Dilated cardiomyopathy is the most common cause of heart failure in young patients and often results from enteroviral myocarditis.\textsuperscript{1,2} Many patients show heart-specific autoantibodies\textsuperscript{2,3} and/or upregulation of activation markers on heart-infiltrating cells.\textsuperscript{4} Interestingly, patients with heart-specific autoantibodies but no evidence for viral genome persistence in heart biopsy samples show improvement of their cardiac function on immunosuppression.\textsuperscript{5} Therefore, it is reasonable to conclude that autoimmunity is involved in the pathogenesis of postinflammatory cardiomyopathy (for review, see Eriksson and Penninger\textsuperscript{6} and Fairweather et al\textsuperscript{7}).

Animal models greatly advanced our knowledge of the pathogenesis of myocarditis and inflammatory cardiomyopathy. In susceptible mice, for example, infection with enteroviruses results in a biphasic myocarditis, with an early acute stage 5 to 8 days after inoculation, followed by a chronic stage of low-grade inflammation.\textsuperscript{8} Interestingly, T cells from mice with enteroviral myocarditis transfer disease in syngeneic severe combined immunodeficiency (SCID) recipients lacking B and T cells, which suggests a crucial role for autoreactive T cells in disease pathogenesis.\textsuperscript{9} Furthermore, immunization of susceptible mice with \(\alpha\)-myosin–derived peptides (MyHC-\(\alpha\)) together with complete Freund’s adjuvant (CFA) results in CD4\textsuperscript{+} T-cell–mediated experimental autoimmune myocarditis.\textsuperscript{6,7,10}

Toll-like receptors (TLRs) belong to the Toll–interleukin-1 (IL-1) receptor superfamily of conserved surface molecules that trigger innate mechanisms of immunity on stimulation with microbial products or endogenous danger signals.\textsuperscript{11,12} In the context of inflammatory heart disease, it has been shown that TLR4 is expressed together with enteroviral replication in hearts from patients with dilated cardiomyopathy.\textsuperscript{13} In addition, mice lacking TLR4 develop markedly reduced myocarditis after infection with enteroviruses such as coxsackievirus B3 (CVB3).\textsuperscript{14} In the experimental autoimmune myocarditis model, activation of TLRs on self-antigen–presenting dendritic cells (DCs) is essential for the induction of myocarditis and heart failure.\textsuperscript{15} Furthermore, IL-1 type 1 receptor signaling on DCs is critical for autoimmune myocarditis development.\textsuperscript{16} During myocarditis development, recruitment of activated bone marrow–derived DCs precedes...
the accumulation of macrophages and T cells in the heart.\textsuperscript{17–19} Accordingly, disease induction by adoptive transfer of heart-specific CD4\textsuperscript{+} T cells requires pretreatment of recipient mice with lipopolysaccharide (LPS) and other strong TLR stimulants that upregulate myosin heavy chain class II expression on heart-resident DCs.\textsuperscript{18}

MyD88 is an essential adaptor molecule that mediates complex proinflammatory pathways that involve a cascade of kinases integrating both TLR4 and IL-1 receptor type 1 activation.\textsuperscript{12} Its role in autoimmune heart disease is not known yet. To assess the role of MyD88 in the expansion of heart-specific CD4\textsuperscript{+} T cells and the development of autoimmune heart disease, we assessed the myocarditis susceptibility of mice that genetically lack MyD88. Here, we describe that MyD88 is required for myocarditis induction after MyHC-\textalpha-/CFA immunization. More specifically, we found that MyD88 signaling in DCs is essential to prime heart-specific CD4\textsuperscript{+} T cells in vivo.

**Methods**

**Mice**

MyD88\textsuperscript{−/−} mice were generated as described\textsuperscript{20} and backcrossed for >9 generations on an H-2\textsuperscript{k} (BALB/c) background. Wild-type or heterozygous littersmates were used as controls. Experiments were performed in accordance with Swiss federal legislation or Austrian law and had been approved by the local authorities.

**Myocarditis Induction**

Mice were injected subcutaneously with 100 \(\mu\)g/mouse of the murine \(\alpha\)-myosin-heavy chain peptide (MyHC-\textalpha/-Ac-SKLMATLFSYASADF-OH) emulsified 1:1 with CFA on days 0 and 7.\textsuperscript{10,21} For myocarditis induction with DCs, immature DCs were pulsed with MyHC-\textalpha and activated for 2 hours with 0.1 \(\mu\)g/mL LPS and 5 \(\mu\)g/mL anti-CD40 before intraperitoneal injection of 250,000 DCs per mouse 3 times every second day. For adoptive transfer, we injected intraperitoneally 10\textsuperscript{4} MyHC-\textalpha–specific in vitro restimulated CD4\textsuperscript{+} T cells per mouse. Depending on the experiment, mice were euthanized after 10, 14, 21, or 28 days.

**Histopathology**

Myocarditis was scored with grades from 0 to 4 [0 = no inflammatory infiltrates; 1 = small foci of inflammatory cells between myocytes; 2 = larger foci of >100 inflammatory cells; 3 = more than 10\% of a cross section involved; and 4 = more than 30\% of a cross section involved].\textsuperscript{15,16}

**Dendritic Cells**

For immunization experiments, bone marrow–derived DCs were generated as described previously.\textsuperscript{15} Naive, primary DCs were obtained from lymph nodes and spleens of immununized mice. Alternatively, we isolated DCs from draining lymph nodes 24 hours after MyHC-\textalpha/CFA immunization using magnetic beads (MACS DC isolation kit, Miltenyi Biotech GmbH) and cell sorting. For analysis of surface molecules, DCs were preincubated for 30 minutes at 4\° with 1\% normal mouse serum in staining buffer (Pharmingen) before staining with the appropriate fluorochrome-labeled antibodies from Pharmingen. Viable cells were assessed in fluorescent-activated cell sorter (FACS) scatterplots by gating on propidium iodine–negative populations. For cytokine analysis, primary DCs were plated at 1 \(\times\) 10\textsuperscript{5}/mL in 96-well plates and activated with LPS at 0.1 \(\mu\)g/mL.

**Cytokine Analysis**

Cytokine levels were measured in culture supernatants with commercially available Quantikine ELISA kits (R&D Biosystems).

**Autoantibodies**

We assessed antibody responses against whole \(\alpha\)-myosin with an ELISA as described previously,\textsuperscript{13} using alkaline phosphatase–labeled goat anti-mouse IgG subclass antibodies (Southern Biotechnology Associates). Antibody titers were determined at half-maximum OD\textsubscript{450}.

**CD4\textsuperscript{+} T-Cell Proliferation**

CD4\textsuperscript{+} T cells were isolated with magnetic beads (CD4\textsuperscript{+} T-cell isolation kit; Miltenyi Biotech GmbH) and cultured in 96-well plates for 72 hours on irradiated (2000 rad) syngeneic splenocytes, with 0.01 to 10 \(\mu\)g/mL of MyHC-\textalpha peptide in RPMI 1640 (Gibco) medium supplemented with 1\% of normal mouse serum. Naive CD4\textsuperscript{+} CD62L\textsuperscript{+} T cells were stimulated in 96-well plates with either 5 \(\mu\)g/mL soluble anti-CD3\textalpha antibody, 50 ng/mL phorbol myristate acetate (PMA) and 500 ng/mL ionomycin, 1 \(\mu\)g/mL concanavalin A (Con A), or 1 \(\mu\)g/mL Con A in the presence of irradiated wild-type DCs.\textsuperscript{18} Alternatively, naive CD4\textsuperscript{+} CD62L\textsuperscript{+} T cells were stimulated with 1 mg/mL Con A on either MyD88\textsuperscript{−/−} or MyD88\textsuperscript{+/+} DCs in the presence or absence of recombinant murine tumor necrosis factor (TNF)-\textalpha (PeproTech) or a TNF-\textalpha–blocking antibody. Proliferation was assessed by measuring [\(\text{H}\)]-methyl-thymidine incorporation. All reagents and media were endotoxin free.

**Statistical Analysis**

Dichotomous data were analyzed by Fisher’s exact test. The Mann-Whitney U test was used for evaluation of severity scores. Proliferation responses and cytokine levels were compared with ANOVA and the t test.

**Results**

**Myocarditis Induction Requires MyD88 Signaling**

To define the role of MyD88 signaling in autoimmune myocarditis, we first compared myocarditis susceptibility of mice lacking MyD88, heterozygous MyD88\textsuperscript{−/+} mice, and MyD88\textsuperscript{−/−} littersmates. As illustrated in Figure 1A (right panel) and Figure 1C and summarized in the Table, both wild-type littersmates and MyD88\textsuperscript{−/+} mice developed severe myocarditis with inflammatory infiltrates that contained granulocytes, eosinophils, and mononuclear cells including macrophages and lymphocytes after 2 immunizations with MyHC-\textalpha/CFA. In contrast, MyD88\textsuperscript{−/−} mice were protected from disease and developed only minimal pericardial calcifications (Figure 1A, left panels; Figure 1C; Table). Differences in disease susceptibility between MyD88\textsuperscript{−/−}, MyD88\textsuperscript{+/−}, and MyD88\textsuperscript{−−} mice were consistently observed at different time points, ie, 14, 21, or 28 days after immunization (data not shown). Therefore, the adaptor molecule MyD88 is crucial for the induction of autoimmune myocarditis after immunization with cardiac self-antigen together with CFA.

**Impaired Expansion of Heart-Specific CD4\textsuperscript{+} T Cells in MyD88\textsuperscript{−/−} Mice**

Autoimmune myocarditis is a CD4\textsuperscript{+} T-cell–mediated disease.\textsuperscript{7,15} In mice lacking MyD88, disease resistance paralleled impaired expansion of heart-specific T cells, as suggested by the impaired in vitro proliferation of MyHC-\textalpha restimulated whole splenocytes (Figure 2A) and more specifically by the absence of in vitro proliferation of MyD88\textsuperscript{−/−} but not MyD88\textsuperscript{+/+} CD4\textsuperscript{+} T cells restimulated with MyHC-\textalpha pulsed irradiated splenocytes (Figure 2B). Accordingly, the production of interferon (IFN)-\gamma was reduced in supernatants of in
MyD88 signaling impairs both the expansion of heart-specific CD4+ T cells and the generation of heart-specific humoral autoimmunity. In conclusion, these findings strongly suggest impaired CD4+ T helper cell function in MyD88−/− mice after immunization with cardiac self-antigen.

**MyD88 Is Not Intrinsically Required for CD4+ T-Cell Activation**

Next, we asked whether MyD88 signaling is intrinsically required for CD4+ T-cell activation. We isolated naïve CD62L− CD4+ T cells from MyD88+/+ and MyD88−/− mice and compared their primary responses on various stimuli. As illustrated in Figure 3, MyD88+/+ and MyD88−/− CD4+ T cells did not differ in their capacity to proliferate on stimulation with anti-CD3ε or PMA/ionomycin. Furthermore, there was no difference in the primary responses between MyD88+/+ and MyD88−/− CD4+ T cells on stimulation with Con A in the presence of irradiated wild-type antigen-presenting cells. In conclusion, we found no in vitro evidence for impaired proliferation of MyD88−/− CD4+ T cells on T-cell receptor stimulation (anti-CD3ε), intrinsic activation (PMA/ionomycin), or antigen-presenting cell-dependent indirect activation (Con A and wild-type DCs) that would explain their impaired expansion in MyD88−/− mice.

**Heart-Specific MyD88+/+ CD4+ T Cells Transfer Myocarditis in MyD88−/− Recipients**

Both IL-1 receptor type 1 and TLRs that share the common adaptor molecule MyD88 are expressed in various tissues, including leukocytes, endothelial cells, and cardiomyocytes. Therefore, the absence of MyD88 might affect cardiac inflammation on many levels. Accordingly, we first asked whether heart-specific CD4+ T cells and other inflammatory cells have any access to the heart in MyD88−/− mice. To address this question, we first created a highly autoreactive and heart-specific CD4+ T-cell line. CD4+ T cells were isolated from immunized MyD88+/+ mice and restimulated several times in vitro with MyHC-α pulsed irradiated splenocytes. A prolonged resting phase followed each restimulation. The resulting CD4+ T-cell line was MyHC-α specific and produced high levels of IFN-γ on restimulation (Figures 4A and 4B). Adoptive transfer of 10⁷ activated, heart-specific CD4+ T cells per mouse induced myocarditis of similar prevalence, albeit slightly reduced severity, in both MyD88+/+ and MyD88−/− mice (Table). These findings suggest that MyD88 signaling is not decisive for the recruitment of autoreactive CD4+ T cells to the heart during myocarditis development.

**MyD88−/− DCs Failed to Prime Wild-Type CD4+ T-Cell Responses**

So far, the present data suggested functionally intact CD4+ T cells in MyD88−/− mice and exclude a relevant role for MyD88 signaling in the recruitment of CD4+ T cells to the heart. We therefore hypothesized that disease resistance of MyD88−/− mice most likely results from the impaired capacity of antigen-presenting cells to prime and expand autoreactive CD4+ T cells in the peripheral compartments in vivo, and we consequently assessed the functionality of MyD88−/− DCs to promote primary T-cell responses. To overcome the...
cytokines after maturation. Their impaired production in DCs fail to produce relevant amounts of proinflammatory mechanisms of antigen processing and presentation, we compared Con A–induced proliferation of naïve wild-type CD4+ T cells in the presence of either MyD88+/− or MyD88−/− DCs. In fact, primary Con A–mediated CD4+ T-cell responses were significantly impaired in the presence of MyD88−/− compared with MyD88+/+ DCs (Figure 5). The impaired functional capacity of MyD88−/− DCs in peripheral lymph nodes, however, did not result from a defect in upregulation of essential costimulatory CD40, CD80, CD86, or myosin heavy chain class II molecules, because we found no difference in the expression of these surface molecules on DCs isolated from draining lymph nodes after immunization (Figure 6A). In contrast, it is well established that MyD88−/− DCs fail to produce relevant amounts of proinflammatory cytokines after maturation. Their impaired production in MyD88−/− mice might contribute to the disease resistance on many levels, including priming, expansion, and maintenance of autoreactive T cells, as well as suppression of regulatory T cells. Given that a very short activation period with TLR stimulants is sufficient to render MyHC−/− cells. Given the central role of TNF-α in autoimmune myocarditis induction, we hypothesized that the absence of the early peak release of TNF-α from MyD88−/− DCs might play an important role in the reduced capacity of MyD88−/− DCs to prime naïve CD4+ T cells. Indeed, in vitro blocking of TNF-α markedly reduced Con A–mediated proliferative responses of naïve wild-type CD4+ T cells in the presence of MyD88−/− DCs to the levels observed in the presence of MyD88+/+ DCs (Figure 6C). On the other hand, addition of recombinant mouse TNF-α restored Con A–induced proliferative responses of naïve CD4+ T cells in the presence of MyD88−/− DCs (Figure 6C). Taken together, these data indicate that MyD88−/− primary DCs are defective in their capacity to prime naïve T-cell responses. Impaired early TNF-α release by DCs lacking MyD88 might explain impaired T-cell priming.

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Figure 2. Impaired CD4+ T-cell expansion and autoantibody production in MyD88−/− mice. A, Impaired proliferation of in vitro MyHC−/− restimulated splenocytes from MyD88−/− mice (△) compared with MyD88+/− mice (▲) 21 days after immunization with MyHC−/− and CFA. Proliferation was assessed by measurement of 3H-thymidine incorporation. Mean±SD of cpm values from 4 to 5 individual mice are shown. B, Impaired proliferation of in vitro MyHC−/− restimulated CD4+ T cells from MyD88−/− and MyD88+/+ mice. CD4+ T cells were isolated 21 days after immunization and restimulated with irradiated syngeneic splenocytes. Mean±SD of cpm values from 5 individual mice are shown. C, Mean±SD of IFN-γ and IL-4 in culture supernatants of MyHC−/− restimulated whole splenocytes from MyHC−/−/CFA-immunized MyD88−/− (solid bars) vs MyD88+/+ (open bars) mice. Each bar represents data from 5 individual mice. D, Impaired humoral IgG subclass responses to whole myosin of MyHC−/−/CFA-immunized MyD88−/− (△) versus MyD88+/+ (▲) mice.
MyHC-α–Loaded Activated DCs Restore Myocarditis in MyD88−/− Mice

If myocarditis resistance of MyD88−/− mice indeed results from the reduced priming capacity of MyD88−/− antigen-presenting DCs in the peripheral compartments, administration of activated MyHC-α–loaded MyD88+/+ antigen-presenting cells would restore the CD4+ T-cell expansion and myocarditis susceptibility of MyD88−/− mice. To test this hypothesis, we injected groups of MyD88−/− and MyD88+/+ mice with mature, CD11c+CD11b+CD8α−, MyHC-α-loaded MyD88+/+ DCs and compared disease susceptibility and the expansion of autoreactive CD4+ T cells in MyD88+/+ versus MyD88−/− mice. Indeed, CD4+ T cells isolated from both MyD88−/− and MyD88+/+ mice after DC immunization resulted in comparable, albeit only mild, myopericarditis after adoptive transfer (Figure I in the Data Supplement). Most importantly, however, both MyD88−/− and MyD88+/+ mice developed severe myocarditis of the same prevalence and severity after injection of activated, self-antigen–loaded MyD88+/+ DCs (Figures 1B and 1C and the Table). We therefore conclude that autoimmune myocarditis resistance of MyD88−/− mice indeed results from a priming defect on the level of antigen-presenting cells. Furthermore, our findings prove that in the presence of activated and competent MyD88+/+ DCs, MyD88 deficiency does not affect the functional capacity of the lymphatic system to provide an environment that allows the generation of autoreactive T cells and autoimmune heart disease.

Discussion

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

In our experiments, we observed upregulation of costimulatory molecules on lymph node–derived DCs after CFA/MyHC immunization in both MyD88−/− and MyD88+/+ mice. These findings contrast with the fact that the in vivo upregulation of costimulatory molecules is impaired in MyD88-deficient pulmonary DCs after TLR activation.22 Obviously, dependent on their mode of generation in vitro or resident location in vivo, DCs have different capacities to engage MyD88-dependent and -independent pathways in response to activation.22 On the other hand, lymph node–derived DCs of MyD88−/− mice showed markedly reduced production of proinflammatory cytokines, such as TNF-α or IL-12, after TLR stimulation. However, even in the absence of exogenous TLR stimulants, we found an impaired capacity of MyD88−/−...
DCs to promote Con A–induced primary T-cell responses. This defect mainly resulted from a lack of TNF-α production in MyD88−/− DCs and might reflect the fact that Con A directly mediates MyD88-dependent proinflammatory pathways. Otherwise, the priming defect of MyD88−/− DCs might result from impaired IL-1β–mediated autocrine/paracrine activation of DCs, because IL-1 type 1 receptor signaling involves MyD88-dependent proinflammatory cascades. It has recently been shown that IL-1 receptor type 1–deficient mice are protected from autoimmune myocarditis.16 The impaired capacity of MyD88-deficient DCs to release specific cytokines certainly contributes to impaired T-cell priming and disease resistance in MyD88−/− mice: TNF-α, IL-12p40, and IL-6, for example, are all essential for autoimmune myocarditis development.21,23,24

Microbial products such as LPS acting on TLR4, CFA predominantly activating TLR2 and TLR4, or endogenous danger signals25,26 are critical for the capacity of antigen-pres-
renting cells to build up effective T-cell responses and to suppress regulatory T cells.27,28 Autoimmunity develops if TLR activation coincides with release and uptake of self-antigen by DCs is supposed to result in tolerogenic rather than autoaggressive T-cell responses.29–31 On the basis of our data, we cannot entirely exclude that the presence of tolerogenic T-cell populations in MyD88−/− mice contributes to their myocarditis resistance. However, it was not possible to overcome disease resistance of MyD88−/− mice by depletion of CD25+ cells before immunization (RRM and UE, unpublished data).

Development of autoimmune myocarditis requires the recruitment of inflammatory cells to the heart.18,19 Interestingly, systemic activation of the innate immune system with TLR stimuli such as LPS results in upregulation of activation markers and myosin heavy chain class II molecules on heart-resident cells.19 Furthermore, LPS injection results in relapses of inflammatory infiltrates and more rapid progression of heart failure in immunized mice.5,15,32 In the context of CVB3-mediated myocarditis, treatment with both LPS33 and IL-1β34 enhances disease susceptibility of resistant mouse strains, most likely by activation of tissue-resident DCs. On the basis of these observations, one would expect that heart-resident antigen-presenting cells interact with autoreactive CD4+ T cells to promote their local expansion and the recruitment of other inflammatory cells, such as macrophages, B cells, and granulocytes.5 Given the fact that MyD88 is a crucial common adaptor molecule that mediates both TLR and IL-1 type 1 receptor activation,11,12 it was tempting to speculate that MyD88 signaling might also be essential for the recruitment and activation of heart-infiltrating cells. The present data, however, clearly show that MyD88 signaling is not required for the development of cardiac infiltrates in the presence of activated autoreactive T cells. In fact, adoptive transfer of activated autoreactive T cells induced myocarditis in both MyD88+/+ and MyD88−/− recipient mice. These findings argue for MyD88-independent mechanisms mediating the recruitment of inflammatory cells to the target organ in the presence of activated heart-specific T cells. Such mechanisms might include, for example, MyD88-independent TLR signaling pathways.35

Several studies suggested that the absence of MyD88 signaling on antigen-presenting cells promotes default Th2-mediated immune responses in the presence of innate activation.36 Therefore, the question arises whether a possible Th1 to Th2 shift in MyD88−/− mice might affect myocarditis susceptibility after MyHC-α/CFA immunization. In fact, the present data show markedly impaired production of the Th1 cytokine IFN-γ in MyD88−/− compared with MyD88+/+ CD4+ T cells. Production of the Th2 cytokine IL-4, however, was uniformly low in both MyD88+/+ and MyD88−/− CD4+ T cells, which suggests that the failure to produce IFN-γ reflects impaired expansion of heart-reactive CD4+ T cells rather than a relevant Th1 to Th2 shift in the MyD88−/− mice.

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

Acknowledgments

This study was supported by the Novartis Foundation and the Swiss Society for Internal Medicine. The authors gratefully thank Regine Landmann and Stephanie Goulet for critical reading of the manuscript. Dr Eriksson holds a Swiss National Foundation professorship for Internal Medicine and Critical Care Medicine.

Disclosures

None.

References


**CLINICAL PERSPECTIVE**

Dilated cardiomyopathy can result from myocarditis after viral and other infections. Many of the affected individuals develop heart antigen–specific autoantibody responses that may contribute to the progression of cardiomyopathy even after clearance of microbes. These observations suggest that the pathogenesis of dilated cardiomyopathy is promoted by postinfectious autoimmunity. Toll-like receptors (TLRs) are ubiquitously present on all cell surfaces and trigger innate mechanisms of immunity after stimulation by endogenous or exogenous signals. In particular, TLRs appear to contribute to the generation of an autoimmune response during viral myocarditis leading to dilated cardiomyopathy. The Toll-receptor adaptor MyD88 integrates both TLR and interleukin-1 receptor type 1 activation, which leads us to hypothesize that it may play a critical role in the development of cardiac inflammation. Using the mouse model of experimental autoimmune myocarditis, we found a crucial role for MyD88 in the priming of heart-specific autoreactive T cells. From a clinical point of view, these findings suggest that treatment strategies targeting MyD88 signaling might lead to the development of novel therapeutic strategies to prevent the development of heart-specific autoimmunity in the presence of TLR-stimulating signals.
MyD88 Signaling Controls Autoimmune Myocarditis Induction
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Circulation. 2006;113:258-265; originally published online January 9, 2006;
doi: 10.1161/CIRCULATIONAHA.105.564294
Circulation is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the
World Wide Web at:
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