Differential Macrophage Polarization in Male and Female BALB/c Mice Infected With Coxsackievirus B3 Defines Susceptibility to Viral Myocarditis

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Rational: Myocardial infiltrating macrophages play an important role in the pathogenesis of viral myocarditis in male BALB/c mice following coxsackievirus B3 (CVB3) infection. Interestingly, comparable macrophage numbers were observed in the myocardium of female mice during acute myocarditis.

Objective: Given CVB3 infection causes severe myocarditis in male but not female mice, we postulated that macrophages infiltrating the myocardium of female mice may display distinct functional properties that contribute to differential susceptibility to CVB3 myocarditis.

Methods and Results: Here, we found that myocardial infiltrating macrophages from CVB3-infected male mice expressed high levels of classically activated macrophages (M1) markers, including inducible nitric oxide synthase, interleukin-12, tumor necrosis factor-α, and CD16/32, whereas those of females showed enhanced expression of arginase 1, interleukin-10, macrophage mannose receptor (MMR) and macrophage galactose type C-type lectin (MGL) that were associated with alternatively activated macrophage (M2) phenotype. Moreover, distinct myocardial-derived cytokines were found to play a critical role in differential macrophage polarization between sexes after CVB3 infection. Adoptive transfer of ex vivo programmed M1 macrophages, as expectedly, significantly increased myocarditis in both male and female mice. Strikingly, transfer of M2 macrophages into susceptible male mice remarkably alleviated myocardial inflammation by modulating local cytokine profile and promoting peripheral regulatory T cell (Treg) differentiation.

Conclusions: Taken together, this study may facilitate the understanding of the mechanism underlying gender bias in susceptibility to CVB3 myocarditis and the development of therapeutic strategies based on macrophage polarization for inflammatory heart disease. (Circ Res. 2009;105:353-364.)

Key Words: coxsackievirus • myocarditis • macrophage polarization

Viral myocarditis is a principal cause of heart failure in young adults and often progresses to chronic myocarditis, dilated cardiomyopathy, and congestive heart failure. Coxsackievirus (CVB3) is believed to be the most common causative agent in human myocarditis, and the same virus strain induced similar inflammatory heart disease in genetically susceptible strains of mice. Previously, using the male BALB/c model of viral myocarditis, which develops acute myocarditis from days 7 to 14 postinfection, we reported that myocardial MCP-1 expression after CVB3 infection led to migration of mononuclear cells into the myocardium. Although CVB3-induced macrophages in the males had been considered to be T lymphocyte–mediated inflammatory heart disease, accumulating data demonstrated that F4/80+ macrophages represented the major inflammatory infiltrates and played a pathogenic role in the development of acute viral myocarditis, because the severity of myocardial inflammation correlated with the intensity of the macrophage infiltration and macrophage-depleted mice failed to develop viral myocarditis. However, substantial macrophage infiltration is not always indicative of severe myocarditis. Huber et al and Frisano-Kiss et al observed an obvious macrophage infiltrate after CVB3 infection in female BALB/c mice which are less susceptible to viral myocarditis. This highlights the heterogeneity of macrophages.

Macrophages are highly versatile, as local environment factors shape their phenotypic and functional properties. Operationally, 2 distinct activation states have been defined for macrophages: the M1 (or classically activated) and M2 (or alternatively activated) macrophages, each being associated with particular phenotypes and functions. M1 macrophages, induced by lipopolysaccharide (LPS) and interferon (IFN)-γ, typically produce copious amounts of proinflammatory cytokines (tumor necrosis factor [TNF]-α, interleukin...
Academy of Sciences. Green fluorescent protein (GFP) transgenic mice of BALB/c background were obtained from The Jackson Laboratory. All animal experiments were performed according to the guidelines for the Care and Use of Laboratory Animals (Ministry of Health, PR China, 1998) and the guidelines of the Laboratory Animal Ethical Commission of Fudan University.

**Methods**

**Mice**

Male and female BALB/c (H-2^b^) mice, 6 weeks of age, were purchased from Shanghai Experimental Animal Centre of Chinese Academy of Sciences.
analysis of iNOS was performed as previously described.23 Regulatory T cells (Tregs) were measured using phycoerythrin (PE) anti-Foxp3 staining set (eBioscience) according to the protocols of the manufacturer.

In Vivo Neutralization of Cytokines
The rat monoclonal antibodies anti-mIFN-γ, anti-mIL-4, anti-mIL-13, anti-mIL-4 plus anti-mIL-13, or rat IgG (R&D Systems) were injected IP (2.5 mg/kg) on days 0, 2, 4, and 6 relative to CVB3 inoculation. Mice were euthanized on day 7 postinfection.

Adoptive Transfer of Macrophages
Mice were injected IV through the tail vein with M0, M1, or M2 macrophages (2.5×10^6) 3 days after recipients were infected with the virus. In other experiments, M2 macrophages (2.5×10^6) were mixed with M1 macrophages (2.5×10^6, 5×10^6, 1×10^7, and 2×10^7) to achieve ratios of M2 to M1 (M2:M1) of 1:1, 1:2, 1:4, or 1:8.

Histopathology
Hearts were cut longitudinally, fixed in 10% phosphate-buffered formalin, paraffin-embedded, sectioned, and stained with hematoxylin/eosin. Stained sections were observed using a Nikon Eclipse TE2000-S microscope (Nikon, Japan) and true color digital images were captured. The myocarditis was determined by identifying both infiltrating mononuclear cells and myocyte necrosis. The severity of myocarditis was judged by percentage area of the myocardium with inflammation as described previously.24,25 Five fields of view (×100 magnification) of each slide were chosen randomly, and three different sections per mouse were examined. The entire areas of the fields and of regions affected by myocarditis were determined with the use of ImageJ software (NIH) and the percent area of the myocardium undergoing inflammation (affected/entire area in percent) was calculated and averaged. All data were analyzed in a blind fashion by 2 independent investigators and averaged.

Immunohistochemistry
The presence of F4/80-positive macrophages in the heart tissue was analyzed by immunohistochemistry as previously described.25

Statistic Analysis
Data are presented as means±SEM. Differences between experimental groups were analyzed for statistical significance by unpaired Student’s t test or by 1-way ANOVA followed by Bonferroni test for the selected pairs using GraphPad Prism version 4.01 (GraphPad Software Incorporated). A value of P<0.05 was considered significant.

Results
Female BALB/c Mice Showed Less Susceptibility to CVB3-Induced Myocarditis Than Males
Age-matched male and female BALB/c mice were infected IP with CVB3. On day 7 postinfection, the mice were euthanized and the incidence and severity of myocarditis were compared. Histopathologic examination showed an obvious inflammatory infiltrate in the myocardial tissue of male mice, whereas only a few inflammatory cells diffuse in the myocardium of females (Figure 1A). Consistently, quantitative analysis revealed that smaller myocardial areas were affected in female mice compared to males (P<0.01) (Figure 1B). Survival rate of male and female BALB/c mice after CVB3 inoculation was also monitored. During the 10-day infection period, mortality occurred in 50% of male mice. In contrast, only 7% of female mice died (Figure 1C). These results indicated that female BALB/c mice developed significantly less CVB3 myocarditis than males.

Male and Female BALB/c Mice Displayed Comparable Cardiac Viral Loads and Macrophage Infiltration During CVB3-Induced Myocarditis
To determine whether differential efficiency of CVB3 infection in male and female BALB/c mice contributed to the gender bias in susceptibility, we examined the levels of viral titer in vivo and in vitro during the early phase (day 3 postinfection) and acute phase (day 7 postinfection) of viral myocarditis by TCID50 assay. There was no significant
difference in viral titers in the hearts ($P>0.05$) and cardiac myocytes ($P>0.05$) between the sexes at day 3 (Figure 2A and 2B). Furthermore, there were even slightly higher myocardial virus titers in female mice as compared to males ($P<0.05$) at day 7 (Figure 2A), although females showed milder myocarditis than male mice. In vitro studies, however, showed that there was no sex difference in viral titer in the cardiac myocytes at day 7 ($P>0.05$) (Figure 2B). Together, decreased inflammation in the myocardium of female mice with viral myocarditis was not attributable to less susceptibility to CVB3 infection.

Accumulating data indicated that abundant macrophages were recruited into the myocardium in male mice after CVB3 infection, which played an indispensable role in the development of viral myocarditis. Given female mice are less prone to acute myocarditis, we speculated that quantitatively and/or functionally distinct macrophages may exist in female mice. Age-matched male and female BALB/c mice were infected with CVB3 and compared for macrophage infiltration by immunohistological analysis. Macrophage-specific F4/80 immunostaining revealed an obvious macrophage infiltration in the myocardium both in males and females (Figure 3A). Interestingly, macrophages in male mice were more frequently clustered, shown as an intense accumulation at the inflamed lesions. As for female mice, however, macrophages tended to be diffusely distributed within the myocardium (Figure 3A). We next determined the absolute numbers of macrophages accumulating in the myocardium using cell yield analyses of F4/80$^+$ cells. No significant differences were observed in the cell counts of infiltrating macrophages per heart between the 2 sexes (male, $7.9±0.4×10^5$ versus female, $7.5±0.6×10^5$; $P>0.05$) (Figure 3B). Comparative analysis demonstrated that sex difference in susceptibility to viral myocarditis was not attributable to quantitative distinction in macrophages.

Myocardial Infiltrating Macrophages Exhibited a Predominant M2 Phenotype in Females During CVB3-Induced Myocarditis

The above data suggested that there were no significant quantitative differences in infiltrating macrophages between male and female BALB/c mice following CVB3 infection, we then determined whether sex differences in macrophage polarization existed by analyzing markers associated with M1 and M2 polarization. It was shown that infiltrating macrophages from male mice significantly overexpressed the M1-associating inflammatory genes Nos2 (iNOS) and Tnfa (TNF-α) compared with female mice (Figure 4A). Strikingly, macrophages derived from females showed increased expression of M2-specific genes Arg1 (Arg-1), Il10 (IL-10), Mrc (MMR), and Mgl (MGL) compared with the macrophages isolated from male mice (Figure 4A). Consistently, FACS analysis revealed significantly increased iNOS and CD16/32 expression in male-derived macrophages, which were markers of M1 phenotype, and dramatically upregulated MMR and MGL expression in females (Figure 4B). As shown in Figure

Figure 2. In vivo and in vitro titration of the myocardial virus in male and female mice following CVB3 infection. A, Age-matched BALB/c males and females received $10^3$ TCID$_{50}$ of CVB3 IP on day 0 and hearts were removed aseptically, weighed, and homogenized on day 3 or day 7 postinfection for TCID$_{50}$ assay. B, Cardiac myocytes ($2×10^5$) from neonatal male and female BALB/c mice were incubated with 10 TCID$_{50}$ CVB3 for 3 or 7 days, and then the viral titers were measured using TCID$_{50}$ assay. Data are presented as the means±SEM of 5 mice per group. $^*P<0.05$.

Figure 3. Quantification of cardiac infiltrating macrophages derived from male and female mice during acute myocarditis. A, Age-matched male and female mice received $10^3$ TCID$_{50}$ of CVB3 IP on day 0 and hearts were collected on day 7 postinfection. Sections were stained with F4/80 antibody to identify macrophages (original magnification: $×100$) in male and female mice. Myocardial infiltrating leukocytes were isolated from the hearts after enzymatic digestion and sorted for F4/80$^+$ cells by magnetic-activated cell sorting (MACS). The number of macrophages per heart is calculated by dividing purified macrophage counts with the number of hearts pooled. B, The absolute numbers of F4/80$^+$ cells per heart are shown. Similar results were obtained in 3 separate experiments using 7 to 10 mice per group. Data show the means±SEM.
4C, higher arginase activity, a typical characteristic of M2 macrophages, was observed in macrophages from female than in male mice. Taken together, we revealed phenotypic differences between myocardial infiltrating macrophages derived from male and female BALB/c mice during acute phase of viral myocarditis, with males being polarized to M1 phenotype, females being of predominantly M2 phenotype.

Myocardial Th2 Cytokine Primed Macrophages Into M2 Phenotype in Female Mice After CVB3 Infection

Given that there was a preferential M1 and M2 polarization under Th1 (IFN-γ) and Th2 cytokines (IL-4, IL-13), respectively, we determined whether distinct cytokine milieu contributed to sex difference in macrophage polarization in BALB/c mice. We first confirmed that male mice had significantly increased levels of myocardial IFN-γ expression compared to females (P<0.01), whereas females produced a larger amount of IL-4 in the hearts (P<0.01) (Figure 5A). Interestingly, another prototypical Th2 cytokine, IL-13, did not show significant difference between sexes (P>0.05) (Figure 5A). To assess the role of these cytokines in macrophage polarization in vivo, male and female BALB/c mice were injected with neutralizing antibody on days 0, 2, 4, and 6 relative to CVB3 infection. After cytokine blockade, macrophage phenotypes were examined by quantitative anal-

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**Figure 4.** Phenotypic analysis of myocardial infiltrating macrophages in male and female BALB/c mice during acute myocarditis. Mononuclear cells were prepared from the hearts of male and female mice inoculated IP with CVB3 7 days earlier. F4/80+ macrophages were freshly purified by magnetic-activated cell sorting and analyzed for the expression of M1 and M2 macrophage-associated markers. A, Total RNA was prepared from purified macrophages, and the mRNA levels of iNOS, TNF-α, Arg-1, IL-10, MMR, and MGL were analyzed by real-time PCR normalized to GAPDH. B, CD16/32, iNOS, MMR, and MGL expression on F4/80+ macrophages was evaluated by FACS. Data are representative of 3 separate experiments. C, Arginase activity was assessed by an assay of urea production from arginine substrate and was normalized to cell counts. Data are expressed as means±SEM of 7 to 10 mice per group. *P<0.05; **P<0.01.
Analysis of Nos2 and Arg1 expression, which were widely viewed as markers of M1 and M2 polarization, respectively. In susceptible male mice, as compared to isotype antibody treatment, macrophages showed decreased Nos2 ($P<0.05$) and increased Arg1 expression ($P<0.05$) after anti–IFN-γ neutralization (Figure 5B). No significant differences were observed after anti–IL-4 and/or anti–IL-13 treatment (Figure 5B). In contrast, Arg1 expression was strongly suppressed in nonsusceptible female mice after treatment with anti–IL-4 ($P<0.01$) or a combination of anti–IL-4 and anti–IL-13

**Figure 5.** Regulation of macrophage polarization by myocardial-derived cytokine in male and female BALB/c mice during acute myocarditis. A, Male and female BALB/c mice were compared for myocardial IFN-γ, IL-4, and IL-13 expression by ELISA at day 7 postinfection. Anti–IFN-γ, anti–IL-4, anti–IL-13, or anti–IL-4 plus anti–IL-13 neutralizing antibodies were injected IP on days 0, 2, 4, and 6 after CVB3 infection. Isotype antibody was used as control. B, Nos2 and Arg1 expression in myocardial infiltrating macrophage was examined by real-time PCR at day 7 postinfection. C, Macrophages obtained from the hearts of male and female BALB/c mice were analyzed for HIF-1α (Hif1a) and PPAR-γ (Pparg) expression by real-time PCR on day 7 postinfection. BMDMs were cultured under the indicated cytokines at a concentration as follows: IFN-γ (7 ng/mL), IL-4 (25 ng/mL), and IL-13 (3 ng/mL). D, After 24 hours, Nos2 and Arg1 expression in BMDMs was analyzed by real-time PCR. Similar results were obtained in 2 separate experiments. Data show the means±SEM *$P<0.05$; **$P<0.01$. 

358 Circulation Research August 14, 2009
Figure 6. Identification of ex vivo–programmed M1 and M2 macrophages. BMDMs were cultured in the presence of IFN-γ (100U/mL) plus LPS (100 ng/mL) or IL-4 (10 ng/mL). A, TNF-α, IL-12, and IL-10 in the supernatant were assayed by ELISA. B and C, Arginase activity (B) and CD16/32, iNOS, MMR, and MGL expression (C) were evaluated as described above. MFI indicates mean fluorescence intensity. D, The frequency of CD4⁺ Foxp3⁺ Treg cells was also evaluated by FACS analysis. Data are representative of three separate experiments. Data show the means±SEM *P<0.05; **P<0.01.
neutralizing antibody (P<0.01), suggesting an essential role of IL-4 in M2 polarization in female BALB/c mice (Figure 5B). IL-13 neutralization alone also slightly reduced Arg1 expression in female mice, indicating less dominant role of IL-13 (Figure 5B). In vitro studies showed that BMDMs cultured at the presence of IFN-γ exhibited M1 phenotype, whereas IL-4, IL-13, or IL-4 plus IL-13 polarized BMDMs toward an M2 phenotype (Figure 5D), consistent with the in vivo data. Interestingly, although IL-13 alone primed macrophages along M2 program, when it was used in combination with high dose of IFN-γ, M2 polarization was markedly impaired, as shown by decreased Arg1 and enhanced Nos2 expression (Figure 5D). Because hypoxia-inducible transcription factor (HIF)-1α and peroxisome proliferator-activated receptor (PPAR)-γ are essential transcription factors for M1 and M2 differentiation, respectively,22,27,28 we further determined whether differential cytokine milieu between sexes regulated macrophage polarization at the transcription level.

As shown in Figure 5C, higher mRNA levels of HIF-1α was observed in male BALB/c mice (P<0.01), whereas PPAR-γ was significantly overexpressed in females on day 7 after CVB3 infection (P<0.01).

Collectively, these data suggest that myocardial Th2 cytokines, notably IL-4, play a bona fide critical role in M2 polarization in female BALB/c mice.

Adoptive Transfer of Ex Vivo–Programmed M2 Macrophages Significantly Ameliorated Myocarditis in Susceptible Male Mice After CVB3 Infection

To explore the role of macrophage polarization in the development of viral myocarditis, we first generated M1 and M2 macrophages in vitro. It was shown that M1 macrophages induced by IFN-γ and LPS produced more proinflammatory cytokines IL-12 (P<0.01) and TNF-α (P<0.01) (Figure 6A), M2 macrophages polarized by IL-4 showed increased IL-10
Figure 8. In vivo effects of polarized macrophages on cytokine expression and Treg differentiation. Male BALB/c mice receiving $10^3$ TCID$_{50}$ CVB3 3 days earlier were injected IV through the tail vein with BMDMs derived from GFP transgenic mice which had been (Continue ...)

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M2 Macrophage Transfer Led to Elevated IL-10 Production and Treg Frequency

Using GFP-positive (GFP⁺) donor macrophages, we examined the migration pattern of ex vivo–polarized macrophages in recipient mice. Comparable percentages of GFP⁺ M0, M1, or M2 macrophages were detected in the spleens on day 2 (Figure 8A) and within myocardium at day 4 after transfer (Figure 8B), indicating that the percentage of GFP⁺ F4/80⁺ among all F4/80⁺ cells, suggesting no preferential myocardial recruitment of either M1 or M2 macrophages into the heart tissue.

Cytokine profiles in the heart homogenate reflect the local immune environment. To better understand the mechanisms responsible for decreased inflammation after M2 transfer, the cytokine profiles of infected male mice that received M0, M1, or M2 macrophages were detected by ELISA at day 4 after adoptive transfer. We found that mice transfused with M1 macrophages showed increased level of proinflammatory cytokine TNF-α (P<0.01), whereas M2 transfusion resulted in significantly less TNF-α (P<0.01) but more antiinflammatory IL-10 production (P<0.01) (Figure 8C) in the heart. We next evaluated the in vivo effect of the donor macrophages on the T cell response. FACS analysis showed that CD4⁺ Foxp3⁺ Treg populations were slightly decreased in the spleen 4 days after M1 transfer, whereas M2 treatment resulted in an increased frequency of CD4⁺ Foxp3⁺ Tregs (Figure 8D), indicating the protective effect of M2 transfer may exert through promoting Treg differentiation.

Discussion

We report here that there were not quantitative but phenotypic differences in the myocardial infiltrating macrophages between males and females following CVB3 infection. To the best of our knowledge, this study is the first to reveal that macrophages of female BALB/c mice are polarized toward an M2 phenotype after CVB3 infection which could effectively suppress the development of CVB3-induced myocarditis.

Several lines of evidence supported the notion that tissue-derived cytokines were responsible for M2 polarization in female BALB/c mice after CVB3 infection. First, our observations that similar percentage of donor polarized macrophages was detected within the myocardium after adoptive transfer suggested that there was no preferential M1 and M2 recruitment into the heart tissue. Secondly, high myocardial IL-4 and IL-13 levels were detected in females, although IL-13 expression was also found in male mice. Thirdly, conditioned medium containing IL-4 and/or IL-13 increased the expression of M2 marker, whereas the medium containing high levels of IFN-γ could potently suppressed the expression of IL-13–induced M2 marker, suggesting high levels of IFN-γ may have an overwhelming effect over IL-13 on macrophage polarization in male mice after CVB3 infection. Most importantly, in male mice, M2 polarization was inhibited by IL-4 and/or IL-13 neutralizing antibody and in male mice treated with anti-IFN-γ neutralizing antibody, macrophages increased M2 marker expression. Furthermore, at the transcriptional level, high expression levels of HIF-1α and PPAR-γ were observed in male and female BALB/c mice on day 7 after CVB3 infection, respectively. These data together indicated that differential myocardial cytokine expression between male and female BALB/c mice after CVB3 infection played a critical role in gender specific macrophage polarization.

Adoptive-transfer experiments using ex vivo programmed macrophages help elucidate the role of macrophage polarization in CVB3 myocarditis. The excessive presence of M1 macrophages may cause damage to the host after CVB3 infection. This is supported by the fact that mice transferred with M1 macrophages developed a more severe inflammation.
in the hearts of both sexes indicating excessive M1 residence caused myocardial damage. The most striking observation is that exogenous M2 macrophages transfer protected against CVB3-induced myocarditis in male mice, suggesting a potentially beneficial function of M2 macrophages in myocarditis. Because M2 macrophages have been found to be protective in many inflammatory conditions, such as obesity, diabetes, and experimental autoimmune encephalomyelitis, etc, adoptive transfer of ex vivo programmed M2 macrophages might be a useful strategy for treating these diseases.

It has been shown that proinflammatory cytokines have an important role in the development of inflammatory myocardial diseases. Suppression of inflammatory cytokines has beneficial effects on ameliorating myocarditis. Our findings that M2 macrophages transfer reduced myocardial TNF-α expression and enhanced IL-10 production in recipient mice may explain, at least partly, the mechanisms underlying M2 macrophages mediated protection. The secretion of IL-10 may help protect against the deleterious effects of TNF-α and related cytokines in myocarditis. The most interesting part of the study was the observation that transferred M2 were capable of migrating into spleen and increasing peripheral Treg cell populations. In the CVB3 myocarditis model, infection with pathogenic variants induces an autoimmune Th1 and CTL response that is directly responsible for cardiac injury in BALB/c mice. In this regard, donor M2 macrophage transfection may facilitate development of Treg cells and prevent immunopathology. Our study highlights the critical role of M2 macrophages in induction of Treg cells in vivo and supports the beneficial role of Treg cells in CVB3 myocarditis.

In conclusion, our findings support that macrophages abundant in myocardium of female BALB/c mice predominantly exhibit alternative activation phenotype, which may confer protection against CVB3-induced myocarditis by limiting the generation of proinflammatory responses and promoting Treg cell differentiation. These findings suggested the possible role of macrophage polarization in defining the sex-related susceptibility to viral myocarditis and emphasized the potential of macrophage polarization in therapeutic intervention of inflammatory heart disease.

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Disclosures
None.

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