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IL-12 Receptor β1 and Toll-Like Receptor 4 Increase IL-1β- and IL-18-Associated Myocarditis and Coxsackievirus Replication[1]

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Th1-type immune responses, mediated by IL-12-induced IFN-γ, protect the host from most viral infections. To investigate the role of IL-12 and IFN-γ on the development of Coxsackievirus B3 (CB3)-induced myocarditis, we examined the level of inflammation, viral replication, and cytokine production in IL-12Rβ1- and IFN-γ-deficient mice following CB3 infection. We report that IL-12Rβ1 deficiency results in decreased viral replication and inflammation in the heart, while IFN-γ deficiency exacerbates CB3 replication. Importantly, decreased IL-1β and IL-18 levels in IL-12Rβ1-deficient hearts correlated directly with decreased myocardial inflammation. Because IL-1β and IL-18 were associated with myocardial inflammation, we examined the effect of TLR4 deficiency on CB3 infection and myocarditis. We found that TLR4-deficient mice also had significantly reduced levels of myocarditis, viral replication, and IL-1β/IL-18, just as we had observed in IL-12Rβ1-deficient mice. This is the first report that TLR4 influences CB3 replication. These results show that IL-12Rβ1 and TLR4 exacerbate CB3 infection and myocarditis while IFN-γ protects against viral replication. The remarkable similarities between the effects of IL-12Rβ1 and TLR4 suggest that these receptors share common downstream pathways that directly influence IL-1β and IL-18 production, and confirm that IL-1β and IL-18 play a significant role in the pathogenesis of CB3-induced myocarditis. These findings have important implications not only for the pathogenesis of myocarditis, but for other autoimmune diseases triggered by viral infections. ***The Journal of Immunology*****, 2003, 170: 4731–4737.**

Interleukin-12 is produced by phagocytic cells and APCs in response to intracellular infection by bacteria and viruses. IL-12 produced during the early phase of an infection promotes the differentiation of T cells to a Th1 phenotype with IFN-γ production, which in turn supports cell-mediated immunity, cytotoxic T cell generation, activation of phagocytic cells, and eventual eradication of intracellular pathogens (1). IL-12 is a heterodimer composed of IL-12p35 and IL-12p40 subunits bound via disulfide bonds and secreted as a biologically active IL-12p70 molecule. IL-12Rs are primarily expressed on activated NK and T cells, and signaling requires coexpression of the IL-12Rβ1 and IL-12Rβ2 chains for the generation of high affinity IL-12p70 binding and maximal IFN-γ production. In the mouse, IL-12R signaling activates STAT1, STAT3, and STAT4, with STAT4 being responsible, through the production of IFN-γ, for most of the biological activities of IL-12 (2, 3).

Recently, IL-18 has emerged as an important cytokine along with IL-12 for increasing IFN-γ production from immune cells. The synergistic effect is mediated through the induction of IL-18Rα on naive T cells by IL-12 and the up-regulation of IL-12Rβ2 by IL-18 (4). In contrast, NK cells constitutively express IL-18R and IL-12Rβ2 and are able to immediately respond to these cytokines (5). The IL-18R has recently been identified as a member of the IL-1R/Toll-like receptor (TLR)[3] superfamily with a signal transduction pathway that is the same as the IL-1R (6). Importantly, IL-18 can also stimulate IFN-γ production through STAT-independent pathways (5, 6). IL-18 is produced by a wide range of immune and nonimmune cells as a biologically inactive precursor that is activated in the same manner as IL-1 by cleavage with caspase-1. Likewise, caspase-1 undergoes proteolytic cleavage to produce its active form after stimulation through TLR4 (7). Recently, respiratory syncytial virus (RSV) was discovered to be a ligand for TLR4, along with LPS, suggesting that activation of TLR may be involved in protecting the host from viral infections (8).

Many cells in the body respond to viral infections by rapidly secreting IFN-α/β, which prevents virus infection and replication. Likewise, IFN-γ released from CTL and NK cells during a Th1-mediated response is also important in purging virus from infected tissues (9). IFNs have been found to be an important defense against Coxsackievirus infections, where they inhibit viral replication in the pancreas and heart, preventing the development of diabetes and myocarditis, respectively (10, 11). Viral myocarditis is frequently the cause of sudden death in young adults, and has been linked to the development of chronic dilated cardiomyopathy (12, 13). Coxsackievirus B3 (CB3) infection of susceptible BALB/c mice results in a disease similar to the clinical heart disease observed in humans, with the development of acute myocarditis from days 7–14 postinfection (p.i.) that later progresses to a chronic, autoimmune phase of disease from day 28 to at least 56 p.i. (12). NK cells and macrophages are abundant in the inflammatory infiltrate during acute CB3 infection (14), where they are believed to clear virus by CTL activity, perforin release, and stimulation of adaptive immunity via IFN-γ production. However,

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[3] Abbreviations used in this paper: TLR, Toll-like receptor; CB3, Coxsackievirus B3; MCMV, murine CMV; p.i., postinfection; RSV, respiratory syncytial virus.

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Th1-mediated immune responses can also cause immunopathology, and have been implicated in a number of autoimmune diseases, such as colitis, type I diabetes, multiple sclerosis, and rheumatoid arthritis (2). Although IFNs are known to protect against CB3 infections, the role of a Th1-mediated immune response and particularly the role of IL-12 and IFN-γ on the development of CB3-induced myocarditis have not been previously investigated.

In this study, we examined the role of IL-12 on the development of acute CB3-induced myocarditis using IL-12Rβ1-deficient mice. We found that decreased levels of IL-1β and IL-18 in the heart following CB3 infection of IL-12Rβ1- or TLR4-deficient mice were directly associated with decreased myocardial inflammation. Unexpectedly, deficiency of either of these two receptors also decreased viral replication in the heart. These results suggest that IL-12Rβ1 and TLR4 share common downstream pathways that directly influence IL-1β and IL-18 production and viral replication. Examination of IFN-γ-deficient mice confirmed that IFN-γ protects against viral replication in the heart. However, IFN-γ deficiency did not affect myocardial inflammation, indicating that the role of the IL-12R in exacerbating acute myocarditis is not via IFN-γ production. Thus, we present in this study novel findings that CB3 stimulates TLR4 and IL-12Rβ1, resulting in increased inflammation, IL-1β and IL-18 production in the heart, and increased viral replication.

Materials and Methods

Mice

Male IL-12Rβ1- and IFN-γ-deficient mice and BALB/cJ (BALB/c) controls were obtained from The Jackson Laboratory (Bar Harbor, ME). TLR4-deficient mice were also obtained from The Jackson Laboratory and had been backcrossed to BALB/c mice from the C3H/HeJ mouse strain, which carries a missense point mutation within the *Tlr4* gene region that prevents functional TLR4 signaling (7). 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 Johns Hopkins University for all procedures.

Myocarditis

Mice, 6–8 wk of age, were inoculated i.p. with a heart-passaged stock of CB3 (Nancy strain) originally obtained from the American Type Culture Collection (ATCC, Manassas, VA). CB3 was diluted in sterile saline, 10^3 PFU was injected i.p. on day 0, and tissues were collected 2 or 12 days p.i. Individual experiments were conducted at least three times with 7–10 mice per group. Hearts were 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

Blood was allowed to clot at room temperature and centrifuged, and sera were collected and frozen at −80°C within 30 min. Heart samples were frozen with dry ice immediately after collection and stored at −80°C until homogenized. Both homogenized and culture supernatants were stored at −80°C until used in ELISAs. Splenocyte cultures were performed essentially as previously described (15). Briefly, viable splenocytes from individual mice were cultured at 1×10^7 cells/well of a 24-well plate in RPMI 1640 medium supplemented with 10% FBS, 15 mM HEPES, 1% L-glutamine, 1% MEM vitamins, 1% nonessential amino acid, 0.1 mM 2-ME, 1% sodium pyruvate, and 100 U/ml of penicillin-streptomycin (Life Technologies, Carlesbad, CA) for 48 h without stimulation. Two days in culture is not the optimal time point for all cytokines, but was chosen as a time when the cytokines of interest could be detected in the heart. Cytokines were measured in sera, homogenized heart supernatants, or cultured splenocyte supernatants using Quantikine cytokine ELISA kits purchased from R&D Systems (Minneapolis, MN), 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-12p70, 2.5 pg/ml; IL-18, 25 pg/ml; and IFN-γ, 2 pg/ml.

Plaque assay

The level of infectious virus was determined in individual pancreas or heart homogenates by plaque assay, according to standard procedures (16, 17). Briefly, pancreas and heart samples were frozen in dry ice immediately after collection 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 the plaque assay. Dilutions of tissue supernatants were incubated on confluent Vero cell (ATCC) monolayers for 1 h at 37°C and 5% CO_2 to allow viral attachment, and then incubated for 3 days to allow plaque formation. Virus titers are expressed as the mean PFU/g tissue ± SEM, and the limit of detection was 10 PFU/g of tissue.

Statistical analysis

Normally distributed data were analyzed by the Student's *t* test; otherwise, the Mann-Whitney *U* test was used. For correlation calculations that were not normally distributed, the Spearman's rank correlation was used. Test values with a $p < 0.05$ were considered significantly different from control values.

Results

IL-12Rβ1 deficiency reduces acute myocarditis and viral replication in the heart

To understand the role of the IL-12R on the development of CB3-induced myocarditis, BALB/c mice deficient in the IL-12Rβ1 (IL-12R$^{-/-}$) were examined for inflammation and viral replication in the heart 12 days after CB3 infection during the acute phase of myocarditis. The IL-12Rβ1 subunit has been found to be essential for functional signaling of the IL-12R, resulting in IFN-γ production (18). We found that IL-12Rβ1 deficiency significantly reduced myocarditis (Fig. 1*A*) and viral replication in the heart at day 12 p.i. (Fig. 1*B*) compared with control BALB/c mice. These results suggest that the IL-12R may exacerbate myocarditis and increase viral replication in the heart following infection with CB3.

IFN-γ deficiency increases viral replication in the heart

Because one of the primary functions of IL-12R signaling is to stimulate IFN-γ production and the development of a Th1-type immune response, we were interested to see the effect of IFN-γ on

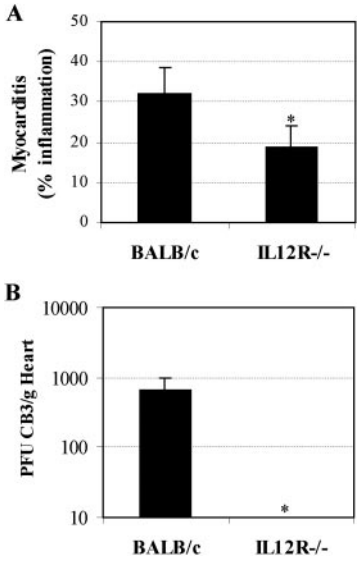

FIGURE 1. IL-12Rβ1 deficiency reduces acute myocarditis and viral replication in the heart. Mice deficient in IL-12Rβ1 (IL-12R$^{-/-}$) were compared with normal BALB/c mice for myocarditis (*A*) and viral replication (*B*) in the heart 12 days after CB3 infection. Mice received 10^3 PFU of CB3 i.p. on day 0, and hearts were collected on day 12 p.i. Individual experiments were conducted at least three times with 7–10 mice per group. Data are presented as the mean ± SEM of 7 mice per group. *, $p < 0.05$.

myocarditis. To investigate the role of IFN-γ, we infected IFN-γ-deficient (IFN-$\gamma^{-/-}$) mice with CB3 and examined the heart at day 12 p.i. Surprisingly, infection of IFN-$\gamma^{-/-}$ mice did not significantly alter myocarditis at day 12 p.i. (Fig. 2*A*), suggesting that factors other than IFN-γ are important in the decreased myocardial inflammation and viral replication in IL-12Rβ1-deficient hearts (Fig. 1). However, the absence of IFN-γ significantly increased viral replication in the heart at day 12 p.i. (Fig. 2*B*), illustrating the contribution of IFN-γ in protecting against CB3 replication in the heart. Hence, the reduced level of inflammation in the heart observed in IL-12R-deficient mice (Figs. 1*A* and 3*A*) is not due to changes in IFN-γ because the level of inflammation in IFN-γ-deficient hearts (Figs. 2*A* and 3*B*) resembles that of control BALB/c mice (Figs. 2*A* and 3*C*). Even though IFN-γ production is important for viral clearance in the heart, it does not influence myocardial inflammation during acute CB3 infection.

CB3 efficiently replicates in the pancreas and heart early after infection

Because the reduction in viral replication observed in IL-12R-deficient hearts was not found to be due to IFN-γ, we wondered whether IL-12R deficiency affected the ability of CB3 to replicate in the pancreas or heart early after infection at day 2 p.i. We examined the ability of virus to infect the pancreas at day 2 p.i. because this is the major initial site of CB3 replication following infection (10), and the heart at day 2 p.i. because CB3 is just beginning to establish infection in the heart at this time (12). IL-12R- and IFN-γ-deficient mice were infected with CB3 at day 0 and compared with control BALB/c pancreas and hearts collected at day 2 p.i. We found that CB3 was able to efficiently replicate in the pancreas (Fig. 4*A*) and heart (Fig. 4*B*) of both knockout strains compared with wild-type BALB/c mice. IFN-γ-deficient mice already had significantly increased levels of viral replication in the pancreas at day 2 p.i., confirming the important role for IFN-γ in preventing viral replication early after infection. However, we could not compare the level of viral replication in the heart to myocarditis at day 2 p.i., because the inflammatory infiltrate does not begin to appear in the heart until about day 7 p.i. (12). Furthermore, CB3 is cleared from the pancreas by day 12 p.i. in all mouse strains used in this study (data not shown). These results show that CB3 replication is not impaired early after infection (day 2) in knockout mice, and so other factors must contribute to the reduced viral replication observed in IL-12Rβ1-deficient hearts later during acute myocarditis (day 12) (Fig. 1*B*).

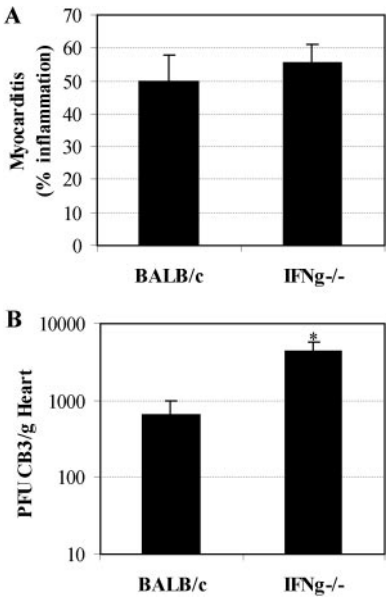

FIGURE 2. Lack of IFN-γ increases viral replication without affecting inflammation in the heart. Mice deficient in IFN-γ (IFN-$\gamma^{-/-}$) were compared with normal BALB/c mice for myocarditis (*A*) and viral replication (*B*) in the heart 12 days after CB3 infection. Mice received 10^3 PFU of CB3 i.p. on day 0, and hearts were collected on day 12 p.i. Individual experiments were conducted at least three times with 7–10 mice per group. Data are presented as the mean ± SEM of 10 mice per group. *, $p < 0.05$.

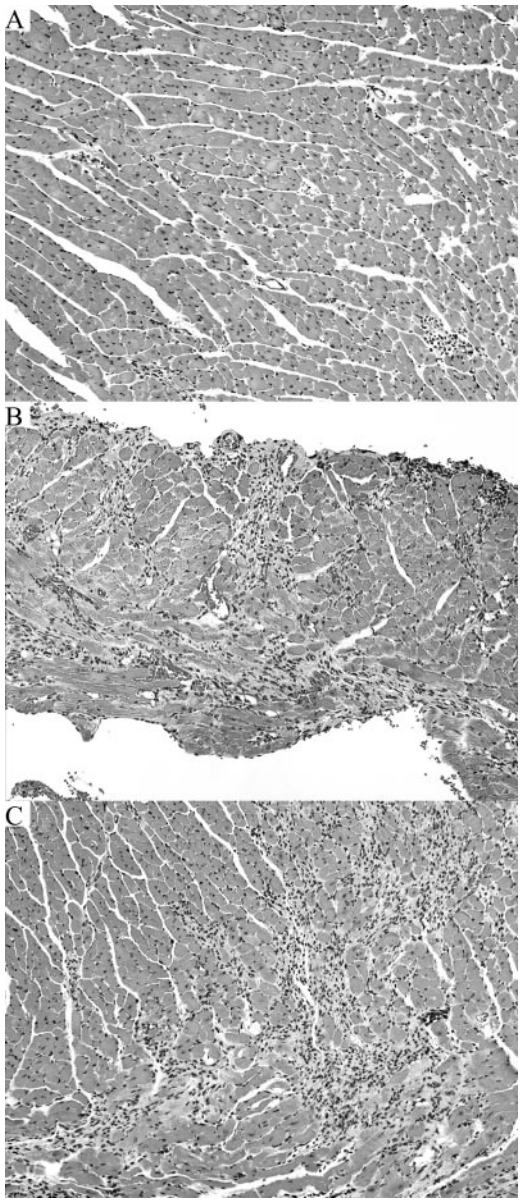

FIGURE 3. Reduced inflammation in IL-12Rβ1-deficient hearts is not due to IFN-γ deficiency. Representative histology sections stained with H&E depict the hearts of IL-12Rβ1 (*A*) or IFN-γ (*B*)-deficient mice compared with normal BALB/c mice (*C*) 12 days after CB3 infection. Mice received 10^3 PFU of CB3 i.p. on day 0, and hearts were collected on day 12 p.i. for histological examination. Magnification ×160.

IL-12Rβ1 deficiency reduces IL-1β and IL-18 in the heart

Because IL-12 is known to influence TNF-α and IFN-γ levels, we measured these proinflammatory cytokines along with IL-1β, IL-12p70, and IL-18 in the sera, splenocyte cultures, and hearts of IL-12Rβ1-deficient mice 12 days after infection with CB3. We

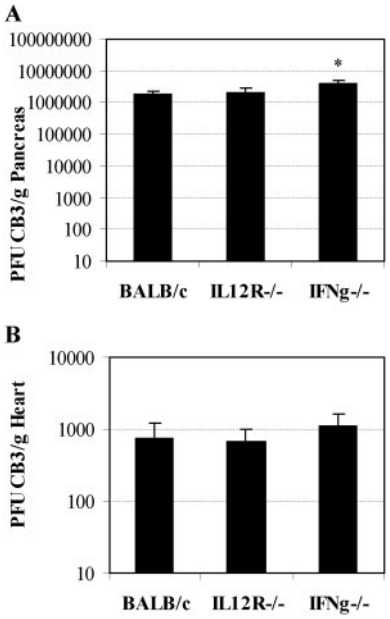

FIGURE 4. CB3 efficiently replicates in the pancreas and heart at day 2 after infection. Mice deficient in IL-12Rβ1 (IL-12R$^{-/-}$) or IFN-γ (IFN-γ$^{-/-}$) were compared with normal BALB/c mice for viral replication in the pancreas (*A*) and heart (*B*) 2 days after CB3 infection. Mice received 10^3 PFU of CB3 i.p. on day 0; pancreas and heart tissues were collected on day 2 p.i. and homogenized; and supernatants were tested for viral replication by plaque assay. Individual experiments were conducted at least three times with 7–10 mice per group. Data are presented as the mean ± SEM of 7 mice per group. *, $p < 0.05$.

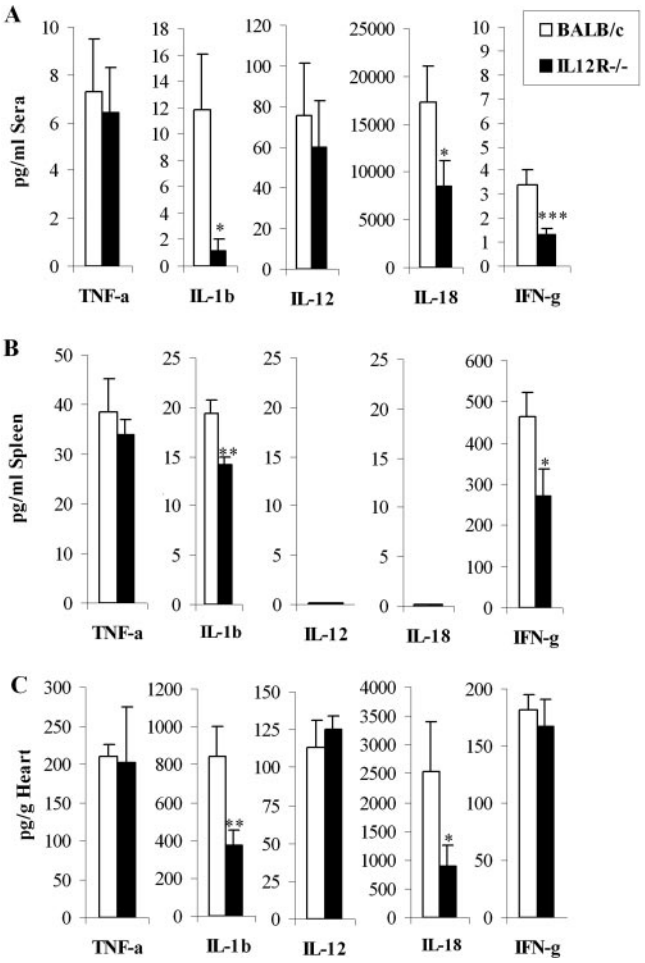

FIGURE 5. IL-12Rβ1 deficiency reduces IL-1β and IL-18 in the heart. Mice deficient in IL-12Rβ1 (IL-12R$^{-/-}$) were compared with normal BALB/c mice for cytokine levels in the sera (*A*), culture supernatants (*B*), and hearts (*C*) 12 days after CB3 infection. Mice received 10^3 PFU of CB3 i.p. on day 0, and sera, splenocytes, and hearts were collected on day 12 p.i. and analyzed for TNF-α, IL-1β, IL-12p70 (IL-12), IL-18, and IFN-γ levels using ELISA kits. Individual experiments were conducted at least three times, and data were presented as the mean ± SEM of 7–10 mice per group. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

found that IL-1β, IL-18, and IFN-γ were significantly reduced in sera from IL-12R$^{-/-}$ mice, whereas TNF-α and IL-12p70 (IL-12) levels were unaffected (Fig. 5*A*). Similar results were obtained when the cytokines released from cultured splenocytes were analyzed, with IL-1β and IFN-γ levels significantly reduced in IL-12R$^{-/-}$ mice (Fig. 5*B*). However, IL-12p70 and IL-18 were not detectable in splenocyte cultures in either mouse strain at the time point examined (48-h culture). Importantly, IL-1β and IL-18 were both significantly reduced in the hearts of IL-12Rβ1-deficient mice (Fig. 5*C*). Surprisingly, IFN-γ was not reduced in IL-12R$^{-/-}$ hearts (Fig. 5*C*). Because TNF-α and IL-12p70 levels in sera, splenocyte cultures, or hearts were not affected in IL-12R$^{-/-}$ mice, the IL-12Rβ1 does not appear to play a significant role in regulating the level of these cytokines during acute CB3 infection. However, both IL-1β and IL-18 were reduced in IL-12R$^{-/-}$ hearts. Importantly, these results demonstrate that IL-12Rβ1 directly influences the production of IL-1β and IL-18, which is a novel finding.

IL-1β and IL-18 levels in the heart correlate with inflammation

Because the reduction in acute myocarditis in IL-12R$^{-/-}$ hearts following CB3 infection was associated with both reduced viral replication (Fig. 1*B*) and reduced IL-1β and IL-18 (Fig. 5*C*), we determined whether the level of inflammation directly correlated with either of these parameters. When the severity of inflammation was compared with the level of IL-1β or IL-18 in the heart of BALB/c mice at day 12 p.i., we found a significant correlation between inflammation and IL-1β, $r = 0.79$ ($n = 14$), and IL-18, $r = 0.83$ ($n = 14$). However, increased myocarditis was not closely associated with increased viral replication ($r = 0.53$) ($n = 14$). Similar results were obtained for IL-12R$^{-/-}$ mice, in which inflammation correlated with IL-1β, $r = 0.80$, and IL-18, $r = 0.75$, but not with viral replication, $r = -0.21$. We were also interested in whether IFN-γ-deficient mice had altered IL-1β or IL-18 levels in the heart because they had increased viral replication, but no change in inflammation (Fig. 2). We found no significant difference between IL-1β or IL-18 levels in IFN-γ-deficient hearts at day 12 p.i. (data not shown), further indicating the association between IL-1β/IL-18 levels and inflammation in the heart. Thus, increased IL-1β and IL-18 levels in the heart during acute CB3-induced myocarditis are directly associated with increased inflammation.

TLR4 deficiency reduces myocarditis, viral replication, and IL-1β/IL-18, similar to IL-12Rβ1 deficiency

Because we found that IL-1β and IL-18 were associated with myocarditis and because TLR4 signaling is known to be a major stimulus of IL-1β and IL-18 production from macrophages (19), we examined whether TLR4 influenced acute CB3 myocarditis. First, we wanted to determine whether TLR4 could influence CB3 replication, as had been shown for RSV (8). TLR4-deficient (TLR4$^{-/-}$) mice, on a BALB/c genetic background, were infected with CB3 as in previous experiments, and pancreas and hearts

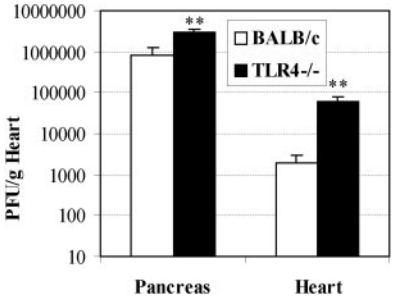

FIGURE 6. TLR4 deficiency increases viral replication in the heart and pancreas early after infection. Mice deficient in TLR4 ($TLR4^{-/-}$) were compared with normal BALB/c mice for the level of viral replication in the pancreas or heart 2 days after CB3 infection. Mice received 10^3 PFU of CB3 i.p. on day 0, and pancreas and hearts were collected on day 2 p.i. Data are presented as the mean ± SEM of 10 mice per group. **, $p < 0.01$.

were collected on days 2 and 12 p.i. We found that TLR4-deficient mice have significantly increased levels of CB3 in the pancreas and heart at day 2 p.i. compared with wild-type BALB/c mice (Fig. 6). Thus, TLR4 reduces CB3 replication early after infection. This is the first report of TLR4 influencing CB3 replication.

We found that myocarditis was significantly reduced at day 12 p.i. in $TLR4^{-/-}$ mice (Fig. 7*A*) similar to IL-12Rβ1-deficient mice (Fig. 1*A*). Likewise, viral replication in the heart was also significantly reduced at day 12 (Fig. 7*B*), while virus was cleared from the pancreas at this time point (data not shown). Importantly, the decrease in viral replication observed in TLR4-deficient hearts at day 12 p.i. (Fig. 7*B*) was not due to a defect in the ability of CB3 to infect the heart of these mice because viral replication actually increased in $TLR4^{-/-}$ hearts at day 2 p.i. (Fig. 6).

Similar to IL-$12R^{-/-}$ mice (Fig. 5*C*), IL-1β and IL-18 levels in the heart were significantly reduced, while TNF-α, IL-12p70, and IFN-γ were unaffected (Fig. 7*C*). The similarity between the effects of IL-12R and TLR4 deficiency on cytokine production suggests that these receptors share common downstream pathways that directly affect IL-1β and IL-18 production, and further confirm the association of IL-1β and IL-18 with the development of acute CB3-induced myocarditis. Furthermore, these findings indicate that TLR4 is an important stimulator of, but not the only source of, IL-1β and IL-18 following CB3 infection because IL-1β and IL-18 production were not completely abolished in $TLR4^{-/-}$ mice. Finally, the remarkable similarity between the effects of IL-12Rβ1 and TLR4 on myocarditis and viral replication shows that IL-1β and IL-18 play an important role in the pathogenesis of CB3-induced myocarditis.

Discussion

In this study, we examined the role of IL-12 and IFN-γ on the development of acute CB3-induced myocarditis using IL-12Rβ1- and IFN-γ-deficient mice. We report that IL-12Rβ1 deficiency results in decreased viral replication and inflammation in the heart, while IFN-γ deficiency exacerbates CB3 replication. IL-12Rβ1 deficiency was found to directly decrease the proinflammatory cytokines IL-1β and IL-18 in the heart, but not to affect TNF-α or IFN-γ levels during acute CB3 infection. In addition, we show for the first time that TLR4 deficiency also decreases CB3 replication and myocarditis by decreasing IL-1β and IL-18 in the heart. The remarkable similarities between the effects of IL-12Rβ1 and TLR4 suggest that these receptors share common downstream pathways that influence IL-1β and IL-18 production, and confirm that IL-1β and IL-18 are important in the pathogenesis of CB3-induced myocarditis.

Although we set out to understand the role of IL-12 and IFN-γ production on the development of CB3-induced myocarditis, we were surprised to find that deficiency in IL-12Rβ1 decreased

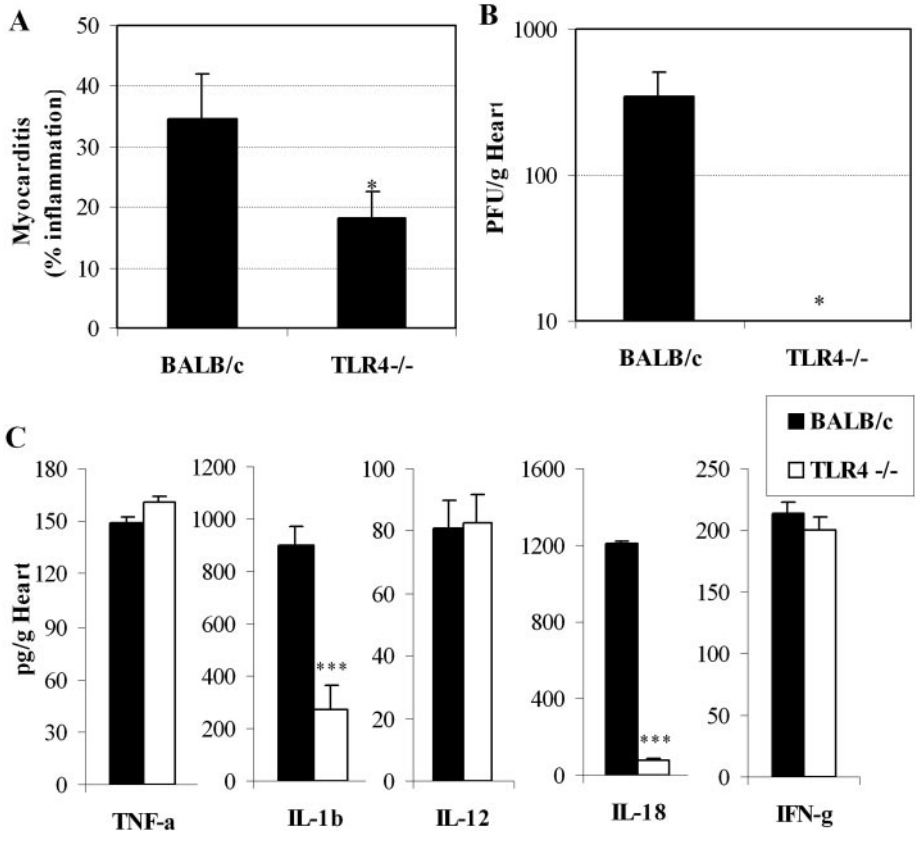

FIGURE 7. TLR4 deficiency reduces myocarditis, viral replication, and cytokines similar to IL-12Rβ1-deficient mice. Mice deficient in TLR4 ($TLR4^{-/-}$) were compared with normal BALB/c mice for myocarditis (*A*), viral replication (*B*), and cytokine levels (*C*) in the heart 12 days after CB3 infection. Mice received 10^3 PFU of CB3 i.p. on day 0, and hearts were collected on day 12 p.i. Individual experiments were conducted three times with 7–10 mice per group. Data are presented as the mean ± SEM of 7 mice per group. *, $p < 0.05$; ***, $p < 0.001$.

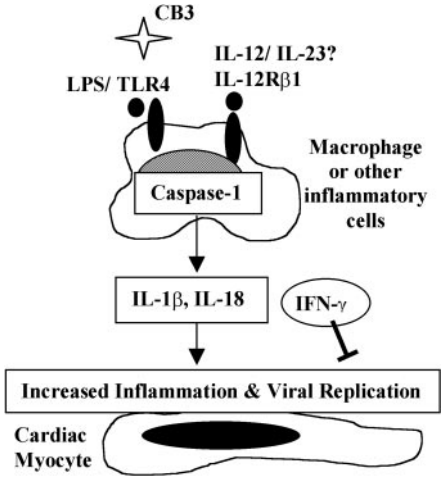

FIGURE 8. Possible mechanisms involved in CB3-induced myocarditis. CB3 infection stimulates TLR4 and IL-12Rβ1 signaling pathways in macrophages that increase IL-1β and IL-18 levels in the heart, resulting in increased inflammation and viral replication. TNF-α production by NK cells may further increase IL-1β and IL-18 levels during acute CB3 infection. Although IFN-γ protects against CB3 replication in the heart, it does not influence inflammation, which is associated with increased IL-1β and IL-18.

IL-1β and IL-18, which are known to be induced following LPS stimulation of TLR4. We have a longstanding interest in the effects of LPS and IL-1 on the development of myocarditis (12, 14), but it has only recently been shown that LPS mediates its effects through TLR4. We first grew interested in the role of LPS when we found that resistant mice inoculated with CB3 and LPS develop myocarditis (20). IL-18 had not yet been described, but at that time we discovered a significant increase in the level of IL-1β and TNF-α in the heart (21, 22). Importantly, the same increase in myocarditis was observed when mice received rIL-1β or rTNF-α with CB3, and this effect could be blocked by administration of IL-1Ra (23, 24). Furthermore, administration of LPS to murine CMV (MCMV)-infected susceptible or resistant mice results in significantly increased myocarditis (25). Interestingly, infection with influenza A or Sendai virus has been reported to increase IL-1β and/or IL-18 levels in patient's sera (26), while RSV has recently been found to stimulate an immune response via TLR4 (8). Thus, similar mechanisms of disease pathogenesis involving LPS/TLR4 and increased IL-1β and IL-18 levels occur after infection with very different viruses (12).

We expected TLR4-deficient mice to have reduced levels of IL-1β and IL-18, but also found this reduction in IL-12Rβ1-deficient mice. The precise mechanisms involved in increasing IL-1β and IL-18 levels by IL-12Rβ1 are not known. A possible mechanism may be through increased TNF-α production. IL-12R signaling is known to increase TNF-α production from macrophages and NK cells, which can then initiate caspase activity either by TNFR signaling or increased Fas/Fas ligand expression (27). Whereas tolerance to LPS can be produced by repeated exposure of macrophages to LPS or IL-1β, exposure to LPS and TNF-α does not have this effect (19). Thus, TNF-α may amplify IL-1β and IL-18 production when virus or bacteria are also present. This may help to explain how, in our earlier findings, TNF-α administration with CB3 or MCMV infection increased myocarditis (23, 25). Although it is possible that IL-12R, TLR4, and TNFR/Fas represent redundant pathways for increased expression of IL-1β and IL-18, the significant reduction in these cytokines observed in either IL-12R- or TLR4-deficient mice suggests that signaling through multiple receptor pathways is necessary for optimal IL-1β and IL-18 production. These findings are not unusual, because other signaling pathways often modulate receptor signaling of a particular cytokine. Recently, remodeling of the IL-12p40 promoter was found to be TLR4 dependent (28). We also found a significant decrease in IL-12p40 expression in the heart of TLR4-deficient mice ($p = 0.02$, data not shown). Thus, although LPS/TLR4 signaling is known to stimulate IL-12 production (1, 28), we report in this work for the first time that the IL-12R can also influence IL-1β and IL-18 production similar to TLR4.

Based on these findings, we propose that CB3 infection stimulates a protective Th1 immune response against the virus. However, virus persists in the heart for at least 14 days following infection, even though it has been cleared from most other organs such as the pancreas by this time (12). Continued stimulation of immune cells activates caspase-1 in macrophages by TLR4, IL-12R, and/or IL-1R and TNFR signaling pathways, resulting in the production of high levels of IL-1β and IL-18 in the heart (Fig. 8). Through mechanisms that are unclear at this time, IL-12Rβ1 and TLR4 increase IL-1β and IL-18, inflammation, and viral replication in the heart. Overall, these findings suggest a contradictory role for IL-12, with production of IFN-γ protecting against viral replication on the one hand, while production of IL-1β and IL-18 increases inflammation on the other.

The direct correlation of IL-1β and IL-18 with the inflammatory infiltrate suggests that the primary source of these cytokines is inflammatory cells, such as macrophages, rather than myocytes, which can also produce IL-1β and IL-18 via TLR4 signaling (6, 29). In addition to its roles in IFN-γ production and NK cell activation, IL-18 is also a potent proinflammatory cytokine (5). Both IL-1β and IL-18 regulate inflammation by modulating migrating immune cell populations and by influencing local tissues such as cardiomyocytes. IL-1β and IL-18 have also been implicated in the development of a number of autoimmune diseases such as diabetes, multiple sclerosis, and rheumatoid arthritis (5, 6, 30, 31). IL-23, a novel cytokine that binds to IL-12Rβ1 and an IL-23R subunit and stimulates IFN-γ production from memory T cells (32), was not investigated in this study. IL-23 is a covalently linked heterodimeric cytokine that shares the p40 subunit of IL-12 and a p19 subunit. It is possible that the effects of IL-12Rβ1 on IL-1β and IL-18 may be due to IL-23 rather than IL-12p70, particularly in light of a recent study that found increased IL-1β and TNF-α levels in p19 transgenic mice (33). The potential effects of IL-23 on IL-12R and TLR4 signaling are not well understood and require further study.

One intriguing question is how IL-12R and TLR4 influence viral replication. We have shown that decreased viral replication in IL-12R- or TLR4-deficient mice is not due to an inability of CB3 to infect the heart or pancreas of these strains (Figs. 4 and 7). Reduced viral replication in IL-12R- and TLR4-deficient hearts could be due to the effects of receptor signaling on NO production. NO is known to be a potent inhibitor of activated caspases and to prevent the release of IL-1β and IL-18 from macrophages (34). Furthermore, we have previously shown that the production of NO following CB3 infection inhibits viral replication in the heart (35). Recent findings indicate that NO production is dependent on both the IL-12R and TLR4 signaling cascades (19, 36), and thus is likely to be affected in mice deficient in these receptors. We are currently investigating this possibility.

We had expected IL-12R stimulation to increase IFN-γ and a protective Th1 immune response, and so were surprised to find that IFN-γ levels were not reduced in IL-12R$^{-/-}$ hearts, even though IFN-γ was significantly reduced in the sera and splenocyte cultures

(Fig. 5). Perhaps the high levels of IL-18 in the hearts of IL-12R-deficient mice allow IFN-γ production to occur via IL-12-independent pathways. But the fact that IFN-γ was not decreased in IL-12R$^{-/-}$ hearts and myocardial inflammation was not affected by IFN-γ deficiency indicates that IFN-γ is not a dominant factor in resolving acute inflammation following CB3 infection. Furthermore, IFN-γ deficiency did not influence IL-1β or IL-18 levels in the heart at day 12 p.i., confirming the association of IL-1β/IL-18 with myocarditis. We are currently investigating the role of IFN-γ in the pathogenesis of chronic CB3-induced myocarditis. Thus, IFN-γ production is important in reducing viral replication at days 2 and 12 after CB3 infection, but does not alter IL-1β or IL-18 levels or inflammation at day 12 p.i. during acute myocarditis.

Many autoimmune diseases are believed to be triggered by viral infections. However, in most cases, the precise virus involved is not known. We are fortunate to study one of the few models of autoimmune disease in which both the virus (CB3) and the autoantigen (cardiac myosin) are known (12). Comparison of the viral and autoimmune models has revealed consistent similarities in the pathogenesis of disease. The findings presented in this study confirm results that we obtained recently in the cardiac myosin-induced model of experimental autoimmune myocarditis. We found that IL-12Rβ1 and STAT4 signaling are required for the development of experimental autoimmune myocarditis in an IFN-γ-independent manner (37). In that study, IL-1β was significantly reduced in splenocyte cultures of IL-12Rβ1-deficient mice (IL-18 was not examined) similar to our observations with CB3-induced myocarditis. Thus, increased IL-1β and IL-18 levels appear to be a common immune mechanism in response to a number of viral infections (e.g., CB3, MCMV, influenza A, Sendai) and Ag-induced models of autoimmune disease. The results presented in this study have important implications not only for the pathogenesis of myocarditis, but for other autoimmune diseases triggered by viral infections.

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References

1. Ma, X., and G. Trinchieri. 2001. Regulation of interleukin-12 production by antigen-presenting cells. *Adv. Immunol. 79:55.*
2. O'Garra, A. 1998. Cytokines induce the development of functionally heterogeneous T helper cell subsets. *Immunity 8:275.*
3. Moser, M., and K. M. Murphy. 2000. Dendritic cell regulation of Th1-Th2 development. *Nat. Immun. 1:199.*
4. Yoshimoto, T., K. Takeda, T. Tanaka, K. Ohkusu, S. Kashiwamura, H. Okamura, S. Akira, and K. Nakanishi. 1998. IL-12 up-regulates IL-18 receptor expression on T cells, Th1 cells and B cells: synergism with IL-18 for IFN-γ production. *J. Immunol. 161:3400.*
5. Nakanishi, K., T. Yoshimoto, H. Tsutsui, and H. Okamura. 2001. Interleukin-18 regulates both Th1 and Th2 responses. *Annu. Rev. Immunol. 19:423.*
6. O'Neill, L. A. J., and C. A. Dinarello. 2000. The IL-1 receptor/Toll-like receptor superfamily: crucial receptors for inflammation and host defense. *Immunol. Today 21:206.*
7. Akira, S. A., K. Takeda, and T. Kaisho. 2001. Toll-like receptors: critical proteins linking innate and acquired immunity. *Nat. Immun. 2:675.*
8. Kurt-Jones, E. A., L. Popova, L. Kwinn, L. M. Haynes, L. P. Jones, R. A. Tripp, E. E. Walsh, M. W. Freeman, D. T. Golenbock, L. J. Anderson, and R. W. Finberg. 2000. Pattern recognition receptors TLR4 and CD14 mediate response to respiratory syncytial virus. *Nat. Immun. 1:398.*
9. Guidotti, L. G., and F. V. Chisari. 2001. Noncytolytic control of viral infections by the innate and adaptive immune response. *Annu. Rev. Immunol. 19:65.*
10. Fairweather, D., and N. R. Rose. 2002. Type I diabetes: virus infection or autoimmune disease? *Nat. Immun. 3:338.*
11. Horwitz, M. S., A. La Cava, C. Fine, E. Rodriguez, A. Ilic, and N. Sarvetnick. 2000. Pancreatic expression of interferon-γ protects mice from lethal Coxsackievirus B3 infection and subsequent myocarditis. *Nat. Med. 6:693.*
12. Fairweather, D., Z. Kaya, G. R. Shellam, C. M. Lawson (James), and N. R. Rose. 2001. From infection to autoimmunity. *J. Autoimmun. 16:175.*
13. Feldman, A. M., and D. McNamara. 2000. Myocarditis. *N. Engl. J. Med. 343: 1388.*
14. Rose, N. R., and M. Afanasyeva. 2003. From infection to autoimmunity: the adjuvant effect. *ASM News 69:132.*
15. Kaya, Z., M. Afanasyeva, Y. Wang, K. M. Dohmen, J. Schlichting, T. Tretter, D. Fairweather, V. M. Holers, and N. R. Rose. 2001. Contribution of the innate immune system to autoimmune myocarditis: a role for complement. *Nat. Immun. 2:739.*
16. Rose, N. R., S. L. Hill, and D. A. Neumann. 1994. Experimental myocarditis. In *Autoimmune Disease Models: A Guidebook*. Academic Press, San Diego, p. 175.
17. Lenzo, J. C., D. Fairweather, V. Cull, G. R. Shellam, and C. M. Lawson. 2002. Characterization of murine cytomegalovirus myocarditis: cellular infiltration of the heart and virus persistence. *J. Mol. Cell. Cardiol. 34:629.*
18. Wu, C.-Y., J. Ferrante, M. K. Gately, and J. Magram. 1997. Characterization of IL-12 receptor β1 chain (IL-12Rβ1)-deficient mice: IL-12Rβ1 is an essential component of the functional mouse IL-12 receptor. *J. Immunol. 159:1658.*
19. Akira, S. 2001. Toll-like receptors and innate immunity. *Adv. Immunol. 78:1.*
20. Lane, J. R., D. A. Neumann, A. LaFond-Walker, A. Herskowitz, and N. R. Rose. 1991. LPS promotes CB3-induced myocarditis in resistant B10.A mice. *Cell. Immunol. 136:219.*
21. Lane, J. R., D. A. Neumann, A. LaFond-Walker, A. Herskowitz, and N. R. Rose. 1992. Interleukin 1 or tumor necrosis factor can promote Coxsackievirus B3-induced myocarditis in resistant B10.A mice. *J. Exp. Med. 175:1123.*
22. Lane, J. R., D. A. Neumann, A. LaFond-Walker, A. Herskowitz, and N. R. Rose. 1993. Role of IL-1 and tumor necrosis factor in Coxsackievirus-induced autoimmune myocarditis. *J. Immunol. 151:1682.*
23. Neumann, D. A., J. R. Lane, G. S. Allen, A. Herskowitz, and N. R. Rose. 1993. Viral myocarditis leading to cardiomyopathy: do cytokines contribute to pathogenesis? *Clin. Immunol. Immunopathol. 68:181.*
24. Huber, S. A., J. Polgar, P. Schultheiss, and P. Schwimmbeck. 1994. Augmentation of pathogenesis of Coxsackievirus B3 infections in mice by exogenous administration of interleukin-1 and interleukin-2. *J. Virol. 68:195.*
25. Lenzo, J. C., D. Fairweather, G. R. Shellam, and C. M. Lawson (James). 2001. Immunomodulation of murine cytomegalovirus-induced myocarditis in mice treated with lipopolysaccaride and tumor necrosis factor. *Cell. Immunol. 213:52.*
26. Pirhonen, J., T. Sareneva, M. Kurimoto, I. Julkunen, and S. Matikainen. 1999. Virus activates IL-1β and IL-18 production in human macrophages by a caspase-1-dependent pathway. *J. Immunol. 162:7322.*
27. Reed, J. C. 1999. Caspases and cytokines: roles in inflammation and autoimmunity. *Adv. Immunol. 73:265.*
28. Weinmann, A. S., D. M. Mitchell, S. Sanjabi, M. N. Bradley, A. Hoffmann, H. C. Liou, and S. T. Smale. 2001. Nucleosome remodeling at the IL-12p40 promoter is a TLR-dependent, Rel-independent event. *Nat. Immun. 2:51.*
29. Frantz, S., L. Kobzik, Y. D. Kim, R. Fukazawa, R. Medzhitov, R. T. Lee, and R. A. Kelly. 1999. Toll 4 (TLR4) expression in cardiac myocytes in normal and failing myocardium. *J. Clin. Invest. 104:271.*
30. Segal, B. M., D. M. Klinman, and E. M. Shevach. 1997. Microbial products induce autoimmune disease by an IL-12-dependent pathway. *J. Immunol. 158: 5087.*
31. Kagari, T., H. Doi, and T. Shimozato. 2002. The importance of IL-1β and TNF-α, and the noninvolvement of IL-6, in the development of monoclonal antibody-induced arthritis. *J. Immunol. 169:1459.*
32. Parham, C., M. Chirica, J. Timans, E. Vaisberg, M. Travis, J. Cheung, S. Pflanz, R. Zhang, K. P. Singh, F. Vega, et al. 2002. A receptor for the heterodimeric cytokine IL-23 is composed of IL-12Rβ1 and a novel cytokine receptor subunit, IL-23R. *J. Immunol. 168:5699.*
33. Wiekowski, M. T., M. W. Leach, E. W. Evans, L. Sullivan, S. C. Chen, G. Vassileva, J. F. Bazan, D. M. Gorman, R. A. Kastelein, S. Narula, and S. A. Lira. 2001. Ubiquitous transgenic expression of the IL-23 subunit p19 induces multiorgan inflammation, runting, infertility, and premature death. *J. Immunol. 166:7563.*
34. Kim, Y.-M., R. V. Talanian, J. Li, and T. R. Billiar. 1998. Nitric oxide prevents IL-1β and IFN-γ-inducing factor (IL-18) release from macrophages by inhibiting caspase-1 (IL-1β-converting enzyme). *J. Immunol. 161:4122.*
35. Lowenstein, C. J., S. L. Hill, A. Lafond-Walker, J. Wu, G. Allen, M. Landavere, N. R. Rose, and A. Herskowitz. 1996. Nitric oxide inhibits viral replication in murine myocarditis. *J. Clin. Invest. 97:1837.*
36. Diefenbach, A., H. Schindler, M. Rollinghoff, W. M. Yokoyama, and C. Bogdan. 1999. Requirement for type 2 NO synthase for IL-12 signaling in innate immunity. *Science 284:951.*
37. Afanasyeva, M., Y. Wang, Z. Kaya, E. A. Stafford, K. M. Dohmen, A. A. Sadighi Akha, and N. R. Rose. 2001. Interleukin-12 receptor/STAT4 signaling is required for the development of autoimmune myocarditis in mice by an interferon-γ-independent pathway. *Circulation 104:3145.*