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IL-12 Protects against Coxsackievirus B3-Induced Myocarditis by Increasing IFN-γ and Macrophage and Neutrophil Populations in the Heart

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Th1-type immune responses, mediated by IL-12-induced IFN-γ, are believed to exacerbate certain autoimmune diseases. We recently found that signaling via IL-12Rβ1 increases coxsackievirus B3 (CVB3)-induced myocarditis. In this study, we examined the role of IL-12 on the development of CVB3-induced myocarditis using mice deficient in IL-12p35 that lack IL-12p70. We found that IL-12 deficiency did not prevent myocarditis, but viral replication was significantly increased. Although there were no changes in the total percentage of inflammatory cells in IL-12-deficient hearts compared with wild-type BALB/c controls by FACS analysis, macrophage and neutrophil populations were decreased. This decrease corresponded to reduced TNF-α and IFN-γ levels in the heart, suggesting that macrophage and/or neutrophil populations may be a primary source of TNF-α and IFN-γ during acute CVB3 myocarditis. Increased viral replication in IL-12-deficient mice was not mediated by reduced TNFRp55 signaling, because viral replication was unaltered in TNFRp55-deficient mice. However, STAT4 or IFN-γ deficiency resulted in significantly increased viral replication and significantly reduced TNF-α and IFN-γ levels in the heart, similar to IL-12 deficiency, indicating that the IL-12/STAT4 pathway of IFN-γ production is important in limiting CVB3 replication. Furthermore, STAT4 or IFN-γ deficiency also increased chronic CVB3 myocarditis, indicating that therapeutic strategies aimed at reducing Th1-mediated autoimmune diseases may exacerbate common viral infections such as CVB3 and increase chronic inflammatory heart disease. The Journal of Immunology, 2005, 174: 261–269.

Viral myocarditis is a frequent cause of sudden death in young adults and can progress to chronic dilated cardiomyopathy (DCM), a major cause of heart failure associated with an autoimmune response (1, 2). Although most people recover from acute viral myocarditis, susceptible individuals develop chronic myocarditis and DCM. Coxsackievirus B3 (CVB3) infection of susceptible mice results in a disease pattern similar to that observed in humans. Acute myocarditis occurs from days 7 to 14 postinfection (p.i.) in all strains of mice, while susceptible mice (100% prevalence) progress to a chronic phase of disease from day 28 to at least 58 p.i. that is associated with the development of DCM (3). Susceptible BALB/c mice respond to CVB3 infection with elevated levels of TNF-α, IL-1β, and IL-12p70 in the heart during the innate response (4). We have shown that increased IL-1β and IL-18 levels in the heart correlate with increased acute CVB3 myocarditis, and are mediated by TLR4 and IL-12Rβ1 signaling (5). Recently, CD4+ Th1 cells were shown to promote CVB3-induced myocarditis in susceptible BALB/c mice by IFN-γ-mediated mechanisms (6). These findings suggest that IL-12-mediated Th1 responses increase myocarditis. This study further examines the role of IL-12 on the development of CVB3-induced myocarditis.

IL-12 is potent at inducing IFN-γ production from NK and T cells and promotes the differentiation of T cells to a Th1 phenotype (7). Th1-mediated immune responses have been implicated in a number of autoimmune diseases, including some forms of colitis, type I diabetes, multiple sclerosis, rheumatoid arthritis, and myocarditis (6, 8, 9). IL-12 is a heterodimer composed of IL-12p35 and IL-12p40 subunits and is secreted as a biologically active IL-12p70 molecule primarily by macrophages, neutrophils, and dendritic cells. Signaling by IL-12 requires coexpression of the IL-12Rβ1 and IL-12Rβ2 chains for high affinity IL-12p70 binding and maximal IFN-γ production. In the mouse, IL-12R signaling activates STAT1, 3, 4, and 5, with STAT4 believed to be responsible for most of the biological activities of IL-12p70 through the production of IFN-γ (7, 10, 11). Similar to IL-12, IL-23 activates STAT1, 3, and 4; induces IFN-γ production; and exacerbates autoimmune disease (12–15). However, the role of IL-12 and IL-23 is functionally distinct, with IL-12 preferentially affecting naïve T cells, while IL-23 induces proliferation of memory T cells (7).

IL-12 is also required for resistance to certain bacterial, viral, and intracellular parasitic infections (16–19). IL-12 exerts its influence over many infectious agents by stimulating the production of TNF-α and IFN-γ, which act synergistically to control infections by recruiting and activating macrophages, NK cells, and T cells to perform their effector functions (17, 20–23). IL-12-induced IFN-γ appears to act on different effector cells in different viral infections. For example, CD8 CTLs have been shown to reduce lymphocytic choriomeningitis virus (LCMV) infection (18),...
while NK cells play a major role in defense against murine cytomegalovirus infection (3, 24). Although clearance of viral infections is usually thought to be mediated by destruction of infected cells by effector immune cells, it is now becoming clear that release of IFNs and TNF at the site of infection is also important in purging virus from infected cells noncytopathically (22, 25, 26). Cytokines can also control viral infections indirectly by modulating the immune response and by up-regulating Ag processing and display of viral epitopes on the surface of infected cells (22). However, the role of IL-12 in the development of CVB3-induced myocarditis has not been previously investigated.

In the present study, we examine the role of IL-12 on the development of CVB3-induced myocarditis using mice deficient in IL-12p35, STAT4, or IFN-γ. We found that IL-12 deficiency did not prevent the development of acute myocarditis, but allowed a significant increase in viral replication in the heart. STAT4 or IFN-γ deficiency also resulted in significantly increased levels of viral replication, indicating that IFN-γ produced by IL-12 and STAT4 transcription is important in reducing CVB3 levels in the heart. IL-12- or IFN-γ-deficient hearts had reduced levels of TNF-α and IFN-γ during acute CVB3 myocarditis, and reduced numbers of macrophages and neutrophils. However, increased viral replication was not mediated by TNFRp55 signaling, because viral replication was unaltered in TNFRp55-deficient mice. These results demonstrate that the IL-12/STAT4 pathway of IFN-γ production is important in limiting CVB3 replication during acute myocarditis, but does not exacerbate myocarditis. Furthermore, STAT4 or IFN-γ was found to protect against the development of chronic CVB3 myocarditis, indicating that therapeutic strategies aimed at reducing Th1-mediated autoimmune diseases may exacerbate common viral infections such as CVB3 and increase chronic inflammatory heart disease.

Materials and Methods

Mice

IL-12p35−/−, STAT4−/−, and IFN-γ−/− deficient mice and wild-type BALB/c (BALB/c) controls were obtained from The Jackson Laboratory. We generated TNFRp55−/− deficient mice on a BALB/c genetic background by backcrossing C57BL/6 TNFRp55−/− deficient mice to susceptible BALB/c mice for 10 generations. TNFRp55−/− deficient mice on a C57BL/6 genetic background were a gift from T. Mak (University of Toronto, Toronto, Canada) (27). TNFRp55 heterozygotes were interbred to generate wild-type and TNFRp55 homozygotes in the Johns Hopkins Animal Facility. Mice were confirmed as deficient for TNFRp55 using a previously published PCR protocol (27). All mice used in these studies were on a BALB/c genetic background. All mice were maintained under specific pathogen-free conditions, and approval was obtained from the Animal Care and Use Committee of the Johns Hopkins University for all procedures.

Myocarditis

Individual experiments were conducted at least three times with 7–10 mice per group. Mice, 6–8 wk of age, were inoculated i.p. with a heart-passaged stock of CVB3 (Nancy strain) originally obtained from the American Type Culture Collection (ATCC). CVB3 was diluted in sterile saline; 103 PFU was injected i.p. on day 0, and tissues were collected on day 12 (acute myocarditis) or day 35 (chronic myocarditis) p.i. This model of myocarditis is subacute, and thus no deaths occurred during the acute or chronic phase of disease (data not shown). Mice inoculated i.p. with PBS or uninfected heart homogenate did not develop acute or chronic myocarditis (data not shown). Hearts were cut longitudinally, fixed in 10% phosphate-buffered formalin, and embedded in paraffin. Sections 5-μm thick were cut at various depths in the section and stained with H&E. 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 with the overall size of the heart section, with the aid of a microscope eyepiece grid.

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 ELISAs or plaque assays. Cytokines were measured in heart supernatants using Quantikine cytokine ELISA kits purchased from R&D Systems, according to manufacturer’s instructions. The limits of detection for the cytokine kits were as follows: TNF-α, 5.1 pg/ml; IL-1β, 3 pg/ml; IL-12, 2.5 pg/ml; IL-18, 25 pg/ml; and IFN-γ, 2 pg/ml. Cytokines were below detectable levels in the 2% MEM used to homogenize samples (data not shown). Cytokines were expressed as pg/g heart tissue ± SEM.

Plaque assay

The level of infectious virus was determined in individual homogenates by plaque assay according to standard procedures (5, 28). Samples were processed in the same manner as for cytokine analysis. Dilutions of tissue supernatants were incubated on confluent Vero cell (ATCC) monolayers for 1 h at 37°C and 5% CO2 to allow viral attachment, and then incubated for 3 days to allow plaque formation. Virus titers were expressed as the mean PFU/g tissue ± SEM, and the limit of detection was 10 PFU/g tissue.

Heart digestion and FACS analysis

The mice were anesthetized, the chest cavity was opened, and the root of the aorta was exposed and cannulated with a 27-gauge needle connected to a DynaMax peristaltic pump (Rainin Instrument). The heart was perfused at a constant flow of 14 ml/min with cold PBS (Biofluids) for 2 min, and then digested with collagenase II (1 mg/ml; Sigma-Aldrich) and protease XIV (0.5 mg/ml; Sigma-Aldrich) in PBS for 7 min at 37°C (29). The heart was then removed from the chest cavity, and single cell separation was completed using razor blades to dislodge immune cells from the tissue.

Individual cell suspensions from seven mice were pooled by group, and leukocytes were separated from heart cells using anti-CD45 paramagnetic beads (30F11.1; Miltenyi Biotec) on a magnetic column (Miltenyi Biotec). Leukocytes were stained with the following mAbs (BD Pharmingen) diluted in 1% PBS (Invitrogen Life Technologies) in PBS: FITC anti-CD3 (total T cells, clone 17A2), PE anti-CD4 (Th cells, GK1.5), CyChrome anti-CD8 (T cells, clone 53-6.7), CyChrome anti-CD49b (DX5, NK, and NKT cells), and PE anti-Gr1 (granulocytes, clone RB6-8C5). FITC anti-F4/80 to measure macrophages was purchased from eBioscience (clone BM8). Cell fluorescence was measured using a FACS Calibur flow cytometer (BD Biosciences), and data were analyzed using CellQuest software (BD Biosciences). The same results were obtained in three separate experiments.

Statistical analysis

Normally distributed data were analyzed by Student’s t test; otherwise, the Mann-Whitney U test was used. Test values with a p < 0.05 were considered significantly different from control values. *, p < 0.05; **, p < 0.01; ***, p < 0.001.

Results

IL-12p35−/− deficient mice develop acute myocarditis similar to wild-type BALB/c mice

CVB3-induced myocarditis in BALB/c mice is believed to be mediated by pathogenic mechanisms involving a Th1 response (6). Previously, we found that signaling via IL-12Rβ1 increases both inflammation following CVB3 infection in BALB/c mice and is similar to the acute myocarditis observed at day 21 in the EAM model (3, 5, 30). We found, by histological examination, that IL-12 deficiency did not reduce the level of inflammation in the heart at this time point compared with wild-type BALB/c controls (Fig. 1). Thus, although IL-12Rβ1 signaling increases acute CVB3 myocarditis, IL-12 is not required for the development of myocardial inflammation.
IL-12p35 deficiency increases viral replication in the heart

Because virus infection initiates myocarditis (31), we were interested in the effect of IL-12p35 deficiency on viral replication in the heart during acute CVB3 myocarditis. We found that IL-12p35 deficiency (or the lack of IL-12p70) significantly increased the amount of infectious virus in the heart compared with wild-type BALB/c controls at day 12 p.i. (Fig. 2). Previously, we showed a strong correlation between the level of inflammation during acute CVB3 myocarditis and IL-12/H9252/IL-18 levels in the heart (r = 0.80) (5). When the severity of inflammation (Fig. 1) was compared with the level of infectious virus (Fig. 2) in this study, we found no significant correlation in either mouse strain (wild-type BALB/c, r = 0.20; IL-12p35−/−, r = 0.16). Because increased infectious virus in the heart did not directly result in increased myocardial inflammation, factors other than viral replication are decisive in attracting inflammatory cells to the heart (5). These results show that IL-12 reduces CVB3 replication in the heart during acute myocarditis.

IL-12p35 deficiency reduces macrophage and neutrophil populations in the heart

To understand the mechanisms involved in the increase in CVB3 replication in the hearts of IL-12p35-deficient mice, we examined specific cell populations in the inflammatory infiltrate of IL-12p35-deficient mice to determine whether they were altered following infection. To investigate this question, we isolated immune cells (contained in the CD45+/H11001/fraction) from the hearts of IL-12p35-deficient mice and compared the relative proportions of macrophages (F4/80), neutrophils (Gr1 low), eosinophils (Gr1 high), NK cells (DX5), Th cells (CD4), CTLs (CD8), and B cells (B220) with wild-type BALB/c controls at day 12 p.i. (Fig. 3). We found that the total numbers of CD45+ cells in the heart at day 12 were similar between IL-12p35-deficient mice and wild-type controls, confirming the histological analysis of the percentage of inflammation in the heart (see Fig. 1). However, the inflammatory infiltrate in the heart of IL-12p35-deficient mice consistently contained ~12% fewer macrophages and ~10% fewer neutrophils than CVB3-infected wild-type BALB/c mice (Fig. 3). All other cell populations were unaffected.

FIGURE 1. IL-12p35 deficiency does not prevent acute CVB3 myocarditis. Mice deficient in IL-12p35 (IL-12p35−/−) (A and C) were compared with wild-type BALB/c (A and B) controls for the development of myocarditis. Mice received 103 PFU of CVB3 i.p. on day 0, and hearts were collected on day 12 p.i. Myocarditis was assessed as the percentage of the heart section with inflammation compared with the overall size of the heart section stained with H&E, with the aid of a microscope eyepiece grid. Individual experiments were conducted three times with seven mice per group, with one representative heart shown for each group (B and C; original magnification ×400). Data are presented as the mean ± SEM.

FIGURE 2. IL-12p35 deficiency increases CVB3 replication in the heart. Mice deficient in IL-12p35 (IL-12p35−/−) were compared with wild-type BALB/c controls for the level of viral replication in the heart 12 days after CB3 infection. Mice received 103 PFU of CVB3 i.p. on day 0, and hearts were collected on day 12 p.i. for analysis by plaque assay. Data are presented as the mean ± SEM of seven mice per group from one experiment of three. **, p < 0.01.

FIGURE 3. Macrophage and neutrophil populations decrease in IL-12p35-deficient hearts. The composition of the inflammatory infiltrate during acute CVB3 myocarditis was compared between wild-type BALB/c mice and mice deficient in IL-12p35 (IL-12p35−/−). Mice received 103 PFU of CVB3 i.p. on day 0, and CD45+ immune cells were isolated from the heart on day 12 p.i. by enzymatic digestion. Individual cell types were separated using magnetic beads, and the percentage of cells was analyzed by FACS. The relative proportion of each of the following cell types was evaluated: macrophages (F4/80), neutrophils (NEU; Gr1 low), eosinophils (EOS; Gr1 high), NK cells (DX5), Th cells (CD4), CTLs (CD8), and B cells (B220). Similar results were obtained in three separate experiments using seven mice per group. Data are presented as the mean ± SEM of three experiments.
populations that we examined either remained the same (EOS and DX5) or increased in number in the heart infiltrate (Fig. 3). Overall, the percentage of inflammatory cells in the heart remained similar between the two strains. Our results suggest that in the absence of IL-12, fewer macrophages and neutrophils are recruited to the heart following CVB3 infection. Because neutrophils and macrophages are important in reducing viral replication (22), reduced numbers of these cells could result in increased viral replication in the heart. Thus, IL-12 is important in inducing and/or maintaining appropriate macrophage and neutrophil function following CVB3 infection.

**IL-12p35 deficiency reduces TNF-α and IFN-γ levels in the heart**

We previously reported that IL-12Rβ1-deficient BALB/c mice have significantly reduced acute myocarditis that correlates with reduced levels of IL-1β and IL-18 in the heart following CVB3 infection (5). However, TNF-α, IL-12, and IFN-γ levels are not significantly altered by IL-12Rβ1 signaling (5). In this study, we examined the levels of TNF-α, IL-1β, IL-12, IL-18, and IFN-γ in the hearts of IL-12p35-deficient and wild-type BALB/c control mice that had been infected i.p. with 10^7 PFU of CVB3 12 days earlier. We found that IL-12p35-deficient mice had significantly decreased levels of TNF-α, IL-12, and IFN-γ in the heart compared with wild-type controls (Fig. 4). Significant changes in IL-1β and IL-18 levels were not observed in IL-12p35-deficient mice (Fig. 4). These findings were in stark contrast to those obtained in IL-12Rβ1-deficient hearts (5). Thus, the effect of IL-12 on cytokine production in the heart following CVB3 infection operates primarily via a signaling pathway other than IL-12Rβ1. Because IL-12Rβ1 binds the IL-12p40 component of IL-12 or IL-23 and IL-12Rβ2 binds only IL-12p35 (7), our findings suggest that IL-23 (or another IL-12p40-related cytokine) leads to increased IL-1β and IL-18 levels (5), while IL-12 increases TNF-α and IFN-γ in the heart during acute CVB3 myocarditis. Because IL-12Rβ2-deficient mice on a BALB/c genetic background are not currently available, we are unable to determine whether the IL-12p35 effect on TNF-α and IFN-γ occurs via IL-12Rβ2 signaling. In preliminary results, we have detected IL-23 transcripts by RT-PCR in the hearts of CVB3-infected mice at day 12 p.i. (S. Fri-sancho-Kiss and D. Fairweather, unpublished observations). We are currently investigating the role of IL-23 on the pathogenesis of CVB3-induced myocarditis. Thus, IL-12 significantly alters the production of TNF-α and IFN-γ in the heart during acute CVB3 myocarditis.

**TNFrp55 deficiency does not increase viral replication or myocarditis**

Several clinical studies have found increased TNF-α levels in the sera of patients with congestive heart failure (32–34). We also found that increased TNF-α levels in the heart during the innate response to viral infection are associated with the development of chronic myocarditis (4, 35–37). In contrast, TNF-α has been found to play a critical role in the control of certain viral infections such as murine cytomegalovirus (38, 39), but not for other viruses such as LCMV (40). However, the role of TNF on the replication of CVB3 during acute myocarditis is not known. Because IL-12 deficiency led to significantly decreased levels of TNF-α in the heart during acute CVB3 myocarditis (Fig. 4), we wanted to determine whether reduced TNF signaling was responsible for the increase in viral replication (see Fig. 2). The biological activities of TNF are mediated through two distinct receptors, TNFRp55 and TNFRp75, that are expressed on many cell types (41). Most of the immune responses classically associated with TNF function are mediated by TNFRp55 (41). We backcrossed TNFRp55-deficient C57BL/6 mice to a susceptible BALB/c genetic background for 10 generations. BALB/c mice homozygous for TNFRp55 deficiency (TNFRp55−/−) were infected i.p. with 10^7 PFU of CVB3 and compared with wild-type BALB/c littermates for the level of myocarditis and viral replication in the heart. We found that TNFRp55 deficiency did not significantly alter the level of viral replication in the heart at day 12 p.i. (Fig. 5A). Furthermore, TNFRp55 deficiency did not significantly reduce the level of inflammation in the heart during acute myocarditis (Fig. 5B), similar to the results obtained with IL-12-deficient mice (Fig. 1). These results show that the increased viral replication observed in IL-12p35-deficient hearts was not due to a requirement for TNFRp55 signaling. However, we did not examine the effect of TNFRp75 signaling on CVB3 viral replication or myocarditis because this strain is not yet available on a BALB/c background. Thus, the actions of TNF-α that are mediated by TNFRp55 signaling do not directly influence viral replication or inflammation during acute CVB3 myocarditis in BALB/c mice.

**STAT4 or IFN-γ deficiency increases viral replication without increasing myocarditis**

Because the biological activities of IL-12 influencing IFN-γ production are primarily mediated via STAT4 (7, 10, 11), we were interested in determining whether STAT4 was also involved in protecting mice from CVB3 infection. We previously found that IFN-γ deficiency did not alter acute myocarditis after CVB3 infection, but significantly increased viral replication in the heart at day 12 p.i (5), similar to the effect of IL-12p35 deficiency reported in this study (see Figs. 1 and 2). In this study, we further evaluated the role of STAT4 and IFN-γ on the development of acute myocarditis following CVB3 infection using STAT4−/− and IFN-γ−deficient mice. We found that STAT4 or IFN-γ deficiency significantly increased viral replication in the heart 12 days after CVB3 infection compared with BALB/c controls (Fig. 6A), but that myocarditis was not significantly altered (Fig. 6B). These results were similar to the effects on myocarditis and viral replication observed in IL-12p35-deficient mice (Figs. 1 and 2). These findings indicate that activation of STAT4 transcription is important for IFN-γ-
mediated reduction of viral replication in the heart during acute CVB3-induced myocarditis.

STAT4 or IFN-γ deficiency reduces TNF-α and IFN-γ in the heart

Because TNF-α and IFN-γ were significantly reduced in IL-12p35-deficient hearts (Fig. 4) and associated with increased viral replication (Fig. 2), we next determined the cytokine environment in STAT4- and IFN-γ-deficient hearts following CVB3 infection. Mice were infected with 10^3 PFU of CB3 i.p., and hearts were analyzed 12 days later for TNF-α, IL-1β, IL-12, IL-18, or IFN-γ levels, as before. STAT4 (Fig. 7A) or IFN-γ (Fig. 7B) deficiency significantly reduced the level of TNF-α and IFN-γ in the heart, like IL-12p35-deficient mice (see Fig. 4). In addition, STAT4-deficient mice had significantly lower levels of IL-12 (Fig 7A), similar to IL-12p35-deficient mice (Fig. 4). IL-1β was somewhat reduced in IFN-γ-deficient hearts at day 12 p.i., but not significantly (p = 0.06) (Fig. 7B). Thus, the significant reduction in TNF-α and IFN-γ in IL-12p35-, STAT4-, and IFN-γ-deficient hearts further supports the view that this cytokine pathway is involved in reducing viral replication in the heart during acute CVB3 myocarditis.

IFN-γ deficiency results in reduced macrophages and neutrophils in the heart

To determine whether reduced IFN-γ levels were responsible for the reduced macrophages and neutrophils we observed in the hearts of IL-12p35-deficient mice (see Fig. 4), we examined the percentages of individual cell populations within the infiltrate of IFN-γ-deficient hearts, as before. We found that macrophage and neutrophil populations were decreased in IFN-γ-deficient hearts at day 12 p.i. (data not shown), similar to IL-12p35-deficient mice (see Fig. 3). The primary difference between mice deficient in IL-12p35 or IFN-γ compared with wild-type BALB/c mice was that IFN-γ-deficient mice had increased numbers of eosinophils in the acute infiltrate (wild-type BALB/c 4.4% compared with IFN-γ-deficient mice with 12.7%). The increase in eosinophils may reflect the skewing of the immune response to an IL-4-mediated Th2 phenotype, which is known to occur in the absence of IFN-γ (42, 43). Thus, IL-12-induced IFN-γ results in increased numbers of macrophages and neutrophils in the heart during acute CVB3-induced myocarditis.
STAT4 or IFN-γ deficiency increases chronic, autoimmune CVB3-induced myocarditis

Because we had established that STAT4-induced IFN-γ production in the heart was important in reducing CVB3 replication during acute myocarditis, we next investigated the effect of this pathway on the development of the chronic, autoimmune phase of disease. We have previously shown that IFN-γ protects against the development of EAM by reducing activated T cells (30). Recently, we found that IFN-γ also protects against the development of chronic CVB3 myocarditis by reducing inflammation, fibrosis, and the profibrotic cytokines TGF-β1, IL-1β, and IL-4 in the heart (44, 45). In contrast, IL-12 signaling does not influence the development of chronic CVB3 myocarditis (44). Because we have not previously examined the role of STAT4 on the development of chronic myocarditis, in this study we infected STAT4- or IFN-γ-deficient mice with 10^5 PFU of CVB3 i.p. and analyzed hearts for the development of myocarditis at day 35 p.i., during the peak of chronic disease (3). CVB3 is cleared from the heart by day 14 p.i. and does not reactivate due to the lack of IFN-γ throughout the chronic phase of disease (day 21 through 35 p.i.) (44). We found that STAT4 or IFN-γ deficiency resulted in significantly increased levels of inflammation in the heart at day 35 p.i. (Fig. 8). Thus, not only does the lack of STAT4-induced IFN-γ protect against CVB3 replication during acute myocarditis, but this signaling pathway also reduces the severity of chronic inflammatory heart disease. Thus, strategies aimed at reducing IFN-γ-induced Th1 responses to prevent autoimmune diseases may actually aggravate viral replication and chronic myocarditis.

Discussion

IL-12 is believed to exacerbate many autoimmune diseases by increasing IFN-γ levels and Th1 responses. In this study, IL-12 deficiency reduced IFN-γ levels in the heart during acute viral CVB3 myocarditis. However, myocardial inflammation was not decreased, suggesting that IL-12 is not necessary for the development of acute myocarditis. Analysis of the cellular composition of the infiltrate revealed that macrophage and neutrophil populations were decreased. These populations were also decreased in IFN-γ-deficient hearts during acute CVB3 myocarditis, indicating that IL-12 may influence both viral replication and inflammation via IFN-γ. Thus, IL-12-induced IFN-γ is important in inducing and/or maintaining macrophage and neutrophil function in the heart during acute CVB3 myocarditis. Reduction of these cell populations has important ramifications for the development of myocarditis due to their role in preventing viral replication. Indeed, mice deficient in IL-12, STAT4, or IFN-γ had significantly increased levels of CVB3 in the heart during acute myocarditis compared with wild-type controls. We conclude that IL-12/STAT4-mediated IFN-γ production increases macrophage and neutrophil numbers in the heart and the ability to reduce viral replication during acute CVB3 myocarditis (Fig. 9).

Studies examining the role of IL-12 on the development of autoimmune immunity have provided conflicting reports (15, 30, 46–50). IL-12-induced/IFN-γ-mediated Th1 responses have been shown to be necessary for the development of many autoimmune diseases (6–9, 50–53). With the recent understanding that IL-12 is part of a family of heterodimeric cytokines that include IL-23 and IL-27, a dichotomy between IL-12Rβ1 and IL-12Rβ2 signaling has emerged (5, 7, 15, 49). Previous studies in experimental animals that concluded that IL-12 exacerbates autoimmune disease, using
neutralizing Abs or genetically deficient mice for the various components of IL-12 or IL-12R signaling, need to be reinterpreted in light of our current understanding of the divergent roles of these cytokines (54, 55). For example, IL-23, which signals via IL-12Rβ1, stimulates a strong proinflammatory response that has been shown to exacerbate experimental autoimmune encephalomyelitis (12–15). Likewise, we have found that signaling via IL-12Rβ1 increases both EAM- and CVB3-induced acute myocarditis (5, 30, 45). In this study, we found that IL-12 did not exacerbate acute CVB3 myocarditis. That is, although certain immune cell populations were increased in the heart, there was no overall increase in inflammation (Figs. 4 and 8). It is likely that IL-12 influences macrophage and neutrophil populations via IFN-γ because these populations were also reduced in IFN-γ-deficient mice. Thus, this study further separates the role of IL-12 from IL-12Rβ1 signaling, showing that IL-12 protects against CVB3 replication, but does not increase the severity of acute myocarditis.

Not only are there conflicting reports on the effect of IL-12 on the development of autoimmune disease, but the role of IFN-γ has also been controversial. After investigating the role of IFN-γ in experimental models of autoimmune disease, many researchers, including us, have found a protective role for IFN-γ in exacerbating autoimmune diseases may be explained, at least in part, by the fact that proinflammatory responses involving TNF-α and/or IL-1β often lead to greater IFN-γ production (22, 61–63). Rather than reducing CVB3 infection, TNF may be more important in inducing apoptosis of lymphocytes, thereby preventing a chronic accumulation of inflammatory cells in the heart. We have not yet examined the effect of TNFRp55 deficiency on the development of chronic CVB3 myocarditis.

It is not surprising that IFN-γ protects against viral infections. IFNs, including IFN-α, IFN-β, and IFN-γ, are essential for effective clearance of many viral infections (21, 22, 25, 26, 40, 64). IFN-γ reduces viral replication directly, by inhibiting protein synthesis and degrading RNA (65, 66), and indirectly, by inducing NO production and apoptosis (67, 68). TNF-α is also an important antiviral provider, acting as a primary function by reducing immune cell activation and chronic inflammation (30, 44, 45, 57, 60). The apparent role of IFN-γ in exacerbating autoimmune diseases may be explained, at least in part, by the fact that proinflammatory responses involving TNF-α and/or IL-1β often lead to greater IFN-γ production (22, 61–63). Rather than reducing CVB3 infection, TNF may be more important in inducing apoptosis of lymphocytes, thereby preventing a chronic accumulation of inflammatory cells in the heart. We have not yet examined the effect of TNFRp55 deficiency on the development of chronic CVB3 myocarditis.

The results of this study highlight the difficulty in delineating the contribution of inflammation and viral replication to the development of viral myocarditis. Clinicians struggle with determining whether patient treatment should be aimed at reducing viral replication in the heart or reducing inflammation and proinflammatory cytokines. In our model of CVB3-induced myocarditis, immune mechanisms appear to be more important than viral replication in determining the development of chronic myocarditis (5, 31, 35, 36, 44, 45). In this study, we demonstrate for the first time that the IL-12/STAT4-induced IFN-γ pathway is not responsible for acute CVB3-induced myocarditis, because mice deficient in this pathway developed myocarditis at the same level as wild-type controls.
But rather, elevated levels of the proinflammatory cytokines TNF-α and IL-1β are associated with susceptibility to disease (4, 5, 31), and can induce disease in resistant strains of mice (35, 36). Because treatments to reduce Th1 responses in patients with autoimmune diseases are being considered (52), it is important to clarify the role of IL-12 and IFN-γ in viral myocarditis. The possibility exists that reduction of Th1 responses may exacerbate common viral infections such as CVB3 and increase chronic inflammatory heart disease.

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