-3-C motif chemokine receptors
Cy3	cyanine
DAMP	damage-associated molecular pattern
DAPI	4′,6-diamidino-2-phenylindole
DCF	2′,7′-dichlorodihydrofluorescein diacetate, succinimidyl ester
DCM	dilated cardiomyopathy
DEPC	diethyl dicarbonate

Dextrin	(2-hydroxpropyl)-β-cyclodextrin
DMEM	Dulbecco’s Modified Eagle medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DPBS	Dulbecco’s phosphate-buffered saline
DPN	diphosphopyridine nucleotide
E2	17β-estradiol
EAM	experimental autoimmune myocarditis
ECL	extracellular chemiluminescence
ECG	electrocardiogram
EDTA	ethylene diamine tetra-acetic acid
e.g.	for example
ELISA	enzyme-linked immunosorbent assay
ER	estrogen receptor
ERK	extracellular signal-regulated kinase
FBS	fetal bovine serum
FCCP	carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone
FEM	Forschungseinrichtung für Experimentelle Medizin
FITC	fluorescein
FoxP3	forkhead box P3
FSH	follicle-stimulating hormone
Fyn	proto-oncogene tyrosine-protein kinase fyn
GBD	global burden of disease
GM-CSF	granulocyte monocyte – colony-stimulating factor
GPER	g-protein-coupled estrogen receptor
HCL	hydrogen chloride
Hepes	n-2-hydroxcethylpiperazine-n-2-ethane sulfonic
HPRT	hypoxanthine guanine phosphoribosyl transferase
IGF	insulin-like growth factor
IgG	immunoglobulin g
IL	interleukin
ILR	interleukin receptor
IFN-y	interferon y
iNOS	induced nitric oxide synthase

IRF	interferon regulatory factor
JAK	Janus kinase
kDa	kilo dalton
LH	luteinizing hormone
LPS	lipopolysaccharide
M0	not polarized macrophage
M1	proinflammatory macrophage (classically activated)
M2	not proinflammatory macrophage (alternatively activated)
M2b	immunoregulatory macrophage
M2c	tissue-repairing macrophage
mABs	monoclonal antibodies
MCP-1	monocyte chemoattractant protein 1
M-CSF	monocyte – colony stimulating factor
MHC	major histocompatibility complex
miRNA	micro ribonucleic acid
MMR	macrophage mannose receptor
MRI	magnetic resonance imaging
mRNA	messenger ribonucleic acid
MyD88	myeloid differentiation primary response 88
n.s.	not significant
NaCl	natrium chloride
NaOH	sodium hydroxide
NEAA	non-essential amino acid
NFκB	nuclear factor kappa-light-chain-enhancer of activated b cells
NK-Cell	natural killer cell
P38	p38 mitogen-activated protein kinases
PAMP	pathogen-associated molecular pattern
PBMC	peripheral blood mononuclear cell
PCR	polymerase chain reaction
PDGF	platelet-derived growth factor
pERK	phosphorylated extracellular signal-regulated kinase
PI3K	phosphoinositid-3-kinase
PIC	protein inhibitor cocktail
pP38	phosphorylated p38 mitogen-activated protein kinases

PPAR	peroxisome proliferator activated receptor
PRR	pattern recognition receptor
Relm Alpha	resistin-like alpha
RNA	ribonucleic acid
r.t.	room temperature
rpm	rounds per minute
RPMI	Roswell Park Memorial Institute
SDS	sodium dodecyl sulfate
SEM	standard error of the mean
Sec	second
STAT	signal transducer and activator of transcription
TBS-T	tris buffered saline + tween-20
TEMED	n, n, n′, n′-tetramethylethane-1,2-diamine
TGF-β	transforming growth factor beta
TH	t helper cell
TIM3	t-cell immunoglobulin and mucin-domain containing 3
TIMP	tissue inhibitor of matrixmetalloproteinase
TLR	toll-like receptor
TNF-α	tumour necrosis factor alpha
Treg	regulatory t-cell
TRIF	tir-domain-containing adapter-inducing interferon-β
Tris	tris(hydroxymethyl)aminomethane
YM1	chil3 chitinase like 3

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4 Abstract

Male sex is a risk factor for higher rates of cardiac fibrosis, stronger cardiac inflammation and the development of dilated cardiomyopathy (DCM) due to myocarditis. The chronic phase of the disease, with risk of DCM development due to cardiac fibrosis, is characterized by the absence of the primal trigger (e.g., viral progeny). It is remarkable that this form of cardiac autoimmunity is more prevalent in males than in females, although the overall prevalence of autoimmune diseases is higher in women due to estrogen's influence on immune responses. The majority of immune cells in the resting heart are macrophages. Mosser et al. described macrophage polarization using a spectrum of subtypes with heterogeneous purposes (e.g., tissue repair, immune regulation). Male rodents show stronger fibrosis in heart samples in experimental autoimmune myocarditis (EAM). My study aims to show sex differences in macrophage polarization in EAM, as macrophages are important regulators of fibrosis.

In EAM animals, we found more pro-inflammatory macrophage populations in male tissues and an increased presence of macrophage populations from the anti-inflammatory M2 spectrum in female tissues. Male EAM rats presented with a decreased ejection fraction in contrast to female rats. Moreover, male heart sections showed stronger fibrosis formation compared to female ones. *In vitro* experiments with macrophages and cardiac fibroblasts from wild type mice showed a stronger polarization into pro-inflammatory macrophages (M1) in male cells following LPS treatment as well as a stronger polarization of male cells into a pro-fibrotic M2 subtype following IL-4/IL-13 treatment. In co-culture experiments, conditioned medium from male M1 macrophages more strongly activated male cardiac fibroblasts compared to conditioned medium from female cells applied to female cardiac fibroblasts. Moreover, male cardiac fibroblasts reacted to TNF-α treatment with stronger activation. A consistent effect of estrogen on macrophage polarization into M1 or pro-fibrotic M2 subtypes could not be demonstrated in *in vitro* experiments.

Conclusion: we found sex differences in EAM regarding a predominant anti-inflammatory M2 polarization of macrophages in female cardiac tissue compared to male animals. Based on these *in vitro* experiments, we conclude that the origin of increased fibrosis formation in male EAM animals is the preference of male macrophages to polarize more strongly into pro-fibrotic phenotypes of the spectrum.

Kurzdarstellung:

Männliches Geschlecht ist ein Risikofaktor für die Entwicklung von ausgeprägter kardialer Fibrose, stärkerer kardialer Entzündung und dilatativer Kardiomyopathie (DCM) aufgrund von Myokarditis. Die chronische Phase der Erkrankung mit kardialer Fibrose ist gekennzeichnet durch das Fehlen des ursprünglichen Auslösers (e.g. virale Partikel). Es ist auffällig, dass diese Form der kardialen Autoimmunität häufiger bei Männern vorkommt, obwohl die Prävalenz von Autoimmunkrankheiten höher bei Frauen ist. Die Mehrheit der Immunzellen im Herzen sind Makrophagen. Mosser et al. beschrieben die Polarisierung von Makrophagen in Subklassen als einen Vorgang, der zu Zellen mit sehr heterogenen Aufgaben (u.a. Gewebereparatur und Immunregulation) führt. Männlichen Ratten mit experimenteller autoimmuner Myokarditis (EAM) zeigen mehr kardiale Fibrose. Da Makrophagen wichtige Regulatoren von Fibrose Prozessen sind, versucht meine Studie Geschlechtsunterschiede in der Polarisierung von Makrophagen in EAM zu zeigen.
EAM Tieren zeigten mehr inflammatorische Makrophagen in männlichem Gewebe und eine vermehrte Präsenz von nicht entzündlichen Makrophagen des M2 Spektrums in weiblichem Gewebe. Männliche EAM Ratten zeigten eine stärker reduzierte Herzleistung als weibliche Tiere. In histologischen Herzschnitten zeigte männliches Herzgewebe eine stärkere Fibrosierung als weibliches Herzgewebe. In vitro Experimente mit Makrophagen und kardialen Fibroblasten von Wildtyp Mäusen zeigten eine stärkere Polarisierung nach LPS Behandlung von männlichen Zellen in einen inflammatorischen M1 Typ und eine stärkere Polarisierung nach IL-4/IL-13 Behandlung in einen profibrotischen M2 Subtyp. Ko-Kultur Experimente konnten zeigen, dass das von männlichen M1 Makrophagen konditionierte Medium männliche Fibroblasten stärker aktivieren konnte, als das ebenso konditionierte Medium weiblicher M1 Makrophagen weibliche Fibroblasten. Außerdem zeigten männliche kardiale Fibroblasten eine stärkere Aktivierung nach Behandlung mit TNF-α. Ein konsistenter Effekt von Östrogen auf die Polarisierung von Markophagen in den M1 oder den Fibrose induzierenden M2 Subtyp konnte nicht gezeigt werden.

Zusammenfassung: Es konnten Geschlechterunterschiede in EAM gezeigt werden hinsichtlich einer verstärkten M2 Polarisierung von Makrophagen in weiblichem Herzgewebe. In vitro Experimente konnten zeigen, dass die verstärkte kardiale Fibrose von männlichen EAM Tieren wahrscheinlich auf die Präferenz männlicher Makrophagen sich in profibrotische Subtypen des Spektrums zu polarisieren, zurückgeführt werden kann.

5 Introduction

5.1 Myocarditis

Myocarditis Definition

Myocarditis is an inflammatory state of the heart muscle characterized by the infiltration of several types of immune cells into the heart tissue. This inflammatory infiltrate consists of different immune cells. [1] Predominantly, macrophages and their differently polarized subtypes, namely T-cells like TH1 and TH2, TH17-type T-cells, and regulatory T-cells (FoxP3+/CD4+) infiltrate the tissue. [1] Many factors influence the infiltrate composition, such as the cause of infection or the stage of the disease (acute, subacute and chronic). [1] Failing regulation of the immune system's response to the primary trigger leads to ongoing inflammation followed by fibrosis, DCM and heart failure in the end stage of this disease. [2] Several pathogens have been described as inducing an inflammatory state in the human heart muscle. Three inflammatory triggers can be distinguished to classify myocarditis: infectious agents, immune-mediated myocarditis processes and cardiotoxins. Infectious myocarditis is the most common form of myocarditis, and coxsackieviruses are the leading cause. [3] Treatment with several drugs used in the chemotherapy of oncologic patients, for example, can also cause myocarditis. This would be referred to as myocarditis due to cardiotoxins. [4] The immune-mediated myocarditis form is a burden especially in patients after having received heart transplantation. [5] Furthermore, there is strong evidence that the pathomechanism of autoimmune reactions of the human body against myocardial proteins plays a key role in the development of a post-viral chronic stage of the disease with ongoing inflammation, fibrosis and the development of DCM. [2] Most insights about myocarditis and post-myocarditis complications, such as autoimmunity, fibrotic remodelling and dilated cardiomyopathy, are obtained from animal models. [6] Rose and colleagues observed that mice with coxsackievirus b3 (CVB3)-caused myocarditis developed a chronic stage of disease after the resolution of the viral cause. This post-viral inflammatory state was characterized by the appearance of antimyosin autoantibodies and fibrotic remodelling of the heart, leading to DCM. [7] This gave rise to a new animal model, which models the chronic stage of post-viral heart inflammation. Mice or rats are immunized with cardiac myosin and Freund's Complete Adjuvant (CFA), which is an immunopotentiator derived from mycobacterium tuberculosis, and develop chronic myocarditis in order to model this chronic inflammatory state. [8]

Myocarditis – chronic inflammation, cardiac autoimmunity and DCM

There is broad agreement that viral infections are the number one cause of myocarditis. [9] Nevertheless, it is not easy to determine the leading virus species that cause myocarditis or the most common one that leads to DCM development. [1] Using molecular biological methods to examine biopsies taken from patients with myocarditis or DCM, different viral species were detected, such as enteroviruses, parvovirus b19 and adenovirus. [9] Parvovirus b19 is the most commonly detected virus in endomyocardial biopsies of myocarditis and DCM patients, and CVB3 is the virus whose pathology is best understood from animal studies. [1] DCM can be an end stage of myocarditis regardless of the original cause of myocarditis. [2,10] It is difficult to determine the most common cause of myocarditis that leads to DCM development. It is estimated that myocarditis causes 9% to 16 % of non-ischaemic DCM cases. [2,11,12] In 25%-40 % of DCM patients, viral genome is found in endomyocardial biopsies. [13]

During the disease, two pathomechanisms lead to damage of heart tissue: the direct effects of the virus and its replication on the host cells, and damage caused by the host's own immune system, which is dealing with the infection. [14] The response of the immune system towards the viral threat consists of two phases. The first response uses the innate immune system, which is nonspecific and adaptive. The innate immune system can recognize highly evolutionary conserved patterns of pathogens by using so-called pattern recognition receptors like the class of toll-like receptors (TLR). Interestingly, it has been demonstrated that a TLR polymorphism is a risk factor for severe forms of enterovirus-caused myocarditis with increased cases of DCM. [15] TLR3- and TLR4-deficient mice, which are not able to establish an adequate response of the immune system towards the viral infection, show lower rates of DCM. [16,17] Macrophages and natural killer cells are important cells in the innate immune system. These are the first cells that infiltrate the infected tissue. [18,19] Several pattern recognition receptors are known to detect CVB3 antigens and start a signal cascade in macrophages. [20] Activated macrophages are an important link to the adaptive immune system, as they function as antigen-presenting cells. [18] The second phase of the immune system response to the viral infection is characterized by lymphocytes of the adaptive part of the immune system. These cells do not recognize environmentally conserved pathogen antigen patterns, but specifically react to distinct viral antigens. TH1-type lymphocytes directly cause damage to viral infected cells, while TH2-type lymphocytes stimulate B-lymphocytes to produce antibodies against viral proteins.[18,19]

An important marker of disease progression into a stage of chronic inflammation and fibrotic remodelling is the development of IgG autoantibodies binding to cardiac myosin. These antibodies are similar in the mouse myocarditis model, the EAM animal model and also in patients suffering from myocarditis as well as dilated cardiomyopathy. [21–23] In 80% of cases of myocarditis-caused DCM, antibodies against myocardial antigens can be detected in patients' blood. [24,25,26] It is thought that ongoing inflammation and self-sustained cardiac autoimmunity are required for disease progression into this chronic stage, which leads to DCM. [2,11,12] The development of autoimmunity occurs via two pathways: molecular mimicry between viral proteins and cardiac proteins, and the exposure of cardiac proteins to the host's immune system during the inflammation and harming of cardiac cells. [27,28] Cardiac myosin is a protein of the sliding filament apparatus of cardiomyocytes, which provides the contractile nature of these cells. Cardiac myosin is a motor protein consisting of two heavy protein chains and four light ones. [29] After the immunization of rats, it takes two weeks for myocardial inflammation to occur. After three weeks, the inflammation reaches its maximum. [8,30] Only the Lewis rat strain is susceptible to developing EAM after immunization. [30] In EAM, the pathology of the disease is driven by CD4+ T-cells. [31] Other than B-Cell deficient mice, T-cell deficient mice are resistant to developing DCM after EAM treatment, which shows the importance of T-cells in EAM pathology. [32] The antigen presentation of injected cardiac myosin depends on an endogenous population of antigen-presenting cells in the heart tissue. These bone marrow-derived cells present processed cardiac myosin using major histocompatibility complex 2 (MHC II) to interact with CD4+ T-cells. [31] Toll-like receptors such as TLR4 are needed to generate long-lasting cardiac autoimmunity. [33] In human patients, myocarditis can be divided into several histopathological subtypes, such as acute and chronic lymphocytic myocarditis. [34] Of these subtypes, giant cell myocarditis has the worst outcome and the highest rates of DCM. [35] The subtype of giant cell myocarditis is characterized by a pathology strongly driven by cardiac autoimmunity. [34] The composition of immune infiltrates in the heart tissue of giant cell myocarditis patients strongly resembles those in the heart tissue of EAM animals. [35] In both the EAM model [36] and human giant cell myocarditis, [12] an early and strong infiltration of macrophages can be observed.

Clinical Relevance: Myocarditis

Myocarditis is a heterogeneous disease in its presentation, with a broad range of symptoms during disease progression. [37] Patients with myocarditis often present with only minor symptoms, including flu-like symptoms. Patients suffering from a more aggressive form of the

disease present with signs of myocardial ischemia and heart failure, such as palpitations, angina pectoris, dyspnoea, and oedema of the lungs and lower limbs. [38] The gold standard of myocarditis diagnostics is the histopathologic examination of a biopsy taken from the patient's heart. [39]

The basic therapy includes the therapy of heart failure (for example, the usage of beta blockers, ACE inhibitors and the achievement of a protective fluid balance) and the therapy of arrhythmic disorders (if these occur during the process of disease). [39,40] Specific treatment of the underlying cause by using antiviral drugs in viral myocarditis, or immunosuppressive drugs if an autoimmune cause is found, should only follow a diagnostic biopsy confirming a specific cause and subtype of myocarditis. The overall prognosis of viral myocarditis is good, and a complete restitution is usually observed even without any specific treatment. Still, some subtypes have very poor outcomes, such as fulminant lymphocytic myocarditis, [41] giant cell myocarditis [42] and necrotic granulomatous myocarditis. [43] There also is a risk of developing a chronic stage of disease with ongoing inflammation following acute viral myocarditis. [3]

Risk factors

Several risk factors have been found to increase the chance of suffering from a more fatal form of this disease. In particular, these include the underlying trigger of myocarditis, the patient's age and condition, and the patient's sex. [1] A Finnish study that evaluated 3,274 Finnish hospital treatments found a higher prevalence of myocarditis in male compared to female patients. [1] In addition, it showed a higher rate of severity in males, including the development of DCM, fibrosis and heart failure. There is a sex bias in many of the common heart diseases regarding the response of the heart tissue towards detrimental causes (such as ongoing inflammation, chronic ischemia and toxic agents). Often, there is an increased incidence and severity in men and a better outcome in women. [44,45,46] Female heart tissue seems to react to harmful triggers in a more physiologic way. [45] Sex differences in cardiac remodelling and cardiac response to stress are probably important causes of the male sex being a risk factor of developing heart failure. [47] A different response of heart tissue towards ischemia, as in coronary heart disease, or towards inflammation, as in myocarditis, was found. Cardiac remodelling differs between male and female patients. Differences in cardiac remodelling are characterized by the fibrosis rate of heart tissue and the macroscopic morphology and structure of the remodelled organ. Differences in cardiac remodelling mechanisms between male and female patients are thought to be of high importance for the risk of developing DCM and heart failure. [47,48] Chronic myocarditis can cause DCM, which often leads to heart failure and the necessity for heart

transplantation. [49] The prevalence of DCM and myocarditis is higher in men than in women. [50,51] Recently, it has been shown that in patients suffering from myocarditis and acute cardiomyopathy, the cardiac recovery and outcome are better in women than in men, and they lead to less heart failure, a longer transplant-free time and higher survival rates in women. [46]

5.2 Sex differences in autoimmune diseases

Autoimmune diseases are a significant burden for the public health system. Over 8% of the population is affected by an autoimmune disease during their lifetime. Interestingly, more than 75% of these are women. [52,53,54] There is a widespread sex bias in autoimmune diseases. [55] The pathologies of different autoimmune diseases resemble each other: there is a phase with an acute inflammatory response to a trigger, followed by a chronic phase of ongoing immunological response in the absence of the initial trigger, which then leads to fibrosis in the affected organ. However, there are differences in the prototypes of female and male immunopathology regarding the cytokines involved and the cell types, as well as differences in the common age of onset of autoimmune diseases in male and female patients. [56] The onset of autoimmune diseases in women is usually after 50, while autoimmune diseases in men mostly start before age 50. [57–59,56] Important examples of autoimmune diseases with a female-like pathology are multiple sclerosis, myasthenia gravis, rheumatoid arthritis, Graves' disease, systemic lupus erythematous and Hashimoto's thyroiditis. Meanwhile, granulomatosis with polyangiitis, diabetes type one, ankylosing spondylitis, autoimmune gastritis and autoimmune myocarditis are more common in men. [54,58,59]

Sex differences in immune responses

Women's response to infection, trauma and vaccination is driven by a TH2 immune response, leading to increased production of antibodies. Conversely, men react to triggers like infection with a stronger TH1-driven, pro-inflammatory response, leading to stronger acute inflammation. [60,61] Consistently, autoimmune diseases with higher prevalence in male patients show a TH1-driven pathology with a strong, acute inflammation and the appearance of autoantibodies in later stages of the disease. Autoimmune diseases, which are more prevalent in women, are mostly antibody-mediated and show a TH2-driven pathology. [56]

Sex-based differences of the human immune system are thought to be regulated by sex hormones such as estrogen, testosterone and progesterone. Thus, these hormones are also likely to play an important role in the different pathologies of male and female autoimmune diseases. [55,62,63] The male sex hormone testosterone is produced in Leydig cells of the testis, although in

much lower doses, it is also found in the female organism. Female and male sex hormones share a connected synthesis. Progesterone and estrogen are known as female sex hormones. Nevertheless, estrogen in particular is also present in the male organism, as it is produced in testicular tissue and fatty tissue. Adipose male patients have increased estrogen levels due to the enzymatic processing of testosterone to estrogen by the enzyme aromatase (CYP19A1) in fatty tissue cells. [64] There are receptors for androgen and estrogen on immune cells. [65] There are cytokine receptors on sex hormone-producing tissues. Thus, the immune system is also likely to regulate sex hormone production and secretion. [63] Parenchymatous cells of inflamed tissue can also be the target of sex hormones. For example, human cardiac cells such as cardiomyocytes, endothelial cells and fibroblasts are targets of sex hormones. Although these are not cells of the immune system, sex hormone effects on these cells can dramatically change the tissue response to inflammation between males and females. [66,67] Important pathways of estrogen receptor signalling are intracellular p38 and ERK signalling. [68]

There is evidence that, in the female system, estrogen stimulates a TH2 response while inhibiting the TH1 arm, leading to the stimulation of antibody production. Furthermore, estrogen has been shown to elevate the levels of IL-4, IL-10 and TGF-β, which are also cytokines from the spectrum of alternatively activated macrophages. In addition, the estrogen-dependent stimulation of Foxp3 and CD80 gene expression gives rise to a population of regulatory T-cells. [33,63,69–73] On a cellular level, the effects of estrogen on immune cells are not as consistent and highly depend on the estrogen concentrations used in cell culture experiments. Estrogen is known to induce the proliferation of TH2 T-cells at high levels, but it decreases T-cell proliferation at low levels. Furthermore, estrogen is known to decrease TNF-α expression in peripheral blood mononuclear cells (PBMC). TNF-α is an important marker for M1 polarization. The decreased expression of TNF-α is found in male and female PBMCs only if estrogen is administered together with LPS to the cells. [63,73] Similar results have been obtained from treating murine cells with LPS and estrogen. [72] There is evidence that estrogen can downregulate NFκB and TH1 signalling in various male, female, murine and human immune cell types via estrogen receptor-alpha signalling. [74–78] Regarding the fibrotic character of autoimmune diseases, the profibrotic effects of estrogen by increasing IL-4 and TGF-β levels should be kept in mind. [63,79] The androgen effects on the human system have been investigated far less than the estrogen effects on the immune system. There is evidence that androgens support a TH1-type immune response in both humans and rodents. [60,69,70,80–83]

5.3 Role of macrophages in chronic inflammation

Origin of macrophages

Macrophages are the major cells in the resting heart, [115] and they are important regulators of inflammation-triggered fibrosis, as in myocarditis. [116,117] Macrophages are phagocytosing cells that are present in most tissues. They infiltrate the tissue from the peripheral blood stream. The origin of macrophages is the bone marrow and its haemopoietic stem cells. For the maturing process from haemopoietic stem cells to mature monocytes, the colony-stimulating factors M-CSF and GM-CSF are highly important. [118] The maturing process of macrophages is not exclusively limited to bone marrow, however. Monocytes in the peripheral blood change their receptor status depending on how long they have been in the blood before being recruited to the tissue. [73,119] Murine macrophages are characterized by the expression of the surface markers CD 68, [120] F4/80 [121] and CD11b. [122]

Role of macrophages in inflammation

Many different macrophage phenotypes are known. They have functions in the inflammatory response towards infections, immune regulation, phagocytosis of sterile debris and tissue repair. Processed debris is loaded with highly conserved patterns of distinct pathogens. Macrophages can recognize these so-called pathogen-associated molecular patterns (PAMPS) [123] by using pattern recognition receptors (PRR) such as the group of TLR or IL-1 receptors. The activation of these receptors leads to an extreme change in macrophage cell polarization. The activated form of macrophages produces pro-inflammatory mediators and cytokines. [124,125,126] This pro-inflammatory state of macrophages is called M1 type, or classically activated macrophages, since other ways of macrophage activation and their other roles in inflammation have been revealed. The M2 type of macrophages, or alternative activated macrophages, are not pro-inflammatory but play key roles in the resolution of active inflammatory states using immune regulation and tissue repair. [127,128] The model of only two types of macrophage subspecies (M1 and M2) was revised after the discovery of additional subtypes of macrophages, which are not pro-inflammatory but also cannot be assigned to one general type of M2 macrophages. [129] Mosser and Edwards thus proposed macrophage polarization as a spectrum with three basic functions: pro-inflammatory (classically activated), wound-healing (tissue repair) and immune-regulatory. In this context, “spectrum” means that macrophages never obtain a definite state of one of these three; rather, they are always on this spectrum with more or fewer characteristics of each subtype. [73]

The spectrum of macrophage polarization

Macrophages fulfill many tasks during inflammation. Tissue-resident macrophages recognize pathogens such as viruses and bacteria using TLR. This triggers a pathway to polarize macrophages into a pro-inflammatory phenotype. By using cytokines and chemotaxines, these macrophages boost the local inflammation and recruit more cells to the affected tissue. As antigen-presenting cells, macrophages also play an important role as a link between the non-adaptive and adaptive immune system. After the pathogen is cleared, another type of macrophage is needed to regulate and shut down the inflammatory response as well as to induce tissue repair. The dysregulation of these polarization processes can lead to ongoing inflammation and fibrotic remodelling, suggesting that macrophages play key roles in several diseases. [130,73]

Classically activated macrophages

The classically activated macrophage is the pro-inflammatory first line of defense in inflammation. TNF-α is produced by macrophages after recognizing PAMPs such as lipopolysaccharide (LPS) via TLR4. Usually, macrophages need another pro-inflammatory stimulus to maintain the M1 phenotype. This is usually IFN-γ, which is mostly produced by TH1 T-cells and natural killer cells. [131,132] TH1 T-cells secrete IFN-γ while interacting with macrophages. [133] LPS is one of the PAMPs and can activate macrophages into an M1 state independently from IFN-γ secreted by lymphocytes or NK cells. [134] Both activated p38 and ERK are known downstream-signalling targets of LPS and are important in M1 macrophage polarization signalling. [135–137] TLR4 can also recognize patterns of CVB3. [16] The downstream transcription factor of LPS/TLR4 signalling is Nuclear Factor Kappa B (NFκB). [131] Further downstream of TLR4 signalling, myeloid differentiation primary response 88 (MyD88) leads to NFκB activation and TNF-α secretion. Another important downstream transcription factor of LPS signalling is c-fos. [135] Other established markers of M1 macrophages include nitric oxide synthase (iNOS), which is a highly antimicrobial enzyme, [138] and the cytokine IL-1β. [73]

After the responsible trigger (e.g., bacteria) is cleared, a phase of immune regulation and tissue repair is necessary after the initial pro-inflammatory phase. [139] Macrophages have an inbuilt mechanism to prevent overwhelming inflammation by ongoing classic activation - this is called LPS tolerance. Experiments have shown the inability of LPS to activate M1 pathways after cells have been exposed to LPS for several hours. [140] However, in this state, these macrophages still can express many genes of the M2 spectrum. Thus, they have some kind of inbuilt switch from an M1- to an M2-like phenotype. [141]

Alternatively activated macrophages

Experiments with the effect of IL-4 showed a subspecies of macrophage with decreased pro-inflammatory abilities. The macrophage mannose receptor (MMR) is upregulated in these cells in mice. Later, IL-13 was demonstrated to be another cytokine that induces the M2 status of macrophages. [142] Mosser and Edwards have suggested thinking of a spectrum between being primarily pro-inflammatory, tissue repairing or immune regulatory as more subspecies with characteristics of more than one subspecies are described. [73,143] Wound-healing macrophages are needed after the clearance of pathogens to support tissue restitution by stimulating fibrotic remodelling. IL-4 and IL-13 are the signature cytokines to induce such a macrophage phenotype. [73] In later stages of inflammation, TH2 T-cells secrete IL-4 and IL-13. [144,145] In IL-4 treated macrophages, the activity of arginase 1, which contributes to the production of the extracellular matrix, is upregulated. [146] Furthermore, this macrophage phenotype shows decreased characteristics of pro-inflammatory macrophages, as the expression of the classic pro-inflammatory cytokines, chemotaxins and nitrogen radicals is decreased. [129] Other tissue repair macrophage markers in murine macrophages are chinitase-like proteins such as YM1. [147] These seem to have a function in reorganizing the extracellular matrix, as they have the ability to bind to extracellular matrix proteins. [148,149] RELMα expression is also induced by IL-4 signalling in murine macrophages. RELMα promotes the deposition of the extracellular matrix. [147] The signalling pathways for alternatively activated macrophages have been investigated less than the classic M1 pathways. The heterogeneity of the non-inflammatory spectrum of macrophages is complex and not as homogenous as the classic M1 type. [73] IL-4 and IL-13 signalling activates the Janus Kinase (JAK) JAK-STAT6 pathway. STAT6 is a transcription factor that regulates the genes of many M2 macrophage signature proteins, such as arginase 1, macrophage mannose receptor 1 (CD206), Fizz 1 and YM1. [150] Another subtype of the spectrum of M2 polarization Mosser and Edwards have suggested are regulatory macrophages, [73] a subspecies that is anti-inflammatory and not tissue repairing. [129] IL-10 is one of the signature cytokines of regulatory macrophages. [151,128]

Macrophage-related fibrosis regulation

Macrophages play a key role in the regulation of fibrosis. Microphages also usually reside in close range to accumulations of connective tissue-producing cells. They activate fibroblasts by using cytokines such as TGF-β, platelet-derived growth factor (PDGF) and TNF-α. [152,116] They also directly intervene in the remodelling of the extracellular matrix by using matrix

metalloproteinase (MMP) to degrade it and tissue inhibitors of matrix metalloproteinase (TIMP) to attenuate the degradation of the extracellular matrix. [153]

Role of macrophages in cardiac fibrosis

Cardiac macrophages have a spindle-like shape and are usually close to endothelial cells. [154,155] They interact with other cardiac cells and thereby regulate different phases of cardiac diseases: acute inflammation, immune regulation and resolution, the processing of cellular debris and cardiac remodelling. Macrophages regulate the response of cardiac tissue to cardiac stress. [156] During phases of cardiac stress (e.g., myocardial infarction or myocarditis), the local macrophage population is expanded through recruitment from the blood stream as well as through local proliferation. [157–159] A major trigger for macrophage tissue infiltration is monocyte chemoattractant protein 1 (MCP-1). [160] The inhibition of MCP-1 signalling by antibody usage reduced vascular inflammation and myocardial fibrosis in a murine animal model of hypertension-induced cardiac disease. [161] The ingestion of apoptotic cells by macrophages promotes a tissue-repairing and immune-regulating M2 type of macrophages. This was shown for apoptotic cardiomyocytes following acute cardiac inflammation as an important mechanism of stimulating cardiac repair following infarction. [162] A key cellular event in the development of cardiac fibrosis is the transformation of cardiac fibroblasts into activated fibroblasts. [163–165] Activated fibroblasts generate an extracellular matrix and scar tissue following cardiac damage. [166,167] An important cellular marker for the activation of fibroblasts is the expression of MCP-1. [168] After the activation of fibroblasts, an increased production of extracellular matrix proteins can be observed. [169] TGF-β, IL-1β and TNF-α have been shown to be able to activate fibroblasts. [116] All three are also effector cytokines of macrophages, indicating that macrophages play an important role in regulating fibroblast activation in damaged cardiac tissue. [170] The depletion of monocytes and macrophages in the myocardium following cardiac stress decreases fibroblast activation and collagen production. [155]

5.4 Working hypothesis and aims of this study

The preliminary data outlined above indicate significant differences between male and female immune responses. Furthermore, macrophages are the major immune cell class in the resting heart, and there is a broad range of macrophage subtypes. Therefore, our study aimed to test the following theses:

1. There are sex differences in the composition of myocardial infiltrates in heart sections of EAM animals regarding macrophages and their differently polarized subtypes.
2. There are sex differences in macrophage polarization, fibroblast activation and macrophage-fibroblast cell-cell interaction, which could explain sex differences in EAM animals.
3. Estrogen signalling contributes to sex differences in macrophage activation and the composition of myocardial infiltrates.

6 Materials

6.1 Devices

Binocular microscope, Wild M8	Leica Biosystems GmbH (Nussloch)
Camera, Canon PowerShot G5	Canon GmbH (Krefeld)
Centrifuge 5417R	Eppendorf (Hamburg)
Centrifuge 5430R	Eppendorf (Hamburg)
Fast Prep	Mpbio (Santa Ana, USA)
Fluorescence microscope, LSM 510	Zeiss (Göttingen)
Freezer, -20°C	Liebherr (Ochsenhausen)
Freezer, -80°C	Sanyo (Munich)
Fridge, 4°C	Bosch (Gerlingen-Schillerhöhe)
Gemini EM Microplate Reader	Molecular devices (Sunnyvale, USA)
Hargreaves electronic timer (Model 336)	IITC Life Sciences (Woodland Hills, USA)
Homogenizer	Roth GmbH (Karlsruhe)
Incubator, BBD 6220	Heraeus (Hanau)
Incubator model 3000	Jane Schütz GmbH (Hammelburg)
Magnet mixer, KMO 2 basic	IKA Werke GmbH (Staufen)
Microscope, Axiophot	Zeiss (Göttingen)
Microwave, NN-E205W	Panasonic (Munich)
Multiwave photometer	Fisher Scientific (Langenselbold)
NanoDrop ND-100 spectophotometer	Peqlab Biotechnologie (Erlangen)
Neubauer cell chamber	Celeromics (Valencia, Spain)
pH meter, pH 300	Hanna Instruments (Kehl am Rhein)
Pipetting devices (1-1000 µl)	Eppendorf (Hamburg)
Power supply unit, Power Pac 3000	Bio-Rad Laboratories GmbH (Munich)
Sterile hood	Hera Safe Heraeus Instruments (Hanau)
StepOne Real-Time PCR System	Life Technologies (Darmstadt)
Suction machine	HLC Biotech (Bovenden)
Teflon pestle	Roth GmbH (Karlsruhe)
Thermomixer comfort	Eppendorf (Hamburg)

Tweezers (sharp and curved)	Dumont & Fils (Montignez, Switzerland)
Tweezers	Carl Roth GmbH (Karlsruhe)
Ultracentrifuge, Coulter Optima L-100 XP (SW40 rotor)	Beckmann (Fullerton, USA)
VictorX Multilabe Plate Reader	PerkinElmer (Waltham, USA)
Vortex Genie 2	Scientific Industries (Bohemia, USA)
Water bath, R19:S54h	Medax GmbH (Neumünster)

6.2 Consumables

Cell culture flasks	Nunc (Wiesbaden)
Cover slips (24 mm)	MatTEK Corporation (Ashland, USA)
Cryogenic vials	VWR International GmbH (Darmstadt)
Eppendorf tubes (0.5, 1.5, 2.0 ml)	Eppendorf (Hamburg)
Falcon tubes (15 and 50 ml)	BD (Franklin Lakes, USA)
MicroAmp Fast Optical 48-well plate	Life Technologies (Darmstadt)
MicroAmp Reaction Tube/Cap, 0.2 ml	Life Technologies (Darmstadt)
Needle (25 gauge)	B. Braun (Melsungen)
Nitrocellulose membrane	Bio-Rad (Munich)
Pasteur pipettes	Sigma-Aldrich (Steinheim)
Petri dishes, Nuclon surface	Nunc (Wiesbaden)
Pipet tips (0.5-10, 10-100, 100-1000 µl)	Eppendorf (Hamburg)
Serological pipets (2, 5, 10 and 25 ml)	BD (Franklin Lakes, USA)
Pipet tips	Sarstedt AG (Nümbrecht)
Syringe (1 ml)	B. Braun (Melsungen)
Ultracentrifuge tube	Beckmann (Fullerton, USA)
Six-well plate	Sarstedt (Nümbrecht)
96-well plate	Sarstedt (Nümbrecht)
Whatman paper	Whatman International Ltd. (Maidstone, UK)

6.3 Chemicals, media, buffers and solutions

All solutions were prepared using ultrapure water (resistivity: 18.2 MΩ) and analytical-grade reagents. If required, the pH was adjusted using sodium hydroxide or hydrochloride acid.

5x first-strand buffer	Life Technologies (Darmstadt)
6x DNA loading dye	Fisher Scientific (Schwerte)
17-β estradiol	Sigma-Aldrich (Steinheim)
β-mercaptoethanol	Sigma-Aldrich (Steinheim)
Antimycin	Sigma-Aldrich (Steinheim)
Bisacrylamide	Roth (Karlsruhe)
Bromophenol blue	Sigma-Aldrich (Steinheim)
BSA	Sigma-Aldrich (Steinheim)
Dapi Fluoromount G	Southern Biotechnology (Birmingham, USA)
DEPC	Fisher Scientific (Schwerte)
Dextrin	Sigma-Aldrich (Steinheim)
Dioxyglucose	Sigma-Aldrich (Steinheim)
Donor horse serum	Sigma-Aldrich (Steinheim)
D-glucose	Sigma-Aldrich (Steinheim)
D-mannitol	Sigma-Aldrich (Steinheim)
DMEM with phenol red	Gibco (Karlsruhe)
DMEM without phenol red	Gibco (Karlsruhe)
Ethanol	Roth (Karlsruhe)
FCCP	Sigma-Aldrich (Steinheim)
Fetal bovine serum	Biochrom (Berlin)
Formaldehyde (35%)	Sigma-Aldrich (Steinheim)
Glycerol	Sigma-Aldrich (Steinheim)
Glycine	Sigma-Aldrich (Steinheim)
Histofix, 4%	Roth (Karlsruhe)
Hydrochloric acid (HCl, 37%)	Roth (Karlsruhe)
IL-4	Sigma-Aldrich (Steinheim)
IL-13	Sigma-Aldrich (Steinheim)
Isoflurane	Sigma-Aldrich (Steinheim)
Isopropanol	Fluka Chemie GmbH (Buchs, Switzerland)
LB broth	Sigma-Aldrich (Steinheim)
L-Glutamine	Sigma-Aldrich (Steinheim)
LPS	Sigma-Aldrich (Steinheim)
Nuclease-free water	Life Technologies (Darmstadt)

Penicillin/Streptomycin solution	Biochrom (Berlin)
Protein inhibitor cocktail (PIC)	Sigma-Aldrich (Steinheim)
Ponceau red	Sigma-Aldrich (Steinheim)
Potassium chloride	Merck Millipore (Darmstadt)
Potassium cyanide	Sigma-Aldrich (Steinheim)
Potassium dihydrogen phosphate	Merck Millipore (Darmstadt)
RNA-Bee	Amsbio (Abbingdon, UK)
Rotenon	Sigma-Aldrich (Steinheim)
RPMI-1640	Sigma-Aldrich (Steinheim)
Sodium chloride (NaCl)	Roth (Karlsruhe)
Sodium hydroxide (NaOH)	Merck Millipore (Darmstadt)
TEMED	Sigma-Aldrich (Steinheim)
TNF-α	Sigma-Aldrich (Steinheim)
TGF-β	Sigma-Aldrich (Steinheim)
Tri-sodium citrate dihydrate	Merck Millipore (Darmstadt)
Triton X-100	Sigma-Aldrich (Steinheim)
Trypan blue	Fisher Scientific (Schwerte)
Trypsin/EDTA without phenol red	Sigma-Aldrich (Steinheim)
Trypsin phenol red-free	Sigma-Aldrich (Steinheim)
Tween 20	Merck Millipore (Darmstadt)

6.4 Buffers and solutions for western blotting

Laemmli buffer

Tris-HCl, pH 6.8	87.5 mM
SDS	0.1% (w/v)
Glycerol	5% (w/v)
Bromophenol blue	0.1% (w/v)
β-mercaptoethanol	0.1% (w/v)

Stacking gel (5%)

Tris-HCl, pH 6.8	87.5 mM
SDS	0.1% (w/v)
Acrylamide	5% (w/v)
Bisacrylamide	0.1% (w/v)
Ammonium persulfate	0.1% (w/v)
TEMED	0.1% (w/v)

Separation gel (12%)

Tris-HCl	375 mM
SDS	0.1% (w/v)
Acrylamide	12 % (w/v)
Bisacrylamide	0.24% (w/v)
Ammonium-persulfate	0.1% (w/v)
TEMED	0.1% (w/v)

4x Western blot-loading buffer

Tris-HCl, pH 6.8	200 mM
SDS	8% (w/v)
Glycerol	40% (v/v)
Bromophenol blue	0.01% (w/v)
Dithiothreitol	40% (v/v)

SDS running buffer

Tris-HCl	25 mM
SDS	0.1% (w/v)
Glycine	192 mM

Transfer buffer

Tris-HCl, pH 8.5	25 mM
Glycine	192 mM
Methanol	10% (v/v)

Washing buffer/TBS-T

Tris-HCl, pH 8.5	20 mM
Sodium chloride	140 mM
Tween 20	0.1% (v/v)

Blocking buffer

BSA	5% (w/v)
TBST-buffer	95% (v/v)

Stripping buffer, pH=2.0

Glycine	250 mM
10% SDS	10% (w/v)

6.5 Buffers and solutions for immunofluorescence

PBS-T

Disodium phosphate	4.3 mM
Potassium dihydrogen phosphate	1.4 mM
Sodium chloride	137 mM
Potassium chloride	2.7 mM
Tween 20	0.1%

1% BSA/PBS-T

BSA	1%
Disodium phosphate	4.3 mM
Potassium dihydrogen phosphate	1.4 mM
Sodium chloride	137 mM
Potassium chloride	2.7 mM
Tween 20	0.1%

3% BSA/PBS-T

BSA	3%
Disodium phosphate	4.3 mM
Potassium dihydrogen phosphate	1.4 mM
Sodium chloride	137 mM
Potassium chloride	2.7 mM
Tween 20	0.1%

6.6 Cell culture media

Medium for L929 cell line

Cultivation medium

RPMI-1640 (with phenol red, cat. 11875093)	88%
Penicillin/Streptomycin	1 mM
Non-essential amino acids (NEAA)	1 mM
Fetal bovine serum (FBS, cat. S0115)	10%

Media for bone marrow-derived mouse macrophages

Washing medium (for isolation protocol)

DMEM (with phenol red, cat. 41965)	88%
Fetal bovine serum (FBS, cat. S0115)	10%
Penicillin/Streptomycin	1 mM
Sodium pyruvate	1 mM
Fetal bovine serum (FBS, cat. S0115)	10%

Differentiation medium

DMEM (with phenol red, cat. 41965)	68%
β-mercaptoethanol	50 µM
Non-essential amino acids	1%
Penicillin/Streptomycin	1 mM
Sodium pyruvate	1 mM
Donor horse serum (cat. H1138)	20%
Fetal bovine serum (FBS, cat. S0115)	10%

Starving medium

DMEM (phenol red free, cat. 31053)	94.5%
β-mercaptoethanol	50 µM
Penicillin/Streptomycin	1 mM
Sodium pyruvate	1 mM
Fetal bovine serum (FBS, cat. S0115), charcoal stripped	2.5%

Charcoal-stripped starvation medium

The remaining hormones were removed from the fetal bovine serum by charcoal stripping before adding it to the starvation medium.

Medium for fibroblasts

Cultivation medium

DMEM (with phenol red, cat. 41965)	88%
Penicillin/Streptomycin	1 mM
Sodium pyruvate	1 mM
Glutamine	1 mM
Fetal bovine serum (FBS, cat. S0115),	10%

Starving medium

DMEM (phenol red free, cat. 31053)	94.5%
Penicillin/Streptomycin	1 mM
Sodium pyruvate	1 mM
Glutamine	1 mM
Fetal bovine serum (FBS, cat. S0115), charcoal stripped	2.5%

6.7 Oligonucleotides

Oligo_(dT)-primers for the qRT-PCR were designed using the Roche Universal Probe Library and purchased from Eurofins Scientific (Luxembourg City, Luxembourg).

Table 1: Used Oligo_(dT)-Primers, sequences, species, product length

Name	Sequences	Species	Product length (bp)
HPRT	FW: GCT TTC CCT GGT TAA GCA GTA CA RV: ACA CTT CGA GAG GTC CTT TTC AC	Mouse	49

C-fos	FW: CGG TTC CTT CTA TGC AGC AG RV: GTA CAG GTG ACC ACG GGA GT	Mouse	80
NFκB	FW: TGT TCA GCT TCG GAG GAA AT RV: TCC TTT GCA CTT CCT CTT TGT C	Mouse	78
IL-1β	FW: AGT TGA CGG ACC CCA AAA G RV: GAA GCT GGA TGC TCT CAT CA	Mouse	42
TNF-α	FW: CCA AAT GGC CTC CCT CTC AT RV: AGC TGC TCC TCC ACT TGG	Mouse	178
TLR 4	FW: GAA GGT TGA GAA GTC CCT GCT RV: CCC ATT CCA GGT AGG TGT TTC	Mouse	167
MCP 1	FW: CCC AAT GAG TAG GCT GGA GA RV: TCT GGA CCC ATT CCT TCT TG	Mouse	125
IL-10	FW: GGT TGC CAA GCC TTA TCG GA RV: ACC TGC TCC ACT GCC TTG CT	Mouse	191
TIM 3	FW: TTT TCA GGT CTT ACC CTC AAC TG RV: CAT AAG CAT TTT CCA ATG ACC TT	Mouse	76
STAT 6	FW: ACA AAT ACT TCA AGG GGA GAA AAA RV: CCT TCC GAG GGC TGT AGT AA	Mouse	65
RELM α	FW: GCA CTA GTG TCA AGA CTA TGA ACA GAT RV: AGC ACA CCC AGT AGC AGT CA	Mouse	68
YM 1	FW: GGT CTG AAA GAC AAG AAC ACT GAG RV: GAG ACC ATG GCA CTG AAC G	Mouse	88
COL1A1	FW: CAT GTT CAG CTT TGT GGA CCT RV: GCA GCT GAC TTC AGG GAT GT	Mouse	44
TGF-β	FW: CCA AGG AGA CGG AAT ACA GG RV: GTT CAT GTC ATG GAT GGT GC	Mouse	76
Arginase	FW: TAT GGT CCA CAG CTG CCA TTC RV: CCA AAG TCT TTA GGT GGC ATC	Mouse	87
HPRT	FW: GTC CCA GCG TCG TGA TTA GT RV: CTC GAG CAA GTC TTT CAG TCC	Rat	140
C-fos	FW: CCT GCA AGA TCC CCA ATG	Rat	65

	RV: AGT CAA GTC CAG GGA GGT CA		
STAT 1	FW: TGT TAT CCG ATC GCA CCT T RV: GTC GTT CCA CCA CAA AGG A	Rat	66
iNOS	FW: ACC ATG GAG CAT CCC AAG T RV: CAG CGC ATA CCA CTT CAG C	Rat	60
IL-1β	FW: TGT GAT GAA AGA CGG CAC AC RV: CTT CTT CTT TGG GGT ATT GTT TGG	Rat	70
MCP-1	FW: AGC CAT CCA CGT GTC GTC TC RV: GAT CAT CTT GCC AGT GAA TGA G	Rat	78
COL1A1	FW: GGT CCT AAT GGC CCA CCT RV: ACC AGG GAA GCC AGT CAT AC	Rat	41
COL3A1	FW: CTC AAG AGC GGA GAA TAC RV: ATC TGT CCA CCA GTG CTT	Rat	150

6.8 Antibodies

The following antibodies were used for western blotting. The sources and dilutions used are stated. All antibodies were diluted in 5% BSA-TBST solution.

Table 2: Antibodies used for western blotting, band size, catalog number; species: appropriate for mouse and rat

First antibody	**First antibody, dilution**	**Second antibody (DaM), dilution**	**Detected band (kDA)**	**Company, catalog number of first antibody**	**Company, catalog number of second antibody**
α-Tubulin	1:10,000	1:20,000	55	Sigma, T9026	Dianova, 115-165-003
ERK	1:200	1:5,000	44	Santa Cruz, sc93	Dianova, 115-165-003

pERK	1:100	1:5,000	42	Santa Cruz, sc16982	Dianova, 115-165-003
p38	1:200	1:5,000	38	Santa Cruz, sc7149	Dianova, 115-165-003
pp38	1:200	1:5,000	38	Santa Cruz, sc17852	Dianova, 115-165-003
NFκB	1:100	1:5,000	50	Santa Cruz, sc7178	Dianova, 115-165-003

The following antibodies were used for immunostaining. The sources and dilutions used are stated. All antibodies were diluted in 5% BSA-TBST solution.

Table 3: Antibodies used for immunostaining, catalog number; species: all appropriate for mouse

First antibody	**First antibody, dilution**	**Second antibody (FITC/CY3), dilution**	**Company, catalog number of first antibody**	**Company, catalog number of second antibody**
F4/80	1:100	1:100	Abcam, ab240946	Dianova, 111-095-045
CD11b	1:100	1:100	Abcam, ab133357	Dianova, 111-095-045

6.9 Kits

BCA™ Protein Assay Reagent Kit	Fisher Scientific (Schwerte)
SuperScript® VILO™ cDNA Synthesis Kit	Life Technologies (Darmstadt)
SYBR Green PCR Master Mix	Life Technologies (Darmstadt)
Pierce™ ECL Western Blotting Substrate	Fisher Scientific (Schwerte)

6.10 Cell lines

The male mouse fibroblast cell line (L929) was a gift from Dr. Dorette Freyer from the Zentrum für Schlaganfallsforschung (Charité – Universitätsmedizin Berlin).

6.11 Animals and animal housing

Wild type mice for cell culture experiments

Eight- to 12-week-old male and female C57/BL6J mice were purchased from the Forschungseinrichtung für Experimentelle Medizin (FEM), Charité – Universitätsmedizin Berlin (Tötungsanzeige: T0333/08). All experimental procedures were performed according to the established guidelines for the care and handling of laboratory animals, and were approved by the Animal Care Committee of the Senate of Berlin. The mice were housed in groups of six to eight animals in a cage with ground corncob bedding. The room temperature was maintained at 22 ± 0.5°C, with a relative humidity between 40% and 60%. A 12h/12h light and dark cycle was applied. Water and food were provided *ad libitum*.

6.12 Software

Endnote 7	Thomson Research Software (New York, USA)
Mendeley	Mendeley Ltd., Elsevier
Image Lab	Bio-Rad
Microsoft Office 2010	Software (Germany)
Prism 5 GraphPad	Software (San Diego, USA)

7 Methods

7.1 Cytological techniques

7.1.1 Cell culture and treatments

The cell culture work was performed in the tissue culture rooms of the Institute for Gender in Medicine at the Charité-Universitätsmedizin Berlin. The cells were kept in incubators at 37°C in a humidified atmosphere (5% (v/v) of water and a constant CO_2 concentration of 5% (v/v) or 10% (v/v) in the case of bone marrow-derived macrophages.

7.1.2 Counting of cells

25 µl of cell suspension was mixed with the same amount of trypan blue and placed under a cover slip on a Neubauer cell chamber. The cells in 16 small quadrants were counted. The total volume of these quadrants was 0.1 µl. The number counted in this volume was multiplied by the *chamber factor of 10^4 , resulting in the number of cells present in 1 ml of cell suspension.

7.2 Bone marrow-derived macrophages – cell culture experiments

Bone marrow isolation and BMM differentiation

Bone marrow-derived macrophages (BMMs) were isolated from eight- to 12-week-old male and female C57/BL6J mice. Briefly, the mice were sacrificed following anesthetization with isoflurane. The abdomen and hind legs were sterilized using 70% ethanol solution. An incision was made in the midline of the abdomen. Subsequently, all muscle tissues were removed from the bones with scissors. The bones were cut at both ends without opening the bone canals and were stored in ice-cold DPBS for further processing. After sterilizing the bones with 70% ethanol, the bone canals were sliced on both ends in a sterile environment. The bone marrow cells were collected by flushing the femurs and tibias with the washing media using a 1 ml syringe with a 20-gauge needle. The cells were pipetted up and down to obtain single-cell suspension and passed through a 70 µm cell strainer. The cells were then centrifuged (1,200 rpm, 5 min, 22°C), resuspended in the differentiation medium and counted in a Neubauer cell chamber. Subsequently, $1x10^7$ cells were diluted in 40 ml differentiation medium and 10 ml L929-preconditioned medium. The cell suspension was transferred to Teflon-coated polypropylene (polythene) bags. Bone marrow cells were cultivated for 10 days in a humidified

incubator with 10% CO_2 at 37°C to promote differentiation into BMMs. At day 10, the BMMs were harvested and used for further experiments.

Harvesting and seeding into six-well plates

After 10 days, the cells were removed from the bags and centrifuged at 1,200 rpm for 5 minutes. The supernatant was discarded, and the cells were resuspended into the macrophage-starving medium. One million cells were put into one well with 3 milliliters of macrophage-starving medium. The cells were seeded with 1 million cells in each well of a six-well plate. The macrophages were stimulated in passage 1.

Cultivation of L929 cell line for pre-conditioned medium

During the conditioning process, L929 cells release cytokines such as M-CSF and GM-CSF (colony-stimulating factors), which signal macrophage precursors to duplicate and differentiate.[171] The cells were cultured in a supplemented RPMI-1640 medium in a 5% CO_2 incubator at 37°C. The cells were grown for two weeks, until 30% of the cells were necrotic. The supernatant was collected and sterile filtered through a 0.45 µm filter. 50 ml aliquots were frozen and stored at -20°C.

7.3 Isolated murine cardiac fibroblasts – cell culture experiments

Fibroblast isolation

Murine cardiac fibroblasts were isolated from eight- to 12-week-old male and female C57/BL6J mice. Briefly, the mice were sacrificed following anesthetization with isoflurane, and the hearts were then removed. The hearts were stored in DMEM medium on ice for up to 1 hour. All the following steps were done on ice until resuspending and seeding the cells. First, the mice hearts were washed with PBS to remove erythrocytes until there was no longer a reddish color in the PBS used. The hearts were then cut into small pieces on a cell culture disc using a scalpel. The pieces were collected, put into a falcon and washed again with PBS. The PBS was removed, and 2.5 milliliters of digestion solution was added to each falcon. Falcons were shaken by hand for 30 seconds and then put into the water bath for two minutes at 37 °C. This procedure was repeated five times. The supernatant was removed carefully and put into falcons with ice-cold fibroblast growth medium. The cells were centrifuged at 1,200 rpm at four degrees for five minutes. The cell pellet was resuspended in PBS at 4 °C and then centrifuged at 1,200 rpm at

4 °C for five minutes. The cells of each heart were resuspended in fibroblast growth medium and cultivated in a T75 cell culture flask.

Harvesting and seeding into six-well plates

The medium was changed every three days until a 90%-100% cell confluence was reached. Next, the fibroblasts were transferred to a six-well plate with 50,000 cells in each well. The cells were washed with PBS and then trypsinized. Fibroblast growth medium was placed in the flask, and the cell suspension was transferred into a falcon. The falcons were then centrifuged at 1,200rpm for five minutes. The cell pellet was resuspended in fibroblast growth medium, and the cells were put into a six-well plate, with 50,000 cells to each well.

7.4 Cell culture experiments – stimulation protocols

7.4.1 Macrophage polarization in M1 and M2 state

Macrophage polarization into M1 and M2 states was established as part of this work. For M1 polarization, the macrophages were treated with LPS (10 ng/ml) for 24 hours. For M2 polarization, they were treated with IL-4 and IL-13 (both 10 ng/ml) for 24 hours. Before stimulation, the macrophages were starved for 24 hours using a macrophage-starving medium.

Estrogen treatment

Before E2 stimulation, the macrophages were starved for 24 hours. E2 stimulation was done together with polarization into M1 and M2 cell states. For the E2-treated group, estrogen was added at a concentration of 10 nM. For the vehicle-treated group (negative group), Dextrin was added at a concentration of 10 nM.

7.4.2 Fibroblast activation with TNF-α for 24 hours

For future experiments with estrogen effects on fibroblast activation, a stimulation protocol with TNF-α was established. Cells were first starved for 24 hours with fibroblast-starving medium. After 12 hours, 20ng/ml TNF-α was pipetted into fibroblast-starving medium for 12 hours. The cells were stimulated for 24 hours overall. The medium for the negative group was not supplied with a stimulant.

Estrogen treatment

Cells were starved for 24 hours with fibroblast-starving medium. First, the cells were stimulated for 12 hours with 10 nM E2 or 10 nM Dextrin. After 12 hours, 20ng/ml TNF-α was pipetted into the cells for another 12 hours.

7.4.3 Cell culture experiments – co-culture of fibroblasts/macrophages

For the co-culture experiments, the supernatant of stimulated macrophages (LPS, LPS and E2, IL-4 and IL-13, IL-4 and IL-13, and E2) was used to stimulate isolated murine cardiac fibroblasts. For the stimulation, macrophage-conditioned supernatant was mixed with fibroblast-starving medium at a ratio of 1:1 and placed on the cells. For the conditioned medium, macrophages were stimulated for 24 hours. For the negative group, fibroblast-starving medium and macrophage-starving medium were mixed at a ratio of 1:1, and then placed on the fibroblasts. The macrophage-conditioned medium was filtered from the remaining macrophages and cell debris in the medium prior to adding to the fibroblast culture.

7.5 Biochemical techniques

7.5.1 Protein extraction

Proteins were extracted using Laemmli buffer. The protein solution was cooked for 10 minutes at 96°C and stored at -80°C until further processing.

Determination of protein concentration

The amount of total protein was evaluated with the Pierce™ 660 nm Protein Assay according to the instructions of the manufacturer. The concentration was measured in a flat bottom 96-well plate using a multiwave photometer at 660 nm. The concentration determined was used in western blot analysis.

7.5.2 Western blot analysis

The Criterion™ Vertical Electrophoresis Cell System was used for the SDS-PAGE. The glassware was washed with water and acetone. An SDS-separating gel (10% or 12%) was first prepared, and then covered with isopropanol. After a complete polymerization, isopropanol was aspirated with Whatman paper, and a 5% SDS-sacking gel was then poured on top. The protein extracts were run on the SDS-PAGE for 80 min at 100 volts and 400 mA for one hour with the

power supply unit Power Pac 3000. A protein ladder was used as a control for the protein weight. After gel electrophoresis, the separating gel and the nitrocellulose membrane were equilibrated for five minutes at room temperature in Millipore water and then in transfer buffer. The gel and the membrane were placed between three layers of Whatman paper in a tank blot transfer cell. The transfer was run for 1.5 hours at 100 volts. The transfer was verified by staining the membrane with Ponceau red, and the membrane was then washed three times for five minutes with TBST. The membranes were blocked with 5% BSA solution for one hour and incubated overnight at 4°C with the primary antibodies. The dilution of antibodies was performed according to the manufacturer's protocol. After one hour of incubation with secondary antibodies, specific bands were visualized through chemiluminescence using an enhanced chemo luminescence (ECL) kit. To analyze protein expression, the protein level of the target protein was normalized to tubulin using ImageLab software.

7.6 Molecular biological techniques

7.6.1 RNA isolation and cDNA synthesis

For RNA isolation, 1 ml per $1x10^6$ of the extraction reagent RNA-Bee was applied to the cells. 0.2 ml chloroform were added per 1 ml of cell suspension and firmly shaken for two minutes at room temperature. The solution was incubated for five minutes on ice and then centrifuged at 14,000 rpm at 4°C for 10 minutes. The upper phase containing the RNA was transferred to a new tube. The equal volume of cooled isopropanol was applied, and samples were firmly shaken. The RNA was precipitated at -20°C overnight. After precipitation, the samples were centrifuged at 14,000 rpm at 4°C for 30 minutes. The supernatant was discarded, and the pellet was washed with 80% ethanol. The samples were centrifuged at 8,000 rpm at 4°C for five minutes, and the supernatant was discarded. The pellet was air-dried at room temperature and carefully resuspended in ultra-pure diethyl pyrocarbonate (DEPC)-H_2O. The RNA concentration was measured using the NanoDrop ND-100 spectrophotometer. The total RNA content was determined photometrically at a wavelength of λ= 260 nm. The purity of the RNA was determined via the A260nm/A280nm ratio. The samples were stored at -80°C until further processing. The reverse transcription reaction from 500 ng of RNA template was performed using the SuperScript® VILO™ cDNA Synthesis Kit as per the manufacturer's instructions.

7.6.2 Quantitative real-time polymerase chain reaction

Real-time polymerase chain reaction (PCR) amplification was performed in a StepOne Real-Time PCR System using the Brilliant SYBR Green qPCR Master Mix (Applied Biosystems, Foster City, CA). PCR amplifications were conducted as follows: two minutes at 95°C; 40 cycles of 35 sec at 95°C, 45 sec at an annealing temperature of 62°C and 1:30 minutes at 72°C; and finally, five minutes at 72°C. The relative amount of target mRNA was determined using the comparative threshold (Ct) method by normalizing target mRNA Ct values to those for Hypoxanthine phosphoribosyltransferase (HPRT; ΔCt). Statistical analyses of real-time PCR data were performed using ΔCt values. All real-time results were expressed as fold changes in mRNA expression with respect to the control cells. All results were normalized to the expression of the housekeeping gene HPRT in the PCR reactions.

7.6.3 Immunofluorescence of formaldehyde fixed cells

The presence of macrophage-characterizing receptors (CD11b, F4/80) was confirmed via immunofluorescence. The cells were stained with CD11b, F4/80 and CD31 antibodies according to the manufacturer's protocol. $1.5x10^4$ cells were seeded in eight-chamber slides and cultured in DMEM in a 5% CO_2 incubator at 37°C. 24 h after seeding, the cells were washed with DPBS to remove dead cells, and the growth medium was replaced by a starving medium. Next, cells were fixated with Histofix (4%) for 20 min at room temperature and washed with PBS. Afterwards, the cells were permeabilized by applying Triton X-100 diluted in PBS (0.2%) for five minutes and then washed three times for five minutes with PBS-T. The cells were blocked for one hour in 3% BSA/PBS-T, and the primary antibody, which was diluted in 1% BSA/PBS-T according to the manufacturer's protocol, was applied overnight at 4°C. Following this, the cells were washed three times for 10 minutes each with PBS-T. The secondary antibody, diluted in 1% BSA/PBS-T according to the manufacturer's protocol, was applied to the cells and incubated for one hour at room temperature. The secondary antibody was removed, and DAPI staining (1:50,000) was directly performed for five minutes. Afterwards, the cells were washed three times for 10 minutes each with PBS-T and air dried afterwards. Finally, the cells were covered with a glass cover slip. Imaging was conducted with a 40x immersion objective lens and a BZ-9000E fluorescence microscope.

7.6.4 Sirius red staining of paraffin sections

First, the paraffin sections were heated for one hour at 60 °C. To remove the paraffin, the sections were overlaid with Neo-Clear for 15 minutes, after which they were alternatingly overlaid with alcohol solutions in decreasing concentrations (96%, 80%, 70%, and 50%, each at 20 seconds) and distillated water. For the staining, the sections were covered with Sirius red solution and then washed for one minute using distillated water. The sections were then overlaid with Neo-Clear for another 10 minutes; after this was removed, the cells were covered with a glass cover slip. To prevent the sections from drying out, they were covered with Vecta Mount, and the glass slips were sealed with nail polish. On the day of the experiment, imaging was conducted with a 40x immersion objective lens. The amount of red staining was measured using Visi-View software.

7.7 EAM treatment protocol

Young male and female Lewis rats were immunized with porcine myocardial myosin by subcutaneous injection into the paw in two 1-mg doses on day 0 and day 7 of the animal model treatment schedule. The end of the treatment schedule was on day 21 after the first immunization of animals, with the scarification of rodents for further biochemical analysis. Dr. Sarah Jeuthe from the Charité – Universitätsmedizin Berlin performed the treatment of animals. For further tissue processing and biochemical analysis, cryo-conserved rodent hearts were provided for this work.

Immunohistological analysis of EAM heart sections

First, paraffin was removed from the heart sections for 30 minutes, and they were then incubated with RT anti-ArgI 1:100 (Santa Cruz sc-18357) for 30 minutes, washed and again incubated with RT biotinylated rabbit anti-goat 1:1,000 (Dianova) for another 30 minutes. Next, RT AP-Streptavidin rtu (Agilent) was used for 30 minutes on the heart sections, followed by 16 minutes of incubation with RT FastRed Chromogen. The probes were then cooked for one minute in HIER-Puffer at pH8. This was followed by 30 minutes of RT anti-CD68 1:250 (Amsbio PA-1518) incubation, the washing of the sections, and another 30 minutes of RT Alexa488 donkey anti-rabbit 1:100 (Invitrogen) incubation. Cell nucleus staining was performed using RT DAPI 1:1,000 for five minutes. Dr. Sarah Jeuthe from the Charité – Universitätsmedizin Berlin performed heart section immunostaining to calculate the replacement fibrosis score and percentage of infiltrates in the myocardium. PD Dr. Anja Kühl

of the Charité – Universitätsmedizin Berlin performed heart section immunostaining to show sex differences in myocardial infiltrates.

7.8 Statistical analysis

The data are given as means ± SEM, where means indicate biological replicates. Comparisons of the means between the groups were performed using the Mann-Whitney test. For two-group comparisons, two-way ANOVA was performed. All analyses were done using PRISM (GraphPad, San Diego, CA). *P* values were two-sided, and the statistical significance level was set at 0.05.

8 Results

8.1 Sex differences in EAM

An animal model was used to study sex differences in autoimmune myocarditis. The validation of this animal model included proving the effectiveness of the treatment protocol (triggering an inflammatory state of heart tissue, which leads to fibrotic remodelling) and verifying sex-dependent differences between male and female rodents. Dr. Sarah Jeuthe from the Charité – Universitätsmedizin Berlin performed the EAM treatment protocol. For further tissue processing and biochemical analysis, I was provided with cryoconserved rodent hearts.

The ejection fraction of male EAM rodents decreases over time

Male and female rats were treated with CFA and cardiac myosin, and the ejection fraction was then measured via MRI at day 14 and day 21 after immunization. The ejection fraction was significantly decreased in male but not in female rats 14 and 21 days after immunization ($p<0.05$ and $p>0.05$; Fig. 1A). Dr. Sarah Jeuthe from the Charité – Universitätsmedizin obtained the data for the analysis of ejection fraction by performing MRI signaling on EAM animals.

A

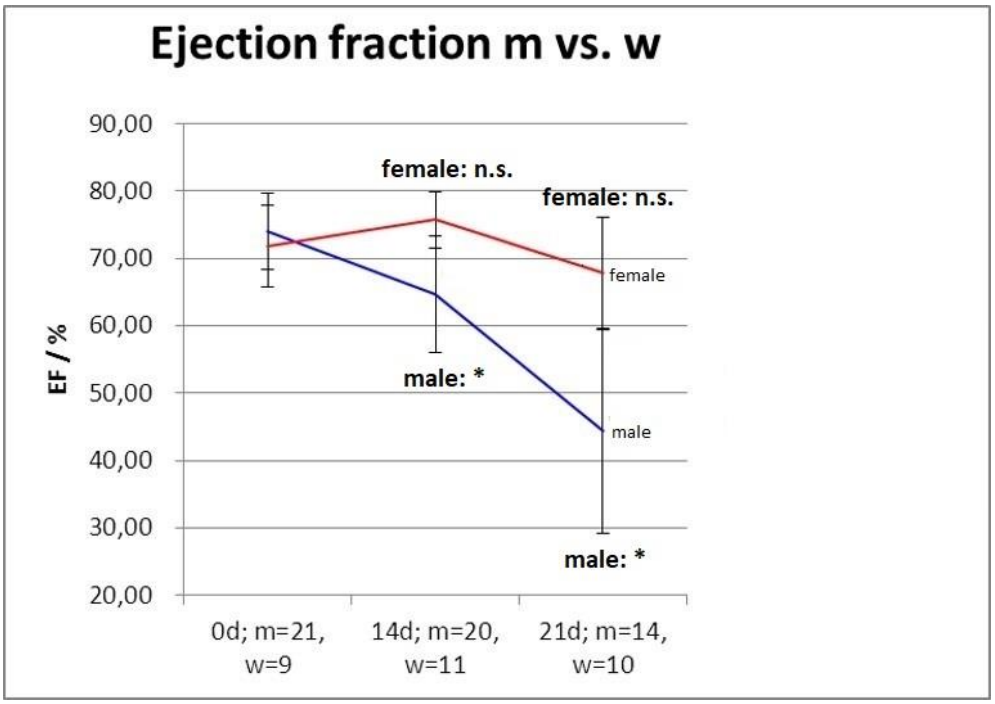


Figure 1: Ejection fraction of male EAM rodents decreases over time.

(A) The ejection fraction was determined at days 0, 14, and 21 after immunization. Shown above are the means and SE from male and female rats after immunization (*p<0.05; n.s.: not significant, Mann Whitney test, n= 9-21/group).

Inflammatory infiltrates are found in male and female EAM heart sections, but fibrotic remodelling is only found in male ones

The analysis of the percentage of immune infiltrates in the myocardium showed a significant increase in both male and female immunized animals after 21 days compared to healthy rats (p<0.001 and p<0.05; Fig. 2A). Male rats tended to show an increased percentage of infiltrates compared to female rats 21 days after immunization (p>0.05; Fig. 2A). The replacement fibrosis score showed a significantly increased fibrosis formation in male rodent hearts, but not in female hearts (p<0.001 and p>0.05; Fig. 2B). Dr. Sarah Jeuthe from the Charité – Universitätsmedizin Berlin obtained the data for the analysis of infiltrates in myocardium and the replacement fibrosis score by performing immunostaining of EAM heart sections.

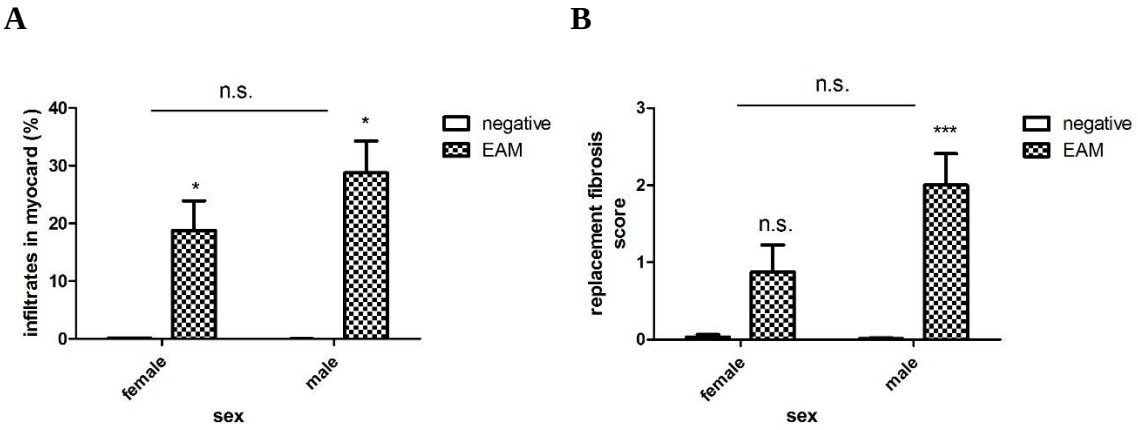


Figure 2: Inflammatory infiltrates are found in male and female EAM heart sections, but fibrotic remodelling is only found in male ones.

(A) Percentage of immune infiltrates in myocardium. (B) Replacement fibrosis score of heart sections from male and female rats 21 days after immunization. Values are shown as mean ± SEM, n=4-9/group (*p<0.05; **p<0.01; ***p<0.001; n.s.: not significant; two-way ANOVA with Bonferroni correction).

Increased fibrosis formation in male EAM heart sections

In order to analyze sex differences in fibrosis formation in male and female EAM heart sections, Sirius red staining of heart sections was performed. The analysis of this experiment showed

significantly increased fibrosis formation in male rodent heart sections compared to female ones ($p<0.05$; Fig. 3A).

A

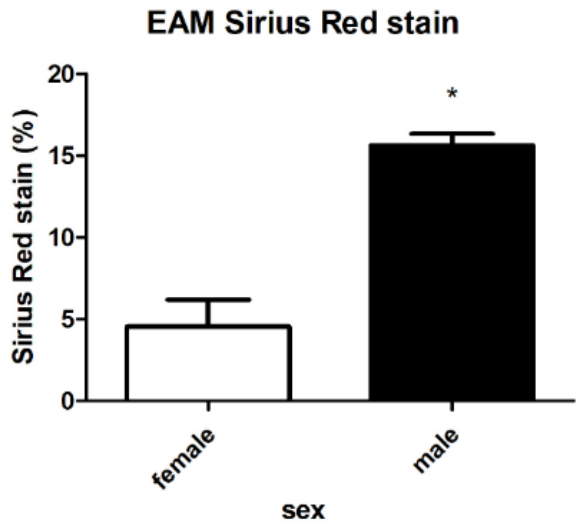


Figure 3: Increased fibrosis formation in male EAM heart sections.
(A) Intensity of Sirius red staining (percent) in EAM heart paraffin sections. Values are shown as mean ± SEM, n=4/group (*$p<0.05$, Mann Whitney test).

The population of CD68 and arginase-positive macrophages (M2) is higher in female EAM heart section infiltrates than in male ones

Immunostaining of general macrophage marker CD68 and M2 subpopulation marker arginase showed an increased population of M2-differentiated macrophages in female EAM heart sections compared to male ones. PD Dr. Anja Kühl of the Charité – Universitätsmedizin Berlin performed the immunostaining of general macrophage marker CD68 and M2 subpopulation marker arginase in EAM heart sections by immunostaining the EAM heart sections.

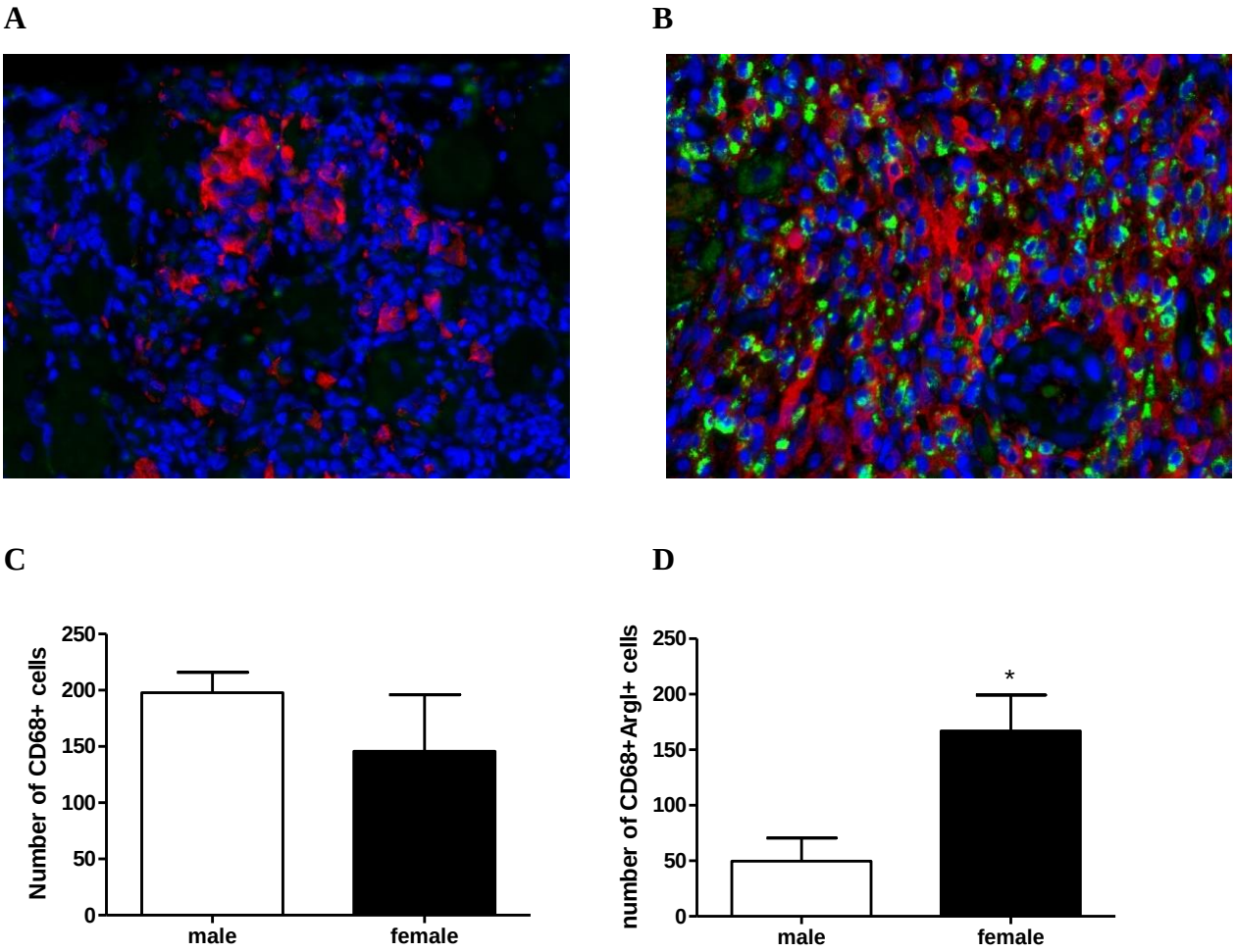


Figure 4: The population of CD68 and arginase-positive macrophages (M2) is higher in female EAM heart section infiltrates than in male ones.

(A) Immunostaining of male heart section with the antibody against arginase (green) and CD68 (red). (B) Immunostaining of female heart section with the antibody against arginase (green) and CD68 (red). A, B: 40x magnification; C: number of CD68+ cells; D: number of CD68+ Arg+ cells. Values are shown as mean ± SEM, n=4-9/group (*$p<0.05$, Mann Whitney test).

Upregulation of inflammatory markers iNOS, c-fos and fibrosis marker COL3A1 in male EAM heart samples

In order to investigate sex differences and the effectiveness of EAM immunization, quantitative real-time PCR analysis of heart tissue was performed.

Two-way ANOVA analysis with Bonferroni correction

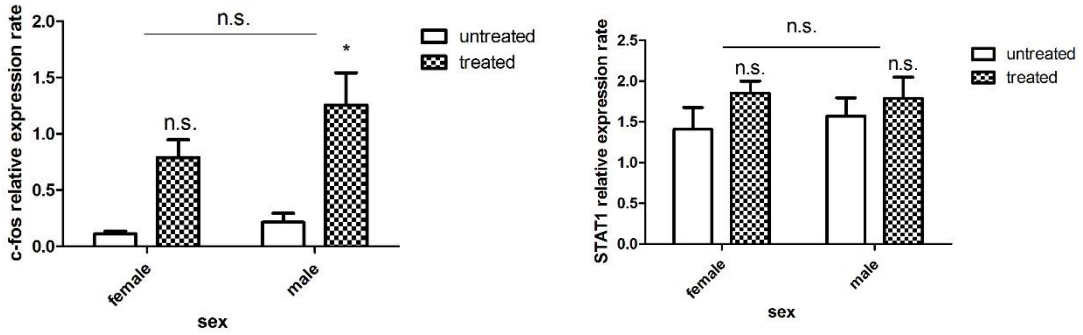
For c-fos, a pro-inflammatory marker and transcription factor, a significant increase was found in male but not in female tissue ($p<0.05$ and $p>0.05$; Fig. 5A). For STAT1, another transcription

factor of the pro-inflammatory M1 spectrum, no significant increase was found in either male or female tissue after EAM treatment ($p>0.05$; Fig. 5B). A significant increase of the pro-inflammatory marker iNOS was found in male but not in female tissue ($p<0.05$ and $p>0.05$; Fig. 5C). For IL-1β, a cytokine of the M1 spectrum, no significant increase was found in either male or female tissue after EAM treatment ($p>0.05$; Fig. 5D). In addition, for the fibrosis marker COL3A1, a significantly increased expression was found on the RNA level in male tissue but not in female tissue ($p<0.05$ and $p>0.05$; Fig. 5E). For the fibrosis marker COL1A1 and MCP-1, no significant increase was found in male or female tissue ($p>0.05$; Fig. 5 F and G).

Two-way ANOVA analysis of overall data variation

Two-way ANOVA analysis was used to identify sex and treatment as well as a possible interaction between the two as significant sources of data variation in each single experiment. Two-way ANOVA analysis revealed the treatment of rats with the EAM protocol to be a significant source of data variation for inflammatory markers c-fos ($p<0.01$; Fig. 5A) and iNOS ($p<0.05$, Fig. 5C). Moreover, for the increase of fibrosis marker COL3A1 ($p<0.05$, Fig. 5E), COL1A1 ($p<0.05$, Fig. 5 F) and MCP-1 ($p<0.05$, Fig. 5 G), two-way ANOVA analysis showed treatment to be a significant source of data variation. For STAT1 (Fig. 5B) and IL-1β (Fig. 5D), no significant difference in animals treated by EAM protocol could be shown. In contrast to treatment, sex could not be determined to be a significant source of data variation in two-way ANOVA analysis of experiments 5A to 5F.

A **B**



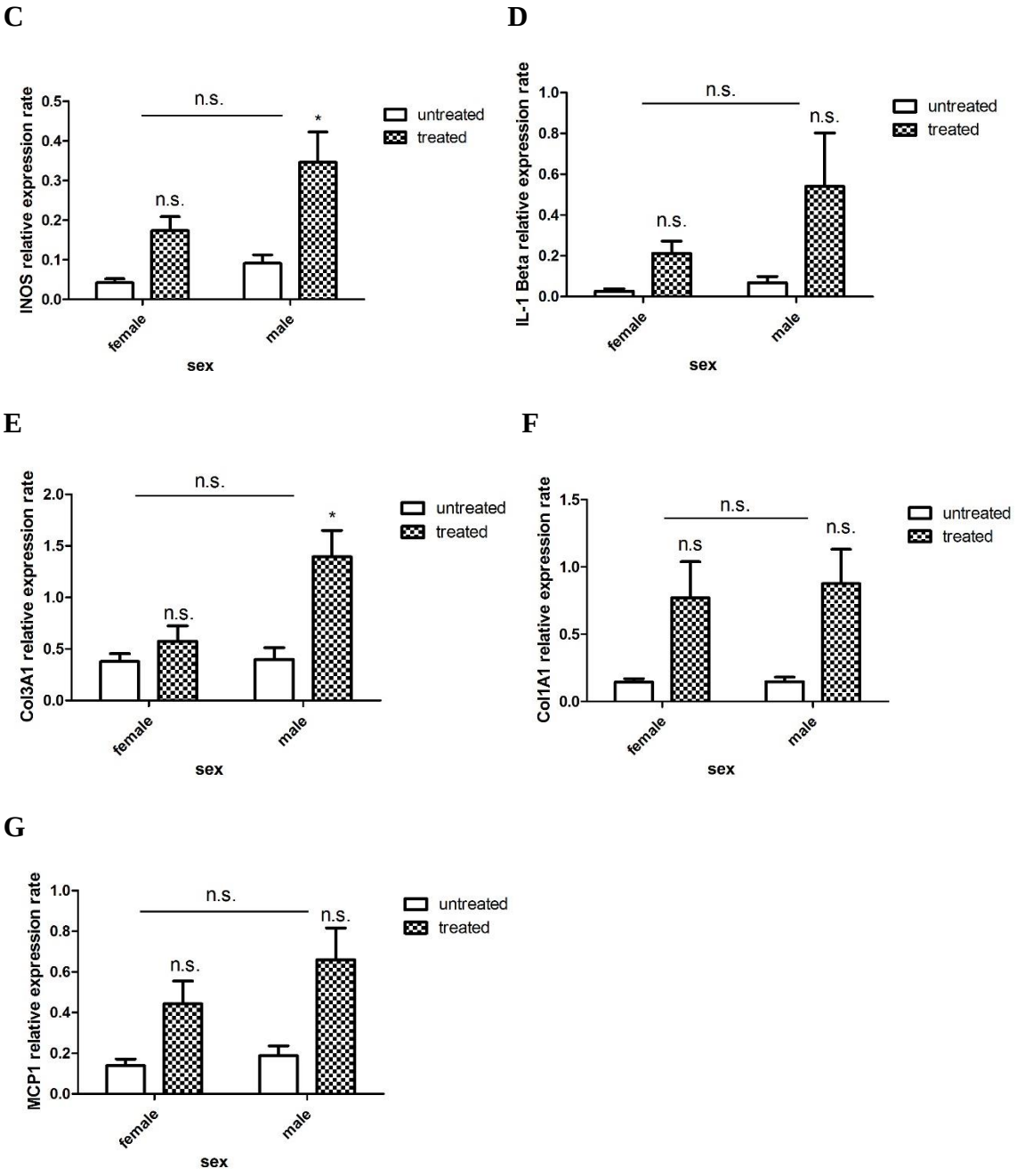


Figure 5: Upregulation of inflammatory markers iNOS and c-fos and fibrosis marker COL3A1 in male EAM heart samples.

qRT-PCR analysis of the relative expression rate of (A) c-fos, (B) STAT 1, (C) iNOS, (D) IL-1β, (E) COL3A1, (F) COL1A1 and (G) MCP-1. (*) indicates significance between immunized and non-immunized groups. Values are expressed as mean ± SEM; n=4-9/group (*$p<0.05$; n.s.: not significant; two-way ANOVA with Bonferroni correction).

Activation of pERK (male only) and pp38 (male and female) pathways in EAM heart samples

Two-way ANOVA analysis with Bonferroni correction

In order to investigate sex differences and the effectiveness of EAM immunization, western blot analysis of heart tissue was performed to investigate the activation (by phosphorylation) of MAP Kinases ERK-1/2 and p38. The pERK/ERK ratio was significantly increased in male tissue but not in female heart tissue ($p<0.05$ and $p>0.05$; Fig. 6A). The pp38/p38 ratio was significantly increased in both male and female heart tissue ($p<0.01$; Fig. 6B).

Two-way ANOVA analysis of overall data variation

Two-way ANOVA analysis showed that treatment was a significant source of data variation for the pERK/ERK ratio ($p<0.01$) and the pp38/p38 ratio ($p<0.01$). Sex could not be determined to be a significant source of data variation by two-way ANOVA analysis ($p>0.05$; Fig. 6 A and B).

A **B**

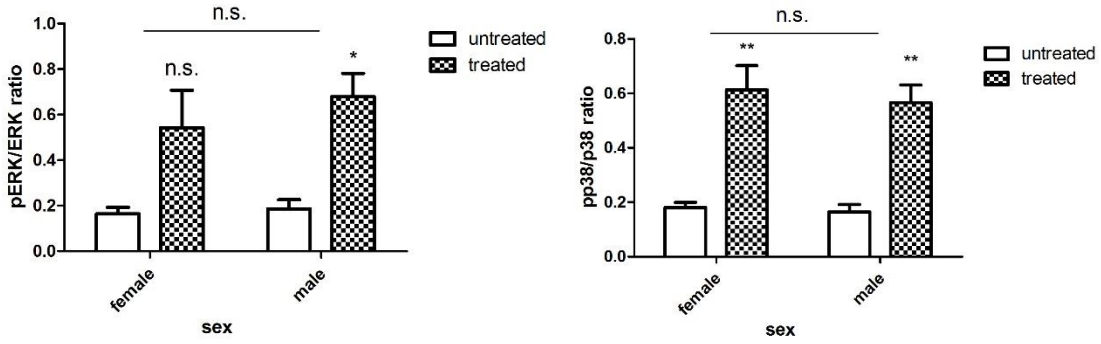


Figure 6: Activation of pERK (male only) and pp38 (male and female) pathways in EAM heart samples.

Western blot analysis of (A) pERK/ERK ratio and (B) pp38/p38 ratio. (*) indicates significance between treated and untreated groups. Values are expressed as mean ± SEM; n=4-9/group (*$p<0.05$; **$p<0.01$; n.s.: not significant; two-way ANOVA with Bonferroni correction).

Sex Differences in the EAM Model

It was shown that there are sex differences in the clinical outcome of EAM animals. Only males showed significantly decreased ejection fractions. On the tissue level, this was associated with increased amounts of myocardial infiltrates in both sexes, but there was a significantly increased fibrosis replacement score only in males. This finding was supported by the stronger fibrosis formation in male EAM sections compared to female ones revealed in the analysis of the Sirius red staining of EAM heart sections. On a cellular level, sex differences in the composition of

myocardial infiltrates were demonstrated. The amount of M2-polarized macrophages was higher in female heart sections than in male ones. On a molecular level, the heart tissue of male EAM animals showed a significant increase of key pro-inflammatory markers of the M1 spectrum. Molecular markers of fibrotic remodelling were also significantly increased only in male EAM heart tissue.

8.2 Cell culture experiments

8.2.1 Cell culture experiments – macrophages

Cell culture experiments with murine bone marrow-derived macrophages were used to show sex differences in macrophage polarization in order to support the thesis that sex differences relating to these cells could partially explain the sex differences found in the EAM model.

Macrophage characterization

Macrophage marker CD11b and F4/80 are expressed on BMMs

In order to analyze whether the cells isolated from murine bone marrow were macrophages, they were immune stained with antibodies against CD11b and F4/80, specific macrophage markers. Immunostaining showed CD11b and F4/80 immune-reactive cells (Fig. 7).

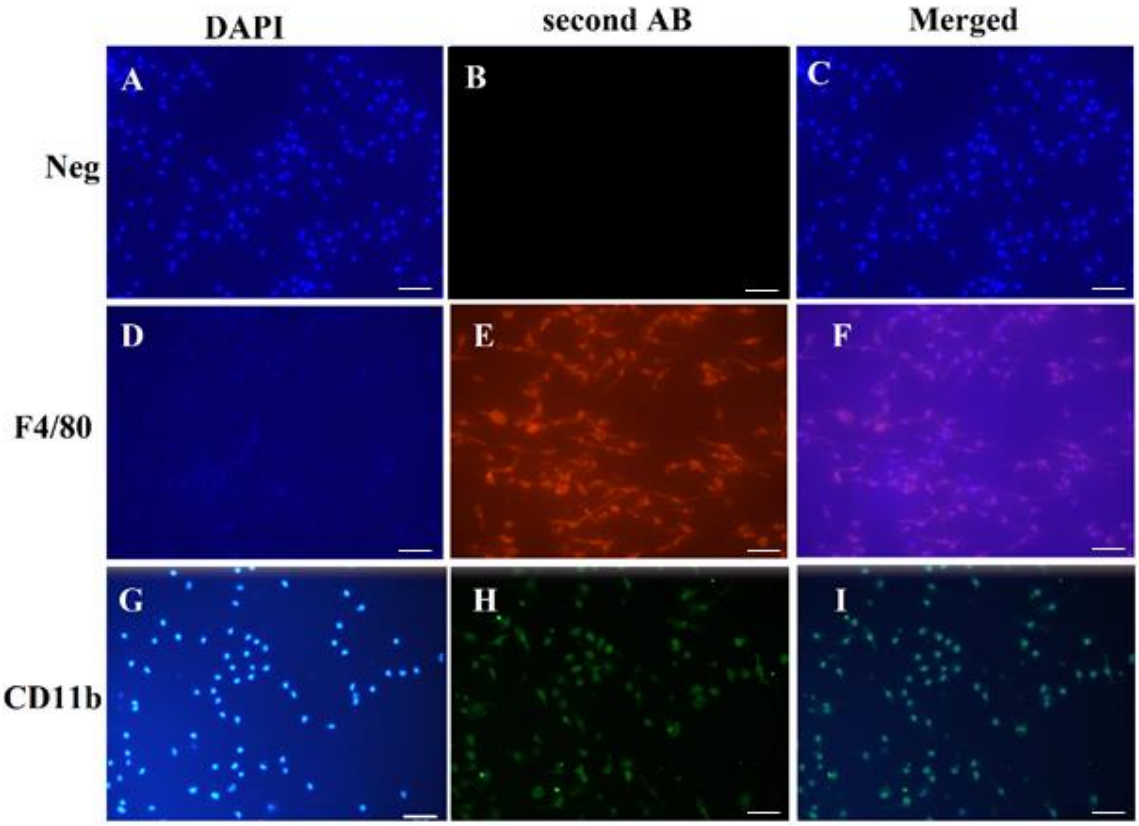


Figure 7: Macrophage markers CD11b and F4/80 are expressed on isolated cells (BMM). (A-C) Negative control (without primary antibody); (D-F) Immunostaining with antibody against F4/80; (G-I) Immunostaining with antibody against CD11b (A-I: 40x magnification; scale bar: 50 μm), n=3.

Macrophage activation with LPS

LPS promotes a stronger pro-inflammatory phenotype (M1) in male compared to female murine BMMs

In order to investigate sex differences in the activation of pro-inflammatory macrophages, male and female BMMs were treated with LPS for 24 hours.

Two-way ANOVA analysis with Bonferroni correction

The M1 markers c-fos ($p<0.001$ and $p<0.05$), NFκB ($p<0.001$ and $p<0.01$), IL-1β ($p<0.001$ and $p<0.05$), TNF-α ($p<0.001$ and $p<0.05$) and TLR 4 ($p<0.001$) were upregulated in LPS-treated male and female cells (Fig. 8 A-E). The M2 marker MCP-1 ($p<0.01$) and the M2 (immune-regulatory) marker IL-10 ($p<0.001$ and $p<0.01$) were also upregulated in LPS-treated male and female macrophages (Fig.8 F and G). LPS treatment leads to the downregulation of TIM3 expression in male and female murine BMMs ($p<0.001$; Fig. 8H).

Two-way ANOVA analysis of overall data variation

For all experiments, two-way ANOVA analysis showed that treatment was a significant source of data variation ($p<0.001$; Fig. A-H). Two-way ANOVA analysis showed a significant interaction between sex and treatment for NFκB ($p<0.001$), IL-1β ($p<0.001$) and TNF-α ($p<0.001$). Two-way ANOVA analysis showed that sex was a significant source of data variation for a stronger M1 response in male murine BMMs, namely NFκB ($p<0.001$), IL-1β ($p<0.01$) and TNF-α ($p<0.001$). The downregulation of TIM3 was stronger in male BMMs (two-way ANOVA: sex was a significant source of data variation, $p<0.001$; significant interaction between sex and treatment, $p<0.001$).

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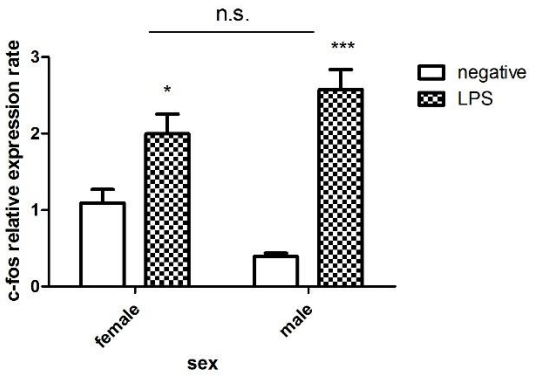
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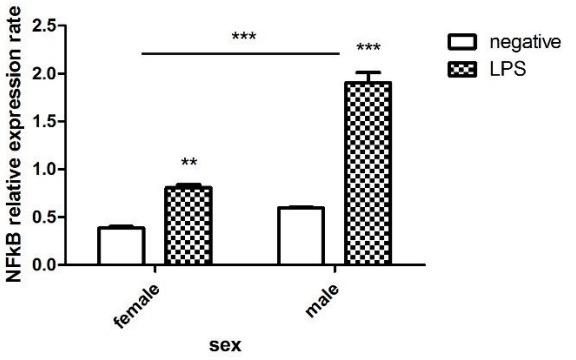
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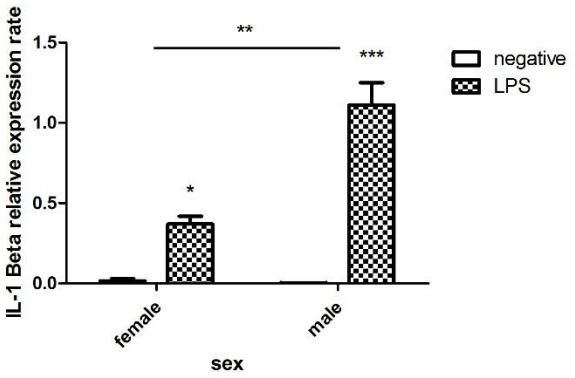
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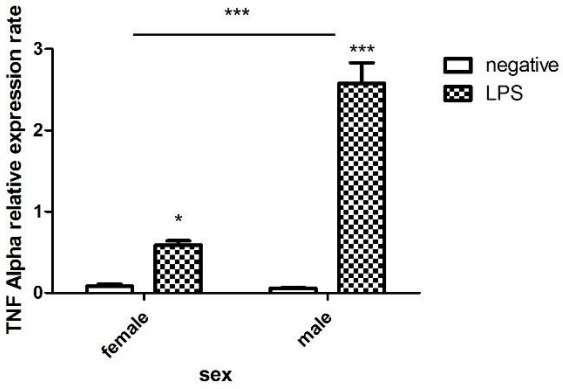
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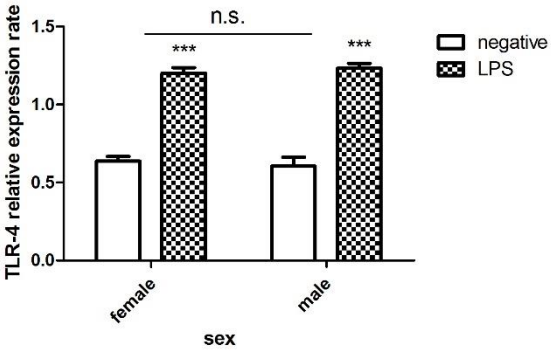
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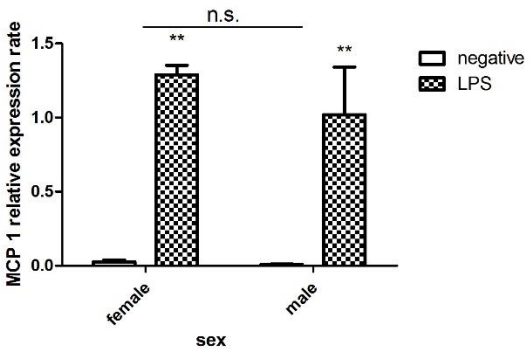
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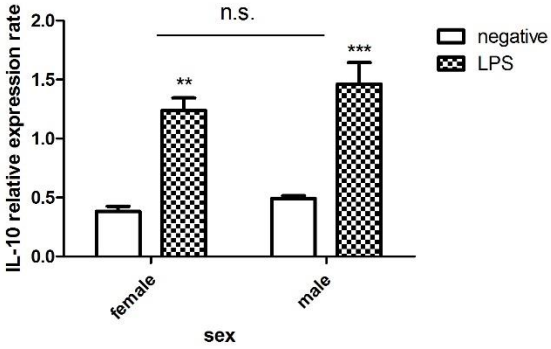
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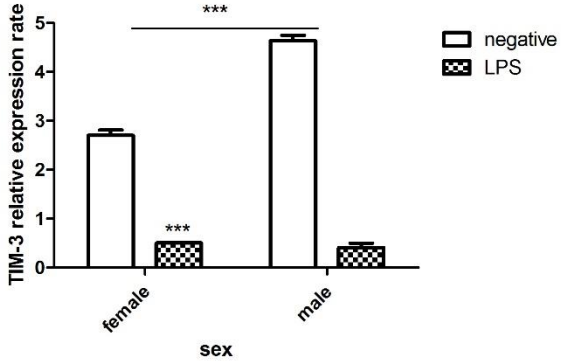
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Figure 8: LPS promotes a stronger pro-inflammatory phenotype (M1) in male compared to female murine BMMs.

qRT-PCR analysis of the relative expression rate of (A) c-fos, (B) NFκB, (C) IL-1β, (D) TNF-α, (E) TLR 4, (F) MCP-1, (G) IL-10 and (H) TIM3. (*) indicates the significance between treated and untreated groups. Data is shown as mean ± SEM; n=3/group; experiments were performed in duplicate (*p<0.05; **p<0.01; ***p<0.001; n.s.: not significant, A-H: two-way ANOVA with Bonferroni correction).

LPS treatment increases the expression of NFκB and promotes activation of p38 in male murine BMMs

In order to analyze sex differences in the inflammatory signal and in the activation of signal cascades, the protein expression of NFκB, which is a prominent pro-inflammatory marker, and the activation of the MAP Kinases ERK and p38 were analyzed.

Two-way ANOVA analysis with Bonferroni correction

At the protein level, LPS-treated male murine BMMs showed an increased expression of NFκB in comparison to non-treated male BMMS (p<0.05; Fig. 9A). In contrast, LPS treatment did not affect the expression of NFκB in female murine BMMs (p>0.05; Fig. 9A). LPS treatment did not influence the activation of the ERK pathway in either male or in female murine BMMs (p>0.05; Fig. 9B). LPS activated the p38 pathway only in male and not female murine BMMs after 24h treatment (p<0.001 and p<0.05; Fig. 9C).

Two-way ANOVA analysis of overall data variation

Furthermore, two-way ANOVA analysis revealed sex (p<0.001) and treatment (p<0.05) as significant sources of data variation for NFκB. The interaction between sex and treatment could be shown to be a significant source of data variation for the stronger M1 response regarding NFκB increase (p<0.05).

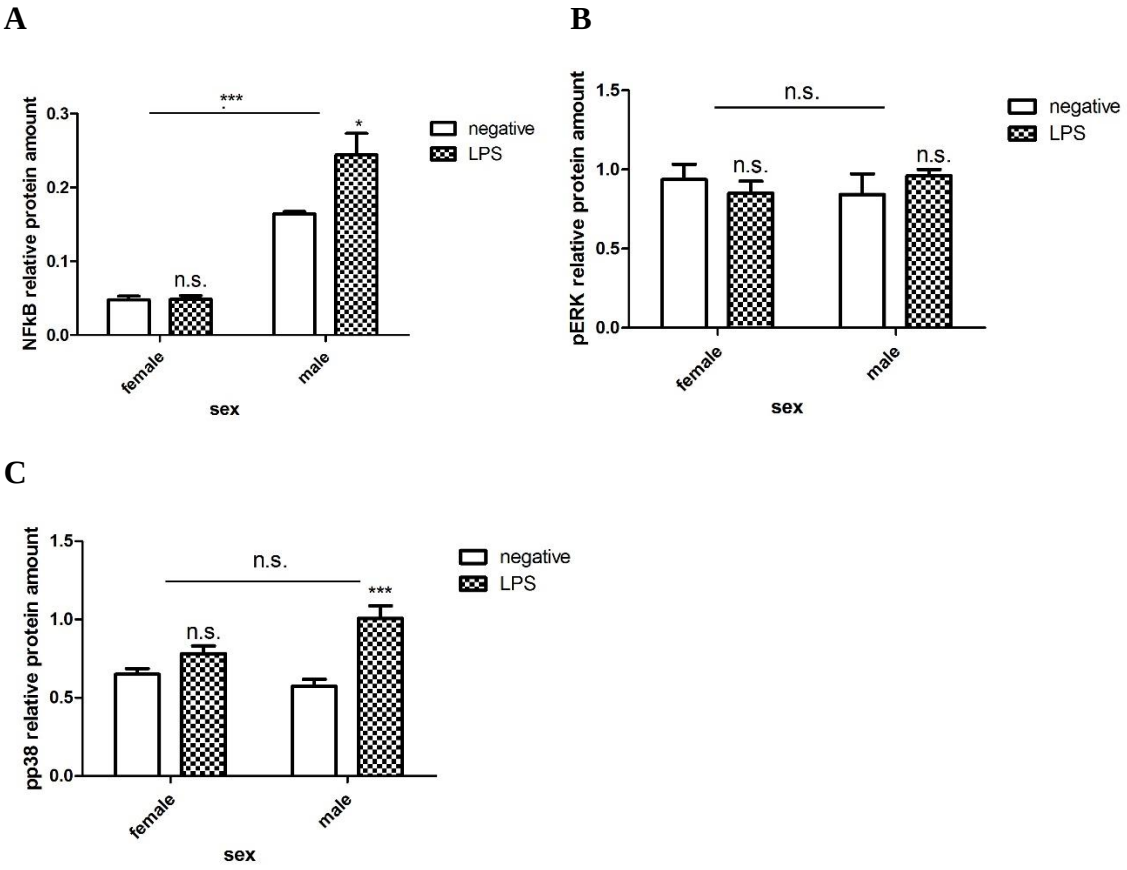


Figure 9: LPS treatment increases the expression of NFκB and promotes activation of p38 in male murine BMMs.

Western blot analysis of the relative protein amount of (A) NFκB, (B) pERK/ERK ratio and (C) pp38/p38 ratio. (*) indicates significance between treated and untreated groups. Values are expressed as mean ± SEM; n=3/group; experiments were performed in duplicate (*$p<0.05$; ***$p<0.001$; n.s.: not significant, A-C: two-way ANOVA with Bonferroni correction).

Macrophage activation with IL-4 and Il-13

IL-4 and IL-13 treatment promotes a stronger M2 phenotype in male BMMs

In order to analyze sex differences in the polarization of anti-inflammatory macrophages (M2), male and female murine BMMs were treated with IL-4 and IL-13 for 24 h.

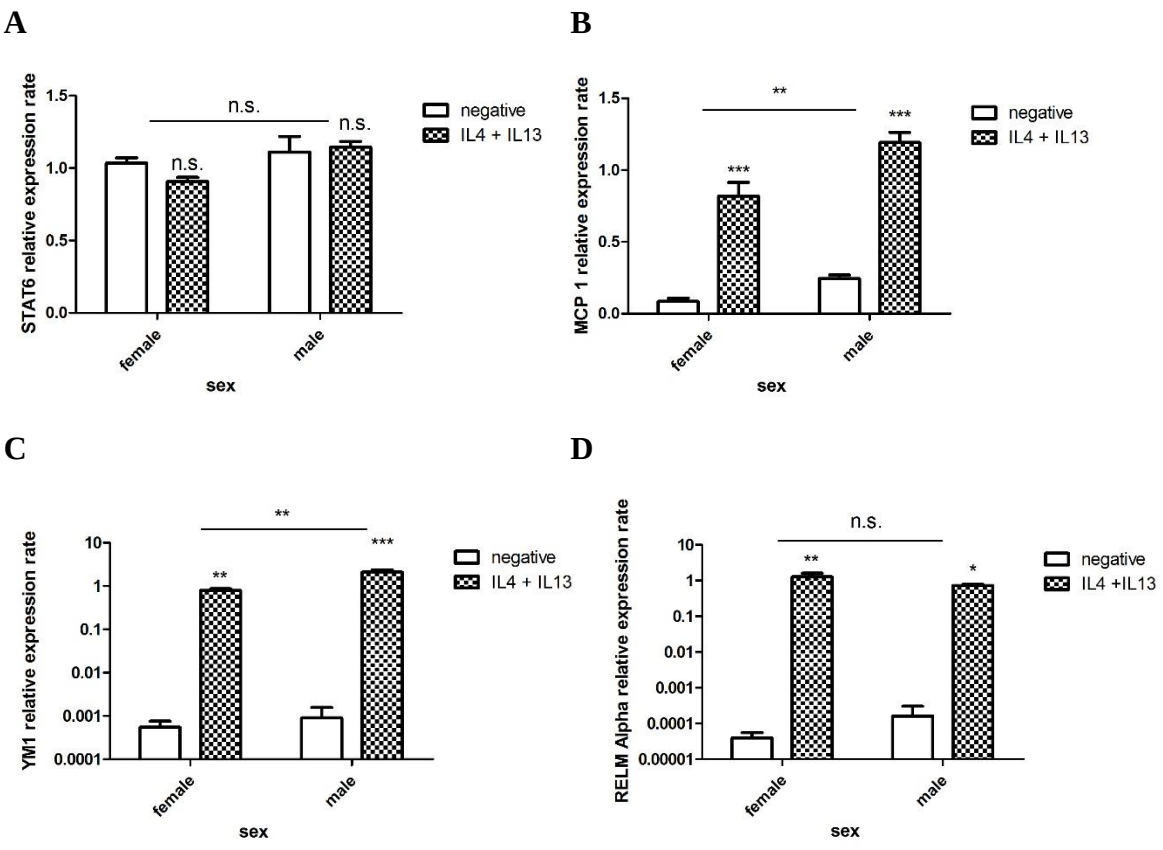
Two-way ANOVA analysis with Bonferroni correction

IL-4/IL-13 long-term treatment significantly increased the expression of MCP-1 at the RNA level in male and female BMMs compared to untreated macrophages ($p<0.001$; Fig. 10B). YM1 ($p<0.001$ and $p<0.05$) and RELM-α ($p<0.05$ and $p<0.01$) markers were significantly increased in male and female BMMs after long-term treatment with IL-4/IL-13 (Fig. 10 C and D). IL-

4/IL-13 long-term treatment did not affect the expression of the M2 spectrum transcription factor STAT6 at the RNA level in either male or female BMMs ($p>0.05$; Fig. 10A). Furthermore, long-term treatment with IL-4 and IL-13 promoted a decreased expression of TNF-α in male and female BMMs ($p<0.01$; Fig. 10E), as well as IL-1β in male and not in female BMMs ($p<0.001$ and $p>0.05$; Fig. 10F).

Two-way ANOVA analysis of overall data variation

Two-way ANOVA analysis revealed an interaction between sex and treatment for MCP-1, YM1 and IL-1β ($p<0.05$, $p<0.01$, $p<0.01$), with sex being a significant source of data variation ($p<0.01$, $p<0.01$, $p<0.05$), and treatment also being a significant source of data variation ($p<0.05$, $p<0.001$, $p<0.001$)



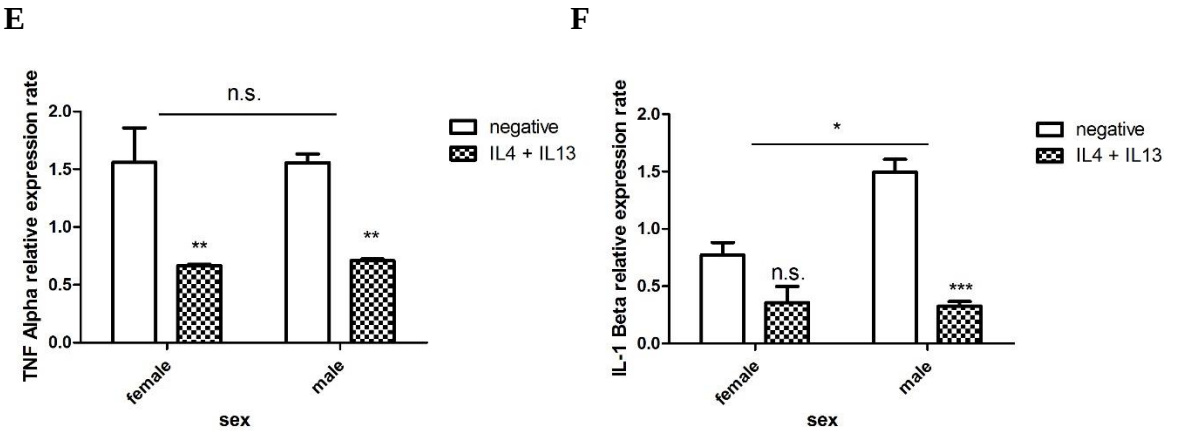


Figure 10: IL-4 and IL-13 treatment promotes a stronger M2 phenotype in male BMMs. qRT-PCR analysis of the relative expression rate of (A) STAT6, (B) MCP-1, (C) RELM-α, (D) YM1, (E) TNF-α and (F) IL-1β. (*) indicates significance between treated and untreated groups. Values are shown as mean ± SEM; n=3/group; experiments were performed in duplicate (*p<0.05; **p<0.01; ***p<0.001; n.s.: not significant, A-F: two-way ANOVA with Bonferroni correction).

ERK and p38 are not activated by IL-4 and IL-13 treatment in murine BMMs

In order to analyze sex differences in the activation of downstream signal cascades, the phosphorylation of ERK and p38 was analyzed**.** On the protein level, IL-4/IL-13 long-term treatment did not show any effect on the phosphorylation of ERK and p38 in male or female murine BMMs (p>0.05; Figure 11 A and B).

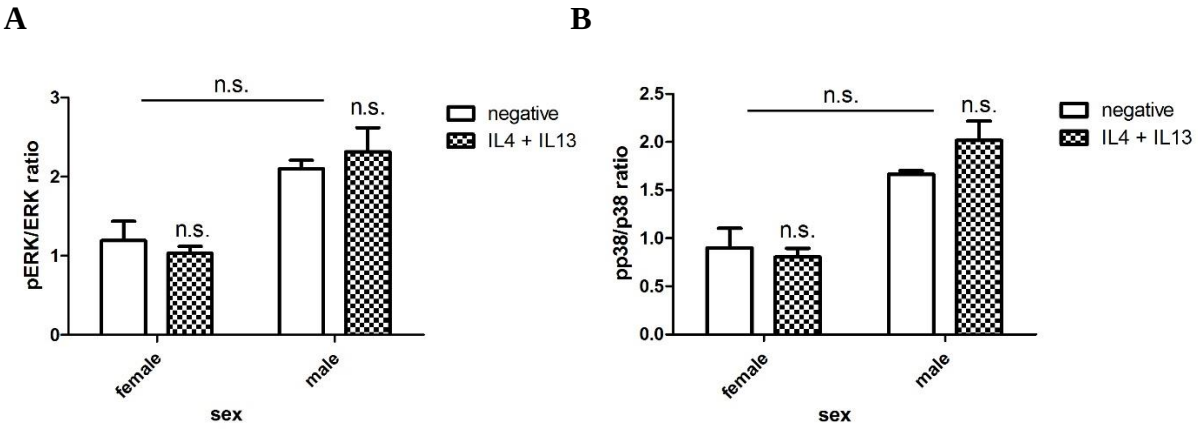


Figure 11: ERK and p38 are not activated by IL-4 and IL-13 treatment in murine BMMs. Western blot analysis of (A) the pERK/ERK ratio and (B) the pp38/p38 ratio. Values are expressed as mean ± SEM; n=3/group; experiments were performed in duplicate (n.s.: not significant; A, B: two-way ANOVA with Bonferroni correction).

Macrophage co-stimulation with LPS and estrogen

Estrogen treatment supports a pro-inflammatory phenotype (M1) in LPS-treated murine male BMMs

In order to investigate the effects of E2 on pro-inflammatory macrophages, male and female murine BMMs were co-treated with E2 and LPS.

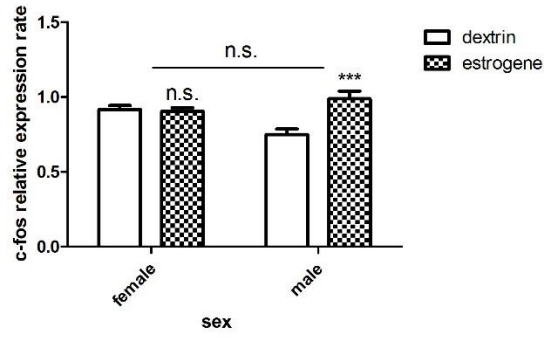
Two-way ANOVA analysis with Bonferroni correction

E2 increased the c-fos expression at the RNA level in male but not in female LPS-treated BMMs ($p<0.001$ and $p>0.05$; Fig. 12A). The RNA expression of NFκB and TNF-α was also significantly increased in males after long-term E2 treatment of LPS-treated BMMs ($p<0.001$; Fig. 12 B and C). In contrast, E2 treatment significantly decreased the expression of TIM3, a prominent anti-inflammatory factor, at the RNA level in male and female BMMs co-treated with LPS and E2 ($p>0.05$; Fig. 12D).

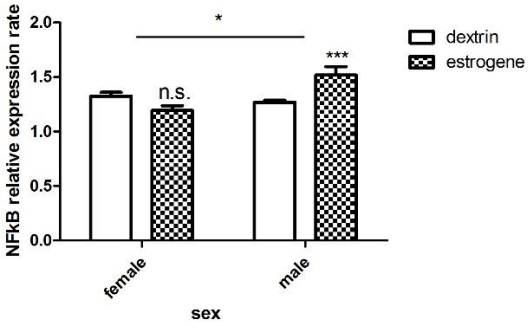
Two-way ANOVA analysis of overall data variation

Two-way ANOVA analysis showed an interaction between treatment and sex for TNF-α ($p<0.01$), with sex being a significant source of data variation ($p<0.001$), and treatment also being a significant source of data variation ($p<0.01$).

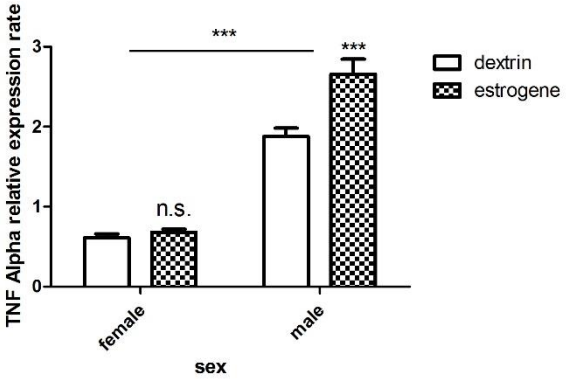
A



B



C



D

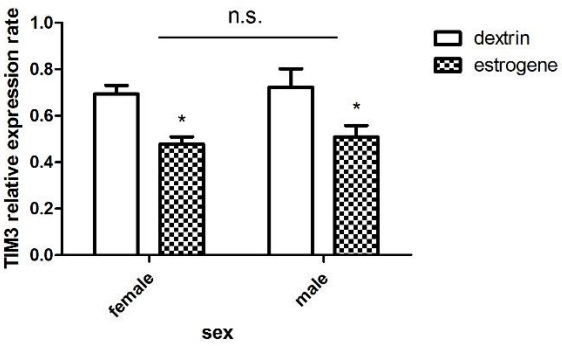


Figure 12: Estrogen treatment supports a pro-inflammatory phenotype (M1) in LPS-treated murine male BMMs.

qRT-PCR analysis of the relative expression rate of (A) c-fos, (B) NFκB, (C) TNF-α and (D) TIM3. (*) indicates significance between treated and untreated groups. Values are expressed as mean ± SEM; n=6/group; experiments were performed in duplicate (*p<0.05; **p<0.01; ***p<0.001; n.s.: not significant; A-H: two-way ANOVA with Bonferroni correction).

Macrophage co-stimulation with IL-4/IL-13 and estrogen

Estrogen treatment inhibits polarization of IL-4- and IL-13-treated male BMMs into anti-inflammatory macrophages (M2)

In order to investigate the effects of E2 on anti-inflammatory macrophages, male and female murine BMMs were co-treated with E2 and IL-4/IL-13. Long-term E2 treatment significantly decreased the RNA expression of MCP-1 in male but not in female BMMs co-treated with IL-4/IL-13 and E2 (p<0.001 and p>0.05). The expression of TNF-α at the RNA level did not change after E2 treatment of male and female IL-4/IL-13-treated BMMs (p>0.05; Figure 13B)

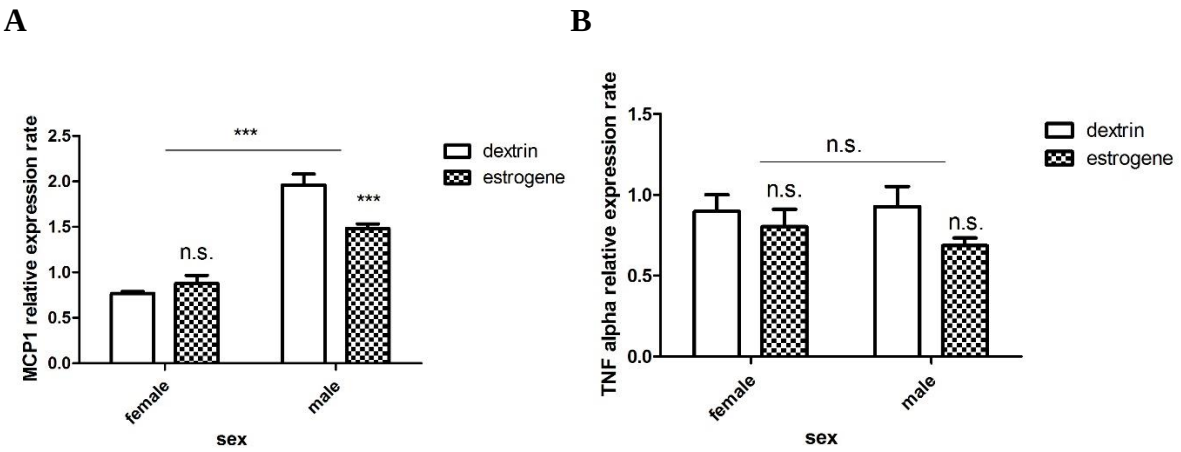


Figure 13: Estrogen treatment inhibits polarization of IL-4- and IL-13-treated male BMMs into anti-inflammatory macrophages (M2).

qRT-PCR analysis of the relative expression rate of (A) MCP-1 and (B) TNF-α. (*) indicates significance between treated and untreated groups. Values are shown as mean ± SEM; n=6/group; experiments were performed in duplicate (***p<0.001; n.s.: not significant; A, B: two-way ANOVA with Bonferroni correction).

Sex differences in macrophage polarization

Sex differences in macrophage polarization were shown. Male murine macrophages reacted to LPS treatment with a stronger polarization into the M1 phenotype. The upregulation of the signature molecular markers of the M1 phenotype was stronger in male cells than in female ones. This stronger polarization into the M1 phenotype in male macrophages was associated with a significant upregulation of the downstream signalling molecules NFκB and p38 only in male cells. Treatment of macrophages with IL-4/IL-13 resulted in a stronger polarization of male cells into the M2 phenotype. Co-stimulation experiments with LPS and estrogen as well as IL4/Il-13 and estrogen only showed significant estrogen effects in male cells and not in female ones. In male macrophage polarization, estrogen promoted the polarization into the M1 phenotype and inhibited polarization into the M2 phenotype.

8.2.2 Cell culture experiments – fibroblasts

Cell culture experiments with murine cardiac fibroblasts were used to show that TNF-α (which can be secreted by macrophages) can activate cardiac fibroblasts.

TNF-α activates primary murine adult cardiac fibroblasts

In order to investigate sex differences in the activation of cardiac fibroblasts, murine adult cardiac primary fibroblasts were treated with TNF-α for 24 hours.

Two-way ANOVA analysis with Bonferroni correction

Long-term treatment with TNF-α significantly increased the RNA expression of MCP-1 in male and female adult cardiac primary fibroblasts ($p<0.001$; Fig. 14A). In addition, the RNA expression of IL-1β and TGF-β, prominent pro-fibrotic factors, was significantly increased in male and female fibroblasts after TNF-α treatment ($p<0.001$; Fig. 14 B and C). TNF-α did not influence the expression of COL1A1 at the RNA level in male and female murine adult cardiac fibroblasts ($p>0.05$; Fig. 14D).

Two-way ANOVA analysis of overall data variation

Two-way ANOVA analysis revealed an interaction between sex and treatment for IL-1β ($p<0.001$), with sex being a significant source of data variation ($p<0.001$); treatment was also a significant source for data variation ($p<0.001$).

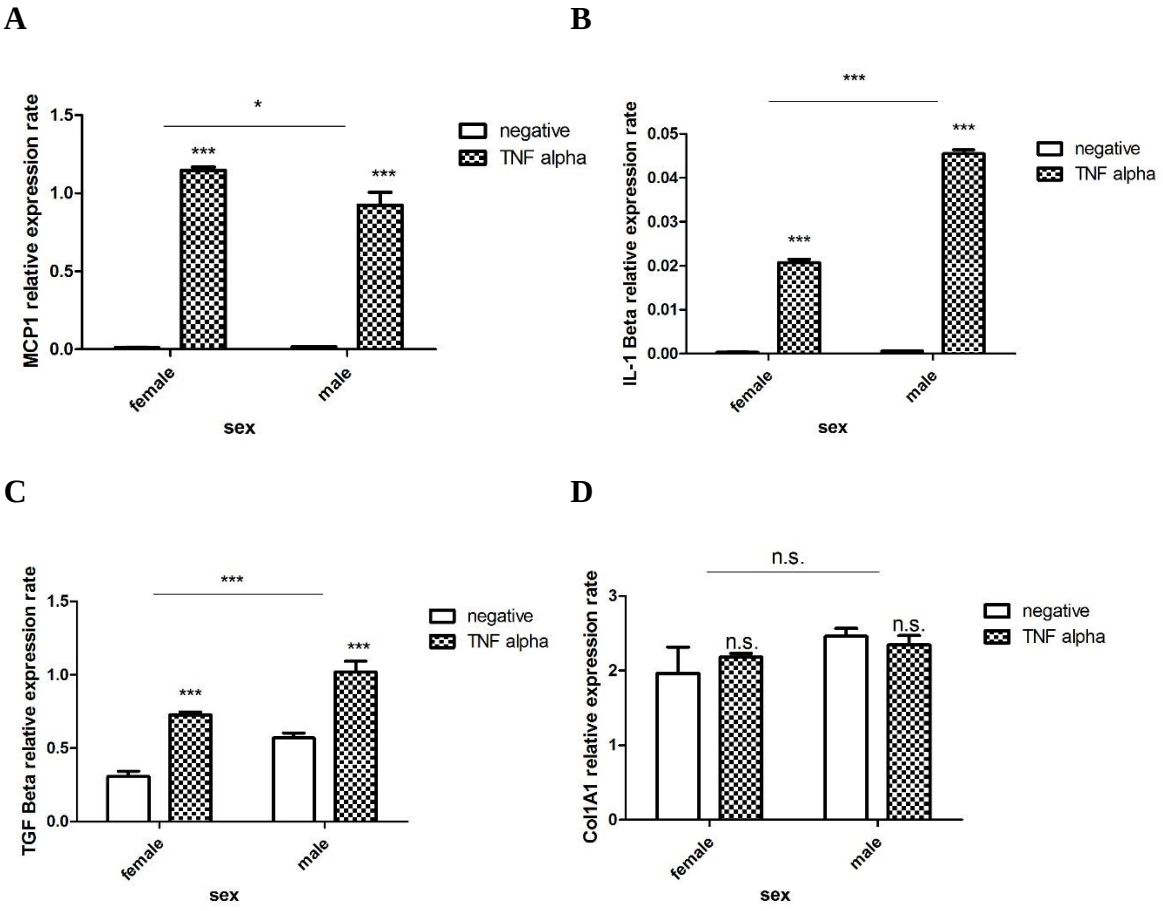


Figure 14: TNF-α activates primary murine adult cardiac fibroblasts.

qRT-PCR analysis of the relative expression rate of (A) MCP-1, (B) IL-1β, (C) TGF-β and (D) Col1A1. (*) indicates significance between treated and untreated groups. Values are shown as mean ± SEM; n=3/group; experiments were performed in duplicate (***p<0.001; n.s.: not significant; A-D: two-way ANOVA with Bonferroni correction).

Cardiac fibroblast activation

It was shown that TNF-α (a cytokine derived by macrophages) is able to activate fibroblasts.

8.2.3 Cell culture experiments – co-culture of fibroblasts/macrophages

In order to investigate sex differences in the ability of macrophages to activate fibroblasts, differently treated male and female murine BMMs (LPS, IL-4/IL-13, LPS/E2, IL-4/IL-13/E2) were used to condition the cell culture medium, which was then used to activate isolated murine cardiac fibroblasts.

The conditioned medium from male pro-inflammatory macrophages is a stronger pro-fibrotic activator of isolated murine cardiac fibroblasts compared to medium conditioned by female pro-inflammatory macrophages

In order to investigate whether a pro-inflammatory M1 environment has effects on the activation of male and female adult cardiac fibroblasts, male and female cardiac primary fibroblasts were treated with conditioned medium from BMMs treated with LPS for 24 h.

Two-way ANOVA analysis with Bonferroni correction

Male and female cardiac fibroblasts expressed significantly more MCP-1 after long-term treatment with conditioned M1 medium when compared with fibroblast growth under normal conditions ($p<0.001$; Fig. 15A). In addition, the RNA expression of arginase, a prominent pro-fibrotic marker, was significantly increased in male and female fibroblasts treated with conditioned M1 medium ($p<0.01$ and $p<0.05$; Fig. 15B). Furthermore, the expression of the pro-fibrotic factor IL-1β was significantly increased in male but not in female cardiac fibroblasts after treatment ($p<0.01$ and $p>0.05$; Fig. 15C). Pro-inflammatory factors, such as NFκB and TNF-α, were also significantly increased after long-term treatment with male and female conditioned M1 medium ($p<0.001$, $p<0.01$ and $p<0.001$, $p<0.05$, respectively; Fig. 15 D and E).

Two-way ANOVA analysis of overall data variation

In addition, two-way ANOVA analysis showed sex to be a significant source of data variation for MCP-1 ($p<0.001$), NFκB ($p<0.001$), TNF-α ($p<0.001$) and IL-1β ($p<0.05$). Two-way ANOVA analysis showed treatment to be a significant source of variation for MCP-1 ($p<0.001$), NFκB ($p<0.001$), TNF-α ($p<0.001$) and IL-1β ($p<0.05$). For these markers, the increase was stronger in male cells compared to female cells. Two-way ANOVA analysis showed a significant interaction between sex and treatment for MCP-1 ($p<0.001$), NFκB ($p<0.001$), TNF-α ($p<0.001$) and IL-1β ($p<0.05$).

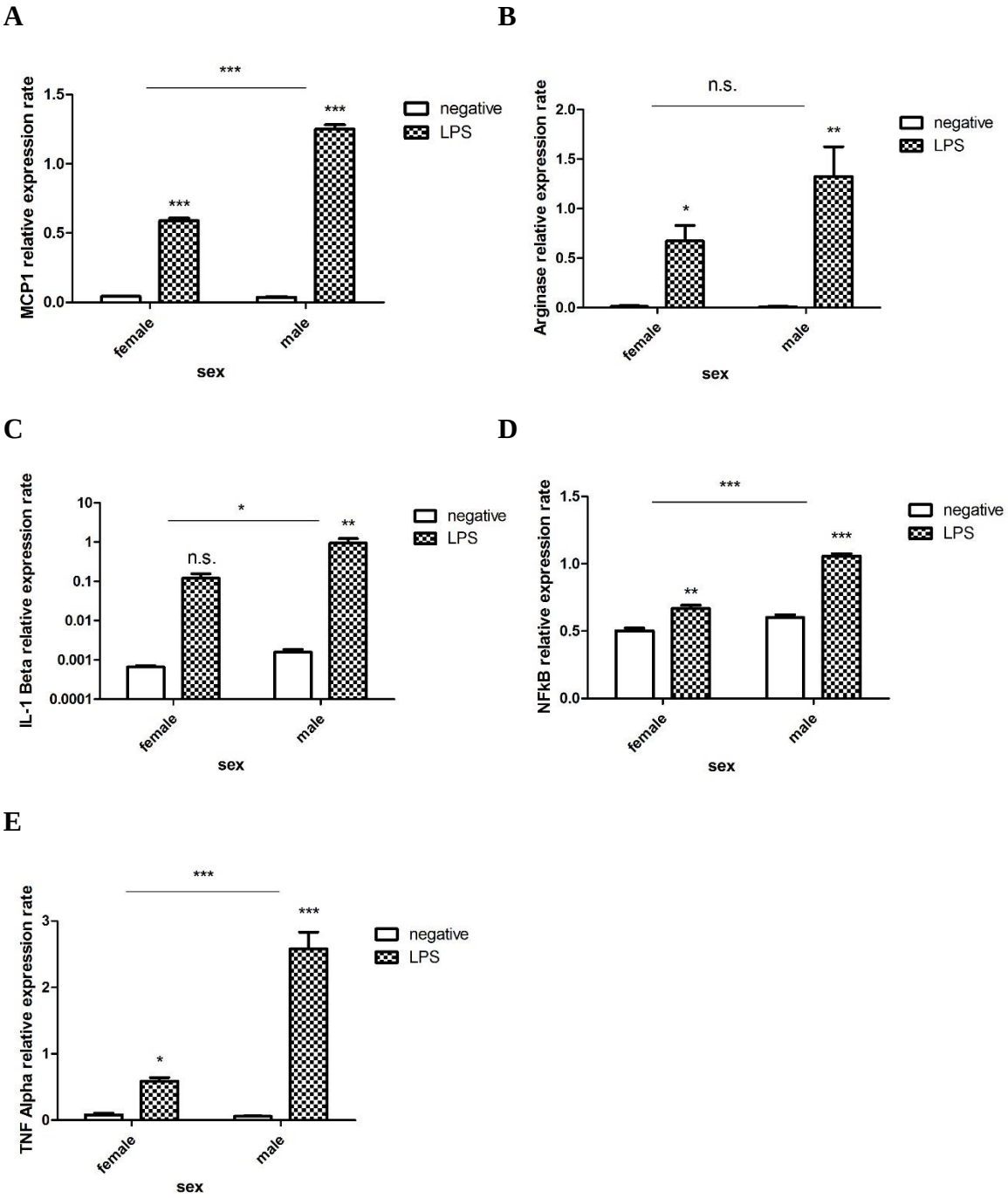


Figure 15: Conditioned medium from male pro-inflammatory macrophages is a stronger pro-fibrotic activator of isolated murine cardiac fibroblasts compared to medium conditioned by female pro-inflammatory macrophages.

qRT-PCR analysis of the relative expression rate of (A) MCP-1, (B) Arginase, (C) IL-1β, (D) NFκB and (E) TNF-α. (*) indicates significance between treated and untreated groups. Values are shown as mean ± SEM; n=3/group; experiments were performed in duplicate (*p<0.05; **p<0.01; ***p<0.001; A-E: two-way ANOVA with Bonferroni correction).

Male and female M2-conditioned medium activates primary murine adult cardiac fibroblasts

In order to investigate whether an anti-inflammatory M2 environment has effects on the activation of male and female adult cardiac fibroblasts, both were treated with conditioned medium from BMMs treated with IL-4/IL-13 for 24 h.

Two-way ANOVA analysis with Bonferroni correction

The treatment with conditioned M2 medium significantly increased the expression of MCP-1 in male and female adult primary cardiac fibroblasts in comparison to untreated ones ($p<0.001$; Fig. 16A). Accordingly, the pro-fibrotic marker arginase was also significantly increased in male and female fibroblasts after treatment ($p<0.01$ and $p<0.001$; Fig. 16B). In addition, the RNA expression of the pro-fibrotic factor IL-1β was significantly increased in male and female treated cardiac fibroblasts compared to untreated ones ($p<0.001$; Fig. 16C).

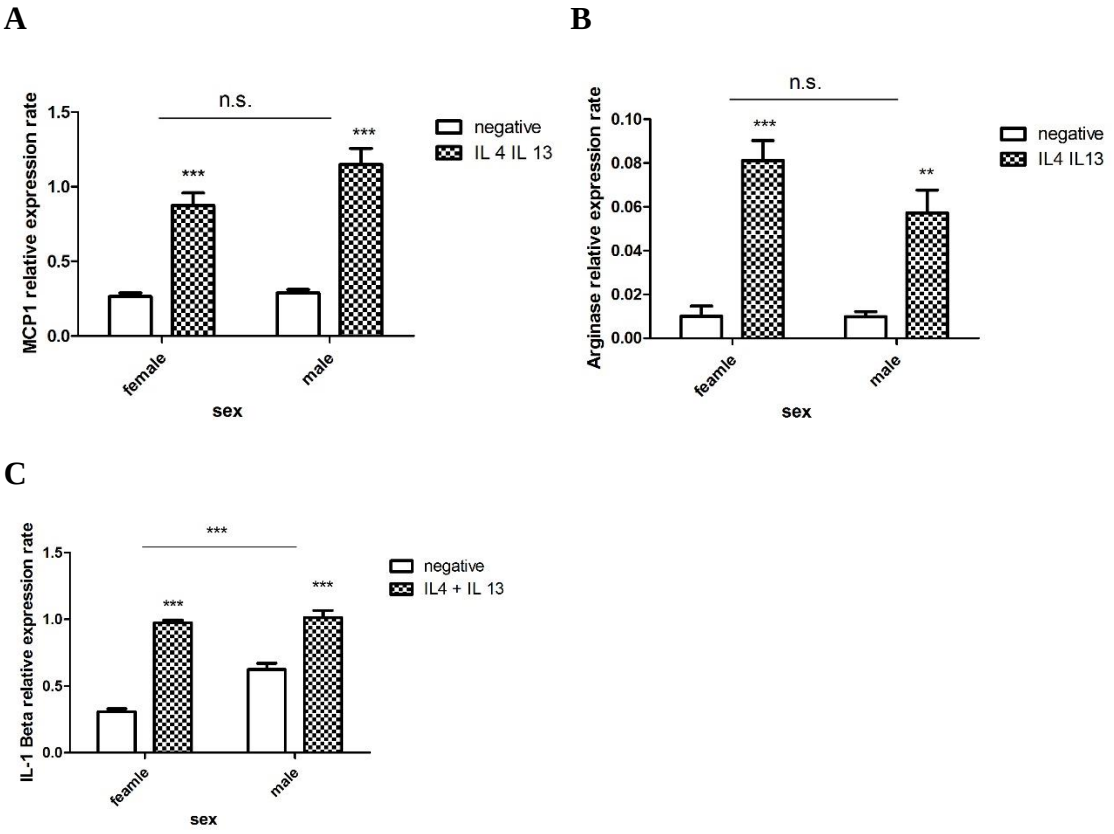


Figure 16: Male and female M2-conditioned medium activates primary murine adult cardiac fibroblasts. qRT-PCR analysis of the relative expression rate of (A) MCP-1, (B) arginase and (C) IL-1β. (*) indicates significance between treated and untreated groups. Values are shown as mean ± SEM; n=3/group; experiments were performed in duplicate (**$p<0.01$; ***$p<0.001$; n.s.: not significant; A: two-way ANOVA with Bonferroni correction).

Estrogen has no effects on the pro-inflammatory M1 environment regarding the ability to activate fibroblasts

In order to investigate if E2 has effects on the pro-inflammatory M1 environment, male and female primary cardiac fibroblasts were treated with conditioned M1 (LPS/E2 co-treatment) medium for 24h. An E2 supplementation to the conditioned M1 medium did not affect the expression of MCP-1, arginase or IL-1β in male or female cardiac fibroblasts ($p>0.05$; Fig. 17 A-C).

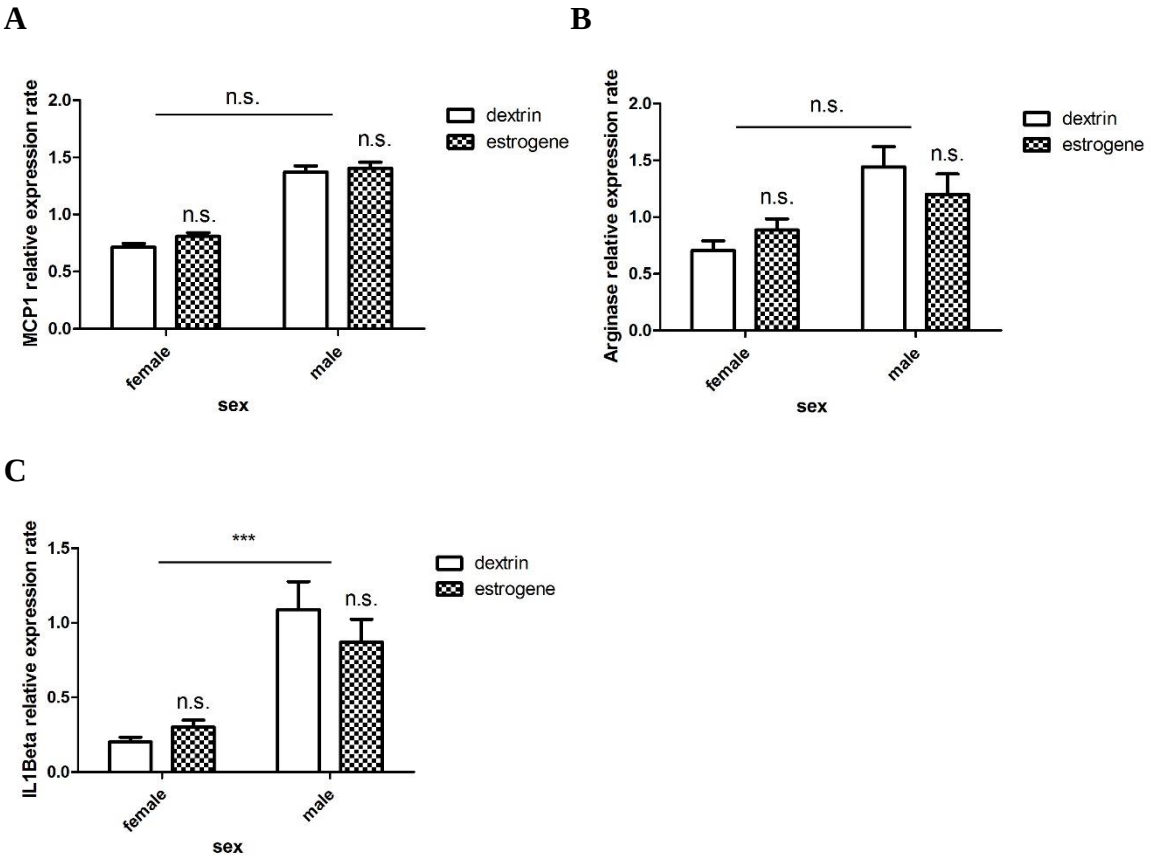


Figure 17: Estrogen has no effects on the pro-inflammatory M1 environment regarding the ability to activate fibroblasts.

qRT-PCR analysis of the relative expression rate of (A) MCP-1, (B) arginase and (C) IL-1β. Values are shown as mean ± SEM, n=6/group; experiments were performed in duplicate (n.s.: not significant; A: two-way ANOVA with Bonferroni correction).

Estrogen has no effects on the anti-inflammatory M2 environment regarding the ability to activate fibroblasts

In order to investigate whether E2 has effects on the anti-inflammatory M2 environment, male and female primary cardiac fibroblasts were treated with conditioned M2 (IL-4/IL-13/E2 co-treatment) medium for 24 h. An E2 supplementation to the conditioned M2 medium did not affect the expression of MCP-1 or arginase in male or female cardiac fibroblasts ($p>0.05$; Figure 18 A and B).

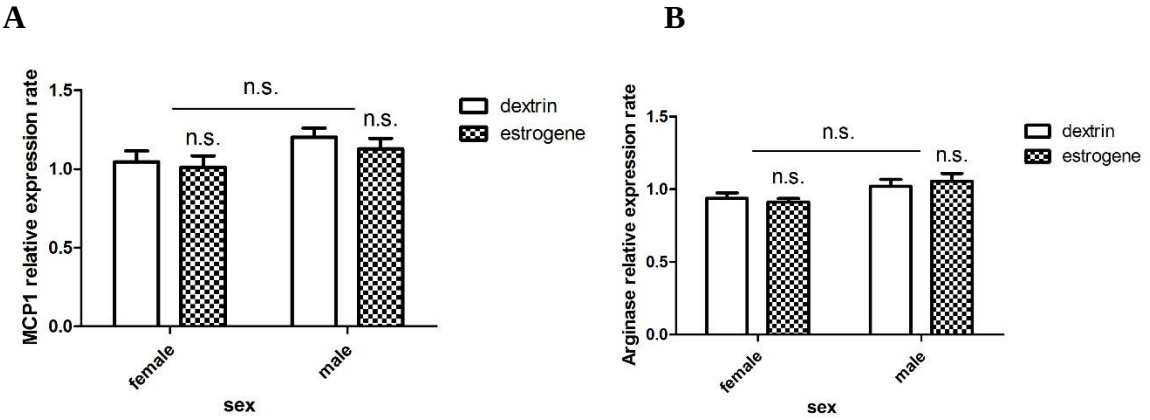


Figure 18: Estrogen has no effects on the anti-inflammatory M2 environment regarding the ability to activate fibroblasts.

qRT-PCR analysis of the relative expression rate of (A) MCP-1 and (B) arginase. Values are expressed as mean ± SEM; n=6/group; experiments were performed in duplicate (n.s.: not significant; A: two-way ANOVA with Bonferroni correction).

Sex differences in macrophage-fibroblast cell-cell interaction

Sex differences in macrophage-derived fibroblast activation were shown. The capability of male pro-inflammatory macrophage-conditioned medium to activate male murine cardiac fibroblasts was stronger than that of medium conditioned by female pro-inflammatory macrophages used on female murine cardiac fibroblasts.

9 Discussion

This study showed that the worse clinical outcome and severe formation of cardiac fibrosis in male EAM animals compared to female ones is associated with sex differences in the composition of myocardial infiltrates (regarding macrophages and their differently polarized subtypes) in the heart sections of EAM animals. Myocardial infiltrates of female EAM animals showed a higher amount of M2 macrophages, while the heart tissue of male EAM animals showed a significantly increased expression of signature markers of the M1 spectrum. Subsequently, cell culture experiments with murine macrophages and murine cardiac fibroblasts were used to support the thesis that sex differences found in the EAM model could be based on sex differences in macrophage polarization and macrophage pro-fibrotic signalling. Male macrophages polarized more strongly into a pro-inflammatory M1 phenotype after LPS stimulation compared to female ones. The medium conditioned by these cells is a stronger activator of fibroblasts compared to medium conditioned by female cells, suggesting that sex differences in macrophage activation may lead to sex differences in cardiac remodelling in EAM.

Sex differences in the EAM model are associated with sex differences in myocardial infiltrates: male sex promotes a stronger M1 response, while female sex promotes a higher quantity of M2 macrophages

In an EAM rat model, Schmerler et al. showed that male rodents developed autoimmune myocarditis 21 days after immunization with cardiac myosin and CFA in the paw. [172] These rats showed an increased amount of myocardial immune cell infiltrates, CD68 immune-reactive cells, and cardiac fibrosis. [172] The EAM immunization protocol that was used models the chronic inflammatory phase of post-viral myocarditis, whose best-characterized strain is CVB3. [6] This phase of chronic inflammation is characterized by ongoing inflammation, fibrotic remodelling, the appearance of antimyosin antibodies, and the development of DCM as an end stage of the disease. [114] Given that the majority of the immune cells in the resting heart are macrophages, [115] this study attempted to demonstrate their role in the reported sex differences in autoimmune myocarditis.

This study investigated sex differences in the amount of myocardial immune infiltrates. Male EAM rats presented more immune cell infiltrates in the heart than female rats 21 days after immunization. The amount of CD68 immune-reactive cells was similar in the hearts of male and female EAM rats; however, female EAM rats had more CD68+ ArgI+ cells, suggesting that the macrophages in the cardiac tissue of female EAM animals are M2 anti-inflammatory

macrophages. [173] In the context of recent findings on the spectrum of macrophage polarization, this is an interesting result. Mosser et al. characterized the different subpopulations of macrophages as a fluent spectrum with distinctive roles in inflammation, tissue repair and immune regulation, whose effects may even contradict each other. The M2 response counteracts the detrimental effects of the pro-inflammatory macrophage polarization during acute inflammation, suggesting that a predominant M2 response in female EAM rats is cardio-protective. [73] Fairweather et al. showed that sex differences in viral myocarditis and post-myocarditis complications, e.g., the development of cardiac autoimmunity and the development of DCM, are not caused by the virus itself, which is cleared 14 days after infection in both sexes, but by different female and male immune responses. [6] They also showed that, for a viral myocarditis model, the detrimental immune response in male individuals is driven by a predominantly M1 response, as compared to a stronger M2 response in female animals. [87] Using the EAM model, this study confirms findings from the CVB3 animal model that the sex bias in myocarditis is not driven by the viral noxa, but rather by the differing immune responses of female and male organisms. Using the EAM model, this study shows that macrophages and their polarization are likely to be highly important for the origin of sex differences in cardiac inflammation. In accordance with the stronger M1 response in male animals, sex differences in the expression of prominent pro-inflammatory mediators such as c-fos and iNOS were shown. Male EAM rats showed an increased expression of c-fos and iNOS compared to non-immunized animals, while in female EAM rats the expression of both factors was unchanged, suggesting that female rats did not develop a pro-inflammatory response after immunization with cardiac myosin and CFA. C-fos [174] is a transcription factor of the M1 spectrum, and iNOS [73] is a signature M1 enzyme. PCR analysis was performed on overall processed cardiac tissue (not only on immune cell infiltrates), and this inaccuracy probably interfered with the characterisation of the distinct expression patterns of the macrophage populations involved. Consequently, a larger number of EAM animals were used to overcome this inaccuracy, in order to show sex differences in more molecular markers of macrophage subpopulations.

Recently, estrogen was shown to regulate macrophage polarization in different pathological tissue states, [175–177] suggesting that E2 is directly involved in the polarization into M2 macrophages in female EAM rats. The next step would be to investigate the role of sex hormones in macrophage polarization in the EAM model. Fairweather et al. noted that the sex bias in cardiac inflammation is driven by sex hormones. [178] Testosterone is associated with increased myocardial inflammation and an M1 response of macrophages in male individuals. [85] Furthermore, Regitz-Zagrosek et al. showed that estrogen benefits a cardio-protective female

tissue response towards harmful noxa in general by reducing cardiac myocyte apoptosis, inhibiting reactive oxygen species-induced cardiac damage, and by counteracting fibrosis and hypertrophy-inducing cellular pathways. [45] The activation of ERK and p38 was increased in EAM rats in comparison to non-immunized rats; however, no sex differences in the phosphorylation rate of ERK and p38 were found in this study, suggesting that other cascades are involved in the increased expression of pro-inflammatory mediators in male EAM rats. Furthermore, this study showed an increased expression of Col3A1 in the cardiac tissue from male EAM rats, while female EAM rats expressed similar amounts of Col3A1 compared to non-immunized female rats. In the study by Schmerler et al., male rats showed severe fibrosis formation in EAM heart sections. [172] Accordingly, this study demonstrates that male but not female EAM rats develop pathological rates of fibrosis in the heart after immunization, indicating that female rats undergo a different immune response that is not involved in fibrosis formation. Gonadectomized EAM rats could be compared to gonadectomized EAM rats receiving E2 treatment during the EAM protocol to differentiate the protective E2 effects on the acute immune response from long-lasting, cardio-protective E2 effects on female heart tissue prior to EAM treatment. However, physiologically, estrogen does not occur in high concentrations in the male organism (only in the pathologic state of adipositas), [64] which is why, in male EAM rodents, the effects of testosterone on disease progress should be further studied. It has been reported that testosterone affects the immune system. [81,179] Furthermore, Frisancho-Kiss et al. showed that testosterone increases cardiac inflammation. [69] Future research could analyze myocardial cell infiltrates and their distinct composition regarding macrophage subtypes in these animals (gonadectomized female EAM rodents: negative group vs. estrogen-treated group, and gonadectomized male EAM rodents: negative group vs. testosterone-treated group). Schmerler et al. demonstrated that male EAM rats had decreased stroke volume and ejection fraction.[172] Accordingly, the ejection fraction was decreased in male EAM rats in the animals used for this study (Sarah Jeuthe from the Charité – Universitätsmedizin obtained preliminary data). Interestingly, in female rats, the ejection fraction was not affected after immunization, suggesting that the different immune response in female animals preserved the cardiac function. However, the statistical analysis did not show sex differences to be significant, although a high number of animals were included in the experiment's treatment protocol. Variations in the ejection fraction have also been observed in healthy subjects, and many factors other than a rodent's sex influence them. [84] This could explain the difficulty in showing a significant difference between male and female EAM animals.

The findings that male macrophages tend to polarize into an M1 phenotype with an increased pro-inflammatory character and a stronger capability to activate fibroblasts of the same sex could contribute to the observed sex differences in the EAM model.

The processed cardiac tissue from EAM rats only showed the overall reaction of the inflamed tissue. For this reason, the distinct reaction of single-cell types, such as macrophages and fibroblasts, could not be distinguished satisfactorily. Thus, sex differences in macrophage polarization and fibroblast activation were investigated *in vitro*. Therefore, the second part of this study focused on cell culture experiments by using murine bone marrow-derived macrophages and murine cardiac fibroblasts. In this study, the *in vitro* experiments with macrophages demonstrated that male macrophages express a stronger pro-inflammatory M1 phenotype, leading to a prominent activation of fibroblasts after treatment with medium conditioned by M1 macrophages. These results suggest that the myocardial macrophages found in male EAM rats have a pro-inflammatory phenotype. The observation that the M1 markers c-fos and iNOS are significantly increased in male EAM samples not in female ones indicates this. [137,138] The appearance of autoantibodies is an important risk factor for detrimental progress with myocarditis. [2,50] Autoantibody-related autoimmune diseases are more common in females because estrogen promotes a TH2-type immune system response with B-cell activation. [180] However, this must be confirmed for the EAM model. The development of autoantibody-driven autoimmunity occurs in two ways: molecular mimicry between viral proteins and cardiac proteins, and the exposure of cardiac proteins to the host's immune system during inflammation and the harming of cardiac cells. [27,28] Thus, increased M1 polarization in male macrophages, which was observed in cell culture experiments, could lead to enhanced cardiomyocyte damage as well as the strong, long-lasting exposure of myocardial proteins to the host's immune system, giving rise to the formation of cardiac autoantibodies. This mechanism could even be enhanced by M1 macrophages having a stronger antigen presentation activity compared to M2 macrophages, leading to the stronger induction of cardiac autoimmunity. [181] On the cell culture level, the increased M1 polarization of male murine BMMs was regulated by a more strongly increased expression of c-fos and NFκB transcription factors, which are important downstream targets of LPS signalling. [135] Conclusively, M1 signature cytokines TNF-α and IL-1β were also more strongly upregulated in male cells, whereas the downregulation of immune-regulatory marker TIM3 [33] was stronger in male LPS-treated murine BMMs. This is also an interesting finding, as it was demonstrated that TIM3 expression correlates negatively with the severity of cardiac inflammation. [33] Western blot protein analysis showed that the p38 pathway was significantly upregulated in LPS-treated male murine BMMs, but not in female ones. Activated

p38 is known as a downstream signalling target of LPS and is important in M1 macrophage polarization signalling. [135–137] TLR 4 activation is a strong inductor of M1 polarization; [136] it is also important for CVB3 cell invasion, and its genetic variations are associated with stronger CVB3-mediated cell damage. [15,16] Moreover, the polymorphism of TLR was found to be an important risk factor in myocarditis. [182] However, LPS treatment increased TLR 4 expression in female and male murine BMMs with no significant sex difference, suggesting that this mechanism makes cells of both sexes more vulnerable to viral cell invasion during the acute phase of viral myocarditis. This study attempted to show an M2 macrophage phenotype, which is characteristic of the female immune response during the EAM immunization protocol. The spectrum of M2 type macrophages is broad, and some M2 subspecies are also pro-fibrotic, such as the M2 (tissue repair) macrophages. [73,183] Notably, IL-4 and IL-13 treatment of murine BMMs also resulted in a stronger M2 (tissue repair) subtype activation in male murine BMMs. In addition, male murine BMMs reacted with stronger upregulation of distinctive M2 markers such as MCP-1. [73] However, pro-inflammatory M1 markers such as TNF-α were also downregulated more strongly in male murine BMMs. The co-culture experiments indicate that the M2 subtype is capable of activating fibroblasts. Therefore, it can also be assumed that male rodents not only show a stronger M1-driven immune response but also benefit pro-fibrotic subtypes of the M2 spectrum, rather the immune-regulatory and inhibitory ones. The next step would be to investigate whether there is an M2 polarization treatment protocol that leads to a stronger expression of distinct subtype markers in female murine BMMs. These could be markers of the immune-regulatory M2 subtype, which can be obtained by stimulating macrophages with IL-10 or apoptotic cells. [73,137,184]

To investigate the role of estrogen in macrophage polarization, estrogen co-stimulation was performed together with LPS or IL-4 and IL-13. Estrogen co-treatment of macrophages only showed effects in male-treated cells. The only exception was the downregulation of the immuno-inhibitory marker TIM3 by estrogen treatment in male and female murine BMMs. Frisancho-Kiss et al. demonstrated that TIM3 is a key marker for determining sex differences in inflammatory heart disease. TIM3 was shown to be associated with a less detrimental disease progress. [69] Other workgroups have reported that estrogen effects on female macrophages drive polarization into M2 phenotypes. [185] The lack of estrogen effects in female primary cells could be explained by the fact that the female cells used in this study underwent estrogen-mediated cell modifications for a long period of time while still being part of the host's body, before being used for estrogen stimulation experiments. Longer estrogen treatment would perhaps overcome this pre-stimulation and show effects in female murine BMM polarization. However,

male murine BMMs reacted to estrogen treatment during LPS stimulation with an even stronger M1 polarization. The estrogen treatment aggravated the expression of TNF-α as well as of c-fos and NFκB transcription factors, which are important downstream targets of M1 polarization in LPS signalling. [135] The effects of estrogen on M2 polarization of BMMs in a co-treatment experiment with IL4 and IL13 did not show results that were as distinct as those of M1 polarization experiments. Additional treatment with estrogen did not affect the polarization of female murine BMMs. On the other hand, for male murine BMMs, additional treatment with estrogen during IL-4- and IL-13-mediated polarization resulted in the attenuated expression of M2 marker MCP-1. Other studies have reported that the estrogen effects on immune cells highly depend on the estrogen concentrations used. [63] It should be noted that estrogen is not a hormone that physiologically exists in high concentrations in the male system, except in non-physiological conditions such as adipositas. [64] Further studies of estrogen effects on both male and female macrophage polarization could include experiments to verify which ER is involved in the macrophage activation and polarization. PPT or DPN (estrogen receptor alpha and beta subtype selective ligands[186]) supplementation could be used to investigate which estrogen receptor contributes to macrophage polarization, and in what way. After not finding estrogen effects - which could be protective in EAM disease progress - on female murine BMM polarization, the focus of further research should be on the effects of testosterone on male macrophage polarization in particular. Such research is important because testosterone is known to negatively affect inflammatory heart disease. [49] This study only tried two different treatment protocols to produce macrophage subspecies: M1/pro-inflammatory (LPS) and M2/tissue repairing (IL-4/IL-13). The M2 immune-regulatory subtype, described above, would also be a suitable target for estrogen co-stimulation experiments. In addition to differences in the immune response, it should also be noted that the tissue of the heart itself and its reaction to harmful triggers are likely responsible for sex differences in the EAM model. As stated above, Regitz-Zagrosek et al. showed that estrogen benefits a cardio-protective tissue response in female individuals regarding cardiac tissue damage due to different triggers. [100] There is evidence on the cellular level that this general cardio-protective tissue response towards stress in females is also mediated by estrogen, as the sex hormone was shown to be a direct regulator of fibroblast activation. [187,188] TNF-α, an important pro-fibrotic cytokine, was used to investigate fibroblast activation. [116] Activated fibroblasts increasingly expressed fibroblast activation markers such as MCP-1, and pro-fibrotic cytokines such as IL-1β. [189–191] Following TNF-α treatment, isolated cardiac fibroblasts expressed these markers more strongly than untreated cells. This also connects macrophage polarization to fibrotic remodelling, as TNF-α is an important cytokine

in crosstalk between macrophages and fibroblasts. [116] Subsequent experiments included the co-stimulation of fibroblasts with TNF-α and estrogen. As with the observations of the estrogen stimulation of murine BMMs, estrogen treatment did not affect female cells. Interestingly, male fibroblasts reacted to additional estrogen treatment during TNF-α activation with a decreased expression of fibroblast activation marker MCP-1 and autocrine-acting pro-fibrotic cytokine IL-1β, suggesting the anti-fibrotic direct effects of estrogen on male fibroblasts. Other studies have reported a similar effect and postulated the cardio-protective effect of estrogen in male and female cells. [192] The lack of estrogen effects on female cells in this study could again be explained by the fact that female cells were under long-term estrogen influence for a long period while still being part of the host's body. It is possible that longer estrogen treatment would also show effects in female fibroblast activation. Macrophages were described as important regulators of fibrotic remodelling and tissue repair. [73,193] Several cytokines from the macrophages' cytokine arsenal mediate this cell-cell interaction, with macrophages as regulators of fibroblasts. Of these pro-fibrotic cytokines secreted by macrophages, IL-1β, TGF-β, and TNF-α are important. [73,194,195] Supporting the idea of different subspecies of macrophages, [128] it was observed that different macrophage subtypes can promote fibroblast activation or inhibit it. [183] To investigate sex differences in macrophage-related fibroblast activation, co-culture experiments with both cell types were established. Macrophage-conditioned cell culture medium (conditioned by male and female as well as differently polarized macrophage subtypes) was used to activate fibroblasts. In addition, the effects of estrogen on the macrophage-related conditioning of the cell culture medium were investigated. The analysis of fibroblasts treated with conditioned medium from LPS-treated macrophages showed that this medium could activate the isolated murine cardiac fibroblasts. The treated cells increasingly expressed fibroblast activation markers such as MCP-1, the pro-inflammatory cytokines TNF-α and IL-1β, and the pro-inflammatory transcription factor NFκB. Furthermore, a sex bias for the fibroblast activation by female and male LPS-treated, macrophage-conditioned medium was found. Male fibroblasts treated with male murine BMM-conditioned (LPS-activated) medium expressed fibroblast activation marker MCP-1 more strongly than female ones. A stronger expression was also observed for the pro-fibrotic and pro-inflammatory cytokines IL-1β and TNF-α. The medium conditioned by IL-4- and IL-13-activated macrophages also activated fibroblasts, emphasizing that this subtype of macrophages plays a crucial role in tissue repair and wound-healing. [73] Finally, the effect of estrogen on the fibroblast activation following this indirect cell-cell interaction was investigated. However, the estrogen

treatment of both LPS- or IL-4- and IL-13-treated macrophages showed no difference in the capability of macrophage-conditioned medium to activate isolated murine cardiac fibroblasts.

9.1 The need for subsequent experiments

The findings that male macrophages react to treatment protocols with stronger polarization into M1 and M2 (tissue repair) subtypes are interesting in view of the fact that macrophages are key regulators of fibrotic remodelling. [183] It was shown that male murine M1 macrophages react with stronger pro-inflammatory polarization, and also that medium conditioned by this subtype activates treated fibroblasts of the same sex more strongly when compared to female murine BMM-conditioned medium. These are intriguing results given that macrophages are key cells in regulating fibrosis and the fibrotic remodelling of the heart. [153] Subsequent studies could include further analysis of cytokine patterns in macrophage-conditioned medium using ELISA analysis. In addition to macrophages, this study also focused on sex differences and estrogen effects on fibroblast activation. Successful activation of isolated murine cardiac fibroblasts was achieved by stimulation with TNF-α, which is known to be a fibroblast-activating cytokine. [191,196] TNF-α is a signature cytokine of the M1 spectrum and is known to be pro-fibrotic. [116] Higher expression of TNF-α in LPS-treated male macrophages was observed in this study, making it a suitable target for further analysis of sex differences in fibroblast activation. TNF-α treatment of fibroblasts showed increased expression of MCP-1 as a marker of fibroblast activation. [197] Interestingly, MCP-1 is a key cytokine for stimulating macrophages and monocytes to migrate to the place of highest concentration of MCP-1 (place of secretion). [198] This emphasizes that the cell-cell interaction of macrophages and fibroblasts is not unidirectional; rather, macrophages are additionally influenced by fibroblasts. Further studies could include cell culture experiments that investigate sex differences in chemotaxis as well as the cell migration of differently stimulated macrophages and fibroblasts (e.g., by using MCP-1 stimulation of macrophages). TNF-α-treated fibroblasts showed sex differences regarding the expression of pro-fibrotic cytokines IL-1β and TGF-β. Male fibroblast expression of these cytokines was more increased after stimulation, suggesting both that male murine macrophages react with stronger M1 polarization to LPS treatment and that male fibroblasts also react with stronger pro-fibrotic signalling to macrophage-derived TNF-α. Similarly to the observations of the estrogen treatment of female macrophages, female fibroblasts did not react differently to co-stimulation with estrogen during TNF-α activation. In summary, sex differences in macrophage polarization and macrophage-related fibroblast activation were found. These are likely to be a contributing factor to the faster and stronger progress of EAM in male rodents

compared to female ones. However, no effects of estrogen on female murine BMM polarization and their capability to activate fibroblasts were found. The next step would be to connect findings in the animal model and cell culture experiments to human biology. Warraich et al. demonstrated that high autoantibody levels against myosin correlate with clinically suspected myocarditis and DCM. Antibody levels correlated with the reduced ejection fraction of patients; [114] however, this must be confirmed for the EAM model. Cell culture experiments should be repeated with human cell lines that resemble macrophages or fibroblasts. The analysis of samples from myocarditis patients could also transfer knowledge from animal models and cell culture to human pathology. It would be interesting to analyze heart biopsies and cellular myocardial infiltrates. Analysis could include sex differences in inflammation and fibrosis with a special focus on distinct macrophage subspecies in myocardial infiltrates. In addition, future studies could measure estrogen and testosterone levels in patients, and compare these levels with markers of detrimental disease progress, such as the ejection fraction of patients. Macrophage subspecies could also be analyzed in PBMCs. These cells from healthy subjects could also be analyzed in cell culture experiments by treating them according to the treatment protocols for murine BMMs used in this study.

Regarding estrogen effects on macrophage polarization, this study only showed effects on male cells (male cells reacted with an increased expression of pro-inflammatory markers in LPS co-stimulation experiments). It is likely that the protocol needs to be optimized. As mentioned above, it is difficult to show estrogen effects on female cells because true estrogen deprivation requires time. Before the experiments, the female cells were part of the female murine host body and thus had a strong estrogen influence before being used for polarization experiments (in contrast to male murine cells). This could be overcome by treating the cells for a period of 48 or 72 hours, rather than only 24 hours.

9.2 Translational science – future therapeutic attempts

Macrophage polarization[73] has been found to be important for the modulation of several pathological tissue states, such as in cancer, [199] the formation of insulin resistance due to obesity, [200] cardiac remodelling, [156] and in several fibrotic diseases. [116,183] This study attempted to investigate the role of macrophage polarization in a specific form of myocarditis. It may be valuable for future research to influence the polarization of macrophage infiltrates into tissue restitution-supporting subtypes. In the case of ongoing, unregulated inflammation and fibrotic remodelling, this could be the regulatory M2 subtype Mosser and Edwards described, which downregulates the local immune response, and is thus anti-fibrotic. [116] However, the fact that

macrophages are highly important regulator cells in numerous organs as well as in physiological and pathological tissue states makes it difficult to find a specific treatment. The signature cytokine of regulatory macrophages is IL-10. Interestingly, IL-10 was also demonstrated to be anti-fibrotic in several animal fibrosis models. [201–203] However, treatment with recombinant IL-10 is still not implemented in everyday medicine, as the treatment effects have not met the initial high expectations. [204,205] Severe side effects often result from trying to modify the immune system by using cytokines or antibodies against distinct cytokines. The inhibition of IL-1β, which was also an important pro-fibrotic target in this study, showed a significant reduction in the reoccurrence of clinical manifestations of atherosclerosis, but it also led to an increased incidence of severe infections. [206] When boosting the inhibitory arm of the immune system, an increase of cancer manifestations is a general side effect that should be expected, as well. [207] Thus, a suitable treatment should be heart-specific. Harel-Adar and colleagues demonstrated a promising way of doing this with intravenous injections of phosphatidylserine (PS)-presenting liposomes, mimicking the anti-inflammatory effects of apoptotic cells in the heart. This led to a population of macrophages that supported heart tissue restitution after myocardial infarction of animals. This macrophage population was characterized by high levels of IL-10 and TGF-β as well as low expression of the pro-inflammatory marker TNF-α. [208] Some progress has been made in the field of antiviral treatment. Interferon β, an antiviral cytokine, has been in clinical use for some time, but is reserved for special cases. [209] However, one problem with these antiviral therapeutic approaches is that the trigger of cardiac inflammation (e.g., cardiotropic viruses) only exists in the host's body for a short period of time. [94] During this period, the disease usually does not present the severe symptoms that justify further diagnostics to introduce a specialized treatment. [209] Thus, a treatment for patients suffering from ongoing inflammation and fibrotic remodelling in a post-viral state of the disease would still be needed. Patients with the formation of cardiac antibodies are an interesting group for specialized treatment. Recently, immunosorbent procedures came to the attention of clinical users. The removal of cardiac autoantibodies seems to have a positive effect on disease outcomes. [210] Regarding the focus of this study, it should be questioned whether female DCM patients could benefit from estrogen treatment, or male patients from antiandrogen treatment. However, this study did not find a consistent role of estrogen effects in myocarditis pathomechanisms regarding *in vitro* macrophage polarization experiments. Furthermore, the role of sex hormones and sex differences of the human immune system is still only understood vaguely. [54,211] Again, it must be noted that the hormonal treatment of patients leads to systemic treatment effects, but also to systemic side effects.

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11 Eidesstattliche Versicherung

Ich, Maximilian Heinrich Niehues, versichere an Eides statt durch meine eigenhändige Unterschrift, dass ich die vorgelegte Dissertation mit dem Thema: „Sex differences in macrophage polarization in experimental autoimmune myocarditis (EAM)/Geschlechterunterschiede in der Makrophagen Polarisation bei experimenteller autoimmuner Myokarditis (EAM)“ selbstständig und ohne nicht offengelegte Hilfe Dritter verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel genutzt habe.
Alle Stellen, die wörtlich oder dem Sinne nach auf Publikationen oder Vorträgen anderer Autoren/innen beruhen, sind als solche in korrekter Zitierung kenntlich gemacht. Die Abschnitte zu Methodik (insbesondere praktische Arbeiten, Laborbestimmungen, statistische Aufarbeitung) und Resultaten (insbesondere Abbildungen, Graphiken und Tabellen) werden von mir verantwortet.

Ich versichere ferner, dass ich die in Zusammenarbeit mit anderen Personen generierten Daten, Datenauswertungen und Schlussfolgerungen korrekt gekennzeichnet und meinen eigenen Beitrag sowie die Beiträge anderer Personen korrekt kenntlich gemacht habe (siehe Anteilserklärung). Texte oder Textteile, die gemeinsam mit anderen erstellt oder verwendet wurden, habe ich korrekt kenntlich gemacht.

Meine Anteile an etwaigen Publikationen zu dieser Dissertation entsprechen denen, die in der untenstehenden gemeinsamen Erklärung mit dem/der Erstbetreuer/in, angegeben sind. Für sämtliche im Rahmen der Dissertation entstandenen Publikationen wurden die Richtlinien des ICMJE (International Committee of Medical Journal Editors; www.icmje.og) zur Autorenschaft eingehalten. Ich erkläre ferner, dass ich mich zur Einhaltung der Satzung der Charité – Universitätsmedizin Berlin zur Sicherung Guter Wissenschaftlicher Praxis verpflichte.

Weiterhin versichere ich, dass ich diese Dissertation weder in gleicher noch in ähnlicher Form bereits an einer anderen Fakultät eingereicht habe.

Die Bedeutung dieser eidesstattlichen Versicherung und die strafrechtlichen Folgen einer unwahren eidesstattlichen Versicherung (§§156, 161 des Strafgesetzbuches) sind mir bekannt und bewusst.“

Datum Unterschrift

12 Anteilserklärung an erfolgten Publikationen

Ich, Maximilian Heinrich Niehues, hatte folgenden Anteil an den folgenden Publikationen:

Präsentation von Daten meiner Dissertation in Form eines Posterbeitrags auf vier verschiedenen Kongressen (es handelt sich um die Präsentation derselben unten aufgeführten Daten):

Barcena de Arellano ML, Niehues M, Jeuthe S, Messroghli D, Regitz-Zagrosek V, "Sex differences in the cardiac inflammatory response in rats with experimental autoimmune myocarditis", DZHK partner site retreat, 2019

Barcena de Arellano ML, Niehues M, Jeuthe S, Messroghli D, Regitz-Zagrosek V, "Sex differences in the cardiac inflammatory response in rats with experimental autoimmune myocarditis", World Cogress on Inflammation (WCI), 2019

Barcena de Arellano ML, Niehues M, Jeuthe S, Messroghli D, Regitz-Zagrosek V, "Sex differences in the cardiac inflammatory response in rats with experimental autoimmune myocarditis". International Society for Hear Research (ISHR), 2019.

Barcena de Arellano ML, Niehues M, Jeuthe S, Messroghli D, Regitz-Zagrosek V, "Sex differences in the cardiac inflammatory response in rats with experimental autoimmune myocarditis", I & I, 2019

Die EAM Protokolle selbst wurden nicht von mir durchgeführt. Ebenfalls nicht von mir stammen die Erhebungen der klinischen Daten von EAM Versuchstieren (Ejektionsfraktion und Schlagvolumen). Von mir kommen die Sirius Red Färbungen und die Berechnung der Fibrosebildung in Herzschnitten von EAM Versuchstieren. Nicht von mir stammen die CD68+ ArgI+ immunhistochemischen Färbungen der Herzschnitte, sowie die Berechnung des Anteils myokardialer Infiltrate.Von mir durchgeführt wurden die PCR-Untersuchungen der Genexpression und Western Blot Untersuchungen in Herzgewebe von EAM Versuchstieren.
Darüber hinaus sind von mir durchgeführt worden: die Stimulationsexperimente mit aus Mäuseknochenmark entnommenen Makrophagen, und aus Mäuseherzen entnommenen Fibroblasten und die Untersuchung der Genexpression in diesen Zellen.

Von mir stammen die Durchführung und Auswertung der:

- Sirius Red Färbungen der Herzschnitte, Berechnung des Anteils an Fibrose (Figure 3 A und B)
- Western Blot Untersuchung von Col3A1 in EAM Ratten Herzgewebe (Figure 3C)
- PCR Untersuchungen der c-fos und IL-6 Expression in EAM Ratten Herzgewebe (Figure 4 A und B)
- PCR Untersuchungen der IL1 Beta, TNF-α und NFκB Expression in LPS und IFN-y behandelten Makrophagen (Figure 5 A – C)
- PCR Untersuchungen der MCP-1, IL1 Beta, TGF-β1, Expression in TNF-α und TGF-β behandelten Makropahgen (Figure 6 A – F)

Maria Luisa Barcena, Sarah Jeuthe, Maximilian Heinrich Niehues, Sofya Podzniakova, Anja Andrea Kühl, Daniel Messroghli, Vera Regitz-Zagrosek, “Sex-specific differences of the inflammatory stage in experimental autoimmune myocarditis” Journal: Frontiers in Immunology, 2021

Von mir stammen die Durchführung der Experimente und Auswertung aus denen die Figure 3A – 3C, Figure 4A und 4B, 5A – 5F hervorgegangen sind.

_____
Unterschrift, Datum und Stempel des/der erstbetreuenden Hochschullehrers/in

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Unterschrift des Doktoranden/der Doktorandin

13 Curriculum Vitae

Mein Lebenslauf wird aus datenschutzrechtlichen Gründen in der elektronischen Version meiner Arbeit nicht veröffentlicht.

14 Publikationen

Maria Luisa Barcena, Sarah Jeuthe, Maximilian Heinrich Niehues, Sofya Podzniakova, Anja Andrea Kühl, Daniel Messroghli, Vera Regitz-Zagrosek, “Sex-specific differences of the inflammatory stage in experimental autoimmune myocarditis” Journal: Frontiers in Immunology, 2021

Barcena de Arellano ML, Niehues M, Jeuthe S, Messroghli D, Regitz-Zagrosek V. (2019). “Sex differences in the cardiac inflammatory response in rats with experimental autoimmune myocarditis.” DZHK partner site retreat 2019.

Barcena de Arellano ML, Niehues M, Jeuthe S, Messroghli D, Regitz-Zagrosek V. (2019). “Sex differences in the cardiac inflammatory response in rats with experimental autoimmune myocarditis.” WCI 2019.

Barcena de Arellano ML, Niehues M, Jeuthe S, Messroghli D, Regitz-Zagrosek V. (2019) “Sex differences in the cardiac inflammatory response in rats with experimental autoimmune myocarditis.” ISHR 2019.

Barcena de Arellano ML, Niehues M, Jeuthe S, Messroghli D, Regitz-Zagrosek. (2019). “Sex differences in the cardiac inflammatory response in rats with experimental autoimmune myocarditis.” I & I 2019.

Barcena de Arellano ML, Niehues M, Pannwitz D, Beling A, Regitz-Zagrosek V. (2017). “Sex differences in oestrogen-dependent macrophage activation in cardiac chronic inflammation.” ESC 2017.

Barcena de Arellano ML, Niehues M, Beling A, Regitz-Zagrosek V. (2017). “Sex differences in oestrogen-dependent macrophage activation in cardiac chronic inflammation.” ISHR 2017.

Barcena de Arellano ML, Niehues, Berchtold D, Beling A, Regitz-Zagrosek V. (2017). “Sex differences in oestrogen-dependent macrophage activation in cardiac chronic inflammation.” DGK Kongress 2017.

Barcena de Arellano ML, Niehues M, Berchtold D, Regitz-Zagrosek V. (2016). “Sex differences in oestrogen-dependent macrophage-fibroblast interaction in cardiac inflammation.” IASP 2016

15 Danksagung

Ich möchte die folgenden Sätze nutzen um den Menschen zu danken, die mich bei meinen ersten Schritten im wissenschaftlichen Arbeiten unterstützt haben. Sei es durch wertvolle fachliche Ratschläge, die mich vor dem Betreten einiger Sackgasse bewahrt haben. Oder durch motivierende Worte, wenn ich mich doch einmal in einer solchen verirrt habe.

Zuerst möchte ich hier Prof. Dr. med. Dr. h. c. Regitz-Zagrosek danken. Dafür, dass Sie mir die Chance gegeben hat in dem hochinteressanten und vor allem relevanten Gebiet der Geschlechter Medizin zu forschen. Während meiner Laborarbeit in ihrer Arbeitsgruppe durfte ich die Schnittmengen aktueller kardiologischer und immunologischer Forschung vor dem Hintergrund der Geschlechter Medizin kennen lernen. Die Erkenntnis, dass Männer und Frauen unterschiedliche krank werden möchte ich auch in meine klinische Tätigkeit als Internist einfließen lassen.

Die wichtigste Ansprechpartnerin während der Durchführung der Experimente, aber auch bei der eigentlichen Erstellung der Dissertation war für mich Dr. Luisa Maria Barcena. Ich möchte ihr dafür danken, dass Sie zu jeder Zeit ein offenes Ohr für meine Fragen und Anregungen hatte (davon gab es einige). Und, dass Sie bei den Durststrecken (auch davon gab es einige) immer die richtigen Worte gefunden hat meine Aufmerksamkeit wieder auf das Ziel der Anstrengungen zu richten.

16 Bescheinigung Statistik

CHARITÉ

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Bescheinigung

Hiermit bescheinige ich, dass Herr Maximilian Niehues innerhalb der Service Unit Biometrie des Instituts für Biometrie und klinische Epidemiologie (iBikE) bei mir eine statistische Beratung zu einem Promotionsvorhaben wahrgenommen hat. Folgende Beratungstermine wurden wahrgenommen:

- Termin 1: *16.08.2019*
- Termin 2: *22.10.2019*

Folgende wesentliche Ratschläge hinsichtlich einer sinnvolle Auswertung und Interpretation der Daten wurden während der Beratung erteilt:

- *Mehrfache Verwendung der Two-Way ANOVA*
- *Hinweis zur Adjustierung des Signifikanzniveaus im multiplen Testproblem*
- *Deskription der Daten*

Diese Bescheinigung garantiert nicht die richtige Umsetzung der in der Beratung gemachten Vorschläge, die korrekte Durchführung der empfohlenen statistischen Verfahren und die richtige Darstellung und Interpretation der Ergebnisse. Die Verantwortung hierfür obliegt allein dem Promovierenden. Das Institut für Biometrie und klinische Epidemiologie übernimmt hierfür keine Haftung.

Datum: 22.10.2019

Name des Beraters/ der Beraterin: Kerstin Rubarth

Unterschrift BeraterIn, Institutsstempel