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Research Report

Sex differences in coxsackievirus B3-induced myocarditis: IL-12Rβ1 signaling and IFN-γ increase inflammation in males independent from STAT4

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ARTICLE INFO

Article history:
Accepted 3 August 2006
Available online 1 September 2006

Keywords:
Sex
Cytokine
Heart disease
Inflammation
Virus

ABSTRACT

Cardiovascular disease is the number one killer of men and women in North America. Male BALB/c mice infected with coxsackievirus B3 (CVB3) develop more severe inflammatory heart disease compared to female mice, similar to the increased heart disease that occurs in men. We show here that increased inflammation in male mice is not due to increased viral replication in the heart, but associated with increased proinflammatory cytokines IL-1β, IL-18 and IFN-γ. We have previously reported that IL-12Rβ1 signaling increases CVB3-induced myocarditis and IL-1β/IL-18 levels in males, while IL-12(p35)/STAT4-induced IFN-γ does not alter the severity of acute disease. However, whether differences exist between males and females in these two cytokine signaling pathways is unknown. In this study, we examined sex differences in 1) IL-12Rβ1 signaling or 2) STAT4/IFN-γ pathways following CVB3 infection in BALB/c mice. We found that male and female mice deficient in IL-12Rβ1 had decreased inflammation and viral replication in the heart, indicating that IL-12Rβ1 signaling increases myocarditis in both sexes. In contrast, STAT4 deficiency did not alter the sex difference in myocarditis, with males maintaining increased inflammation over females. IFN-γ deficient males, however, had decreased myocarditis and viral replication compared to females. Thus, IFN-γ increases inflammation in males independent from STAT4. These results demonstrate that sex differences greatly influence viral replication and the severity of acute CVB3-induced myocarditis.

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doi:10.1016/j.brainres.2006.08.003

Abbreviations:
CVB3, coxsackievirus B3
DC, dendritic cells
DCM, dilated cardiomyopathy
IL, interleukin
i.p., intraperitoneal
IFN, interferon
MC, mast cells
NK, natural killer
p.i., post-infection
PFU, plaque forming units
R, receptor
STAT, signal transducer and activator of transcription
Th, T helper
TLR, Toll-like receptor
TNF, tumor necrosis factor
Treg, regulatory T cells

1. Introduction

Cardiovascular disease is the leading cause of death in men and women in the U.S., accounting for 38% of all deaths in 2002 (American Heart Association, 2006; Liu et al., 2003), while 16.6 million people worldwide die of heart disease each year (Mieres, 2006; Yusuf et al., 2001). Myocarditis, or inflammation of the heart, is a principal cause of heart failure in young adults often leading to chronic heart disease and dilated cardiomyopathy (DCM) (Dec, 2003; Feldman and McNamara, 2000). Recent studies have reinforced sex-related differences in the pathology of cardiovascular disease, with an increased incidence and mortality in men (American Heart Association, 2006; Fabre and Sheppard, 2006; Liu et al., 2003; Mieres, 2006). The lower incidence of heart disease in women has been attributed to the cardioprotective effects of estrogen (Mendelsohn and Karas, 1999). However, recent clinical trials of hormone replacement therapy found an increased risk of heart disease with estrogen and progestin treatment (Rossouw et al., 2002), indicating that many of the effects of sex hormones on the pathogenesis of heart disease remain unclear. In previous experiments, we found that gonadectomy reduces CVB3-induced myocarditis in males and females, indicating that both testosterone and estrogen can increase inflammation in the heart (Fairweather and Rose, 2004a).

Coxsackievirus B3 (CVB3) infection is believed to be a principle etiologic agent in human myocarditis (Dec, 2003; Feldman and McNamara, 2000). CVB3 infection of susceptible A/J or BALB/c mice results in a disease similar to that observed in humans, with the development of acute myocarditis from days 7 to 14 after infection that progresses to chronic myocarditis and DCM from day 28 after infection (Fairweather et al., 2005a; Rose et al., 1986). Both viral replication and immune-mediated mechanisms increase inflammation in the heart (Fairweather et al., 2003, 2004a, 2005a,b). Immune mechanisms include cell-mediated and antibody-mediated pathology (Huber and Lodge, 1986; Rose, 2002). For example, immunoglobulin (Ig)G and complement are deposited in the hearts of patients with DCM and in A/J mice inoculated with CVB3 (Fairweather et al., 2006; Neumann et al., 1990, 1994). Proinflammatory cytokines are critical for the development of myocarditis. Increased interleukin (IL)-1β and IL-18 levels in the heart directly correlate with increased inflammation in susceptible strains of mice (Fairweather et al., 2003, 2005a). We have shown previously that Toll-like receptor (TLR)4 and IL-12 receptor (R)β1 signaling increase inflammation and IL-1β/IL-18 levels in the heart of males (Fairweather et al., 2003, 2005a). In contrast, IL-12p35, signal transducer and activator of transcription (STAT)4 or interferon (IFN)-γ deficiency, does not alter the severity of acute CVB3-induced myocarditis (Fairweather et al., 2003, 2005b; Fairweather and Rose, 2005), indicating that IL-12Rβ1 signaling and IL-12-induced IFN-γ have separate effects on inflammation in the heart.

To investigate the mechanisms leading to increased inflammatory heart disease in males, we examined sex differences in the development of CVB3-induced myocarditis in IL-12Rβ1, STAT4 or IFN-γ deficient male and female BALB/c mice. We found that increased inflammation in WT BALB/c males was not due to increased viral replication in the heart since CVB3 replicated at the same level in males and females. IL-12Rβ1 deficiency reduced inflammation and viral replication in the heart of both sexes. STAT4 deficiency did not alter the sex difference in myocarditis, but increased viral replication in males, while IFN-γ deficient males had decreased myocarditis and viral replication compared to females. Thus, sex differences greatly influence viral replication and inflammation in the heart, with IL-12Rβ1 signaling and IFN-γ increasing acute CVB3-induced myocarditis in males.

2. Results

2.1. *BALB/c males develop increased CVB3-induced myocarditis*

Male BALB/c mice develop more severe CVB3-induced myocarditis compared to females (Huber and Pfaeffle, 1994), but the reasons inflammation is increased in males are unclear. To investigate the mechanisms responsible for increased heart disease in males, we examined the cellular infiltrate of

male and female BALB/c mice after CVB3 infection. We confirmed by histological (Figs. 1A, C, D) and FACS (Fig. 1B) analyses that BALB/c males develop significantly increased acute myocarditis compared to females (Mann–Whitney *U* test, $p=0.0001$). Thus, we confirmed that male BALB/c mice respond to CVB3 infection with an increased inflammatory infiltrate in the heart.

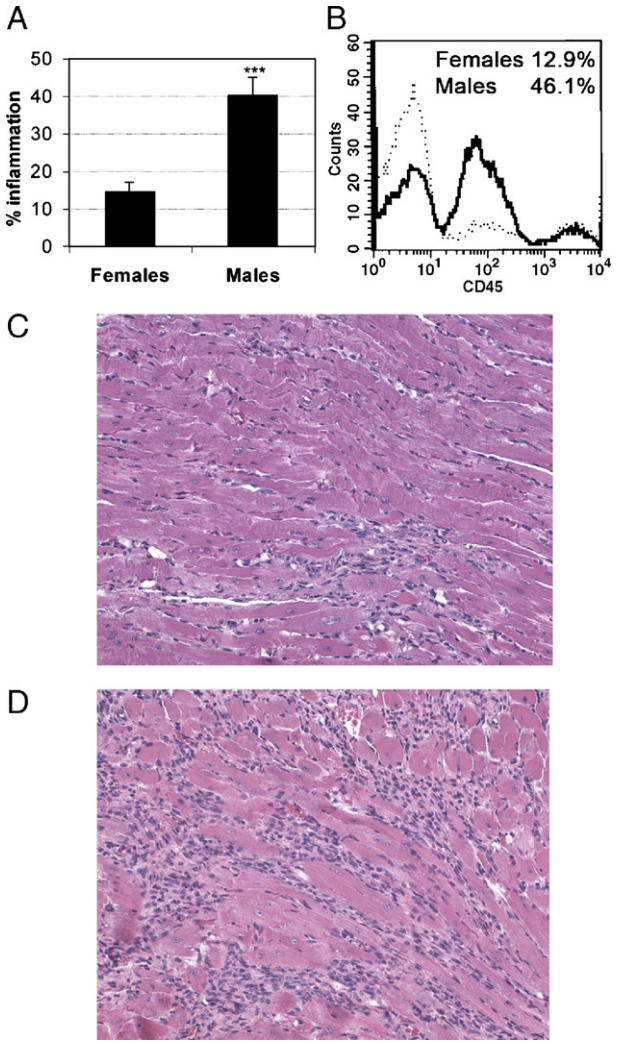

Fig. 1 – Males develop increased acute CVB3-induced myocarditis. Female BALB/c mice (A, B, C) were compared to males (A, B, D) for the development of acute myocarditis by histological (A, C, D) or FACS (B) analysis. Mice received 10^3 PFU of CVB3 i.p. on day 0, and hearts were collected on day 8 (B) or 12 (A, C, D) p.i. Myocardial sections were stained with H&E to detect inflammation and assessed as the percentage of the heart section with inflammation compared to the overall size of the heart section, with representative sections shown for each group (original magnification ×64) (C, D). Data are presented as the mean ± SEM, $^{***}p=0.0001$ (A). Total lymphocytes ($CD45^+$ cells) were isolated from the heart at day 8 by enzymatic digestion. Females (dotted line) were compared to males (solid line) by FACS for $CD45^+$ cells (B). Similar results were obtained in three separate experiments for histology and three separate experiments for FACS using 7 to 10 mice per group.

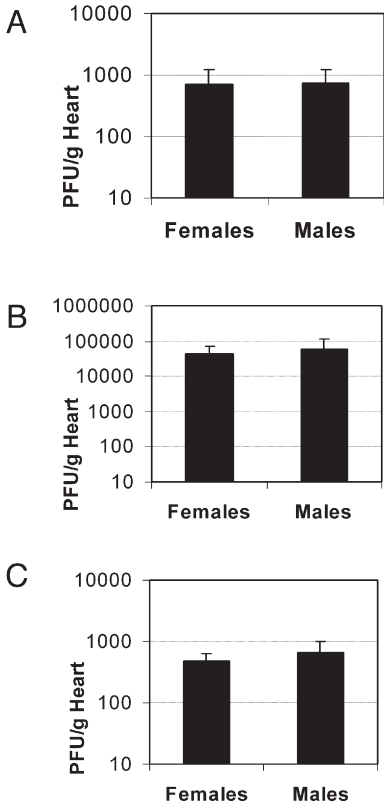

Fig. 2 – Viral replication is not increased in the heart of males. BALB/c males and females received 10^3 PFU of CVB3 i.p. on day 0 and were compared for the level of viral replication in the heart during the innate immune response at day 2 (A) or during acute myocarditis at day 8 (B) or day 12 (C) p.i. Data are presented as the mean PFU/g ± SEM of 7 to 10 mice per group.

2.2. *Viral replication is not increased in the heart of males*

Since increased myocarditis in males could be due to increased viral replication in the heart (Fairweather et al., 2005b; Huber, 2005), we examined the level of viral replication in the heart of males and females at day 2, 8 and 12 p.i. by plaque assay. We did not find a significant difference in viral replication in the heart between the sexes at any time-point examined by the Mann–Whitney *U* test for non-parametric data at day 2 $p=0.38$ (Fig. 2A), day 8 $p=0.32$ (Fig. 2B) or day 12 $p=0.42$ (Fig. 2C). Thus, increased inflammation in the heart of males in our model of CVB3-induced myocarditis is not due to increased viral replication in the heart, indicating that other factors contribute to increased myocarditis in males.

2.3. *Males develop a proinflammatory, Th1 response to CVB3 infection, while females develop a Th2 response*

Male BALB/c mice are known to develop a T helper (Th)-type 1, IFN-γ response to CVB3 infection, while females develop a Th2, IL-4 response (Huber and Pfaeffle, 1994). We confirmed increased IFN-γ in males (Student's *t* test, $p=0.02$) and increased IL-4 in females (Student's *t* test, $p=0.0007$) (Fig. 3A). We found that male BALB/c mice also have significantly

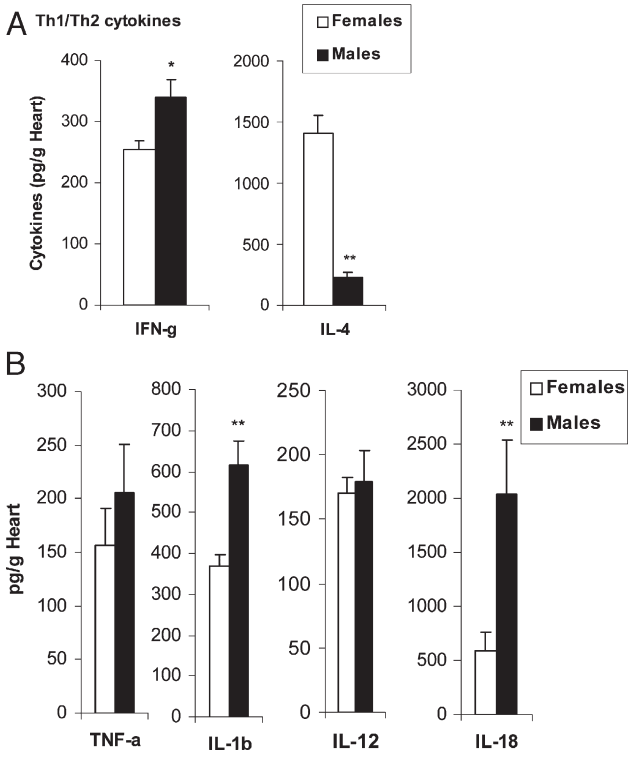

Fig. 3 – Males produce increased IFN-γ, IL-1β and IL-18 during acute myocarditis. Male and female BALB/c mice were compared for the level of Th1/Th2 cytokines (A) or proinflammatory cytokines (B) in the heart during acute myocarditis. Mice received 10^3 PFU of CVB3 i.p. on day 0, and hearts were collected on day 12 p.i. for analysis of cytokine levels by ELISA. Individual experiments were conducted at least three times using 7 to 10 mice per group. Data are shown as the mean ± SEM. *$p<0.05$; **$p<0.01$.

increased levels of IL-1β and IL-18 in the heart compared to females (Student's *t* test, IL-1β $p=0.006$, IL-18 $p=0.009$) (Fig. 3B). Since tumor necrosis factor (TNF)-α and IL-12 levels were not increased in male hearts (Student's *t* test, TNF $p=0.14$, IL-12 $p=0.09$) (Fig. 3B), these cytokines are not likely to directly contribute to the raised IFN-γ levels in males (Fig. 3A). IL-1 and IL-18 are known to increase IFN-γ via MyD88 signaling (IL-18 was previously known as IFN-γ-inducing factor) (Kanda et al., 2000; Suzuki et al., 2003) and so could be responsible for the elevated IFN-γ levels and Th1-type response observed in males during acute myocarditis.

2.4. *Myocarditis in IL-12Rβ1, STAT4 and IFN-γ deficient males and females*

We have shown previously that IL-12Rβ1 signaling (a receptor for IL-12 and IL-23) increases viral replication and inflammation in the heart during acute CVB3-induced myocarditis in males (Fairweather et al., 2003). In contrast, IL-12/STAT4-induced IFN-γ does not alter the severity of myocarditis in males during acute myocarditis but reduces viral replication in the heart (Fairweather et al., 2003, 2005b). IFNs, including IFN-α/β and IFN-γ, are essential for the effective clearance of many viral infections, including CVB3 (Guidotti and Chisari, 2001;

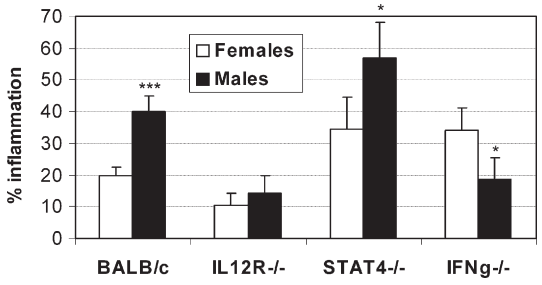

Fig. 4 – Effect of IL-12Rβ1, STAT4 or IFN-γ deficiency on acute myocarditis in male and female BALB/c mice. Mice received 10^3 PFU of CVB3 i.p. on day 0, and hearts were collected on day 12 p.i. as previously. Data are shown as the mean ± SEM of 7 to 10 mice per group. *$p<0.05$, ***$p<0.001$.

Horwitz et al., 2000). Thus, we have shown previously that IL-12Rβ1 signaling increases CVB3-induced myocarditis while IL-12/STAT4-induced IFN-γ reduces viral replication. Since these two cytokine signaling pathways critically influence the severity of myocarditis and viral replication in the heart of males, we wanted to examine whether sex differences existed in IL-12Rβ1 and STAT4/IFN-γ deficient mice. We found that IL-12Rβ1 deficiency (–/–) decreased inflammation in the heart compared to WT BALB/c mice regardless of sex (Student's *t* test, BALB/c vs. IL-12Rβ1–/– females, $p=0.05$; BALB/c vs. IL-12Rβ1 males, $p=0.003$) (Fig. 4). However, there was no significant difference in the severity of inflammation in IL-12Rβ1 deficient males and females (Student's *t* test, $p=0.31$). STAT4 deficient males had significantly increased myocarditis compared to female STAT4 deficient mice (Mann–Whitney *U* test, $p=0.05$) (Fig. 4), similar to WT BALB/c males and females (Figs. 1 and 4). In contrast, the sex difference was reversed in IFN-γ deficient mice, with males developing significantly lower myocarditis compared to females (Mann–Whitney *U* test, $p=0.04$) (Fig. 4). Thus, conclusions made regarding the effects of deficiencies in Th1 cytokine signaling pathways on myocarditis depend on the sex of the animal used for the experiments.

2.5. *Viral replication in IL-12Rβ1, STAT4 and IFN-γ deficient males and females*

Viral replication in the heart of IL-12Rβ1, STAT4 or IFN-γ deficient males and females during acute myocarditis (Fig. 5)

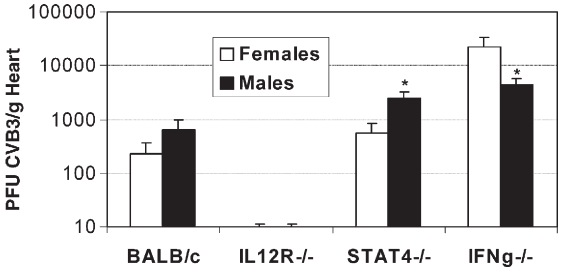

Fig. 5 – Effect of IL-12Rβ1, STAT4 or IFN-γ deficiency on viral replication in the heart of males and females. Mice received 10^3 PFU of CVB3 i.p. on day 0, and hearts were collected on day 12 p.i. for plaque assay. Data are presented as the mean PFU/g ± SEM of 7 to 10 mice per group. *$p<0.05$.

was similar to the severity of inflammation in cytokine deficient mice (Fig. 4). There was no detectable infectious virus in the hearts of IL-12Rβ1 deficient males and females (Fig. 5). Thus, CVB3 appeared to be cleared more quickly from the heart in IL-12Rβ1 deficient mice (CVB3 is usually cleared by day 16 p.i.) (Fairweather et al., 2003). STAT4 deficient males had significantly increased viral replication at day 12 p.i. compared to STAT4 deficient females (Student's *t* test, $p=0.029$) (Fig. 5), suggesting that STAT4 is more important for viral clearance in males than in females. However, IFN-γ deficiency did not increase, but decreased, viral replication in IFN-γ deficient males compared to IFN-γ deficient females (Student's *t* test, $p=0.048$) (Fig. 5), indicating that factors other than IFN-γ can reduce viral replication in males that are either not present or reduced in females (Fairweather et al., 2005b). Although female BALB/c mice respond to CVB3 infection with a more Th2-type (IL-4) response, they also produce Th1-type cytokines (e.g. IL-12, IFN-γ) (Fig. 3). Thus, females are able to clear CVB3 from the heart similar to males (Fig. 2) unless they are deficient in IFN-γ (Fig. 5). These results show that sex differences in cytokine production following CVB3 infection greatly influence viral replication in the heart.

2.6. *Viral replication in IL-12Rβ1, STAT4 and IFN-γ deficient males and females during innate immunity*

In order to determine whether IL-12Rβ1, STAT4 and IFN-γ deficiency alter viral replication during the innate immune response to CVB3 infection, we examined cytokine deficient males and females at day 2 p.i. for the level of viral replication in the heart. Although viral replication was increased in IL-12Rβ1, STAT4 and IFN-γ deficient females compared to males, the increases were not statistically significant (Mann–Whitney *U* test, IL-12Rβ1–/– $p=0.22$; STAT4–/– $p=0.18$, IFN-γ–/– $p=0.13$) (Fig. 6). Thus, sex differences in IL-12Rβ1 signaling and STAT4/IFN-γ cytokine pathways influence viral replication in the heart more profoundly during the adaptive immune response.

2.7. *Cytokine profiles in the heart of IL-12Rβ1, STAT4 and IFN-γ deficient males and females*

Cytokine profiles in the heart reflect the influence of (1) the inflammatory infiltrate (increased IL-1β and IL-18 levels correlate with increased inflammation) and (2) viral replication (increased levels of TNF-α and IL-12/STAT4-induced IFN-γ are associated with decreased viral replication) (Fairweather et al., 2003, 2005a,b). For this reason, we examined the cytokine profile in IL-12Rβ1, STAT4 or IFN-γ deficient mice during acute CVB3-induced myocarditis. IL-12Rβ1 deficient males had significantly reduced IL-12 (Student's *t* test, $p=0.009$) and IL-4 (Student's *t* test, $p=0.04$) levels compared to females (Fig. 7A). There was no significant difference between inflammation in IL-12Rβ1 deficient males and females (Fig. 4) and no significant difference in IL-1β, IL-18 or IFN-γ levels in the heart of IL-12Rβ1 deficient males or females (Fig. 7A). Thus, deficiency in IL-12Rβ1 signaling reduced IL-1β, IL-18 and IFN-γ in the hearts of males (Figs. 3 and 7A), indicating that IL-12Rβ1 signaling is important in increasing the characteristic male cytokine profile (i.e. elevated IL-1β, IL-18 and IFN-γ).

In contrast, STAT4 deficient males retained a male cytokine profile with elevated IL-1β, IL-18 and IFN-γ levels compared to STAT4 deficient females (Student's *t* test, IL-1β $p=0.03$, IL-18 $p=0.02$, IFN-γ $p=0.0007$) (Figs. 3 and 7B) and increased myocarditis (Fig. 4). The only change in STAT4 deficient (Fig. 7B) compared to WT cytokine profiles (Fig. 3) was that STAT4 deficient females had similar IL-4 levels compared to males (Fig. 7B), indicating that STAT4 deficiency decreased IL-4 levels in females. Thus, STAT4 deficiency does not appear to alter the sex difference in the severity of myocarditis (Fig. 4) or the cytokine profile in males, but decreases IL-4 in females (Fig. 7B). IFN-γ deficient males had significantly increased levels of TNF-α in the heart (Mann–Whitney *U* test, $p=0.002$) (Fig. 7C). TNF is an important antiviral agent that recruits neutrophils, which then release an array of antimicrobial agents (Fairweather et al., 2005b; Nathan, 2006). Thus, increased TNF-α in male IFN-γ deficient mice (Fig. 7C) may reduce viral replication in males (Fig. 5). There was no significant difference in the other cytokines that we examined in IFN-γ deficient males or females (Fig. 7C). Thus, deficiencies in Th1-type cytokines affect male and female BALB/c mice differently following CVB3 infection, impacting the severity of acute inflammatory heart disease.

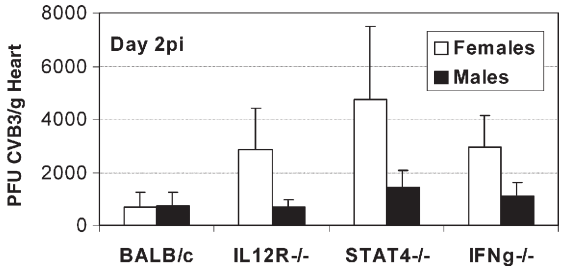

Fig. 6 – Effect of IL-12Rβ1, STAT4 or IFN-γ deficiency on viral replication in the heart during innate immunity. Mice received 10^3 PFU of CVB3 i.p. on day 0, and hearts were collected on day 2 p.i. for plaque assay. Data are presented as the mean PFU/g ± SEM of 7 mice per group.

3. Discussion

In this study, we show that male BALB/c mice respond to CVB3 infection with increased inflammation and proinflammatory cytokines in the heart, and this increase is not due to increased viral replication. In the 1970s, Woodruff reported that male BALB/c mice develop greater CVB3-induced myocarditis (Wong et al., 1977). In studies using the Woodruff strain of CVB3, also called the H3 variant, $CD8^+$ T cells are the major mediator of cardiac damage during acute myocarditis, killing virus infected myocytes resulting in widespread myocyte necrosis and mortality (Guthrie et al., 1984; Huber et al., 2002; Huber, 2005). There are important differences between H3-CVB3-induced myocarditis and the Nancy strain of CVB3 used to induce myocarditis in these studies (Fairweather and Rose, 2004b). Male BALB/c mice in both models have increased myocarditis and an IFN-γ, Th1-type response (Huber and Pfaeffle, 1994). However, IFN-γ is pathogenic in the H3 model; that is, IFN-γ deficient mice do not develop myocarditis and $V\gamma4^+$ T cells require IFN-γ to activate

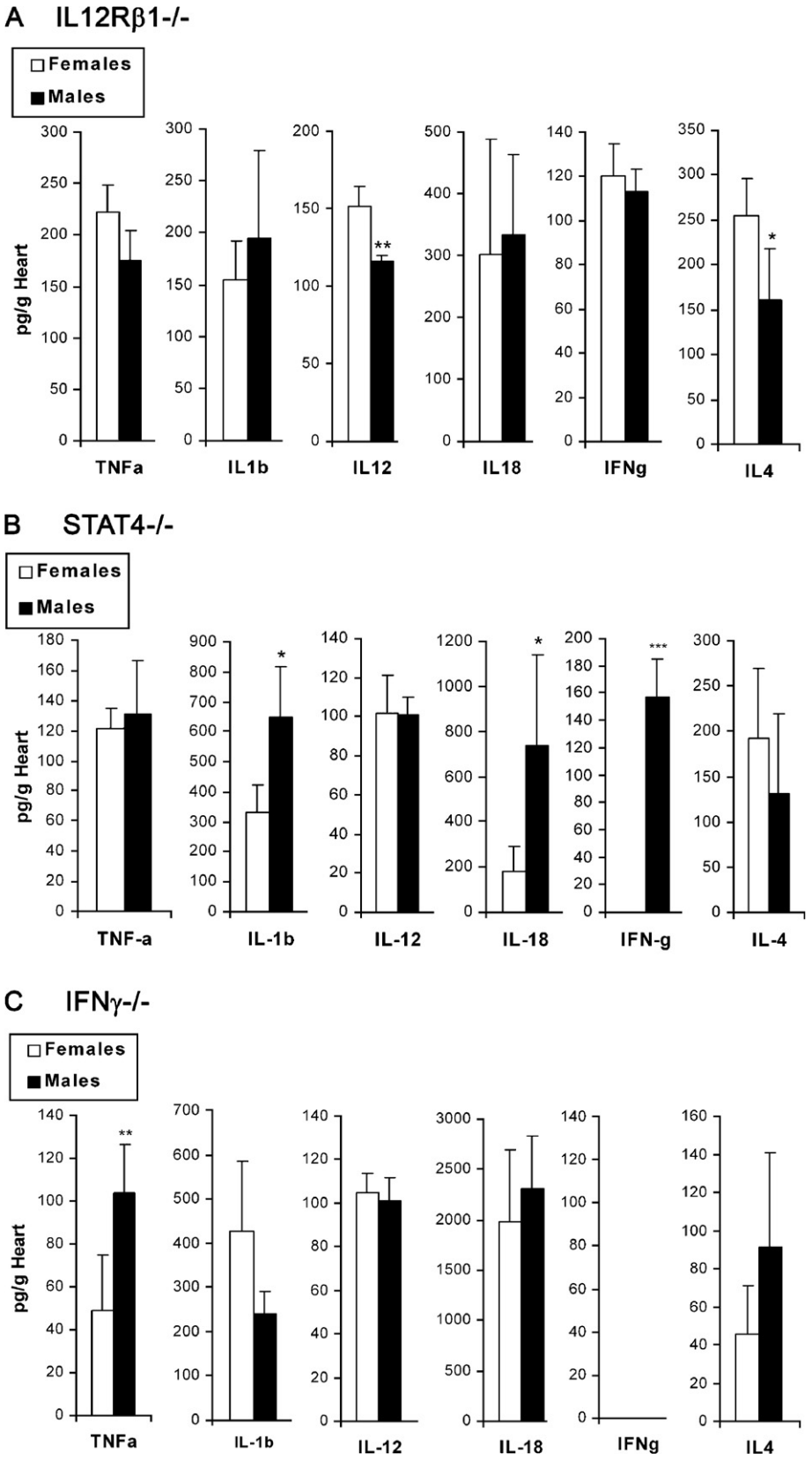

Fig. 7 – IL-12Rβ1 (A), STAT4 (B) or IFN-γ (C) deficiency alters cytokines in the heart of male and female BALB/c mice during acute CVB3-induced myocarditis. Mice received 10^3 PFU of CVB3 i.p. on day 0, and hearts were collected on day 12 p.i. for analysis of cytokine levels by ELISA. Data are shown as the mean ± SEM of 7 to 10 mice per group. $^{*}p<0.05$; $^{**}p<0.01$, $^{***}p<0.001$.

pathogenic CD8+ T cells (Huber et al., 2002). In contrast, we, and others, have shown that IFN-γ protects against acute CVB3-induced myocarditis by reducing viral replication during the innate and adaptive response to CVB3 infection (Horwitz et al., 2000; Fairweather et al., 2005b) and protects against chronic myocarditis by reducing fibrosis and DCM (Fairweather et al., 2004a; Fairweather and Rose, 2005). Thus, the role of a Th1 response in the progression of CVB3-induced myocarditis depends not only on the sex of the mouse but also on the strain of CVB3 used for the studies.

In this study, we found that males compensate for the loss of IFN-γ by increasing TNF-α in the heart (Fig. 7C), a potent antiviral agent (Guidotti and Chisari, 2001), whereas loss of IFN-γ in females leads to increased viral replication and myocarditis (Figs. 4 and 5). Although females respond to CVB3 infection with increased IL-4, IFN-γ is also present in the heart indicating a mixed Th1/Th2 response (Fig. 3A). A number of viruses in addition to CVB3 produce a mixed Th1/Th2 response including respiratory syncytial virus, influenza virus, hepatitis C virus, Sindbis virus, Sendai virus, lymphocytic choriomengingitis virus and murine cytomegalovirus (Fairweather et al., 2004b). Compared to WT males and females (Figs. 2 and 5), IFN-γ deficient males and females have increased viral replication in the heart (Fig. 5) (Fairweather et al., 2003, 2005b). Thus, IFN-γ inhibits CVB3 replication regardless of sex.

Women respond to infection or trauma with less inflammation in the heart compared to men (Kher et al., 2005; Styrt and Sugarman, 1991). Likewise, animal studies have consistently shown that females are protected from acute myocardial injuries such as ischemia, burn and sepsis (Kher et al., 2005). Estrogen decreases proinflammatory cytokines via effects on NFκB and enhances B cell maturation resulting in higher antibody titers in females compared to males (Fox et al., 1991; Schuster and Schaub, 2001; Styrt and Sugarman, 1991; Rossouw et al., 2002). Consistent with these reports, we observed increased B cell numbers (unpublished results, S. Frisancho-Kiss and D. Fairweather) and reduced IFN-γ levels (Fig. 3) in the hearts of females. However, viral replication was not significantly increased in males (Fig. 2), as is observed with the H3-CVB3 variant. Infection with the H3-CVB3 variant results in relatively high virus replication in male hearts (10^7), little inflammation (3%) and widespread necrosis and death (Huber, 2005), while infection with the Nancy strain of CVB3 results in relatively low viral replication in the heart (10^5), high inflammation (30–50%) and little or no necrosis or death (Fairweather et al., 2003, 2004a, 2005b). Thus, different pathogenic mechanisms exist between models of CVB3-induced myocarditis (Fairweather and Rose, 2004b). It is interesting to note that two outbreaks of CVB3 infection in humans found no sex difference in the rate of infection (Schoub et al., 1985; Dechkum et al., 1998) even though a clear increase in incidence and mortality of heart disease occurs in men (Fabre and Sheppard, 2006; Liu et al., 2003). Further evidence that viral replication itself does not increase inflammation in males comes from the finding that myocarditis is increased in male mice in the adjuvant/cardiac myosin-induced model of experimental myocarditis (unpublished results, N.R. Rose) and in men receiving a smallpox vaccination (Feery, 1977; Halsell et al., 2003). Overall, these results suggest that the robust proinflammatory immune response to CVB3 infection in males further increases inflammation in the heart over what is induced by viral replication alone (Fairweather et al., 2005a).

In this study, we found that IL-12Rβ1 signaling increased inflammation and viral replication in males and females. Since IL-12Rβ1 signaling increases IL-1β and IL-18, but not IL-12 or IFN-γ, during acute CVB3-induced myocarditis (Fairweather et al., 2003), this signaling pathway is critical for the development of acute myocarditis in both sexes. We showed previously that IL-12/STAT4-induced IFN-γ does not increase myocarditis in males (Fairweather et al., 2003; 2005b). We found here that STAT4 deficiency did not alter the sex difference in myocarditis, with males maintaining increased inflammation over females (Figs. 1 and 4). Reduced IL-4 levels in the heart of STAT4 deficient females suggest that STAT4 may influence IL-4 production (Fig. 7B). In fact, STAT4 has been shown to be expressed in Th2 cells (IL-4 producing cells), although the precise role for STAT4 in Th2 cells is unclear (Watford et al., 2004). In previous studies of adjuvant/cardiac myosin-induced myocarditis, STAT4 deficient mice were shown to develop significantly reduced acute myocarditis indicating a pathogenic role for STAT4 (Afanasyeva et al., 2001a). However, female BALB/c mice (which develop lower myocarditis) were used in those studies, which may account for the different results in the two models. Alternatively, decreased IL-4 levels in STAT4 deficient mice may reduce inflammation in that model, which depends on IL-4 (Afanasyeva et al., 2001b). Interestingly, the roles for IL-12Rβ1 signaling and IFN-γ are the same in the adjuvant/cardiac myosin- and CVB3-induced myocarditis models suggesting that common mechanisms of disease pathogenesis occur between the two models (Afanasyeva et al., 2001a, 2005; Fairweather and Rose, 2005; Fairweather et al., 2003, 2004a, 2005b).

In this study, we show that differences in the severity of myocarditis between sexes are not due to the level of viral replication in the heart but due to differences in the immune response to infection. Treatment of acute myocarditis and chronic heart disease with antiviral agents and/or immunosuppressive therapies has often produced disappointing results (Frustaci et al., 2006; Hia et al., 2004; Liu et al., 2005). Regulating viral replication and inflammation is further compounded by differences in the immune response to infection in males and females. In order to develop better therapies to treat, or possibly even prevent, inflammatory heart disease, a better understanding of differences in the immune response of males and females is needed.

4. Experimental procedures

4.1. *Mice*

Male and female BALB/c mice or BALB/c mice deficient in IL-12Rβ1, STAT4 or IFN-γ were obtained from the Jackson Laboratory (Bar Harbor, ME). At least three separate experiments using 7 to 10 mice per group were conducted for each procedure and time-point. Mice were maintained under pathogen free conditions in the animal facility at Johns Hopkins School of Medicine, and approval was obtained

from the Animal Care and Use Committee of the Johns Hopkins University for all procedures. Mice determined to be suffering from pain or discomfort at any time during daily observations were immediately euthanized.

4.2. *Myocarditis*

Mice, 6 to 8 weeks of age, were inoculated with an intraperitoneal (i.p.) injection of a heart-passaged stock of CVB3 (Nancy strain) originally obtained from the American Type Culture Collection (ATCC, Manassas, VA) and grown in Vero cells (ATCC, Manassas, VA). CVB3 was diluted in sterile saline and 10^3 plaque forming units (PFU) injected i.p. on day 0 and tissues or cells collected at day 2 post-infection (p.i.) (innate response) or on day 8 or 12 p.i. (acute myocarditis). No deaths occurred in male or female BALB/c mice during acute myocarditis (data not shown). Mice inoculated i.p. with PBS or uninfected tissue homogenate did not develop myocarditis (data not shown). Hearts were cut longitudinally and fixed in 10% phosphate-buffered formalin and embedded in paraffin. Sections 5 μm thick were cut at various depths in the tissue section and stained with H&E to determine the level of inflammation. Sections were examined by two independent investigators in a blinded manner, and myocarditis was assessed as the percentage of the heart section with inflammation compared to the overall size of the heart section, with the aid of a microscope eyepiece grid.

4.3. *Cytokine measurement*

Hearts were frozen in dry ice immediately and stored at −80 °C until homogenized. Tissues were homogenized at 10% weight/volume in 2% MEM, and supernatants were stored at −80 °C until used in ELISA or plaque assays. Cytokines were measured in heart supernatants using Quantikine cytokine ELISA kits (R&D Systems, Minneapolis, MN), as previously (Fairweather et al., 2003). Cytokines were below detectable levels in the 2% MEM used to homogenize samples (data not shown). Cytokines were expressed as pg/g of heart tissue ± SEM.

4.4. *Plaque assay*

The level of infectious virus was determined in individual homogenates by plaque assay, according to standard procedures (Fairweather et al., 2003). Samples were processed in the same manner as for cytokine analysis. Virus titers were expressed as the mean PFU/g ± SEM, and the limit of detection was 10 PFU/g of heart tissue.

4.5. *Heart digestion and FACS analysis*

The heart was perfused at a constant flow of 14 ml/min with cold PBS (Biofluids, Rockville, MD) for 2 min, as previously (Fairweather et al., 2005b, 2006). Individual cell suspensions from 7 mice were pooled by group and immune cells separated from heart cells using a magnetic column and anti-CD45 paramagnetic beads (30F11.1; Miltenyi Biotec, Auburn, CA). Cell fluorescence was measured using a FACScalibur flow cytometer (BD BioSciences, San Jose, CA), and data analyzed using Cell Quest software (BD BioSciences, San Diego, CA).

4.6. *Statistical analysis*

Individual experiments were conducted at least three times with 7 to 10 mice per group. Normally distributed data were analyzed by the Student's *t* test. The Mann–Whitney *U* test was used to evaluate non-parametric data. Differences between male and female WT or deficient mice were considered significant if $p \leq 0.05$. Comparisons of IL-12Rβ1, STAT4 and IFN-γ deficient to WT BALB/c mice are previously published (Fairweather et al., 2003, 2004a, 2005b).

Acknowledgments

This work was supported by National Institutes of Health Grants HL67290, HL70729, AI51835, ES03819 and T32 ES07141 (to J.F.N.).

REFERENCES

Afanasyeva, M., Wang, Y., Kaya, Z., Stafford, E.A., Dohmen, K.M., Sadighi Akha, A.A., Rose, N.R., 2001a. Interleukin-12 receptor/STAT4 signaling is required for the development of autoimmune myocarditis in mice by an interferon-γ-independent pathway. Circulation 104, 3145–3151.

Afanasyeva, M., Wang, Y., Kaya, Z., Park, S., Zilliox, M.J., Schofield, B.H., Hill, S.L., Rose, N.R., 2001b. Experimental autoimmune myocarditis in A/J mice is an interleukin-4-dependent disease with a Th2 phenotype. Am. J. Pathol. 159, 193–203.

Afanasyeva, M., Georgakopoulos, D., Belardi, D.F., Bedja, D., Fairweather, D., Wang, Y., Kaya, Z., Gabrielson, K.L., Rodriguez, E.R., Caturegli, P., Kass, D.A., Rose, N.R., 2005. Impaired up-regulation of CD25 on $CD4^+$ T cells in IFN-γ knockout mice is associated with progression of myocarditis to heart failure. Proc. Natl. Acad. Sci. U.S.A. 102, 180–185.

American Heart Association, 2006. Heart Disease and Stroke Statistics—2006 Update, www.americanheart.org.

Dec, G.W., 2003. Introduction to clinical myocarditis. In: Cooper Jr., L.T. (Ed.), Myocarditis: From Bench to Bedside. Humana Press, Totowa, NJ, pp. 157–281.

Dechkum, N., Pangsawan, Y., Jayavasu, C., Saguanwongse, S., 1998. Coxsackie B virus infection and myopericarditis in Thailand, 1987–1989. Southeast Asian J. Trop. Med. Public Health 29, 273–276.

Fabre, A., Sheppard, M.N., 2006. Sudden adult death syndrome and other non-ischaemic causes of sudden cardiac death. Heart 92, 316–320.

Fairweather, D., Rose, N.R., 2004a. Women and autoimmune diseases. Emerging Infect. Dis. 10, 2005–2011.

Fairweather, D., Rose, N.R., 2004b. Models of coxsackievirus B3-induced myocarditis: recent advances. Drug Discovery Today: Dis. Models 1, 381–386.

Fairweather, D., Rose, N.R., 2005. Inflammatory heart disease: a role for cytokines. Lupus 14, 646–651.

Fairweather, D., Yusung, S., Frisancho(-Kiss), S., Barrett, M., Gatewood, S., Steele, R., Rose, N.R., 2003. IL-12Rβ1 and TLR4 increase IL-1β and IL-18-associated myocarditis and coxsackievirus replication. J. Immunol. 170, 4731–4737.

Fairweather, D., Frisancho-Kiss, S., Yusung, S.A., Barrett, M.A., Gatewood, S.J.L., Davis, S.E., Njoku, D.B., Rose, N.R., 2004a. IFN-γ protects against chronic viral myocarditis by reducing mast cell degranulation, fibrosis, and the profibrotic cytokines TGF-β_1, IL-1β, and IL-4 in the heart. Am. J. Pathol. 165, 1883–1894.

Fairweather, D., Frisancho-Kiss, S., Gatewood, S., Njoku, D., Steele, R., Barrett, M., Rose, N.R., 2004b. Mast cells and innate cytokines are associated with susceptibility to autoimmune heart disease following coxsackievirus B3 infection. Autoimmunity 37, 131–145.

Fairweather, D., Frisancho-Kiss, S., Rose, N.R., 2005a. Viruses as adjuvants for autoimmunity: evidence from coxsackievirus-induced myocarditis. Rev. Med. Virol. 15, 17–27.

Fairweather, D., Frisancho-Kiss, S., Yusung, S.A., Barrett, M.A., Davis, S.E., Steele, R.A., Gatewood, S.J., Rose, N.R., 2005b. IL-12 protects against coxsackievirus B3-induced myocarditis by increasing IFN-γ and macrophage and neutrophil populations in the heart. J. Immunol. 174, 261–269.

Fairweather, D., Frisancho-Kiss, S., Njoku, D.B., Nyland, J.F., Kaya, Z., Yusung, S.A., Davis, S.E., Frisancho, J.A., Barrett, M.A., Rose, N.R., 2006. Complement receptor 1 and 2 deficiency increases coxsackievirus B3-induced myocarditis and heart failure by increasing macrophages, IL-1β and immune complex deposition in the heart. J. Immunol. 176, 3516–3524.

Feery, B.J., 1977. Adverse reactions after smallpox vaccination. Med. J. Aust. 2, 180–183.

Feldman, A.M., McNamara, D., 2000. Myocarditis. N. Engl. J. Med. 343, 1388–1398.

Fox, H.S., Bond, B.L., Parslow, T.G., 1991. Estrogen regulates the IFN-γ promoter. J. Immunol. 146, 4362–4367.

Frustaci, A., Pieroni, M., Chimenti, C., 2006. Immunosuppressive treatment of chronic non-viral myocarditis. Ernst Schering Res. Found. Workshop 55, 343–351.

Guidotti, L.G., Chisari, F.V., 2001. Noncytolytic control of viral infections by the innate and adaptive immune response. Annu. Rev. Immunol. 19, 65–91.

Guthrie, M., Lodge, P.A., Huber, S.A., 1984. Cardiac injury in myocarditis induced by coxsackievirus group B, Type 3 in BALB/c mice is mediated by Lyt 2^+ cytolytic lymphocytes. Cell. Immunol. 88, 558–567.

Halsell, J.S., Riddle, J.R., Atwood, J.E., Gardner, P., Shope, R., Poland, G.A., Gray, G.C., Ostroff, S., Eckart, R.E., Hospenthal, D.R., Gibson, R.L., Grabenstein, J.D., Arness, M.K., Tornberg, D.N., the Department of Defense Smallpox Vacciination Clinical Evaluation Team, 2003. Myopericarditis following smallpox vaccination among vaccinia-naive US military personnel. JAMA 289, 3283–3289.

Hia, C.P., Yip, W.C., Tai, B.C., Quek, S.C., 2004. Immunosuppressive therapy in acute myocarditis: an 18 year systematic review. Arch. Dis. Child. 89, 580–584.

Horwitz, M.S., La Cava, A., Fine, C., Rodriguez, E., Ilic, A., Sarvetnick, N., 2000. Pancreatic expression of interferon-gamma protects mice form lethal coxsackievirus B3 infection and subsequent myocarditis. Nat. Med. 6, 693–697.

Huber, S.A., 2005. Increased susceptibility of male BALB/c mice to coxsackievirus B3-induced myocarditis: role for CD1d. Med. Microbiol. Immunol. 194, 121–127.

Huber, S.A., Lodge, P.A., 1986. Coxsackievirus B3 myocarditis: identification of different pathogenic mechanisms in DBA/2 and BALB/c mice. Am. J. Pathol. 122, 284–291.

Huber, S.A., Pfaeffle, B., 1994. Differential Th1 and Th2 responses in male and female BALB/c mice infected with coxsackievirus group B type 3. J. Virol. 68, 5126–5132.

Huber, S.A., Sartini, D., Exley, M., 2002. $V\gamma4^+$ T cells promote autoimmune CD8+ cytolytic T-lymphocyte activation in coxsackievirus B3-induced myocarditis in mice: role for CD4+ Th1 cells. J. Virol. 76, 10785–10790.

Kanda, T., Tanaka, T., Sekiguchi, K., Seta, Y., Kurimoto, M., Wilson-McManus, J.E., Nagai, R., Yang, D., McManus, B.M., Kobayashi, I., 2000. Effect of interleukin-18 on viral myocarditis: enhancement of interferon-gamma and natural killer cell activity. J. Mol. Cell. Cardiol. 32, 2163–2171.

Kher, A., Wang, M., Tsai, B.M., Pitcher, J.M., Greenbaum, E.S., Nagy, R.D., Patel, K.M., Wairiuko, G.M., Markel, T.A., Meldrum, D.R., 2005. Sex differences in the myocardial inflammatory response to acute injury. Shock 23, 1–10.

Liu, P.Y., Death, A.K., Handelsman, D.J., 2003. Androgens and cardiovascular disease. Endocr. Rev. 24, 313–340.

Liu, C., Chen, J., Liu, K., 2005. Immunosuppressive treatment for inflammatory cardiomyopathy: meta-analysis of randomized controlled trials. Int. Heart J. 46, 113–122.

Mendelsohn, M.E., Karas, R.H., 1999. The protective effects of estrogen on the cardiovascular system. N. Engl. J. Med. 340, 1801–1811.

Mieres, J.H., 2006. Review of the American Heart Association's guidelines for cardiovascular disease prevention in women. Heart 92 (Suppl. III), iii10–iii13.

Nathan, C., 2006. Neutrophils and immunity: challenges and opportunities. Nat. Rev., Immunol. 6, 173–182.

Neumann, D.A., Burek, C.L., Baughman, K.L., Rose, N.R., Herskowitz, A., 1990. Circulating heart-reactive antibodies in patients with myocarditis or cardiomyopathy. J. Am. Coll. Cardiol. 16, 839–846.

Neumann, D.A., Rose, N.R., Ansari, A.A., Herskowitz, A., 1994. Induction of multiple heart autoantibodies in mice with coxsackievirus B3- and cardiac myosin-induced autoimmune myocarditis. J. Immunol. 152, 343–350.

Rose, N.R., 2002. Mechanisms of autoimmunity. Semin. Liver Dis. 22, 387–394.

Rose, N.R., Wolfgram, L.J., Herskowitz, A., Beisel, K.W., 1986. Postinfectious autoimmunity: two distinct phases of coxsackievirus B3-induced myocarditis. Ann. N. Y. Acad. Sci. 475, 146–156.

Rossouw, J.E., Anderson, G.L., Prentice, R.L., LaCroix, A.Z., Kooperberg, C., Stefanick, M.L., Jackson, R.D., Beresford, S.A., Howard, B.V., Johnson, K.C., Kotchen, J.M., Ockene, J., 2002. Risks and benefits of estrogen plus progestin in healthy postmenopausal women: principal results from the Women's Health Initiative randomized controlled trial. J. Am. Med. Assoc. 288, 321–333.

Schoub, B.D., Johnson, S., McAnerney, J.M., Dos Santos, I.L., Klaassen, K.I., 1985. Epidemic Coxsackie B virus infection in Johannesburg. South Africa J. Hyg. (Lond) 95, 447–455.

Schuster, J.P, Schaub, G.A., 2001. Experimental Chagas disease: the influence of sex and psychoneuroimmunological factors. Parasitol. Res. 87, 994–1000.

Styrt, B., Sugarman, B., 1991. Estrogens and infection. Rev. Infect. Dis. 13, 1139–1150.

Suzuki, N., Chen, N.-J., Millar, D.G., Suzuki, S., Horacek, T., Hara, H., Bouchard, D., Nakanishi, K., Penninger, J.M., Ohashi, P.S., Yeh, W.-C., 2003. IL-1 receptor-associated kinase 4 is essential for IL-18-mediated NK and Th1 cell responses. J. Immunol. 170, 4031–4035.

Watford, W.T., Hissong, B.D., Bream, J.H., Kanno, Y., Muul, L., O'Shea, J.J., 2004. Signaling by IL-12 and IL-23 and the immunoregulatory roles of STAT4. Immunol. Rev. 202, 139–156.

Wong, C.Y., Woodruff, J.J., Woodruff, J.F., 1977. Generation of cytotoxic T lymphocytes during coxsackievirus B-3 infection. III. Role of sex. J. Immunol. 119, 591–597.

Yusuf, S., Reddy, S., Ounpuu, S., Anand, S., 2001. Global burden of cardiovascular diseases: part i: general considerations, the epidemiologic transition, risk factors, and impact of urbanization. Circulation 104, 2746–2753.