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# Findings in Murine Viral Myocarditis

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## 1. Introduction

Acute myocarditis may not only develop into congestive heart failure, but it has also been strongly implicated in the pathogenesis of dilated cardiomyopathy. The mechanism of myocardial cell injury involved in acute myocarditis is of great clinical significance, but remained to be clarified for a long period. Because patients with acute myocarditis often show significantly increased virus titer in serum, and the myocardial histological findings of acute myocarditis are similar to those of experimental viral myocarditis, it is believed that most of human acute myocarditis is induced by virus infection. Many studies have been done on the experimental murine viral myocarditis caused by Coxsackievirus (CVB3), which is the most common pathogen of human acute myocarditis. Because maximal inflammation develops after a significant decrease in virus titer, it is thought that immunological mechanisms in addition to the direct cytolytic effects of viruses play a critical role in myocardial injury in viral myocarditis [1]. Furthermore, myocardial necrosis occurs with massive cell infiltration, strongly suggesting that cell-mediated (rather than humoral) cytotoxicity plays an important role.

Using a murine model of viral myocarditis caused by CVB3, we investigated two aspects of cell-mediated immune mechanism involved in myocardial injury. First, we analyzed the characteristics of the infiltrating immune effector cells and their mechanism of cytotoxicity, especially a role of pore-forming protein (perforin), one of the most important cytolytic effector molecules with which killer lymphocytes directly injure target cells. Second, we investigated the mechanism of infiltrating T-cell activation, usage of T-cell receptor (TCR) repertoire, expression of major histocompatibility complex (MHC) antigens, and co-stimulatory signals for T-cell activation, which are mainly mediated by members of the immunoglobulin as well as tumor necrosis factor (TNF) receptor/ligand superfamilies.

## 2. Characteristics of the infiltrating cells

### 2.1. Phenotypic analysis

There were some studies reporting the phenotypes of the immune cells playing a critical role in the development of murine viral myocarditis. These studies showed indirect evidence that T-cells, cytotoxic T-lymphocytes (CTLs), or natural killer cells (NK cells) mediated the inflammation characterized by mononuclear cell infiltration and cardiac myocyte necrosis [1-4]. However, there had been no reports directly showing the phenotypes of the infiltrating mononuclear cells and whether these infiltrating cells directly injure the cardiac myocytes. We analyzed the phenotypes of the infiltrating cells in the heart of murine viral myocarditis by immunohistochemistry with antibodies specific for NK cells, T-cells, T-helper cells (Th-cells), CTLs, and macrophages, which are the major effector cell types in cell-mediated immunity. There were almost no  $\gamma\delta$  T-cells expressing TCR  $\gamma\delta$ . Also, we found that most of the infiltrating cells were NK cells in the early stage (on day 7 after virus infection) when maximal inflammation develops, and T-cells consisting of Th-cells and CTLs represented 10% of the infiltrating cells. The proportion of T-cells increased to 30-40% in the later stage of acute myocarditis [5]. Next, we examined the ultrastructure of the infiltrating cells by electron microscopy, and found them to be large granular lymphocytes [5]. Thus, the phenotypic and morphological analyses revealed that most of the infiltrating cells are NK-like large granular lymphocytes in the early stage when maximal inflammation develops.

### 2.2. Expression of a cytolytic factor perforin

NK cells and CTLs are thought to kill virus-infected cells or tumor cells by means of effector molecules contained in their cytoplasmic granules, one of which and the most important is called pore-forming protein or perforin. Perforin was shown to play a critical role in cytolysis and can be a good marker for killer lymphocytes [6-8]. To investigate whether these infiltrating cells express perforin in their cytoplasmic granules and directly injure cardiac myocytes, we examined the expression of perforin by immunohistochemistry, *in situ* hybridization, and immunoelectron microscopy. We found that about 15% of the infiltrating cells strongly expressed perforin in their cytoplasmic granules, and most of the infiltrating cells expressed perforin gene transcripts [5]. Electron microscopic analysis revealed that the infiltrating cells released massive amount of perforin molecules directly onto the surface of cardiac myocytes. There were also numerous circular lesions, consistent with pores formed by perforin on the membrane of cardiac myocytes [9]. These data clearly showed that the infiltrating cells were NK-like killer cells and directly destroy cardiac myocytes in acute myocarditis *in vivo*. We also showed the expression of perforin in the infiltrating cells in the hearts of patients with acute myocarditis and dilated cardiomyopathy [10]. These data strongly suggested that perforin-expressing killer lymphocytes play a pivotal role in myocardial inflammation. Gebhard, et al. [11] reported that perforin knockout mice infected with CVB3 develop only a mild myocarditis as compared with extensive inflammation of perforin-positive mice, whereas virus titers were indistinguishable between two groups. This supports the role of perforin in inflammation but not in virus clearance, and offers perforin to be a possible therapeutic target. However, because

the strain of mice used in the study is known to develop minimal myocarditis by CVB3, further investigation using virus-sensitive strains of mice may be needed.

### 2.3. T-cell receptor (TCR) repertoire

Phenotypic analysis revealed that NK-like killer lymphocytes infiltrate the heart first, then infiltration by T-cells subsequently increases in the later stage. To investigate the nature of T-cell infiltration, we analyzed the expression of TCR V $\beta$  genes in the heart of acute murine myocarditis. Polymerase chain reaction (PCR)-amplified V $\beta$  gene products were subjected to Southern blot hybridization with a C $\beta$  cDNA probe. We found that in contrast to spleen lymphocytes, the expression of TCR V $\beta$  genes in the heart was restricted [12]. The restricted usage of TCR V $\beta$  genes by infiltrating T-cells indicated that some specific antigens in the heart with viral myocarditis were being targeted. We also demonstrated the restricted usage of TCR V $\alpha$  as well as V $\beta$  genes by infiltrating cells in the hearts of patients with acute myocarditis and dilated cardiomyopathy [10]. This strongly suggested that the infiltration by T-cells recognizing some specific antigens in the heart continued, resulting in persistent myocardial cell damage, which led to the development of dilated cardiomyopathy. Because no enterovirus genomes were detected in the heart tissue by PCR in all patients, it seemed that a T-cell-mediated autoimmune mechanism may be triggered by virus infection and go on to play a pivotal role in the pathogenesis of persistent myocardial cell damage.

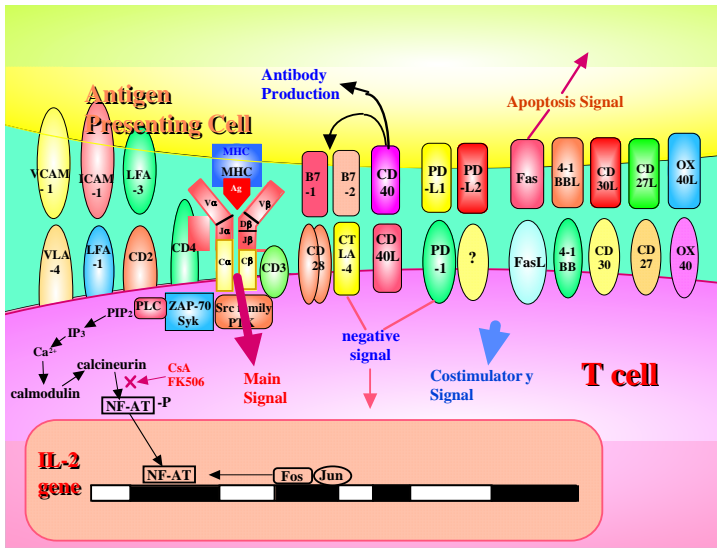
## 3. Interaction between the infiltrating cells and cardiac myocytes

### 3.1. Expression of major histocompatibility complex (MHC) antigens

T-cells expressing TCR  $\alpha\beta$ , consisting of CTLs and Th-cells, are known to recognize foreign antigens, such as virus-derived proteins, by their TCRs, in association with syngeneic MHC antigens on the surface of antigen-presenting cells (APCs). The recognition of MHC antigens by CTLs and Th-cells is restricted MHC classes, in general class I for CTLs and class II for Th-cells [13, 14]. To become target cells for the infiltrating T-cells, virus-infected cells need to express MHC antigens on their surfaces. To examine whether cardiac myocytes, which were reported not to express these antigens under normal conditions [15, 16], really express MHC antigens during acute viral myocarditis, we analyzed the expression of MHC antigens in hearts with acute murine myocarditis induced by CVB3. We found that CVB3-induced acute myocarditis resulted in enhanced expression of MHC class I (H-2K) antigen on cardiac myocytes adjacent to the area of cell infiltration, but undetectable or low levels of MHC class I (H-2D) or Class II (Ia) antigen were seen on cardiac myocytes, respectively [17]. The induction of MHC antigens was confirmed *in vitro* in cultured cardiac myocytes by treatment with interferon (IFN)- $\gamma$  by immunohistochemistry and Northern blot analysis [17]. Induction of MHC class I antigen on cardiac myocytes with acute viral myocarditis strongly supported the interaction between cardiac myocytes and the infiltrating cells, especially CTLs, which may play a significant role in the persistent myocardial damage involved in later phase of myocarditis.

### 3.2. Expression of co-stimulatory molecules

It is necessary for T-cells to receive two signals from the APC for antigen-specific T-cell activation to occur. The first signal is provided by TCR engagement with the antigen-MHC complex. The second signal, that is co-stimulatory signal, is provided by co-stimulatory molecules expressed on both APC and T-cell [18]; they are mainly members of the immunoglobulin as well as TNF receptor/ligand superfamilies. A scheme showing the interaction between T-cell and APC is shown in Figure 1.



**Figure 1.** Interaction between T-cell and antigen-presenting cell (APC). Scheme shows pairs of receptor/ligand co-stimulatory molecules expressed on both T-cell and APC.

#### A. Immunoglobulin superfamily

Intercellular adhesion molecule-1 (ICAM-1): Cell-cell interactions in the immune responses are known to be mediated by cell adhesion molecules expressed on both immune effector cells and target cells. One of the most important cell adhesion molecules is intercellular adhesion molecule-1 (ICAM-1), a ligand for lymphocyte function-associated antigen -1 (LFA-1), is expressed on most lymphocytes and thought to be induced on various target cells at the site of inflammation by cytokines [19]. ICAM-1 is known to provide a co-stimulatory signal for T-cell activation and to play an important role in the recognition, adhesion, and destruction of target cells by killer lymphocytes. Therefore, we analyzed the expression of ICAM-1 in hearts with acute murine myocarditis induced by CVB3. We found that acute myocarditis resulted in enhanced expression of ICAM-1 on cardiac myocytes, and most of the infiltrating cells expressed LFA-1 [20]. Induction of ICAM-1 was also confirmed *in vitro* in cultured cardiac myocytes by treatment with IFN- $\gamma$ /TNF- $\alpha$  by immunohistochemistry, flow cytometry, and

Northern blot analysis [20]. Because both interferon- $\gamma$  and TNF- $\alpha$  were shown to be expressed by the infiltrating cells in the heart by *in situ* hybridization [20], the expression of ICAM-1 as well as MHC class I antigen on cardiac myocytes was thought to be induced by the infiltrating cells *in vivo*. Furthermore, we found that *In vivo* administration of an anti-ICAM-1 monoclonal antibody (mAb) significantly reduced myocardial inflammation without enhancing virus genomes in the heart [20]. We also found the expression of ICAM-1 and MHC class I antigen on cardiac myocytes and infiltration by perforin-expressing killer cells without enterovirus genomes in the heart of patients with acute myocarditis and dilated cardiomyopathy [10]. This suggested that the infiltrating killer cells may recognize some autoantigen and continuous expression of ICAM-1 as well as MHC class I antigen on cardiac myocytes may enable the infiltrating killer cells to cause persistent myocardial damage in an autoimmune phase of myocarditis, leading to dilated cardiomyopathy.

**Vascular cell adhesion molecule-1 (VCAM-1):** Another immunoglobulin family cell adhesion and co-stimulatory molecule, VCAM-1 was also reported to be induced on myocardial cells in acute murine myocarditis. However, the role of VCAM-1 in the myocardial damage seemed to be less important than ICAM-1 [21].

**B7 family molecules (B7-1, B7-2):** Among the immunoglobulin superfamily co-stimulatory molecules, B7-1 and B7-2, which are the ligands for CD28 and cytotoxic T lymphocyte antigen (CTLA)-4 expressed on T-cells, have been extensively characterized and appear to be most critical [22-24]. To investigate the role of B7-1/B7-2 in the development of acute viral myocarditis, we analyzed the expression of B7-1/B7-2 in hearts with acute murine myocarditis induced by CVB3. We found that acute myocarditis strongly induced the expression of both B7-1 and B7-2 on cardiac myocytes, which normally do not express these antigens [25]. The induction of both B7-1 and B7-2 was also confirmed *in vitro* in cultured cardiac myocytes by treatment with interferon- $\gamma$ . *in vivo* administration of an anti-B7-1 mAb markedly decreased myocardial inflammation, whereas an anti-B7-2 mAb-treatment abrogated the protective effect of anti-B7-1 mAb [25], indicating that different roles for B7-1 and B7-2 antigens are involved in the development of acute myocarditis. Using a murine model of chronic ongoing myocarditis, we also found that *in vivo* administration of an anti-B7-1 mAb significantly prolonged the survival of mice with myocarditis, whereas an anti-B7-2 mAb-treatment abrogated the survival-prolonging effect of anti-B7-1 mAb [26]. We found the expression of B7-1 and B7-2 on cardiac myocytes of patients with acute myocarditis and dilated cardiomyopathy [27], strongly suggesting the critical roles of these co-stimulatory molecules as in murine myocarditis. In contrast to the many co-stimulatory molecules, which deliver positive signals for T-cell activation, CTLA-4, a second B7 receptor, delivers a negative signal for T-cell activation competing with CD28. T-cell immunoglobulin mucin (Tim)-3 is highly expressed on Th1 cells, and is known to negatively regulate Th1 responses and affects susceptibility to allergy and autoimmune diseases. Frisanho-Kiss et al. [28] reported that *in vivo* anti-Tim-3 blocking mAb-treatment reduced CTLA-4 levels in Th-cells in the spleen, and significantly increased myocardial inflammation of mice infected with CVB3. This indicates the negative regulatory role of CTLA-4 through Tim-3 signaling in viral myocarditis. Furthermore, Love et al. [29]

showed a negative regulatory role of CTLA-4 in CTLs, using a murine model of myocarditis caused by adoptive transferred antigen-specific CTLs.

**Programmed death-1 (PD-1)/PD-1 ligands (PD-L1, PD-L2):** Among other known co-stimulatory molecules, which mediate negative signals for T-cell activation, PD-1/PD-1 ligands, belonging to the immunoglobulin superfamily, pathway seems to be the most important [30-33]. To investigate roles of PD-1/PD-1 ligands pathway in the development of myocardial damage in murine acute myocarditis, we examined the expression of PD-L1 and PD-L2 in hearts with acute myocarditis induced by CVB3. We found that the expression of PD-L1 (but not PD-L2) was markedly induced on cardiac myocytes with acute myocarditis. The induction of PD-L1 (but not PD-L2) was also confirmed *in vitro* in cultured cardiac myocytes by treatment with IFN- $\gamma$  [34]. Furthermore, *in vivo* treatment with anti-PD-1 blocking mAb significantly increased the myocardial inflammation, whereas anti-PD-1 stimulating mAb-treatment significantly decreased the myocardial inflammation. *In vivo* treatment with anti-PD-L1 blocking mAb increased the inflammation (but statistically not significant), whereas anti-PD-L2 blocking mAb-treatment had no effect [34]. This indicated that PD-1/PD-L1 pathway plays a critical role in suppressing myocardial inflammation induced by CVB3 infection.

#### B. TNF receptor/ligand superfamilies

**Fas and Fas ligand (FasL):** Fas and its ligand FasL, which belong to the TNF receptor/ligand superfamily, are well-characterized co-stimulatory molecules and known to play an essential role in the induction of apoptosis [35-38]. They are also known to play an important role in the cytotoxicity by T-cells and NK cells [39-41]. Because the percentage of cardiac myocytes undergoing apoptosis was too low to explain the mechanism involved in massive myocardial injury in acute murine myocarditis, we investigated the role of Fas/FasL pathway in the activation of the infiltrating immune cells. We found that Fas was markedly induced on cardiac myocytes with acute myocarditis. The induction of Fas expression on cardiac myocytes was confirmed *in vitro* by treatment with IFN- $\gamma$ . *In vivo* administration of an anti-FasL mAb decreased myocardial inflammation as well as virus genomes in the heart. Myocardial inflammation was also decreased in Fas-deficient *lpr/lpr* and FasL-deficient *gld/gld* mice infected by CVB3 as compared with wild type [42]. This strongly suggested that Fas/FasL pathway played a critical role in the development of myocardial necrosis through activation of the infiltrating immune cells, rather than inducing apoptosis of cardiac myocytes.

**CD40/CD40 ligand (CD40L):** Another pathway of co-stimulatory molecules CD40, CD40L, which belong to the TNF receptor/ligand superfamily, is known to induce expression of B7 antigens and cytokine production by APCs, and to initiate T-cell-dependent antibody responses [43-45]. We found that CD40 was clearly induced on cardiac myocytes with acute myocarditis, and that the expression of CD40 on cardiac myocytes was induced by treatment with IFN- $\gamma$  *in vitro*. We also found that the production of interleukin-6 by cultured cardiac myocytes was markedly enhanced by treatment with an anti-CD40 mAb *in vitro*. *In vivo* administration of an anti-CD40L mAb significantly decreased myocardial inflammation, indicating a critical role of CD40/CD40L pathway in the development of acute murine myocarditis [46].

**CD30/CD30L, CD27/CD27L, OX40/OX40L, 4-1BB/4-1BBL:** Other co-stimulatory molecules belonging to the TNF receptor/ligand superfamily include CD30/CD30L, CD27/CD27L, OX40/OX40L, and 4-1BB/4-1BBL [47, 48]. We again investigated the roles of these co-stimulatory molecules in the development of acute murine myocarditis [49]. Acute myocarditis caused by CVB3 clearly induced the expression of 4-1BBL and CD30L on cardiac myocytes *in vivo*, whereas CD27L and OX40L were constitutively expressed on cardiac myocytes. Induction of 4-1BBL and CD30L on cardiac myocytes was confirmed by treatment with IFN- $\gamma$  *in vitro*. Anti-4-1BBL or -CD30L mAb along with IFN- $\gamma$  significantly stimulated the production of interleukin-6 by cultured cardiac myocytes *in vitro*. Furthermore, *in vivo* administration of anti-4-1BBL mAb (but not other mAbs) significantly decreased myocardial inflammation, indicating the critical role of 4-1BB/4-1BBL pathway in the development of acute viral myocarditis. We found a persistent expression of CD40 and CD30L on cardiac myocytes in a murine model of chronic ongoing myocarditis as well [50].

## 4. Therapeutic interventions

### 1. *In vivo* antibody therapy

It is known that immunosuppressant therapy with corticosteroids or cyclosporin [51] may exacerbate acute viral myocarditis by enhancing virus titers. Godeny and Gauntt [3, 4] reported that depleting NK cells by injection of anti-asialo GM1 antiserum exacerbated murine viral myocarditis with increase in virus titers in the heart, indicating the protective role of NK cells against viral myocarditis by limiting virus replication. Therefore, nonspecific immunotherapies inhibiting virus-clearance seem to worsen the course of viral myocarditis, at least in the acute phase when virus genomes have not disappeared yet. We showed that immunomodulation therapy specifically targeting co-stimulatory molecules, such as ICAM-1 and FasL by *in vivo* administration of blocking mAbs, can decrease myocardial damage without inhibiting (or even enhancing) virus-clearance [20, 42]. We also showed that immunomodulation therapy targeting co-stimulatory molecules B7-1, CD40L, 4-1BBL, and PD-1 (with stimulating mAb) can significantly attenuate myocardial inflammation [25, 46, 49, 34]. Although we did not analyze the effects of these therapies on the virus-clearance in the heart, the protective effects against myocardial injury strongly suggested that immunomodulation therapies targeting these co-stimulatory molecules improve the course of myocarditis without inhibiting virus-clearance. The relative effects of immunomodulation therapies targeting co-stimulatory molecules is summarized in Figure 2. Recently, Fousteri et al. reported that *in vivo* administration of anti-OX40L mAb strongly reduced the inflammation of chronic phase of CVB3-induced murine myocarditis, supporting the role of these co-stimulatory molecules in progression to autoimmune phase [52].

### 2. IFNs

IFNs are among the most important antiviral agents, and are clinically used in hematological malignancy, autoimmune disorder, and viral infection such as hepatitis B and C. For viral myocarditis, the effectiveness of IFN- $\alpha$  A/D in a murine model of viral myocarditis

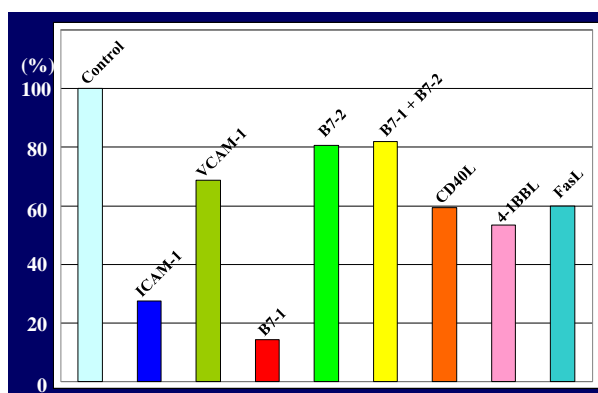
was reported [53, 54]. Yamamoto et al. [55] analyzed the effects of IFN- $\gamma$  and IFN- $\alpha/\beta$  by intranasal and intramuscular routes on murine viral myocarditis. The authors found that both IFN- $\gamma$  and IFN- $\alpha/\beta$  by either route significantly increased the survival rate and that the effect of IFN- $\gamma$  was significantly greater than that of IFN- $\alpha/\beta$ . The survival-prolonging effect of IFN- $\gamma$  was confirmed even when started after virus inoculation. Furthermore, intranasal administration of IFN- $\gamma$  significantly suppressed the virus replication and inflammation in the heart, which in turn dramatically improved the prognosis of acute murine viral myocarditis. The intranasal administration of IFN- $\gamma$  offers a very useful antiviral therapy for acute myocarditis in clinical use.

### 3. TNF- $\alpha$

TNF- $\alpha$  is another major cytokine known to be involved in viral myocarditis. Wada et al. [56] reported that survival rate of TNF- $\alpha$ -deficient mice with acute viral myocarditis was significantly lower than that of wild-type control mice, and *in vivo* administration of recombinant TNF- $\alpha$  improved the survival of TNF- $\alpha$ -deficient mice in a dose dependent manner. Although the authors speculated that TNF- $\alpha$  plays a protective role in acute viral myocarditis through leukocyte recruitment, it is unclear whether administration of TNF- $\alpha$  improves the survival of wild-type mice with acute viral myocarditis.

### 4. Angiotensin II receptor blockers (ARBs)

Angiotensin II has been shown to play an important role in the pathophysiology of various organs, especially the cardiovascular system. The effects of ARB on hypertension, congestive heart failure, and myocardial fibrosis have been well analyzed in human trials as well as animal models. The focus of interest is now directed to its pleiotropic effects especially on the inflammatory disorders. To investigate the effects of the ARB olmesartan on the cell-mediated myocardial injury involved in acute myocarditis, we analyzed the effects of olmesartan on the development of murine acute myocarditis caused by CVB3 [57]. We found that olmesartan



**Figure 2.** Summary of relative effects of immunomodulation therapies targeting co-stimulatory molecules in murine acute myocarditis.



significantly decreased myocardial inflammation as compared with control. Olmesartan also significantly decreased the expression of IFN- $\gamma$ , FasL, inducible nitric oxide synthase (iNOS), perforin as well as CVB3 genomes in myocardial tissue, indicating that olmesartan suppressed activation of the infiltrating killer lymphocytes without inhibiting virus-clearance. This raises a possibility that olmesartan will reduce myocardial injury and improve prognosis of patients with acute myocarditis. Although we did not examine whether other ARBs have also protective effects against myocardial inflammation, there is a possibility that the prognosis of acute myocarditis patients receiving ARBs may be better than those not treated with ARBs.

### 5. Beta-adrenergic receptor blockers ( $\beta$ -blockers)

$\beta$ -blockers, as well as angiotensin-converting enzyme inhibitors (ACEIs) and ARBs, have now been established as the therapy of heart failure. Especially, carvedilol, a non-selective  $\beta_1$ ,  $\beta_2$  (and less potent  $\alpha_1$ )-blocker, is known for its anti-oxidant properties [58]. In murine model of viral myocarditis, carvedilol was shown to attenuate the inflammation and improve left ventricular function through modulating the production of inflammatory cytokines and matrix metalloproteinases [59-61]. Because selective  $\beta_1$ -blocker, metoprolol was much less effective, the cardioprotective effects of carvedilol may be due to pleiotropic effects as well as  $\beta$ -blocking effects, would be potentially useful in the treatment of patients with acute myocarditis.

### 6. Anti-virus therapy

Werk et al. [62] reported the effects of two anti-viral strategies, siRNA to degrade cytoplasmic CVB3 RNA, and a soluble variant of the coxsackievirus-adenovirus receptor fused to a human immunoglobulin (sCAR-Fc) to inhibit cellular uptake of CVB3. The authors demonstrated that combination therapy resulted in a strong synergistic inhibition of an ongoing virus infection. Because the study was done using a cell culture system, further study using an *in vivo* infection model is needed. Moreover, it is unknown whether the combination therapy is effective on patients with acute myocarditis who come to the hospital well after virus infection occurs.

Until now, not a few antiviral compounds have been developed and evaluated in clinical studies. WIN 63843 (pleconaril) is an orally bioavailable antiviral compound, which inhibits the binding of picornaviruses to the cell surface receptors and internalization of the viruses into the cell. In murine viral myocarditis caused by CVB3, pleconaril dramatically reduced the virus titer in the heart and increased the survival rate [63]. For other mechanism of antiviral activity, nitric oxide-releasing compounds such as glyceryl trinitrate (GTN) and isosorbide dinitrate (ISDN) were shown to inhibit proteinases 2A and 3C of CVB3, resulting in inhibition of viral replication and protecting the host cells from the cytopathic effects. Furthermore, GTN and ISDN significantly reduced the myocardial inflammation in murine model of viral myocarditis caused by CVB3 [64]. These antiviral therapeutics seem to be effective in the very early phase of viral myocarditis when viral replication actively occurs. However, in general, patients with acute myocarditis go to hospital after signs of inflammation have appeared when immune response to the virus-infected cells but not cytopathic effects of viruses mainly mediate myocardial injury. Therefore, the effectiveness of these antiviral therapeutics should be evaluated in clinical studies. On the other hand, Foustari et al. reported that nasal admin-

istration of cardiac myosin-derived oligopeptides (CM-peptides) significantly reduced myocardial inflammation and mortality by enhancing regulatory T cells and IL-10 production in murine myocarditis caused by CVB3 [52]. However, the authors started the administration of CM-peptides before CVB3-infection. Because it is impossible to start the treatment at such timing clinically, efficiency of the therapy should be evaluated when started after the onset of inflammation.

## 7. Cell therapy

Mesenchymal stem cells (MSCs) are known to have anti-apoptotic, anti-fibrotic, pro-angiogenic, as well as immunomodulatory features. Linthout et al. [65] demonstrated that MSCs reduced CVB3-infected cardiomyocytes apoptosis and viral production in a nitric oxide-dependent manner *in vitro*, and MSCs required priming via IFN- $\gamma$  to exert their protective effects. Furthermore, *in vivo* administration of MSCs in mice with CVB3-induced myocarditis improved cardiac function through reduction in cardiac apoptosis and myocardial injury. The authors also isolated and identified novel cardiac-derived cells from human cardiac biopsy specimen, that is cardiac-derived adherent proliferating cells (CAPs). CAPs have anti-apoptotic and immunomodulatory features similar to MSCs. Like MSCs, *in vivo* administration of CAPs in mice with CVB3-induced myocarditis improved cardiac function through reduction in cardiac apoptosis and virus proliferation [66].

## 8. MicroRNA

MicroRNAs (miRNAs) are small non-coding RNA molecules endogenously held by many species. It is known that miRNAs repress the expression of mRNAs by binding to 3' untranslated region of their target mRNAs. Corsten et al. [67] analyzed the profiles of miRNA expression in myocardial biopsy specimen from patients with acute myocarditis, and in myocardial tissue from myocarditis-susceptible and non-susceptible strain of mice with CVB3-induced acute myocarditis. They found that expression of microRNA-155, primarily localized in infiltrating cells, was consistently and strongly upregulated during acute myocarditis in both humans and susceptible mice. Inhibition of microRNA-155 by a systemically delivered locked nucleic acid (LNA)-anti-miRNA, a class of miRNA inhibitors, attenuated cardiac cell infiltration and myocardial damage in acute phase of murine myocarditis. MicroRNA-155 inhibition further improved cardiac function and reduced mortality of mice with viral myocarditis in later phase, offering a promising therapy against acute myocarditis. MicroRNA-122 is expressed in the liver, and is implicated as a key regulator of cholesterol and fatty-acid metabolism. Elmen et al. [68] first demonstrated using African green monkeys that *in vivo* administration of LNA-anti-microRNA-122 resulted in long-lasting decrease in plasma cholesterol levels without any toxicities. For anti-microRNA therapy against viral infection in primates, Lanford et al. [69] reported that treatment of chimpanzees chronically infected with hepatitis C virus with LNA-anti-microRNA-122 resulted in long-lasting suppression of viremia and improvement of liver pathology with safety profile. Successful study in primates against virus infection common to a human disease may strongly support clinical trials in patients with hepatitis C virus infection as well as acute myocarditis.

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## References

- [1] Woodruff JF: Viral myocarditis: A review. *Am J Pathol* 1980;101:427-484.
- [2] Guthrie M, Lodge PAA, Huber SA: Cardiac injury in myocarditis induced by coxsackievirus group B type 3 in BALB/c mice is mediated by Lyt 2+ cytolytic lymphocytes. *Cell Immunol* 1984;88:558-567.
- [3] Godeny EK, Gauntt CJ: Involvement of natural killer cells in coxsackievirus B3-induced murine myocarditis. *J Immunol* 1986;137:1695-1702.
- [4] Godeny EK, Gauntt CJ: Murine natural killer cells limit coxsackievirus B3 replication. *J Immunol* 1987;139:913-918.
- [5] Seko Y, Shinkai Y, Kawasaki A, Yagita H, Okumura K, Takaku F, Yazaki Y: Expression of perforin in infiltrating cells in murine hearts with acute myocarditis caused by Coxsackievirus B3. *Circulation* 1991;84:788-795.
- [6] Shinkai Y, Takio K, Okumura K: Homology of perforin to the ninth component of complement (C9). *Nature* 1988;334:525-527.
- [7] Young JDE: Killing of target cells by lymphocytes: A mechanistic view. *Physiol Rev* 1989;69:250-314.
- [8] Young LHY, Klavinskis LS, Oldstone MBA, Young JDE: *In vivo* expression of perforin by CD8+ lymphocytes during acute viral infection. *J Exp Med* 1989;169:2159-2171.
- [9] Seko Y, Shinkai Y, Kawasaki A, Yagita H, Okumura K, Yazaki Y: Evidence of perforin-mediated cardiac myocyte injury in acute murine myocarditis caused by Coxsackievirus B3. *J Pathol* 1993;170:53-58.
- [10] Seko Y, Ishiyama S, Nishikawa T, Kasajima T, Hiroe M, Kagawa N, Osada K, Suzuki S, Yagita H, Okumura K, Yazaki Y: Restricted usage of T cell receptor  $V\alpha$ - $V\beta$  genes in infiltrating cells in the hearts of patients with acute myocarditis and dilated cardiomyopathy. *J Clin Invest* 1995;96:1035-1041.

- [11] Gebhard JR, Perry CM, Harkins S, Lane T, Mena I, Asensio V, C, Campbell IL, Whitton JL: Cocksackievirus B3-induced myocarditis. Perforin exacerbates disease, but plays no detectable role in virus clearance. *Am J Pathol* 1998;153:417-428.
- [12] Seko Y, Yagita H, Okumura K, Yazaki Y: T-cell receptor V $\beta$  gene expression in infiltrating cells in murine hearts with acute myocarditis caused by Cocksackievirus B3. *Circulation* 1994;89:2170-2175.
- [13] Ploegh HL, Orr HT, Strominger JL: Major histocompatibility antigens : The human (HLA-A, -B, -C) and murine (H-2K, H-2D) class I molecules. *Cell* 1981;24:287-299.
- [14] Kaufman JF, Auffray C, Korman AJ, Shackelford, DA, Strominger JL: The class II molecules of the human and murine major histocompatibility complex. *Cell* 1984;36:1-13.
- [15] Daar AS, Fuggle SV, Fabre JW, Ting A, Morris PJ: The detailed distribution of HLA-A, B, C antigens in normal human organs. *Transplantation* 1984;38:287-292.
- [16] Daar AS, Fuggle SV, Fabre JW, Ting A, Morris PJ: The detailed distribution of MHC class II antigens in normal human organs. *Transplantation* 1984;38:293-298.
- [17] Seko Y, Tsuchimochi H, Nakamura T, Okumura K, Naito S, Imataka K, Fujii J, Takaku F, Yazaki Y: Expression of major histocompatibility complex class I antigen in murine ventricular myocytes infected with coxsackievirus B3. *Circ Res* 1990;67:360-367.
- [18] Mueller DL, Jenkins MK, Schwartz RH: Clonal expansion versus functional clonal inactivation: a co-stimulatory signaling pathway determines the outcome of T cell antigen receptor occupancy. *Annu Rev Immunol* 1989;7:445-480.
- [19] Wawryk SO, Novotny JR, Wicks IP, Wilkinson D, Maher D, Salvaris E, Welch K, Fecundo J, Boyd AW: The role of LFA-1/ICAM-1 interaction in human leukocyte homing and adhesion. *Immunol Rev* 1989;108:135-161.
- [20] Seko Y, Matsuda H, Kato K, Hashimoto Y, Yagita H, Okumura K, Yazaki Y: Expression of intercellular adhesion molecule-1 in murine hearts with acute myocarditis caused by Cocksackievirus B3. *J Clin Invest* 1993;91:1327-1336.
- [21] Seko Y, Yagita H, Okumura K, Yazaki Y: Expression of vascular cell adhesion molecule-1 in murine hearts with acute myocarditis caused by Cocksackievirus B3. *J Pathol* 1996;180:450-454.
- [22] Azuma M, Ito D, Yagita H, Okumura K, Phillips JH, Lanier LL, Somoza C: B70 antigens is a second ligand for CTLA-4 and CD28. *Nature* 1993;366:76-79.
- [23] Hathcock KS, Laszlo G, Dickler HB, Bradshaw J, Linsley P, Hodes RJ: Identification of an alternative CTLA-4ligand co-stimulatory for T cell activation. *Science* 1993;262:905-907.

- [24] Freeman GJ, Gribben JG, Boussiotis VA, Ng JW, Restivo VA Jr, Lombard LA, Gray GS, Nadler LM: Cloning of B7-2: a CTLA-4 counter-receptor that costimulates human T cell proliferation. *Science* 1993;262:909-911.
- [25] Seko Y, Takahashi N, Azuma M, Yagita H, Okumura K, Yazaki Y: Effects of *In vivo* administration of anti-7-1/B7-2 monoclonal antibodies on murine acute myocarditis caused by coxsackievirus B3. *Circ Res* 1998;82:613-618.
- [26] Seko Y, Takahashi N, Yagita H, Okumura K, Azuma M, Yazaki Y: Effects of *In vivo* administration of anti-7-1/B7-2 monoclonal antibodies on the survival of mice with chronic ongoing myocarditis caused by coxsackievirus B3. *J Pathol* 1999;188:107-112.
- [27] Seko Y, Takahashi N, Ishiyama S, Nishikawa T, Kasajima T, Hiroe M, et al.: Expression of co-stimulatory molecules B7-1, B7-2, and CD40 in the heart of patients with acute myocarditis and dilated cardiomyopathy. *Circulation* 1998;97:637-639.
- [28] Frisancho-Kiss S, Nyland JF, Davis SE, Barrett MA, Gatewood SJ, Njoku DB, Cihakova D, Silbergeld EK, Rose NR, Fairweather D: Cutting edge: T cell Ig mucin-3 reduces inflammatory heart disease by increasing CTLA-4 during innate immunity. *J Immunol* 2006;176:6411-6415.
- [29] Love VA, Grabie N, Duramad P, Stavrakis G, Sharpe A, Lichtman A: CTLA-4 ablation and interleukin-12-driven differentiation synergistically augment cardiac pathogenicity of cytotoxic T lymphocytes. *Circ Res* 2007;101:248-257.
- [30] Ishida Y, Agata Y, Shibahara K, Honjo T: Induced expression of PD-1, a novel member of the immunoglobulin gene superfamily, upon programmed cell death. *EMBO J* 1992;11:3887-3895.
- [31] Nishimura H, Honjo T: PD-1: an inhibitory immunoreceptor involved in peripheral tolerance. *Trends Immunol* 2001;22:265-268.
- [32] Freeman GJ, Long AJ, Iwai Y, Bourque K, Chernova T, Nishimura H, et al.: Engagement of the PD-1 immunoinhibitory receptor by a novel B7 family member leads to negative regulation for lymphocyte activation. *J Exp Med* 200;192:1027-1034.
- [33] Latchman Y, Wood CR, Chernova T, Chaudhary D, Borde M, Chernova I, et al.: PD-L2 is a second ligand for PD-1 and inhibits T cell activation. *Nat Immunol* 2001;2:261-268.
- [34] Seko Y, Yagita H, Okumura K, Azuma M, Nagai R: Roles of programmed death-1 (PD-1)/PD-1 ligands pathway in the development of murine acute myocarditis caused by coxsackievirus B3. *Cardiovasc Res* 2007;75:158-167.
- [35] Yonehara S, Ishii A, Yonehara M: A cell-killing monoclonal antibody (anti-Fas) to a cell surface antigen co-downregulated with the receptor of tumor necrosis factor. *J Exp Med* 1989;169:1747-1756.

- [36] Trauth BC, Klas C, Peters AM, Matzku S, Möller P, Falk W, et al.: Monoclonal antibody-mediated tumor regression by induction of apoptosis. *Science* 1989;245:301-305.
- [37] Itoh N, Yonehara S, Ishii A, Yonehara M, Mizushima S, Sameshima M, et al.: The polypeptide encoded by the cDNA for human cell surface antigen Fas can mediate apoptosis. *Cell* 1991;66:233-243.
- [38] Suda T, Takahashi T, Golstein P, Nagata S: Molecular cloning and expression of the Fas ligand: a novel member of the tumor necrosis factor family. *Cell* 1993;75:1169-1178.
- [39] Rouvier E, Luciani M-F, Goldstein P: Fas involvement in  $Ca^{2+}$ -independent T cell-mediated cytotoxicity. *J Exp Med* 1993;177:195-200.
- [40] Hanabuchi S, Koyanagi M, Kawasaki A, Shinohara N, Matsuzawa A, Nishimura Y, et al.: Fas and its ligand in a general mechanism of T cell-mediated cytotoxicity. *Proc Natl Acad Sci USA* 1994;91:4930-4934.
- [41] Arase H, Arase N, Saito T: Fas-mediated cytotoxicity by freshly isolated natural killer cells. *J Exp Med* 1995;181:1235-1238.
- [42] Seko Y, Kayagaki N, Seino K, Yagita H, Okumura K, Nagai R: Role of Fas/FasL pathway in the activation of infiltrating cells in murine acute myocarditis caused by coxsackievirus B3. *J Am Coll Cardiol* 2002;39:1399-1403.
- [43] Caux C, Massacrier C, Vanbervliet B, Dubois B, Van-Kooten C, Durand I, Banchereau J: Activation of human dendritic cells through CD40 cross-linking. *J Exp Med* 1994;180:1263-1272.
- [44] Ranheim EA, Kipps TJ: Activated T cells induce expression of B7/BB1 on normal or leukemic B cells through a CD40-dependent signal. *J Exp Med* 1993;177:925-935.
- [45] Kelsall BL, Stuber E, Neurath M, Strober W: Interleukin-12 production by dendritic cells: the role of CD40-CD40L interactions in Th1 T-cell responses. *Ann NY Acad Sci* 1996;795:116-126.
- [46] Seko Y, Takahashi N, Azuma M, Yagita H, Okumura K, Yazaki Y: Expression of costimulatory molecule CD40 in murine heart with acute myocarditis and reduction in inflammation by treatment with anti-CD40L/B7-1 monoclonal antibodies. *Circ Res* 1998;83:463-469.
- [47] Smith CA, Farrah T, Goodwin RG: The TNF receptor superfamily of cellular and viral proteins: activation, costimulation, and death. *Cell* 1994;76:959-962.
- [48] Gruss HJ, Dower SK: Tumor necrosis factor ligand superfamily: involvement in the pathology of malignant lymphomas. *Blood* 1995;85:3378-3404.
- [49] Seko Y, Takahashi N, Oshima H, Shimozato O, Akiba H, Takeda K, et al.: Expression of tumor necrosis factor (TNF) ligand superfamily co-stimulatory molecules CD30L,

- CD27L, OX40L, and 4-1BBL in murine hearts with acute myocarditis caused by coxsackievirus B3. *J Pathol* 2001;195:593-603.
- [50] Seko Y, Takahashi N, Oshima H, Shimozato O, Akiba H, Kobata T, et al.: Expression of tumor necrosis factor (TNF) receptor/ligand superfamily co-stimulatory molecules CD40, CD30L, CD27L, and OX40 in murine hearts with chronic ongoing myocarditis caused by coxsackievirus B3. *J Pathol* 1999;188:423-430.
- [51] O'Connell JB, Reap EA, Robinson JA: The effects of cyclosporine on acute murine Coxsackie B3 myocarditis. *Circulation* 1986;73:353-359.
- [52] Fousteri G, Dave A, Morin B, Omid S, Croft M, von Herrath MG: Nasal cardiac myosin peptide treatment and OX40 blockade protect mice from acute and chronic virally-induced myocarditis. *J Autoimmun* 2011;36:210-220.
- [53] Weck PK, Rinderknecht E, Estell DA, Stebbing N: Antiviral activity of bacteria-derived human alpha interferons against encephalomyocarditis virus infection of mice. *Infect Immun* 1982;35:660-665.
- [54] Matsumori A, Crumpacker CS, Abelmann WH: Prevention of viral myocarditis with recombinant human leukocyte interferon  $\alpha$  A/D in murine model. *J Am Coll Cardiol* 1987;9:1320-1325.
- [55] Yamamoto N, Shibamori M, Ogura M, Seko Y, Kikuchi M: Effects of intranasal administration of recombinant murine interferon- $\gamma$  on murine acute myocarditis caused by encephalomyocarditis virus. *Circulation* 1998;97:1017-1023.
- [56] Wada H, Saito K, Kanda K, Kobayashi I, Fujii H, Fujigaki S, Maekawa N, Takatsu H, Fujiwara H, Sekikawa K, Seishima M: Tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) plays a protective role in acute viral myocarditis in mice : a study using mice lacking TNF-  $\alpha$ . *Circulation* 2001;103:743-749.
- [57] Seko Y: Effect of the angiotensin II receptor blocker olmesartan on the development of murine acute myocarditis caused by coxsackievirus B3. *Clin Sci* 2006;110:379-386.
- [58] Book WM: Carvedilol: a nonselective beta blocking agent with antioxidant properties. *Congest Heart Fail* 2002;8:173-177.
- [59] Nishio R, Shioi T, Sasayama S, Matsumori A: Carvedilol increases the production of interleukin-12 and interferon-gamma and improves the survival of mice infected with the encephalomyocarditis virus. *J Am Coll Cardiol* 2003; 41:340-345.
- [60] Tschöpe C, Westermann D, Steendijk P, Noutsias M, Rutschow S, Weitz A, Schwimmbeck PL, Schultheiss HP, Pauschinger M: Hemodynamic characterization of left ventricular function in experimental coxsackieviral myocarditis: effects of carvedilol and metoprolol. *Eur J Pharmacol* 2004;491:173-179.
- [61] Pauschinger M, Rutschow S, Chandrasekharan K, Westermann D, Weitz A, Peter Schwimmbeck L, Zeichhardt H, Poller W, Noutsias M, Li J, Schultheiss HP, Tschöpe C: Carvedilol improves left ventricular function in murine coxsackievirus-induced

- acute myocarditis association with reduced myocardial interleukin-1beta and MMP-8 expression and a modulated immune response. *Eur J Heart Fail* 2005;7:444-452.
- [62] Werk D, Pinkert S, Heim A, Zeichhardt H, Grunert HP, Poller W, Erdmann VA, Fechner H, Kurreck J: Combination of soluble coxsackievirus-adenovirus receptor and anti-coxsackievirus siRNAs exerts synergistic antiviral activity against coxsackievirus B3. *Antiviral Res* 2009; 83:298-306.
- [63] Pevear DC, Tull TM, Seipel ME, Groarke JM: Activity of pleconaril against enteroviruses. *Antimicrob Agents Chemother* 1999;43: 2109-2115.
- [64] Zell R, Markgraf R, Schmidtke M, Gorchach M, Stelzner A, Henke A, Sigusch HH, Gluck, B: Nitric oxide donors inhibit the coxsackievirus B3 proteinases 2A and 3C *in vitro*, virus production in cells, and signs of myocarditis in virus-infected mice. *Med Microbiol Immunol* 2004;193:91-100.
- [65] van Linthout S, Savvatis K, Miteva K, Peng J, Ringe J, Warstat K,C. Schmidt-Lucke C, Sittlinger M, Schultheiss HP, Tschöpe C: Mesenchymal stem cells improve murine acute coxsackievirus B3-induced myocarditis. *Eur Heart J* 2011;32:2168–2178.
- [66] Miteva K, Haag M, Peng J, Savvatis K, Becher PM, Seifert M, Warstat K, Westermann D, Ringe J, Sittlinger M, Schultheiss HP, Tschöpe C, van Linthout S: Human cardiac-derived adherent proliferating cells reduce murine acute Coxsackievirus B3-induced myocarditis. *PLoS One* 2011;6:e28513.
- [67] Corsten MF, Papageorgiou A, Verhesen W, Carai P, Lindow M, Obad S, Summer G, Coort SL, Hazebroek M, van Leeuwen R, Gijbels MJ, Wijnands E, Biessen EA, De Winther MP, Stassen FR, Carmeliet P, Kauppinen S, Schroen B, Heymans S: MicroRNA profiling identifies microRNA-155 as an adverse mediator of cardiac injury and dysfunction during acute viral myocarditis. *Circ Res* 2012;111:415-425.
- [68] Elmen J, Lindow M, Schutz S, Lawrence M, Petri A, Obad S, Lindholm M, Hedtjarn M, Hansen HF, Berger U, Gullans S, Kearney P, Sarnow P, Straarup EM, Kauppinen S: LNA-mediated microRNA silencing in non-human primates. *Nature* 2008;452:896–899.
- [69] Lanford RE, Hildebrandt-Eriksen ES, Petri A, Persson R, Lindow M, Munk ME, Kauppinen S, Ørum H: Therapeutic silencing of microRNA-122 in primates with chronic hepatitis C virus infection. *Science* 2010; 327: 198-201.