Attenuation of experimental autoimmune myocarditis by blocking activated T cells through inducible costimulatory molecule pathway

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Abstract

Objective: Inducible costimulator (ICOS) is a member of the CD28 family. Although inflammation is an essential pathological feature of myocarditis, the role of ICOS in myocarditis remains unclear. Methods and Results: Lewis rats were immunized on day 0 with purified porcine cardiac myosin to establish experimental autoimmune myocarditis (EAM). Flow cytometry was used to examine expression of ICOS on myocardial infiltrating cells. Anti-ICOS antibody or ICOS-immunoglobulin (ICOSIg) was administered intravenously, and rats were killed on day 14 or 21 to study effects of ICOS/ICOS-ligand (ICOSL) pathway blockade during the antigen priming phase (days 0–14) or immune response phase (days 14–21), respectively. The heart weight to body weight ratio was determined, and histological examination and echocardiogram were performed to evaluate the severity of the disease. Cytokine expression in the heart and T cell proliferation against cardiac myosin were analyzed. Flow cytometry revealed that the majority of infiltrating cells, especially CD4-positive cells, expressed ICOS. Blockade of the ICOS/ICOSL pathway during the immune response phase attenuated EAM development. However, blockade of the ICOS/ICOSL pathway during the antigen priming phase did not attenuate and exacerbate EAM. Blockade of T cell activation through ICOS suppressed expression of cytokines including INF-\(\gamma\), IL-4, IL-6, IL-10, IL-1\(\beta\), and TNF-\(\alpha\) and inhibited T cell proliferation in vitro. Conclusions: Blockade of T cell activation through ICOS during the immune response phase regulates development of EAM, and therefore, ICOS may be an effective target for treating myocarditis.

Keywords: Heart failure; Immunology; Leukocytes; Lymphatic circulation; Myocarditis

1. Introduction

Acute myocarditis is a fatal disease and a major cause of dilated cardiomyopathy \cite{1,2}, however, the etiology of myocarditis is unclear, and an effective treatment does not yet exist. Autoimmunity plays an important role in myocarditis, in particular a reaction to cardiac myosin following viral infection may contribute to development of myocarditis \cite{3}. Experimental autoimmune myocarditis (EAM) in rats is characterized by severe myocardial damage and the appearance of multinucleated giant cells and is used as an animal model of human giant cell myocarditis \cite{4,5}. Previous study reported that this model had two stages. The first stage detected a focal inflammation mainly consisting of macrophages and lasted up to day 14. The next stage detects strong inflammation consisting of macrophages and CD4-positive T cells and the maximal inflammation lasted up to around day 21 \cite{6}. We defined days 0–14 as the antigen priming (Ap) phase and days 14–21 as the immune response (Ir) phase in EAM. EAM is thought to be induced by T cell activation \cite{7}. T cell
activation requires distinct signals from an antigen-pre-
senting cell (APC) [8]. The first signal is antigen-specific
and is mediated by the T cell receptor (TCR), and the
second signal occurs through costimulatory molecules such
as CD28. The interactions of CD28 and CD40L, with their
respective ligands, B7-1/B7-2 and CD40, play a critical
role in T cell activation; therapy with cytotoxic T lympho-
cyte antigen 4 (CTLA4)-Ig prevents development of EAM
[9]. Inducible costimulator (ICOS) was identified as the
third member of the CD28 family [10]. ICOS is expressed
on the surface of T cells activated through the CD28
pathway or by phorbol 12-myristate 13-acetate (PMA)-
inomycin or anti-CD3 [11–13]. Studies of ICOS-deficient
mice have shown that ICOS costimulation is necessary for
activation and function of effector T cells [14–16]. The
ICOS ligand was identified as the third member of the B7
family and was termed B7 homolog (B7-H) [17].

It was previously shown that blockade of the ICOS/
ICOSL pathway plays a significant role in graft-versus-
host disease (GVHD), cardiac allograft survival, and
experimental allergic encephalomyelitis (EAE) [18–20].
However, there have been no reports regarding the role of
this molecule in myocarditis. In the present study, we
investigated the effect of ICOS/ICOSL pathway blockade
in EAM. We used anti-ICOS antibody and ICOSIg to
to examine the effect of ICOS blockade or B7-H blockade,
respectively. We found that ICOS plays an important role
in myocarditis and that blockade of the ICOS/ICOSL
pathway inhibits development of myocarditis.

2. Methods

2.1. Animals

Male Lewis rats (7-weeks-old; body weights 200 to 250 g)
were purchased from Sankyo Laboratories. They were
fed a standard diet and water and were maintained in
compliance with the animal welfare guidelines of the
Institute of Experimental Animals, Tokyo Medical and
Dental University.

2.2. Antigen and immunization

Purified porcine cardiac myosin (Sigma Chemical Co.)
was dissolved in 0.01 M phosphate-buffered saline (PBS)
and emulsified with an equal volume of complete Freund’s
adjuvant (Difco) supplemented with Mycobacterium tuber-
culosis H37RA (Difco) at a concentration of 10 mg/ml.
On day 0, rats were injected subcutaneously in the
footpads with 0.2 ml of emulsion, yielding an immunizing
dose of 1.0 mg/body of cardiac myosin per rat [21,22].

2.3. Reagents

Anti-rat ICOS monoclonal antibody (JMAb50, generated
at JT Frontier Research Laboratory) and isotype-matched
control IgG1k monoclonal antibody against keyhole limpet
hemocyanin (JMAb211) were prepared as described previ-
ously [23]. Biotinylated isotype-matched control IgG,
FITC-conjugated anti-CD4 antibody, FITC-conjugated
anti-CD8 antibody, and streptavidin–PE were purchased
from PharMingen. Human IgG was obtained from Jackson
ImmunoResearch Laboratories.

2.4. Preparation of ICOSIg

Soluble ICOS (ICOSIg) was prepared by constructing an
adenovirus vector containing cDNA encoding an extracellular
domain of human ICOS and the Fc portion of
human IgG. The ICOS cDNA was amplified by reverse
transcription-polymerase chain reaction (RT–PCR) of
mRNA isolated from human peripheral blood leukocytes
stimulated with Con-A. PCR primers were forward, 5’-
GGACTGAATTCTGTTTCTGGCAAACATG-3’ and reverse,
5’-CATGGATCCGGTAACCAGAACTTCAGCTG-3’. The cDNA was inserted into the EcoRI/BamHI site of a plasmid carrying IgG1-Fc DNA [24]. The ICOSIg DNA
was then removed by EcoRI/XbaI digestion, and a blunt-
ended fragment was ligated into the SwaI site of the
pAXCwt cosmid vector to prepare recombinant adeno-
virus AxICOSIg. Preparation of recombiant adenovirus
was then given the Adenovirus Expression Vector Kit
(Takara) according to the manufacturer’s protocol. ICOSIg
protein was purified from lysate of AxICOSIg-infected
COS7 as described [25]. In brief, concentrated COS7 cells
were washed with PBS and cultured with DMEM for 4
days in the presence of AxICOSIg. The ICOSIg protein
was purified from the supernatant with protein A-Sepharose
4FF (Pharmacia) affinity chromatography.

2.5. Treatment

Because human disease is diagnosed on the basis of
clinical symptoms, it is important to determine if a
treatment is effective against progressing or established
disease. It is reported that cellular infiltration of myocar-
dium in EAM occurs around day 14 [7], so we adminis-
tered anti-ICOS antibody either from day 0 or from day 14.

In the first set of experiments, rats in group Ab (n=6)
were injected intravenously with anti-ICOS antibody (3
mg/kg) on days 0, 4, 7, 11, 14, and 18. Rats in group Ir
(n=7) were given 3 mg/kg anti-ICOS antibody and those
in group Ig (n=6) were given 1 mg/kg ICOSIg intravenously
on days 14 and 18. As controls, groups C1 and C2 were
were treated with isotype-matched IgG monoclonal anti-
boby, and group C3 was treated with human IgG (n=9 in
each group). Rats were killed on day 21 to examine the
effect of ICOS/ICOSL pathway blockade.

In the second set of experiments, rats in group Ap (n=6)
were given 3 mg/kg anti-ICOS antibody intravenously on
days 0, 4, 7, and 11 and killed on day 14. Group C4 (n=6)
received isotype-matched IgG monoclonal antibody on
Trans-thoracic echocardiography was performed on animals anesthetized by intraperitoneal administration of pentobarbital sodium on day 21. An echocardiographic machine with a 7.5 MHz transducer (Nemio, Toshiba) was used for M-mode left ventricular echocardiographic recordings. A 2D targeted M-mode echocardiogram was obtained along the short-axis view of the left ventricle at the papillary muscles. Left ventricular fractional shortening (LVFS) was calculated from M-mode echocardiograms over three consecutive cardiac cycles according to the American Society for Echocardiography leading edge method [30,31]. Measurements were made offline by two independent investigators.

### 2.9. Ribonuclease protection assay (RPA)

Trizole (Life Technologies) was used to isolate mRNA according to the manufacturer’s protocol. Probe was synthesized by the in vitro transcription method with a Multi-Probe Template Set (Pharmingen), T7 polymerase, and [α-32P]UTP. Ten micrograms of total RNA was hybridized with probe at 56 °C for 16 h. All samples were then treated with RNase before treatment with proteinase K. Samples were separated by electrophoresis on a 5% acrylamide denaturing gel. mRNA bands were detected with an image analyzer (BAS2000, Fujifilm). Messenger RNA levels were quantified and normalized against levels of GAPDH. The normalized level of mRNA in each control group was expressed as 1.0 [32].

### 2.10. T cell proliferation assay

Spleen cells were isolated from rats with myocarditis on day 18. Cells (5×10^3/well) were cultured in 96-well culture plates with 50 μg/ml purified porcine heart myosin. Anti-ICOS antibody or ICOS Ig was added to each well at various concentrations. Cultures were incubated at 37 °C under 5% CO_2 for 3 days. Similarly, spleen cells isolated from rats in the control group or in the group with ICOS/ICOSL pathway blockade were cultured with purified porcine heart myosin at various concentrations. T cell proliferation was assessed with the Cell Counting Kit-8 (Dojindo). Cell proliferation was expressed as the optical density [33].

### 2.11. Enzyme-linked immunosorbent assay (ELISA)

Supernatant was collected from cultures used for T cell proliferation assays. Concentrations of INF-γ and IL-2 were determined with an ELISA kit (BioSource International) according to the manufacturer’s instructions.

### 2.12. Statistical analysis

Values are given as mean±S.D. Groups were compared...
with Scheffé’s ANOVA (Stat View, SAS Institute, Inc.). We used Student’s t-test for comparisons between two groups in the second experiment. Differences were considered statistically significant at a value of $P<0.05$.

3. Results

3.1. Expression of ICOS by inflammatory cells

ICOS was detected on myocardial inflammatory cells. CD4-positive cells showed stronger expression of ICOS than did CD8-positive cells (Fig. 2A and B). However, ICOS was not detected on either CD4- or CD8-positive spleen cells (Fig. 2C and D).

3.2. ICOS/ICOSL pathway blockade reduced heart weight/body weight ratios

In the first set of experiments, the heart weight to body weight ratios in groups, Ab ($n=6$), Ir ($n=7$), and Ig ($n=6$) were less than those of the control groups ($n=9$ each) (Fig. 3A). In the second set of experiments, the heart weight to body weight ratio of group Ap ($n=6$) was not remarkably different from that of group C4 ($n=6$) (Fig. 3B). The heart weight to body weight ratios in the treatment groups were decreased in the first set of experiments, but that was not changed in the second set of experiments.

Fig. 2. Expression of ICOS on T cells. T cells stained with anti-ICOS antibody are shown as a solid line, and T cells stained with isotype control IgG are shown in black. Panels A and C show CD4-positive cells. Panels B and D show CD8-positive cells.
Fig. 4. Representative cross-sections of heart. Panel A shows a representative cross-section of heart from rats with ICOS/ICOSL pathway blockade (group Ir). Panel B shows little infiltration by inflammatory cells in group Ir. Panel C shows a representative cross-section of heart from control rats (group C2) on day 21. Panel D shows severe myocarditis lesions, including giant cells, in group C2. A representative heart from rats with ICOS/ICOSL pathway blockade (group Ap) on day 14 is shown (E). Panel F shows a representative cross-section of heart from control rats (group C4) on day 14. Panels G and H show the myocarditis-affected area ratios in the respective groups. Original magnification in A, C, E, and F is ×10. Original magnification in B and D is ×400.

3.3. Reduction of inflammatory cells in the heart

In the first set of experiments, myocardial lesions were rarely observed in hearts of rats treated with ICOS pathway blockade (Fig. 4A). In the groups treated with ICOS/ICOSL pathway blockade, there was little infiltration of inflammatory cells or myocardial necrosis (Fig. 4B). However, severe myocardial lesions were observed in
hearts of all control rats on day 21 (Fig. 4C). These lesions were composed of extensive necrosis and infiltration by mononuclear cells and polymorphonuclear neutrophils (Fig. 4D). In the second set of experiments, myocardial lesions were observed in hearts of rats treated with ICOS/ICOSL pathway blockade as well as those of control rats on day 14 (Fig. 4E and F).

In the first set of experiments, the ratios of myocarditis-affected areas in groups Ab (n=6), Ir (n=7), and Ig (n=6) were less than those of the control groups (n=9 each) (Fig. 4G). In the second set of experiments, the ratio of group Ap (n=6) was not significantly different from that of group C4 (n=6) (Fig. 4H). Myocardial lesions were clearly reduced with ICOS/ICOSL pathway blockade in the first set of experiments, but were not in the second set of experiments.

3.4. ICOS/ICOSL pathway blockade improved cardiac function

On day 21, ICOS/ICOSL pathway blockade with anti-ICOS antibody improved LVFS in group Ir (Fig. 5A) in comparison with that in group C2 (Fig. 5B).

In the first set of experiments, LVFS in groups Ab (n=6), Ir (n=7), and Ig (n=6) showed greater improvement than those of each control groups (n=9 each) (Fig. 5C). In the second set of experiments, LVFS in group Ap (n=6) showed no significant change in comparison to that of group C4 (n=6) (Fig. 5D). LVFS was improved with ICOS/ICOSL pathway blockade in the first set of experiments, but was not in the second set of experiments.

3.5. Cytokine mRNA expression during ICOS/ICOSL pathway blockade

RPA was used to examine expression of cytokine mRNAs in hearts. Levels of cytokine mRNAs for IL-4, IL-6, IL-10, IL-1β, and TNF-α in groups Ab (n=4), Ir (n=4), and Ig (n=4) were markedly decreased comparison with that of each control group (n=4 each) (Fig. 6A and B). Expression of cytokine mRNAs in group Ap (n=4) and group C4 (n=4) was enhanced (Fig. 6C and D). In the first set of experiments, expression of cytokine mRNA was reduced with ICOS/ICOSL pathway blockade, but was not reduced in the second set of experiments. RPA revealed that ICOS/ICOSL pathway blockade during only the inflammatory phase (days 14–21) reduced expression of IL-4, IL-6, IL-10, IL-1β, and TNF-α mRNAs as well as ICOS/ICOSL pathway blockade during the antigen priming phase and inflammatory phase. However, ICOS/ICOSL pathway blockade during only the antigen priming phase did not affect expression.

3.6. Suppression of cell proliferation

We performed cell proliferation assays to examine the effect of ICOS/ICOSL pathway blockade on antigen-induced T cell proliferation. Antigen-induced T cell proliferation was suppressed by both anti-ICOS antibody (Fig.
Fig. 6. Expression of cytokine mRNAs in the heart. Panel A shows representative findings on day 21 in groups Ir and C2. Panel C shows representative findings on day 14 in group Ap and C4. Levels of mRNAs were normalized to those of GAPDH as control group (B and D). Data are the mean of four independent experiments. *P<0.05. N.S., not significant.
7A) and ICOSIg (Fig. 7B). Antigen-specific cell proliferation in rats treated with ICOS/ICOSL pathway blockade was reduced in comparison to that in rats in the control group (Fig. 7C).

3.7. Th1-type cytokine change by ICOS/ICOSL pathway blockade

ELISA analysis of supernatants after incubation of spleen cells with cardiac myosin revealed that production of INF-γ by group Ab (n=4) cells was suppressed in comparison to that in group C1 (n=4) (Fig. 8A). Production of IL-2 was not affected by cardiac myosin (Fig. 8B).

4. Discussion

Treatment of acute myocarditis in humans remains a major clinical problem [34]. Here, we clearly show that the ICOS/ICOSL pathway plays an important role in EAM, and blockade of the ICOS/ICOSL pathway with anti-ICOS antibody or ICOSIg suppresses the development of EAM.

It was previously reported that the CD28/CTLA4-B7 and CD40/CD40L pathways play crucial roles in EAM [8]. However, the role of ICOS, which is a novel costimulatory molecule, in EAM is not known. T cell proliferation is enhanced by stimulation of the ICOS/ICOSL pathway [10], and this response is inhibited by blockade of the ICOS/ICOSL pathway [35]. Development of EAM involves myosin-autoreactive T cell proliferation.

We confirmed that ICOS is expressed on myocardial

![Fig. 7. Effects of ICOS/ICOSL blockade on antigen-induced proliferation of splenocytes. Splenocytes were isolated from group C1 on day 18. Both anti-ICOS antibody (anti-ICOSAb) and ICOSIg suppressed T cell proliferation (A and B). Rats in group C1 show greater myosin-specific splenocyte proliferation than those in group Ab (C). *P<0.05.](image)

![Fig. 8. Th1-type cytokine production. Splenocytes from rats in group Ab produced significantly less INF-γ after restimulation with cardiac myosin than did those in group C1 (A). Production of IL-2 in group Ab was similar to that of group C1 (B). *P<0.05.](image)
Th1 and Th2 cytokines are produced by T cells and modulate inflammation in this model. ICOS/ICOSL pathway blockade during the immune response phase decreased expression of proinflammatory cytokines including IL-6, IL-1β, and TNF-α. Suppression of proinflammatory cytokines that produce Th1-type cytokines prevented development of EAM [9]. However, ICOS/ICOSL pathway blockade during the antigen priming phase did not. These results were consistent with those of previous studies that showed that ICOS is expressed on activated T cells [11–13] and that ICOS is not expressed on activated T cells at the site of inflammation during the antigen priming phase.

Changes in expression of Th1-type cytokines, such as INF-γ and IL-2, caused by ICOS/ICOSL pathway blockade in response to cardiac myosin was analyzed in splenocytes, because EAM in Lewis rats is mediated by the Th1 response [6]. We found that production of INF-γ was reduced, whereas that of IL-2 was not changed by ICOS/ICOSL pathway blockade. This result is supported by previous findings that the ICOS/ICOSL pathway promotes production of IFN-γ but not IL-2 in ICOS-deficient mice [10]. Our results suggest that the role of INF-γ is more important than that of IL-2 in the development of EAM. However, some studies in GVHD and lung mucosal inflammation models indicated that ICOS/ICOSL pathway blockade do not reduce expression of Th1-type cytokines and reduce Th2-type cytokines, such as IL-4 and IL-10 [18,36]. Our results in EAM are consistent with data from studies of allograft rejection, EAE, and collagen-induced arthritis models that ICOS/ICOSL pathway blockade suppresses production of Th1- and Th2-type cytokines [19,20,38].

In our model of EAM, myocarditis occurs as early as 14 days after immunization followed by aggressive cellular infiltration. Although this is not a case in mild clinical myocarditis, some clinical cases including giant cell myocarditis show similar clinical course and histopathology as this animal model. It is not clear whether the Th1 response is related to clinical myocarditis; we assume that myocarditis is mediated by the Th1 response irrespective of species including human giant cell myocarditis because of the similarity in the clinical course and histology.

In the present study, we show that ICOS/ICOSL pathway blockade suppresses cytokine production and T cell activation and thus attenuates development of EAM. These results indicate that the ICOS/ICOSL pathway blockade may have potential as a therapy for myocarditis. Further studies are needed to evaluate the clinical usefulness of this novel strategy for treatment of myocarditis.

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