Macrophages Modulate Cardiac Function in Lipotoxic Cardiomyopathy

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Running Head: Macrophages and lipotoxic cardiomyopathy

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Abstract

Diabetes is associated with myocardial lipid accumulation and an increased risk of heart failure. While cardiac myocyte lipid overload is thought to contribute to the pathogenesis of cardiomyopathy in the setting of diabetes, the mechanism(s) through which this occurs is not well understood. Increasingly, inflammation has been recognized as a key pathogenic feature of lipid excess and diabetes. In this study, we sought to investigate the role of inflammatory activation in the pathogenesis of lipotoxic cardiomyopathy using the transgenic MHC-ACS mouse model. We found that several inflammatory cytokines were upregulated in the myocardium of MHC-ACS mice before the onset of cardiac dysfunction and this was accompanied by macrophage infiltration. Depletion of macrophages with liposomal clodrolip reduced the cardiac inflammatory response and improved cardiac function. Thus, in this model of lipotoxic cardiac injury, early induction of inflammation and macrophage recruitment contribute to adverse cardiac remodeling. These findings have implications for our understanding of heart failure in the setting of obesity and diabetes.

key words: Heart failure, diabetes, inflammation,
**Introduction**

Obesity and diabetes are significant risk factors for the development of heart failure (11). In many diabetic patients, this can occur in the absence of underlying coronary artery disease or hypertension, a phenomenon known as diabetic cardiomyopathy (DCM) (1). The pathogenesis of DCM is complex; however, myocardial lipid overload is a pathologic feature of this condition in humans and in animal models (6, 7, 12, 25). The observation that cardiac myocyte lipid overload causes cardiomyopathy in genetic mouse models, without systemic abnormalities of insulin or glucose metabolism, supports the notion that excess lipids can be cardiotoxic (4, 5, 7, 32).

Membrane lipid remodeling, endoplasmic reticulum and oxidative stress, and production of toxic lipid species, such as ceramides, have been implicated as potential mechanism(s) of cardiac lipotoxicity (12, 23).

Systemic inflammation is another hallmark of obesity and diabetes, and has been shown to correlate with the risk of heart failure in patients with these metabolic conditions (2). This raises the intriguing possibility that lipid-induced inflammatory responses might contribute to cardiac dysfunction in obesity and diabetes. Much of what is known regarding the link between diabetes and inflammation has come from investigations of adipose tissue. In humans and animals, obesity triggers the recruitment of macrophages to white adipose tissue, which is followed by release of inflammatory mediators and the onset of insulin resistance (26, 29). When this response is interrupted in mice fed a high fat diet, either through macrophage depletion or genetic knock-out of macrophage chemoattractants, the animals are protected against insulin resistance despite
an equivalent extent of obesity (19, 21, 28). Macrophages co-expressing F4/80 and CD11c appear to be a key leukocyte subset involved in the inflammatory and metabolic effects of a high fat diet (17, 21). Despite the evidence supporting a critical role for adipose tissue macrophages in the pathogenesis of metabolic disease, very little is known about cardiac macrophages in heart failure associated with metabolic stress. However, models of myocardial ischemia and/or infarction have illuminated an important, but complicated, function of these inflammatory cells in cardiac injury and repair. For example, monocytes and macrophages are required for the reparative response to myocardial tissue damage, yet overexuberant inflammation and cytokine production can exacerbate pathologic LV remodeling (8).

In the present study, we sought to investigate the role of inflammation and macrophages in the pathogenesis of lipotoxic cardiomyopathy. To address this issue, we used the well-established MHC-ACS transgenic mouse model of heart failure. In these mice, cardiac-selective overexpression of long-chain acyl CoA synthetase 1 (ACS) leads to marked myocyte lipid accumulation independent of systemic metabolic disturbances and leads to cardiac dysfunction and premature death (5). Our findings reveal that macrophages modulate the myocardial response to this metabolic stress and contribute to adverse cardiac remodeling in response to lipid overload.
Materials and Methods

Reagents. Antibodies for flow cytometry were from BD Pharmingen (CD11b-PE, CD45-FITC, CD11c-APC and CD16 Fc Block) or eBiosciences (F4/80-PerCP-Cy5.5). Antibodies for MAC3 immunohistochemistry and STAT-3/phospho STAT-3 western blot were from Santa Cruz Biotechnology (sc-81729, sc-8059, sc-7179). Clodrolip and control liposomes were produced as described by Dr. Reto Schwendener (34).

Animal Experiments. MHC-ACS mice in FVB inbred genetic background (5) were maintained on a standard chow diet ad libitum (6 % fat). For experiments, MHC-ACS and non-transgenic littersmates were analyzed at the indicated ages. For clodrolip macrophage depletion, mice were given intraperitoneal injections of 100 mg/kg clodrolip or control liposomes in 150 μl volume at 22 days of age, followed by injections of 30 mg/kg in 100 μl volume every 4 days for the duration of the experiment. All animal experiments were conducted in accordance with NIH guidelines for humane treatment of animals and were approved by the Animal Studies Committee of Washington University School of Medicine.

RNA Analysis and Real Time Quantitative PCR (qRT-PCR). Total cellular RNA was isolated from mouse cardiac ventricles using the RNazol B followed by chloroform extraction. Messenger RNA was reverse transcribed using a High Capacity cDNA Reverse Transcription kit according to manufacturer’s instructions (Applied Biosystems). Real time qRT-PCR was performed using SYBER green reagent (Applied Biosystems) and mouse specific primers (Supplemental Table 1) on an ABI Prism 7500 Sequence
Detection system. Signal intensity was normalized to 36B4. For the qRT-PCR array (SA Biosciences, PAMM-011A), RNA samples were further purified following initial RNazol B/chloroform using RNaeasy columns (Qiagen). Relative quantification of gene expression was performed using the delta-delta CT method.

**Echocardiographic Studies.** Transthoracic M-mode and 2-dimensional echocardiography was performed on mice anesthetized with inhaled isofluorane in the Washington University Cardiovascular Phenotyping Core using a Visual Sonics 770 echocardiography system (5).

**Protein Analysis.** Total cellular protein lysates were prepared from mouse hearts using 250 μl modified RIPA buffer (1% NP40, 0.5 % SDS, 0.1 M phenylmethylsulfonyl fluoride, and 1X protease complete (Roche) in PBS) followed by sonication (50% duty cycle, output 4, 30 pulses, Branson Sonifier 250, on ice). Total protein was quantified using the bicinchoninic acid assay method (Thermo Scientific). For western blots, 25 μg of total protein was run on a 7.5% polyacrylamide gel, transferred to a nitrocellulose membrane and probed with antibodies specific to phospho-STAT3 and STAT3 (1:1000 overnight at 4 degrees C).

**Immunohistochemistry.** Heart tissues were fixed overnight at 4° C in 10% formalin. After dehydration in ethanol and paraffin embedding, 5 um slides were stained with hematoxylin and eosin (H&E) or anti-MAC3 antibody (1:50, overnight at 4°) and developed using a biotinylated anti-Rat secondary followed by streptavidin-HRP and
DAB substrate per manufacturer’s instructions (Vector Laboratories). Bright field microscopy was performed using a Zeiss Axioskop.

**Flow Cytometry.** Mice were euthanized by CO$_2$ asphyxiation and retrograde perfusion of hearts was performed using 30 ml of ice cold PBS. Ventricles were dissected, rinsed in ice cold PBS, and minced to approximately 1 mm in size and 5 ml of collagenase mix (collagenase II, Invitrogen) 100 units/ml in HBSS with 60 units/ml of DNase 1 was added to samples. Samples were placed on shaker at 37 °C for 1 hr. Following digestions, the hearts were triturated and passed through a 40 micron filter. The cells were pelleted by centrifugation at 200xg for 5 min after they were washed in 5 ml PBS and counted. Antibody staining was performed in FACS buffer (PBS, 2% FBS, 0.02% sodium azide). All samples were incubated with 0.5 μg Fc blocking antibody for 5 min on ice prior to the addition of primary antibodies. For staining, primary antibodies were added to the cells in a volume of 20 μl and incubated for 15 min on ice in the dark. Following incubation the cells were washed with FACS buffer and resuspended in 300 μl of PBS + 1% paraformaldehyde. The data was collected on a FACSCaliber Flow cytometer and analyzed using FlowJo software.

**Myocardial Triglyceride (TAG) Determination**

Hearts were harvested and perfused with PBS and then frozen at -80 °C until analysis. Tissue was homogenized in chloroform:methanol (2:1), and an aliquot of organic extract was evaporated and analyzed using the Infinity Triglyceride Reagent (Thermo Scientific). Concentrations were normalized per mg of tissue.
Statistics. Statistical analysis was performed using GraphPad Prism software. All results are expressed as means +/- SE where n= number of animals analyzed. Groups were compared by unpaired Student’s t test, 1-way ANOVA or 2-way ANOVA as appropriate. A value of P< 0.05 was considered significant.
Results

*Inflammatory phenotype of mice with lipotoxic cardiomyopathy.*

Increasing evidence suggests that excess lipid can promote toxicity through activation of inflammatory pathways (9). The ACS model of lipotoxic cardiomyopathy is characterized by marked lipid accumulation as early as 18 days (5). Thus, we assessed activation of inflammatory pathways by quantifying myocardial expression of several prototypical inflammatory cytokines by qRT-PCR in 4-week-old transgenic animals and non-transgenic (NTG) littermates. Importantly, ACS mice still have preserved cardiac function at this point despite increased cardiac lipid import, allowing us to identify inflammatory changes that precede the onset of cardiomyopathy (Fig. 1A). ACS mice had significantly higher mRNA expression levels of the inflammatory cytokines IL-6 and MCP-1/CCL2 in the heart; however, neither TNFα nor iNOS transcripts were induced (Fig. 1B). Consistent with elevated levels of IL-6, phopho-STAT3, a read out of IL-6 receptor activation, was markedly increased in ACS hearts compared to NTG littermates (Fig. 1C). Based on these initial findings, we sought to further characterize the inflammatory phenotype of ACS mice using a qRT-PCR array of inflammatory target genes. The results of this analysis revealed significant upregulation of several macrophage chemokines/chemokine receptors and related cytokines including osteopontin (OPN), MCP-2/CCL8, MCP-3/CCL7, MCP-5/CCL12 and CCR5 (Table 1).

*Macrophage infiltration of the myocardium is an early event in MHC-ACS mice*

The inflammatory profile of ACS mice suggested that macrophages might be recruited to heart in response to myocardial lipid overload. Consistent with this prediction, mRNA
expression of the macrophage markers CD68 and F4/80 was significantly elevated in the hearts of ACS mice (Fig. 2A). We also observed a profound increase in the expression of CD11c, a marker of dendritic cells and a subset of activated macrophages. The expression of the macrophage chemoattractant/migration molecules MCP-2 and OPN was also profoundly upregulated in 4-week-old ACS mice compared to NTG littermates, consistent with our analysis from the qRT-PCR array (Fig. 2A, Table 1). To confirm the presence of enhanced cardiac macrophage recruitment, MAC3 immunohistochemical staining of myocardial tissue sections was performed. We observed a marked increase in the number of macrophages within the myocardium of ACS mice prior to the development of heart failure (Fig. 2B). Similar to the crown ringed structures observed with macrophage accumulation in adipose tissue of obese mice, the infiltrating macrophages in ACS hearts existed in distinct clusters distributed throughout the myocardium (Fig. 2B). While robust lipid accumulation in ACS hearts is detected at 3 weeks of age (Fig. 2C), we detected only a small increase in expression of the macrophage marker CD68 at that early time point, compared to marked induction of CD68 at four weeks of age (Fig. 2D). These observations are consistent with a model in which myocardial lipid accumulation leads to macrophage recruitment. Both lipid accumulation and macrophage recruitment precede heart failure, which is not observed until 8 weeks (Fig. 1A).

To further define the phenotype of the infiltrating macrophages, cardiac cells from 4-week-old ACS and NTG mice were disaggregated into single-cell suspensions and analyzed by flow cytometry. CD45+ cells (leukocytes) were analyzed for expression of F4/80, CD11b, and CD11c (Fig. 3). Lipotoxic mice had a higher percentage of
myocardial CD45 cells expressing the myeloid marker CD11b compared to NTG littermates. In line with the qRT-PCR data, there was a substantial increase in CD11b⁺,F4/80⁺ and CD11b⁺,CD11c⁻ cells in ACS mice. Only a small percentage of these myeloid cells co-expressed both CD11c and F4/80 surface markers. Interestingly, neither classic M1 markers, such as TNF and iNOS, nor classic M2 markers such as L-arginase, IL-10, or Chi-313 were significantly upregulated in ACS mice, although M2 markers trended up in ACS mice (Fig 1B, Fig 2A). Thus, the macrophage phenotype in this model of lipotoxicity is not clearly polarized towards M1 or M2.

**Depletion of Macrophages Modulates the Cardiac Inflammatory Response to in MHC-ACS mice**

To determine the contribution of macrophage infiltration to the cardiac inflammatory phenotype of ACS mice, macrophages were depleted by intraperitoneal (IP) injection of liposomal clodronate (clp), which leads to apoptosis of both tissue macrophages and circulating monocytes (33, 34). Compared with vehicle (veh) liposome injected ACS mice, clp treated animals had near complete loss of myocardial F4/80 expression by qRT-PCR (Fig 4A). Both CD68 and CD11c were also significantly reduced in clp injected mice; however, the expression of these genes was still increased in ACS myocardium relative to NTG littermates. Induction of the chemokine MCP-2 was completely abrogated, and OPN was significantly attenuated in clp treated mice (Fig 4A). In contrast, IL-6 and MCP-1 mRNA levels were unaffected in macrophage-depleted mice compared to controls (Fig. 4A). Consistent with the qRT-PCR data, H&E and MAC3 staining of left ventricular tissue from these mice revealed a substantial reduction in the
number of inflammatory cells in clp treated ACS mice (Fig. 4B). In line with the IL-6 expression data, clp depletion did not reduce the increase in phospho-STAT3 observed in ACS mice (Fig 4C). In addition, macrophage depletion did not alter myocardial TAG accumulation in ACS mice (Fig. 4D). Thus, treatment of ACS mice with clp dramatically reduces macrophage infiltration and selectively modulates the cardiac inflammatory profile. Together these findings suggest a model in which macrophages act downstream of myocardial lipid accumulation.

**Depletion of Macrophages Attenuates the Cardiomyopathy in MHC-ACS mice**

To determine the effects of macrophage depletion on the development of lipotoxic cardiomyopathy, echocardiograms were performed on 8-week-old ACS or NTG mice that had been injected with clp or veh as described above. Clp had no effect on baseline cardiac function in NTG mice (Fig 5). Interestingly, ACS mice treated veh or clp had a similar degree of cardiac hypertrophy as assessed by LV mass index (LVMI, Fig. 5A). However, clp-treated ACS mice had a significant improvement in systolic LV fractional shortening (FS) compared to veh-treated ACS mice (Fig. 5B). Moreover, both end-systolic and end-diastolic diameters were reduced in ACS mice treated with clp vs. veh (Table 2). These findings demonstrate that in the face of myocyte lipid stress, macrophage depletion attenuates adverse LV remodeling.
Discussion

Diabetes is a major risk factor for the development of heart failure through mechanisms involving both direct myocardial toxicity and accelerated atherosclerosis. A key feature of the diabetic heart is the accumulation of myocardial lipid, which is thought to contribute to myocyte dysfunction and cell loss (15, 20, 23, 25). However, the mechanisms involved in cardiac lipotoxicity in this disease are unresolved. We hypothesized that activation of inflammatory pathways in the setting of lipid-stressed cardiomyocytes would accelerate progression of cardiac dysfunction. To address this question, we turned to the ACS transgenic model of cardiac lipotoxicity. By 3-weeks of age, these mice reproducibly develop cardiac myocyte lipid overload which leads to systolic heart failure by 8-weeks of age. Using this model, we demonstrated that inflammatory cytokine expression is increased in the hearts of ACS mice prior to the onset of cardiomyopathy. Moreover, this inflammatory response is accompanied by macrophage infiltration of the myocardium. Depletion of macrophages with liposomal clodroplip reduced the expression of several inflammatory cytokines, decreased leukocyte infiltration, and attenuated the progression of LV dysfunction, independent of effects on cardiac lipid accumulation. Together these data are consistent with a model in which myocardial lipid accumulation leads to recruitment of macrophages in the heart. Moreover, the deleterious effects of lipid overload in the heart are dependent, in part, on this lipid-induced inflammatory response.

Evidence is emerging that inflammation is important in the pathogenesis of metabolic disease (10). It is now well established that macrophage recruitment to adipose tissue in the setting of obesity is a key event in the development of insulin resistance (24).
In particular, CD11c-expressing macrophages have been shown to be critical for the metabolic effects of high fat diet (21). Interestingly, ACS mice have significant macrophage accumulation in the myocardium by 4-weeks of age, well before the onset of cardiomyopathy. Because the perturbation of lipid metabolism in ACS mice is cardiac-selective, and because the ACS mice have normal systemic glucose and lipid metabolism, the recruitment of macrophages is unlikely to be related to changes in systemic metabolism. Using qRT-PCR and flow cytometry to analyze the infiltrating leukocytes, we demonstrated a significant enrichment of F4/80$^+$ cells and CD11c$^+$ cells. However, only ~15% of F4/80$^+$ cells co-expressed CD11c. Of note, there was also large population of CD11b$^+$, F4/80$^-$ cells in ACS hearts most likely representing monocytes. While the source of the signal(s) leading to macrophage recruitment to the hearts of ACS mice is not yet clear, several macrophage chemokines are significantly unregulated in ACS heart tissue. MCP-1 is an attractive candidate, as its expression is upregulated in ACS mice and not impacted by the depletion of macrophages. This suggests that a resident, non-macrophage cell type is responsible for its production and potentially is the initial trigger for macrophage infiltration. It is also possible that myocyte cell death or free fatty acids themselves contribute to the influx of macrophages (14). Future studies will be required to identify the cellular source of MCP-1 and other macrophage chemoattractants in this and other models of lipotoxic cardiomyopathy.

At present, the mechanism by which macrophages accelerate the pathogenesis of cardiomyopathy is not clear; however, it is most likely related to paracrine factors released by these inflammatory cells. Macrophages are often subdivided into either M1 (inflammatory) or M2 (reparative) subsets based in part on the expression or secretion of
distinct molecules (3). M1 cells express pro-inflammatory genes such as TNF, iNOS, and IL-6, whereas M2 cells are characterized by expression of L-arginase, Chi3, and IL-10. Interestingly, the cytokine profile in ACS hearts does not clearly implicate either of these phenotypes, although M2 markers did trend higher in ACS mice. Thus, despite the established relationship between classical M1 inflammatory cytokines and LV dysfunction due to TNFα and iNOS, heart failure in this model of lipotoxic cardiomyopathy may involve distinct inflammatory signals. Our cell depletion experiments show that this inflammatory response in the myocardium of ACS mice contributes significantly to adverse myocardial remodeling. The observation that cardiac function is not entirely restored to baseline following macrophage depletion may be consistent with residual myocardial inflammation (e.g., CD68, CD11c, and osteopontin are decreased but not restored to baseline) and/or more direct toxic effects of lipid accumulation, which is not affected by treatment with liposomal clodropl.

Within the unique cytokine profile observed in ACS mice, OPN, a molecule well known to contribute to macrophage recruitment and function, was profoundly upregulated. Previous studies have shown that expression of OPN is increased in diabetes, where it plays a key role in high fat diet-induced macrophage recruitment to adipose tissue and the induction of insulin resistance (18). Moreover, OPN is upregulated in many forms of heart failure in which both gain- and loss-of-function experiments have shown that it plays a pathologic role in cardiac dysfunction (22, 27, 31). Of note, the expression of OPN was decreased by ~ 70% in ACS mice treated with clodropl, suggesting that it may be an important signaling molecule from macrophages
that contributes to heart failure progression in this model. Further investigation of OPN as a pathophysiological mediator of cardiac lipotoxicity is ongoing.

The ACS transgenic model of lipotoxic cardiomyopathy provided an ideal setting in which to evaluate the contributions of inflammation to metabolic stress in the heart. Consistent with our findings in these mice, non-transgenic models of lipid overload using long-term high fat diet or streptozotocin treatment have also demonstrated increased numbers of macrophages within the hearts of insulin resistant and/or diabetic mice (13, 30). However, assessing the influence of cardiac macrophages on LV function using cell depletion strategies with these models is confounded by the concomitant role of macrophages in promoting the primary dysregulation of systemic metabolism, which is further modified by macrophage depletion (16, 21). The ACS model, in which myocardial lipid accumulation is independent of systemic metabolic perturbations, enabled us to investigate the contribution of macrophages to adverse cardiac remodeling in the setting of metabolic stress. On the other hand, the rapid time course of cardiomyopathy in ACS mice at a relatively young age precluded the use of bone marrow and adoptive transfer experiments to further manipulate the myeloid compartment in this model. Future genetic studies will be required to extend our findings from the ACS model to investigate cardiac myocyte-macrophage interactions in the setting of systemic lipid overload and diabetes.

The present study supports a model in which macrophages can influence the response of the heart to lipid-induced injury, contributing to rapid progression of cardiac dysfunction. This model is supported by the observations that macrophages infiltrate the heart prior to the onset of cardiomyopathy and their depletion attenuates cardiac
dysfunction and remodeling. As such, further research investigating the mechanisms of macrophage recruitment to and activation in the myocardium has the potential to yield novel insight towards the pathogenesis of diabetic cardiac disease.
Grants

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Disclosures

None

Author Contributions

J Schilling - primary researcher
H Machkovech – secondary researcher
A Kim - assistant researcher on flow cytometry experiments
R Schwendener – provided clodrolip reagent and significant technical assistance with macrophage depletion
J Schaffer – principal investigator
References


Figure Legends

Figure 1. Inflammatory cytokines are induced in lipotoxic cardiomyopathy. (A) Transthoracic echocardiograms were performed in lightly anesthetized MHC-ACS (ACS) and non-transgenic (NTG) littermates at 4 and 8 weeks of age. The bars represent the mean fractional shortening. (B) Hearts from 4-week-old ACS and NTG littermates were harvested and qRT-PCR was performed on total mRNA to assess the expression of inflammatory cytokines. Graphs display mean expression relative to 36B4 ± standard error (SE) for n=3-4 per group. * p < 0.05 and ** p < 0.005 for ACS vs. NTG; ns, not significant. (C) Total protein was isolated from the hearts of 4-week-old NTG or ACS mice. Phosphorylated and total STAT3 levels were determined by western blotting.

Figure 2. Macrophages are recruited to the myocardium in lipotoxic cardiomyopathy. (A) Hearts from 4-week-old ACS and NTG littermates were harvested and qRT-PCR was performed on total mRNA to assess the expression of macrophage markers. Graphs show mean expression relative to 36B4 ± SE for n=3-4 per group. (B) Formaldehyde-fixed, paraffin-embedded heart tissue from 4 week old NTG and ACS mice was stained for the macrophage marker, MAC3 with detection using immunoperoxidase. Representative images are shown in low power (upper panels) and high power (lower panels). Bars, 100 µm. (C) Myocardial triglyceride (TAG) levels were determined in ACS and NTG littermates at 3 and 4 weeks of age. Each dot represents an individual mouse with the mean shown for n=4 per group. (D) CD68 mRNA expression
in heart tissue was determined by qRT-PCR from 3 and 4 week old ACS and NTG mice. The graph shows mean expression relative to 36B4 ± SE for n=3-4 per group.

*, p < 0.05 and **, p < 0.005 for ACS vs. NTG.

Figure 3. F4/80 and CD11c expressing cells accumulate in the myocardium of MHC-ACS mice. (A) Hearts from 4-week-old NTG and ACS mice were digested to prepare single cell suspensions, stained with fluorochrome-coupled antibodies, and analyzed by flow cytometry. CD45+ cells (leukocytes) were gated (far left) and further analyzed for the expression of CD11b, CD11c, and F4/80. Representative flow plots are shown with the percentage of cells in each quadrant as indicated. (B) Quantification of CD11c+ and F4/80+ myeloid cells in NTG VS. ACS hearts. Graphs display mean ± SE for n=3 mice per group. * p < 0.05 and ** p < 0.005 for ACS vs. NTG.

Figure 4. Macrophage depletion reduces leukocyte influx and modulates the cytokine response in MHC-ACS mice. ACS mice were injected with clodrolip (clp, red bars) or vehicle (veh, black bars) beginning at 22 days of age and continuing every four days until heart tissue was harvested at 4 weeks of age. (A) mRNA was isolated from heart tissue and expression of leukocyte markers and cytokines (relative to 36B4) was determined using qRT-CPR and compared to veh-treated NTG mice (white bars). Graphs display mean expression ± SE for n=4 per group. (B) Formaldehyde-fixed, paraffin embedded heart tissue from veh-ACS and clp-ACS mice was stained by H&E (left panels) or for MAC3 (right panels). Representative images are shown. Bars, 100 µm. (C) Total and phospho-STAT3 levels were determined by western blotting using
myocardial protein extracts from 4 week old ACS and NTG mice treated with clp or veh.

(D) Myocardial TAG content was quantified in clp or veh–treated ACS and NTG mice at 4 weeks of age. * p < 0.05 and ** p < 0.005 for ACS (veh or clp) vs. NTG, and # p < 0.05 and ## p < 0.005 for ACS-veh vs. ACS-clp.

Figure 5. Macrophage depletion improves cardiac function in MHC-ACS mice.

NTG (filled circles) and ACS mice (open inverted triangles) were treated with veh or clp every 4 days, beginning at 22 days of age and continuing until 8 weeks of age at which time cardiac function was assessed by transthoracic echocardiography. (A) Left ventricular mass index (LVMI) and (B) left ventricular fractional shortening are shown for each of the treatment groups. Each symbol represents one mouse and bar indicates mean for each group. * p < 0.05 and ** p < 0.005 for comparisons indicated (n=3 to 8 per group).
Figure 1

A

Fractional shortening (%)

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mRNA expression (fold induction)

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C

NTG     ACS

STAT3-P

STAT3-total
Figure 2

A

mRNA expression (fold induction)

CD68  **  **
F4/80  **  **
CD11c  **  **
MCP-2

mRNA expression (fold induction)

OPN  *
Arg  ns
CHI313  ns
IL-10  ns

B

NTG  ACS

CD68  **  **
F4/80  **  **
CD11c  **  **
MCP-2

mRNA expression (fold induction)

OPN  *
Arg  ns
CHI313  ns
IL-10  ns

C

Myocardial TAG (µg/mg tissue)

3 wk  4 wk

D

mRNA expression (fold induction)

CD68  **  **
Figure 3

A

NTG

ACS

B

CD11b+ CD11c+

CD11b+ F4/80+
Figure 4

A

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Myocardial TAG (µg/mg tissue)

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Figure 5

A

B

NTG
ACS

venh clp veh clp

NTG
ACS

fractional shortening (%)

0 20 40 60 80

0 2 4 6 8

ns

**

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*
Table 1. Inflammatory Markers in MHC-ACS mice at 4 weeks of age

<table>
<thead>
<tr>
<th>cytokine/chemokine</th>
<th>induction</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCL2/MCP-1</td>
<td>3.0</td>
<td>0.027</td>
</tr>
<tr>
<td>CCL3/MIP-1α</td>
<td>4.8</td>
<td>0.020</td>
</tr>
<tr>
<td>CCL6</td>
<td>3.1</td>
<td>0.007</td>
</tr>
<tr>
<td>CCL7/MCP-3</td>
<td>3.1</td>
<td>0.006</td>
</tr>
<tr>
<td>CCL8/MCP-2</td>
<td>47.6</td>
<td>0.0002</td>
</tr>
<tr>
<td>CCL12/MCP-5</td>
<td>3.4</td>
<td>0.022</td>
</tr>
<tr>
<td>CCR3</td>
<td>5.1</td>
<td>0.045</td>
</tr>
<tr>
<td>CCR5</td>
<td>3.6</td>
<td>0.01</td>
</tr>
<tr>
<td>CXCL5</td>
<td>5.9</td>
<td>0.01</td>
</tr>
<tr>
<td>SPP1/OPN</td>
<td>628</td>
<td>0.006</td>
</tr>
</tbody>
</table>

Induction reflects expression in ACS relative to NTG mice. N = 3 animals/group
Table 2. Echocardiographic measurements in NTG and ACS mice following veh or clp

<table>
<thead>
<tr>
<th></th>
<th>NTG (veh)</th>
<th>NTG (clp)</th>
<th>ACS (veh)</th>
<th>ACS (clp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HR, beats/min</td>
<td>587±13</td>
<td>621±19</td>
<td>506±11†</td>
<td>517±5†</td>
</tr>
<tr>
<td>LVEDD (mm)</td>
<td>2.91±.12</td>
<td>2.89±.05</td>
<td>3.75±.06†</td>
<td>3.46±.10†</td>
</tr>
<tr>
<td>LVESD (mm)</td>
<td>1.28±.17</td>
<td>1.43±.20</td>
<td>2.55±.07†</td>
<td>1.94±.08†</td>
</tr>
<tr>
<td>FS (%)</td>
<td>56.3±5.2</td>
<td>57.9±3.1</td>
<td>31.5±2.1†</td>
<td>42.4±1.5†</td>
</tr>
<tr>
<td>LPWT (mm)</td>
<td>0.84±.02</td>
<td>0.88±.01</td>
<td>0.91±.03</td>
<td>0.93±.03</td>
</tr>
<tr>
<td>LVMI (mg/weight)</td>
<td>3.82±.12</td>
<td>3.72±.22</td>
<td>5.89±.19†</td>
<td>5.79±.18†</td>
</tr>
</tbody>
</table>

HR (heart rate); LVEDD (left ventricular end-diastolic diameter); LVESD (left ventricular end-systolic diameter); FS (fractional shortening); LPWT (left ventricular posterior wall thickness); LVMI (left ventricular mass index); † p < 0.05 NTG vs. ACS.