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Immunomodulation by interleukin-4 suppresses matrix metalloproteinases and improves cardiac function in murine myocarditis

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Abstract

Immune response is critically involved in determining the course of viral myocarditis and immunomodulation. Different cytokines may have either deleterious or protective effects. Following acute Coxsackievirus B3 infection, intramyocardial inflammation is associated with altered myocardial matrix metalloproteinase (MMP) expression and left ventricular dysfunction. In this study, we evaluated the effect of exogenous interleukin-4 treatment on myocardial inflammation, MMPs and left ventricular function in Coxsackievirus B3-induced acute murine myocarditis. Eight-week-old inbred male BALB/c (H-2^d) mice (The Jackson Laboratory, Bar Harbor, Maine, USA) were used. Myocardial inflammation was measured by immunohistochemical detection of $CD3^+$-, $CD8a^+$-T-lymphocytes, and $CD11b^+$ macrophages. In situ hybridization was used to detect enteroviral genome in the myocardium. Semi-quantitative reverse transcriptase polymerase chain reaction (RT-PCR) was employed to detect cytokine and MMP mRNA. MMP activity was quantified by zymography analysis. Detection of myocytolysis was performed by Luxol fast blue staining. In the early acute phase, in comparison to infected mice without treatment, interleukin-4 administration (200 ng daily) reduced intramyocardial inflammation ($CD3^+$ lymphocytes: 55.3±7.0 vs. 72.1±13.7 cells/mm^2, $P<0.05$; $CD8a^+$ lymphocytes: 31.7±3.6 vs. 64.2±7.7 cells/mm^2, $P<0.05$; $CD11b^+$ macrophages: 5.1±2.3 vs. 13.2±2.5 cells/mm^2, $P<0.05$). It also down-regulated interleukin-2 (IL) (1.7-fold, $P<0.001$) but increased transforming growth factor-β_1 (TGF) (1.5-fold, $P<0.001$) and IL-4 (1.4-fold, $P<0.001$). IL-4 suppressed MMP-2/-3/-9 transcription and activity. These biochemical alterations were accompanied by a significant improvement of left ventricular function as assessed by Milar tip catheter (left ventricular endsystolic pressure, 1.3-fold, $P<0.01$; dP/dt max, 1.5-fold, $P<0.01$). Immunomodulation by exogenous IL-4 treatment may lead to an anti-inflammatory effect with the inhibition of Th_1 cell phenotypic response, which may further mediate the down-regulation of MMPs. A significant suppression of MMPs may mainly contribute to an improvement of left ventricular dysfunction in acute murine CVB3-induced myocarditis.

Keywords: Myocarditis; IL-4; Matrix metalloproteinases; Cytokines

1. Introduction

A triphasic pathogenic scheme comprising of viral infection, immune response, and cardiac dilatation has been proposed to explain transition of viral myocarditis to dilated cardiomyopathy (Liu and Mason, 2001; Pauschinger et al., 1999). The delicate balance between protective and deleterious immune mechanisms is a decisive factor in the evolution of myocardial remodeling (Liu and Mason, 2001). Matrix metalloproteinases (MMPs) are pivotal for the homeostasis of extracellular matrix

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remodeling, especially collagen (Murphy et al., 1992). MMPs belong to a family of zinc-dependent enzymes, that includes more than 20 members, which share a zinc-binding catalytic domain (Murphy et al., 1992). Among these MMP members, MMP-3 can both activate other MMPs (Ogata et al., 1992) and also degrade a broad range of extracellular matrix components, including collagen type I (He et al., 1989). With regard to the degradation of insoluble collagen, MMP-9 has demonstrated its collagenolytic activities on the cross-linked polymers of collagen (Okada et al., 1995). We recently observed that myocardial inflammation is associated with altered myocardial MMP profiles and left ventricular dysfunction in a Coxsackievirus B3-induced murine acute myocarditis model (Li et al., 2002).

Modulation of immune response has become a focus for controlling or changing the course of viral myocarditis. Exogenous administration of interleukin (IL)-2 was found to increase myocardial inflammation and the severity of myocarditis in mice infected with Coxsackievirus B3 (CVB3) (Huber et al., 1994). Interestingly, administration of IL-10, a cytokine that inhibits Th_1 cells, macrophage function, and production of pro-inflammatory cytokines, was found to be an effective treatment strategy in experimental viral myocarditis (Nishio et al., 1999).

Several studies have reported a regulatory role for cytokines on this proteolytic–antiproteolytic system (Thomas et al., 1998; Murray and Freeman, 1996), so cytokine-mediated therapeutic immunomodulation may influence myocardial MMP expression. A cytokine of particular interest for the treatment of myocarditis is IL-4, a Th_2 cytokine with autoregulatory action (Lacraz et al., 1992; Yoshimoto et al., 1996; Mosmann et al., 1986) that suppresses IL-2 production (Tanaka et al., 1993) and promotes the expression of anti-inflammatory cytokines such as TGF-β_1 (Inobe et al., 1998), which inhibits MMP-1 expression in myocytes (Chen et al., 2003). In addition, IL-4 inhibits the expression of MMP-9 (Chizzolini et al., 2000; Corcoran et al., 1992) and MMP-1 in monocytes and macrophages (Zhang et al., 1998). In light of these considerations, we investigated the effect of exogenous IL-4 on cardiac function and its potential mechanisms involving myocardial inflammatory infiltration, cytokine modulation, and MMPs in acute viral myocarditis.

2. Methods

2.1. Experimental acute myocarditis

The experiments were performed in accordance with the German Law on Animal Protection, 1993.

Eight-week-old inbred male BALB/c (H-2^d) mice (The Jackson Laboratory, Bar Harbor, Maine, USA) were inoculated intraperitoneally with 5×10^5 plaque-forming units of CVB3 (Nancy strain; VR-30, Manassas, USA) in 0.2 ml of buffered saline (infected mice, $n=20$). Control BALB/c mice matched for age and sex received 0.2 ml of buffered saline intraperitoneally ($n=12$).

2.2. Interleukin administration

Recombinant murine interleukin-4 (rmIL-4) was obtained from R&D System. CVB3-infected mice were injected daily intraperitoneally with 200 ng of rmIL-4 in 0.4 ml of phosphate-buffered saline containing 0.1% bovine serum albumin. The dose of the rmIL-4 was chosen on the basis of a previous study in BALB/c mice (Samoszuk and Yang, 1994). Two strategies were used to determine the time-dependent effect of interleukin-4 administration.

For strategy I, a group of infected mice ($n=4$) received rmIL-4 daily for the first five days after infection when T cells remain in the naïve state and viral titers are high in the myocardium (Huber

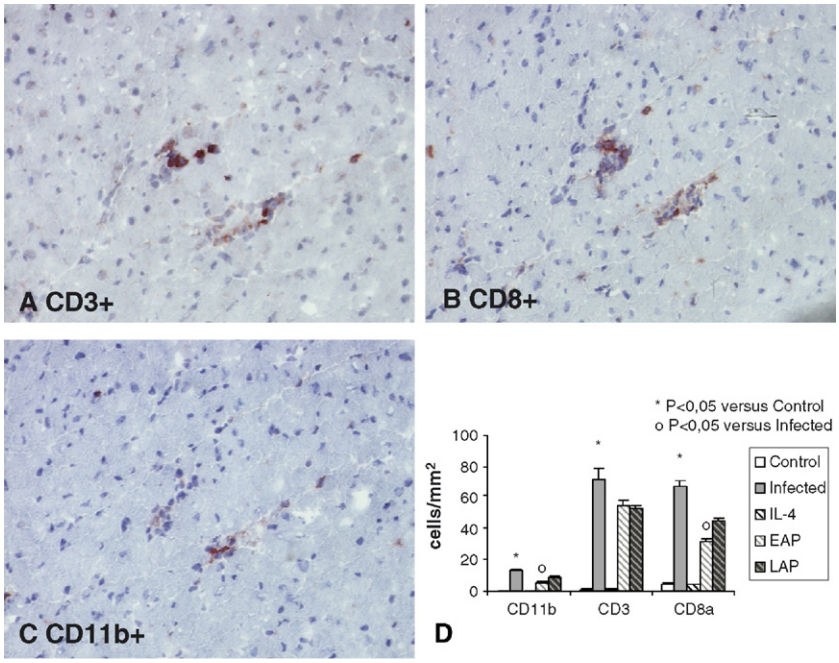

Fig. 1. Immunoperoxidase detection of myocardial $CD3^+$- and $CD8a^+$-lymphocytes and $CD11b^+$ macrophages in infected mice (A–C) (original magnification, 200×). EAP IL-4 treatment reduces infiltrated lymphocytes ($CD3^+$ and $CD8a^+$) and $CD11b^+$ macrophages significantly (1D).

and Job, 1983). This group will be referred to as the Early Acute Phase treatment group (EAP).

For strategy II, another group of infected mice ($n=4$) received rmIL-4 daily on days 5–10 after inoculation when T-cells are activated and viral titers are low in the myocardium (Huber et al., 1994; Huber and Job, 1983). This group is referred to as the Late Acute Phase treatment group (LAP).

Two groups served as controls: one comprising uninfected mice ($n=4$) that received rmIL-4 treatment for 10 days and another including infected mice ($n=4$) that never received interleukin-4 treatment. The day of virus infection was designated as day 0. The animals were sacrificed on the 10th day of virus inoculation, when inflammatory induction and left ventricular dysfunction are significant (Li et al., 2002).

2.3. Immunohistochemical evaluation of intramyocardial inflammation

Transversely dissected myocardial samples for immunohistochemical evaluation of inflammation were embedded in OCT (SLEE) and snap-frozen. Five micrometer cryostat sections were fixed with acetone, and endogenous peroxidase activity was blocked by 10 min of incubation with 0.3% H_2O_2 in phosphate-buffered saline. In order to avoid non-specific binding, antibodies were diluted in PBS containing 1.5% rabbit serum. Sections were incubated with goat anti-CD3 (1:25; Santa Cruz), rat anti-CD8a (1:50; Pharmingen), anti-CD11b (1:50; Pharmingen), rabbit anti-MMP-3 and anti-MMP-9 (Chemicon) antibodies for 30 min. Following brief rinses in PBS, the sections were exposed for 30 min to biotinylated rabbit anti-rat IgG diluted to 1:100 (DAKO); rabbit anti-goat IgG diluted to 1:200 (ABC-Kit). Subsequently, the sections were incubated with VECTASTAIN® ABC reagent (Vector) for 30 min. 3-amino-9-ethylcarbazole (Merck) and hematoxylin were used as the chromogenic substrate and counterstain, respectively. Serial sections subjected to this protocol but without primary antibodies served as the negative control. For quantitative analysis, the coded slides were evaluated in a blinded fashion. The total number of $CD3^+$, $CD8a^+$, and $CD11b^+$ cells in different sections was counted at 200× accounted 0.39 mm^2. For each sample, four tissue sections (2 sections per slide, 2 slides per heart) were evaluated. The mean cell number per mm^2 was calculated and represented as the extent of intramyocardial inflammation.

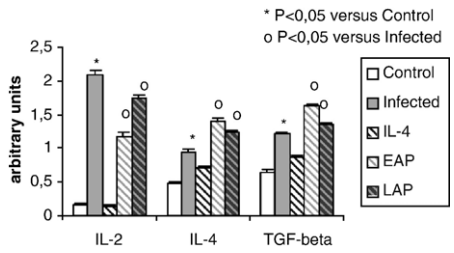

Fig. 2. Myocardial cytokine mRNA expression following IL-4 administration in acute viral myocarditis. Down-regulation of IL-2 and up-regulation of IL-4 and TGF-β_1 are shown in the EAP group and LAP group. IL-4 treatment alone (IL-4) significantly increases IL-4 expression in control mice.

2.4. RNA preparation and cDNA synthesis

Total RNA was extracted from myocardial samples frozen in liquid nitrogen by the Trizol method (GIBCO BRL) as per the manufacturer's instructions. Following treatment with RNase-free DNase I (Roche, Mannheim, Germany), reverse transcription was performed using a first-strand cDNA synthesis kit (Promega, Mannheim, Germany).

2.5. Semi-quantitative PCR analysis for myocardial cytokines and MMPs/TIMPs

Semi-quantitative mRNA detection was performed as previously described (Li et al., 2002). Briefly, an amplification reaction was carried out in 50 μl of 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 50 mM KCl, 0.2 mM dNTP, 0.5 μM of primers, and 1.25 U Taq DNA polymerase (Rapidozym, Berlin, Germany). PCR products were resolved and visualized on 1.2% agarose gel containing 0.4 μg/ml ethidium bromide. Bands were analyzed as relative optical densities using Scion Image, with respect to β-actin. The primers have been described previously (Li et al., 2002) except for interferon-γ (IFN) (sense: 5′-agcaacagcaaggcgaaaaag-3′; antisense: 5′-gttgtatctgggggtgggggga-3′).

2.6. Zymography of MMP activity

Gelatin zymography was performed to determine the gelatinolytic activities of MMP-2 and MMP-9. Forty micrograms of myocardial protein was treated with sampling buffer (0.5 M Tris-HCL, Glycerol, 10% sodium dodecylsulfate (SDS), 0.1% Bromphenolblue) as a final solution of 20 μl. SDS-Gel electrophoresis was performed using 10% polyacrylamid gel with 0.1% gelatin at 125 V for 60 min. The SDS was removed with Triton X-100 for 60 min and the gel was incubated in a developing buffer (Tris-Base, Tris-HCL, NaCl, $CaCl_2$, Brij-35, $ZnCl_2$) overnight. Gels were stained for 3 h with 0.5% Coomassie G250 and destained for 60 min in 7% acetic acid and 35% methanol. The gelatinolytic activities were detected as clear bands against a blue background and analyzed using Scion Image software as relative optical densities.

2.7. Luxol fast blue staining

Luxol fast blue staining was performed to determine myocytolysis in myocard tissue. Paraffin-embedded tissues were deparaffinized and hydrated to 95% alcohol. Tissues were stained for 4 h with Luxol fast blue solution (0.1% Solvent Blue 38, 0.5% acetic acid in alcohol) at 60 °C. Differentiation was performed with 0.005% lithium carbonate solution for 60 s and 70% ethanol for 30 s. After differentiation, the tissues were stained in Nuclear fast red for 4 min and EOSIN Y solution for 10 s and were then dehydrated. The area percent of myocytolysis in different sections was measured under high-power field at 100× magnification using Lucia G software, version 3.51.

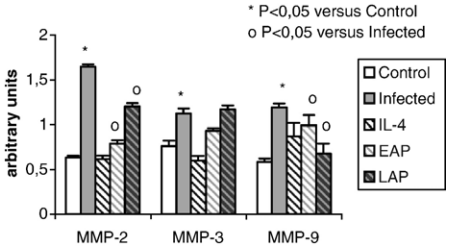

Fig. 3. The increased MMP-2 and MMP-9 mRNA expression following infection is suppressed by interleukin-4 treatment in the EAP and LAP groups, respectively. Additionally, semi-quantitative RT-PCR showed a trend of suppressing MMP-3 in the EAP group. IL-4 alone did not influence MMP-2 or MMP-9 mRNA in the uninfected control group (IL-4).

2.8. In situ hybridization

Ten days after infection, tissue samples were fixed in 2% paraformaldehyde/0.1 M sodium phosphate-buffer add pH 7.2 and embedded in paraffin for in situ detection. In situ detection was performed using a 35S-labeled enterovirus-specific probe, as previously described (Klingel et al., 1992). Tissue sections were counterstained with hematoxylin. The area percent of CVB-3 detection were measured under high-power field at 100× magnification using Lucia G software, version 3.51.

2.9. Hemodynamic evaluation

Prior to animal sacrifice and the gathering of myocardial tissue, anesthetized (thiopental 125 μg/g, i.p.), artificially ventilated, and

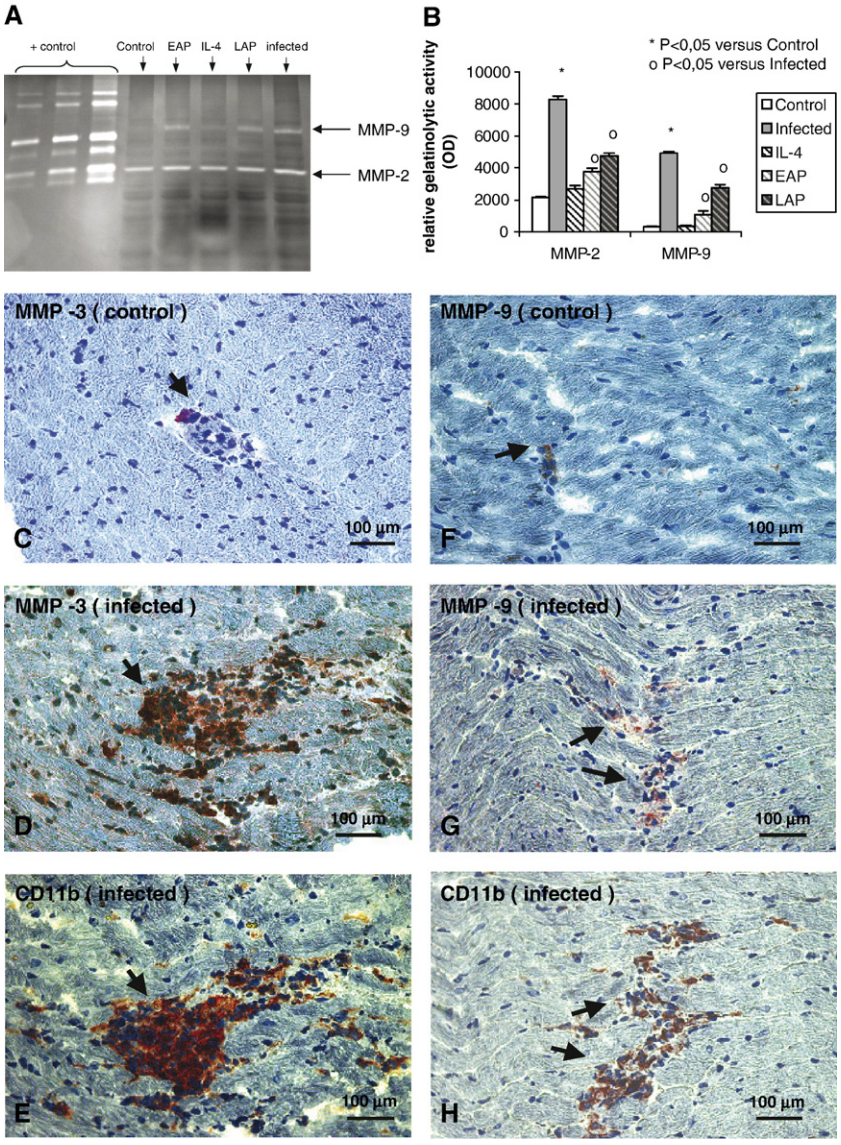

Fig. 4. SDS-Gel electrophoresis using 10% polyacrylamid gel containing 0.1% gelatin. The gelatinolytic activities were detected as clear bands against the background and analyzed using Scion Image software as relative optical densities (OD) (4A). The increased MMP-2 and MMP-9 activity following infection is suppressed by interleukin-4 treatment in the EAP and LAP groups, respectively. IL-4 alone did not influence MMP-2 and MMP-9 mRNA in the uninfected control group (IL-4) (4B). Immunohistochemical staining showed that increased myocardial MMP-3 (4C-4E) and MMP-9 (4F–4H) were located in infiltrated $CD11b^{+}$ macrophages after CVB3 infection.

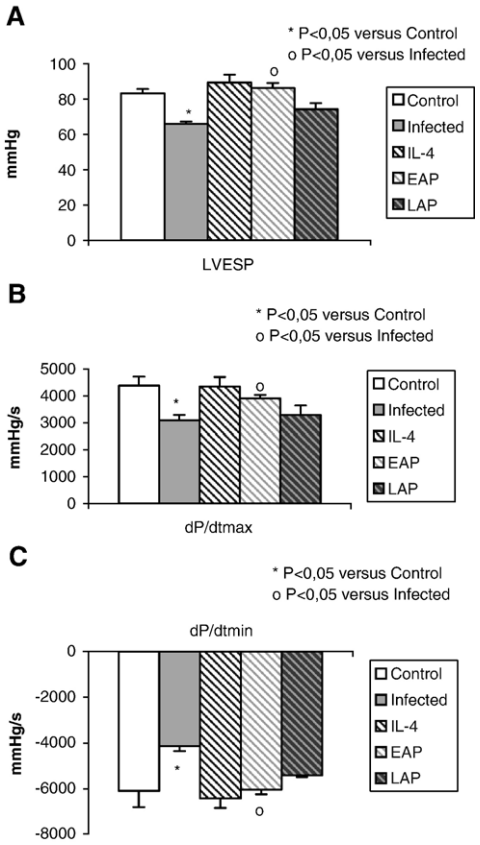

Fig. 5. Anesthetized, artificially ventilated, and open-chest operated mice underwent hemodynamic evaluation via a 1.2 F Milar tip catheter on the 10th day post-infection with different treatment strategies. A–C show left ventricular endsystolic pressure, dP/dt max, and dP/dt min in different treatment groups. A significant improvement of left ventricular endsystolic pressure, dP/dt max, and dP/dt min was observed in the EAP group. IL-4 alone did not influence the left ventricular function in the uninfected control group (IL-4).

open-chest experimental mice underwent hemodynamic evaluation with a 1.2 F Milar tip catheter introduced into the left ventricular chamber. The left ventricular pressure, the maximal rate of pressure increase over time (dP/dt max.), and the minimal rate of pressure decrease over time (dP/dt min.) were recorded.

2.10. Statistical analysis

The data are expressed as mean±S.D. Comparisons of the data on left ventricular function were made with the Mann-Whitney U test and Kruskal-Wallis analysis of variance. The rest of the data was analyzed by post-hoc Bonferroni test following one-way analysis of variance. A P value <0.05 was considered significant.

3. Results

3.1. Reduction of intramyocardial inflammation by interleukin-4

The infiltration of lymphocytes and macrophages were detected by immunoperoxidase labeling, in order to study the extent of myocardial inflammation in response to coxsackieviral infection and interleukin-4 treatment. Ten days after infection, significant infiltration of $CD3^+$-T-lymphocytes and $CD11b^+$ macrophages was demonstrated in the myocardium of infected mice (Fig. 1A–D). IL-4 treatment reduced myocardial infiltration of $CD3^+$, $CD8a^+$ lymphocytes, and $CD11b^+$ macrophages following infection (Fig. 1D). A significant reduction of myocardial inflammation was observed in EAP when compared to infected mice without treatment, as shown by reduced $CD3^+$ lymphocytes (55.3±7.0 vs. 72.1±13.7 cells/mm^2), reduced $CD8a^+$ lymphocytes (31.7±3.6 vs. 64.2±7.7 cells/mm^2, $P<0.01$), and reduced $CD11b^+$ macrophages (5.1±2.3 vs. 13.2±2.5 cells/mm^2, $P<0.01$) (Fig. 1D).

3.2. Influence of IL-4 treatment on cytokine expression in viral myocarditis

Since the pathogenesis of CVB3-induced myocarditis in BALB/c mice is characterized by a predominant Th_1 inflammatory response in the myocardium, we decided to investigate the effects of IL-4 treatment on myocardial cytokines, as related to the differentiation of Th-cells. Semi-quantitative RT-PCR showed a significant increase in the expression of IL-2, a pro-inflammatory Th_1 cytokine, in the myocardium following infection. Interleukin-4 administration attenuated CVB3 infection-induced IL-2 expression. The most significant effect was detected in the EAP group, with a decrease of IL-2 mRNA by 1.7-fold ($P<0.01$, Fig. 2). The IFN-γ and tumor necrosis factor-alpha (TNF-α) expressions in response to CVB3 infection were not influenced by any mode of IL-4 administration (data not shown). By contrast, mRNA expressions of the anti-inflammatory cytokines IL-4 and TGF-β_1 were further increased in the

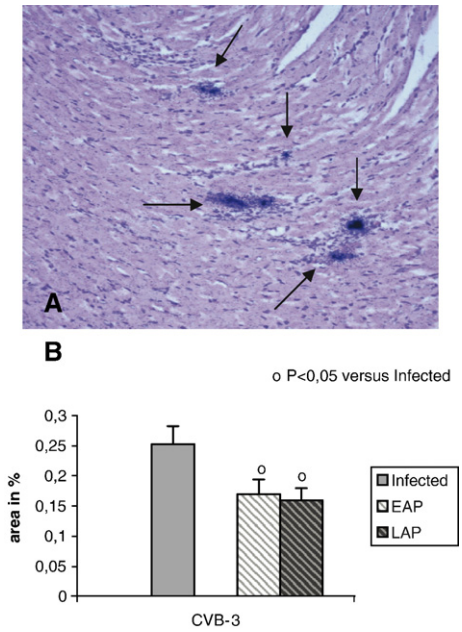

Fig. 6. Positive mRNA signals of CVB-3 were shown in paraffin-embedded cardiac sections by in situ hybridization (6A, arrows). IL-4 administration significantly reduced CVB-3 RNA in mice 10 days post-infection. The area percent of measured CVB-3 signals though showed no significant difference between the EAP and LAP groups. CVB-3 mRNA was not detected in the uninfected control group (6B).

EAP group (1.5-fold, $P<0.01$ and 1.4-fold, $P<0.01$, respectively), and the mRNA expression of IL-4 was increased in the LAP group (1.4-fold, $P<0.05$) (Fig. 2).

3.3. Regulation of myocardial MMPs by IL-4 treatment

We recently observed that altered myocardial MMPs are associated with left ventricular dysfunction in an acute myocarditis model induced by Coxsackievirus (Li et al., 2002). Here we investigated whether exogenous IL-4 influenced myocardial MMPs. The increase in MMP-2 and MMP-9 mRNA expression following infection was suppressed by exogenous interleukin-4 treatment (EAP: 2.1-fold and 1.2-fold, respectively, $P<0.05$; LAP: 1.4-fold and 1.8-fold, respectively, $P<0.05$; Fig. 3). MMP-3 mRNA expression showed only a trend of reduction in the EAP group (1.2-fold). Protein activity analysis by zymography revealed a similar change in MMP-2 and MMP-9 in response to interleukin-4 treatment (EAP: 2.2-fold and 4.6-fold, respectively, $P<0.01$; LAP: 1.7-fold and 1.8-fold, respectively, $P<0.01$) (Fig. 4A, B). Immunohistochemical staining of CVB3-infected heart sections showed that the positive signals for MMP-3 and MMP-9 mRNA were scarcely detected in those interstitial areas in which inflammatory infiltrates occurred (Fig. 4C, F). Using both macrophage marker CD11b and MMP-3/MMP-9, myocardial infiltrating macrophages were found to be the main sources of MMP-3 and MMP-9 production on serially cut myocardial sections (Fig. 4C–H).

3.4. Left ventricular function following IL-4 administration

We have previously shown that a significant left ventricular dysfunction occurs in association with the alterations of myocardial MMPs and cytokine milieu in this murine model of CVB3-induced myocarditis (Li et al., 2002). In the present study we examined whether IL-4 treatment may have an effect on left ventricular function in acute myocarditis.

CVB3-infected mice developed a significant impairment of left ventricular function as evidenced by reduced left ventricular endsystolic pressure, dP/dt max, and dP/dt min (Fig. 5A–C). IL-4 treatment led to a beneficial effect on cardiac function in infected mice, and the effect was profound in the EAP group, in terms of left ventricular endsystolic pressure (86.25±5.68 mmHg vs. 65.74±4.32 mmHg, $P<0.01$) and dP/dt max (6049±419 mmHg/s vs. 4139±637 mmHg/s, $P<0.01$). Trends toward improvement of dP/dt min in the EAP group and of left ventricular endsystolic pressure and dP/dt max in the LAP group was also observed (Fig. 5A–C).

3.5. CVB-3 virus load and myocytolysis

In order to rule out a directly detrimental effect by CVB3 infection on the myocardium, we examined the CVB-3 load and looked for a potential change in myocytolysis. In situ hybridization showed that IL-4 treatment resulted in a reduced level of CVB-3 genes in the myocardium 10 days post-infection. The area percent of measured CVB-3 signal showed no significant differences between the EAP and LAP groups (Fig. 6A/B).

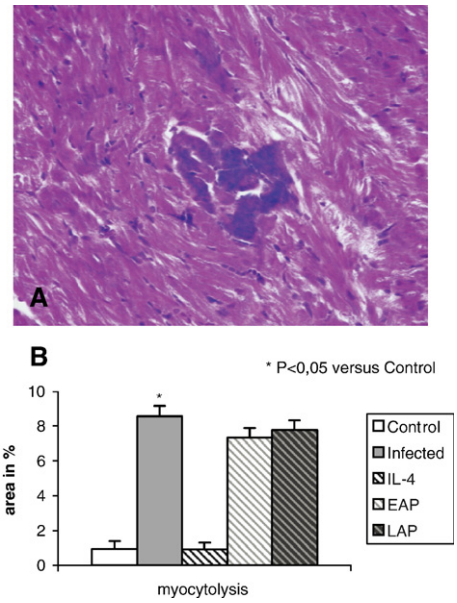

Fig. 7. Luxol fast blue staining shows the myocytolysis in infected mice (7A). Increased myocytolysis was detected in mice after infection. IL-4 administration showed only a trend of reduction in cardiomyocytolysis in the EAP and LAP groups (7B).

Although IL-4 administration did not significantly reduce the cardiomyocytolysis (EAP: 1.2-fold, LAP: 1.1-fold), we did find a significant induced degeneration of cardiomyocytes in infected mice compared to controls (8.9-fold, $P<0.01$), (Fig. 7A, B).

4. Discussion

Inflammatory immune response is the major mechanism for the pathogenesis of CVB3-induced myocarditis in BALB/c mice. Immunomodulation by adequate IL-4 treatment resulted in the up-regulation of the anti-inflammatory cytokines IL-4 and TGF-β1 and in down-regulation of Th_1 inflammatory cytokines such as IL-2. A reduction of IL-2 may then lead to the inhibition of MMPs. A significant suppression of MMPs may mainly contribute to an improvement of left ventricular dysfunction in acute murine CVB3-induced myocarditis.

4.1. Myocardial inflammation in viral myocarditis following IL-4 treatment

Many previous studies have shown that inflammatory induction is a prerequisite for the pathogenesis of CVB3-induced myocarditis in BALB/c mice (Huber et al., 1994; Huber and Job, 1983; Kishimoto et al., 1985). The abnormal remodeling of the myocardial matrix does not occur in athymic/T-cell-deficient BALB/c mice (Kishimoto et al., 1985). Infected, T-lymphocyte-deficient BALB/c mice show either minimal or no cardiac injury, although virus concentrations in the hearts of T-cell-deficient and normal animals are similar (Kishimoto et al., 1985). In this study, the myocardial inflammatory induction in CVB3-infected BALB/c mice was characterized by increased infiltrations of macrophages and lymphocytes, and by the overexpression of inflammatory

cytokines such as IL-2. Although IL-4 and TGF-β1 were up-regulated, the increased inflammatory cytokines may dominate in a predominantly Th_1 cell phenotypic response in CVB3-infected BALB/c mice (Huber et al., 1994; Huber and Job, 1983). IL-4 treatment modulated the expression pattern of myocardial cytokines. The anti-inflammatory cytokines IL-4 and TGF-β1 were up-regulated, and the Th_1 inflammatory cytokine IL-2 was down-regulated in CVB3-infected mice treated with IL-4. Cytokines are important regulators during the differentiation of native Th0 cells toward Th_1 or Th2. IL-4 polarizes precursor Th0 cells to IL-4 secreting Th2 cells; whereas, IL-12 switches Th0 cells to IL-2 secreting Th_1 cells (Yoshimoto et al., 1996; Mosmann et al., 1986). Moreover, IL-4 has been shown to convert Th_1 cells to Th0/Th2 cells in vitro (Murphy et al., 1996) and to induce TGF-β secreting T-cells in vitro (Inobe et al., 1998).

Interestingly, IL-4 treatment for the first five days, contrary to the second five days, was more effective in suppressing myocardial inflammation. This may be explained by several recent findings. It has previously been shown that IL-4 can no longer convert the murine Th_1 to Th0/Th2 cells when Th0 cells are already polarized into Th_1 cells for a long period of time, thus demonstrating that the plasticity of Th_1 cells is lost after a long stimulation (Murphy et al., 1996). Exogenous IL-4 may fail to convert Th_1 cells to Th2 cells in vivo after five days, which would explain its lower effectiveness in suppressing myocardial inflammation.

On the other hand, IL-2 itself is a key stimulator not only for T-cell differentiation, survival, clonal expansion, and infiltration (Vella et al., 1998), but also for monocyte/macrophage proliferation (Taniguchi and Minami, 1993). An in vitro study has shown that IL-4 can block acute IL-2 production of naïve T-cells derived from transgenic mice expressing specific T-cell receptors, but that IL-4 fails to block IL-2 production by such cells after they have been primed (Tanaka et al., 1993). The state of the naïve T-cells may also be relevant for IL-4 production. It has been reported that treatment of mice with neutralizing anti-IL-4 antibodies at the time of immunization causes significant inhibition of the priming of T-cells for the production of IL-4 (Gross et al., 1993). By contrast, several mechanisms may be involved in IL-4-mediated TGF-β_1 induction. For instance, exogenous IL-4 may directly induce the differentiation of TGF-secreting T-cells (Inobe et al., 1998). In addition, IL-4-mediated TGF production can also be further enhanced by TGF itself in both T-cells and non-T-cells (Kim et al., 1989; Van Obberghen-Schilling et al., 1988). Thus, mechanisms related to cytokine production in naïve T-cells and other T-cell subsets may also account for a reduced inflammatory response after IL-4 treatment.

During inflammatory infiltration, IL-2 and other Th_1 cytokines such as the macrophage inflammatory proteins 1α and 1β that are present exclusively in Th_1 cells differentiated in in vitro cultures (Loetscher et al., 1996; Lerner et al., 2000), play a pivotal role for the chemotaxis of lymphocytes and monocyte/macrophages (Loetscher et al., 1996; Natuk and Welsh, 1987). The reduced infiltration of macrophages and lymphocytes after IL-4 treatment may be mainly attributable to an inhibition of these Th_1 cytokines including IL-2, especially in the EAP group.

Based on these findings, it seems that the early IL-4-mediated immunomodulation after CVB3 infection is required for exerting a cardioprotective effect, which may be totally different from the mechanisms behind interferon therapy. It is known that IFN-γ production is important in reducing viral replication at days 2 and 12 after CVB3 infection but does not alter myocardial inflammation including IL-1β expression at day 12 post-infection during acute myocarditis (Fairweather et al., 2003). In addition, the fact that myocardial inflammation was not affected by IFN-γ deficiency indicates that IFN-γ is not a dominant factor in resolving acute inflammation following CVB-3 infection (Fairweather et al., 2003). Because the effect of IFN-γ on regulating T-cell populations appears to occur later during the pathogenesis of chronic disease, a primary role for IFN-γ treatment is probably related to the reduction in mast cell degranulation and myocardial fibrosis during the chronic processes of myocarditis and dilated cardiomyopathy (Fairweather et al., 2004). In contrast, IL-10 and IL-4 may share some immunomodulatory mechanisms similar to IL-2 production, as shown in a murine experimental model of acute viral myocarditis caused by the encephalomyocarditis virus (Nishio et al., 1999).

4.2. Myocardial MMPs and TIMPs in viral myocarditis following interleukin-4

Since $CD11b^+$ macrophages are co-localized with increasing MMP-3 and MMP-9 expression, the most significant suppression of MMPs, as shown in the EAP group, may be achieved mainly by a reduction of macrophage infiltration. Cytokines have been shown to mediate the synthesis of MMPs in vitro (Ihn et al., 2002; Edwards et al., 1987; Saren et al., 1996). Other related mechanisms may include the inhibitory roles of anti-inflammatory cytokines such as IL-4, which potently inhibit the release of MMPs in macrophages and monocytes (Lacraz et al., 1992; Corcoran et al., 1992). IL-1β and TNF-α have been found to be involved in stimulating the synthesis of MMP-9 in cultured macrophages (Saren et al., 1996). In the present study, the expression of IL-1β and TNF-α was unchanged after immunomodulation with IL-4 treatment (data not shown). Nonetheless, we cannot exclude the possibility that IL-4 also played a role in the regulation of MMPs via an early alteration of these cytokines, because induced cytokine mRNA, such as that of IL-1β, TNF-α, occurs as early as 3 days after viral inoculation (Shioi et al., 1996). Additionally, the direct effects of altered cytokines and reduced infiltrated CD8 T-lymphocytes on myocardial injury cannot be completely excluded (Murray and Freeman, 1996).

All members of MMPs participate in myocardial matrix degradation. MMP-3 cleaves many extracellular matrix components including collagen type I and activates other MMPs such as MMP-9 (Okada et al., 1995). MMP-2 and MMP-9 are referred to as gelatinase, due to their ability to effectively degrade gelatin and other collagen subtypes (Okada et al., 1995). In human dilated cardiomyopathy and murine myocarditis, the enhancement of MMP-3 and MMP-9 is associated with myocardial collagen degradation (probably by a posttranslational mechanism), which may then lead to left ventricular

dysfunction (Ogata et al., 1992; Li et al., 2002; Thomas et al., 1998). Conversely, suppression of cardiac MMPs by IL-4 treatment may result in an improvement of left ventricular dysfunction in murine CVB3-induced myocarditis.

The detection of enteroviral genome on the 10th post-infection day showed no significant differences in the EAP compared to the LAP group, suggesting that the positive effects of IL-4 on cardiac function in EAP as compared to LAP should not be due to reduced virus loads. These results are consistent with the analysis of myocytolysis by Luxol fast blue staining. In this assay, no significant change was shown between EAP and LAP groups concerning myocytolysis. Thus, a change in virus-associated toxicity in this treatment model may not contribute to the significant improvement of left ventricular function seen in the EAP group. Nonetheless, a differential effect of IL-4 treatment on viral loads between EAP and LAP groups cannot be completely excluded at a much earlier phase.

4.3. Limitations

One limitation of our study is that we did not expand our experiments with specific IL-4 inhibitors. Furthermore, the study groups were relatively small, although the groups were large enough to provide sufficient statistical power to reveal significant differences between the groups. Hemodynamic measurements were carried out invasively by the use of a Milar tip catheter, but we did not perform echocardiographic measurements of left ventricular-hypertrophy or left ventricular-diameters.

5. Conclusions

Immunomodulation by exogenous IL-4 treatment leads to an anti-inflammatory effect. It inhibits the Th_1 cell phenotypic response, which may further mediate the down-regulation of MMPs. A significant suppression of MMPs may mainly contribute to an improvement of left ventricular dysfunction in acute murine CVB3-induced myocarditis.

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