CVR-2004-1086R2

Immunological molecules expression of cardiomyocytes, inflammatory and interstitial cells in rat autoimmune myocarditis

Tsuyoshi Yoshida, Haruo Hanawa *, Ken Toba, Hiroshi Watanabe, Ritsuo Watanabe, Kaori Yoshida, Satoru Abe, Kiminori Kato, Makoto Kodama, Yoshifusa Aizawa

Division of Cardiology, Niigata University Graduate School of Medical and Dental Science.

A Running Head: Expression of immunologic molecules in EAM

*Correspondence to Haruo Hanawa, Division of Cardiology, Niigata University Graduate School of Medical and Dental Science, 1-754 Asahimachi, Niigata City, 951-8510, Japan
FAX number: +81-25-227-0774
Telephone number: +81-25-227-2185
E-mail: hanawa@med.niigata-u.ac.jp

Total words 5695 words

Keywords
Myocarditis
Dilated cardiomyopathy
Autoimmunity
Chemokines
Cytokines
Quantitative RT-PCR

Abstract

Background: In a heart with myocarditis, there are cardiomyocytes, inflammatory cells and non-inflammatory interstitial cells. Immunologic molecules are thought to influence not only inflammatory cells but also cardiac function and remodeling. Whatever their origin, the cells they target and crosstalk by them remain unclear. Here, we examined native gene expression of immunologic molecules in normal and rat experimental autoimmune myocarditis (EAM) days 18 and 90 after immunization, using real time RT-PCR in cardiomyocytes, $CD11b^+$ cells, αβT cells and non-cardiomyocytic non-inflammatory (NCNI) cells.

Methods and results: Cells were isolated by collagenase perfusion on a Langendorff apparatus and purified by passing through a stainless-steel sieve followed by magnetic bead column separation using appropriate monoclonal antibodies. Most immunologic molecules were expressed in inflammatory cells. However, some of them were expressed in NCNI cells or cardiomyocytes. Interestingly, most of Interleukine (IL)-10, monocyte chemoattractant protein (MCP)-1 or tumor necrosis factor (TNF)-α receptor was found in NCNI cells and most of fractalkine was found in NCNI cells and cardiomyocytes. Moreover, TNF-α significantly upregulated fractalkine and MCP-1 mRNA in cultivated cells from EAM hearts.

Conclusion: In rat experimental myocarditis heart, inflammatory cells express many immunologic molecules. Some of them are thought to influence NCNI cells or cardiomyocytes directly via receptors on these cell types. It is further suggested that fractalkine, IL-10 and MCP-1 expressed in NCNI cells or cardiomyocytes regulate inflammatory cells.

1. Introduction

Rat experimental autoimmune myocarditis (EAM) resembles human giant cell myocarditis [1] and recurrent forms lead to dilated cardiomyopathy (DCM) [2]. $CD4^+$ αβT cells play important roles in initiating the disease process, while macrophages and $CD4^+$αβT cells infiltrate the heart during the acute phase [3, 4]. Gene expression of immunologic molecules in EAM heart changes diversely from acute phase to recovery phase [5]. For example, Th1 cytokine increases in the acute phase and decreases during the recovery phase. On the other hand, Th2 cytokine increases during the recovery phase [5, 6]. Immunologic molecules, in addition to their influence on inflammatory cells, are believed also to affect cardiac function and mediate myocardial damage [7]. It is thought that inflammatory cells and non-inflammatory cells in EAM heart engage in crosstalk by means of immunologic molecules. Thus far, in vivo analysis of immunologic molecules and their receptor-expressing origin cells has not been performed satisfactorily. It is important to determine which cells express these molecules or their ligands and receptors in order to understand deterioration of cardiac function and remodeling in myocarditis.

In this study, we investigated native gene expression of immunologic molecules by using real time RT-PCR in cardiomyocytes, αβT cells, $CD11b^+$ cells (macrophages/ granulocytes/ dendritic cells in part) or non-cardiomyocytic non-inflammatory (NCNI) cells without cultivation. Cells were isolated and purified by passing through a stainless-steel sieve and using appropriate monoclonal antibodies on a magnetic bead column separation system. Moreover, we examined the effect of tumor necrosis factor (TNF)-α on immunologic molecules in cultivated cells from EAM hearts. We aimed to elucidate implications on the potential crosstalk in EAM.

2. Methods

2.1. Animals

Lewis rats were obtained from Charles River, Japan (Atsugi, Kanagawa, Japan) and were maintained in our animal facilities until they reached 7 weeks of age. Throughout the studies, all the animals were treated in accordance with the guidelines for animal experiments of our institute and the guide for the care and use of laboratory animals published by the US National Institutes of Health.

2.2. Induction of EAM

Whole cardiac myosin was prepared from the ventricular muscle of porcine hearts as previously described [1]. It was dissolved in PBS at a concentration of 10 mg/ml and emulsified with an equal volume of complete Freund's adjuvant supplemented with 10 mg/ml of Mycobacterium tuberculosis H37RA (Difco, Detroit, Michigan). On day 0, the rats received a single immunization at 2 subcutaneous sites with a total of 0.2 ml of emulsion for each rat. Normal rats (n=5), EAM rats were killed on day 18 (n=6) (acute phase), and EAM rats were killed on day 90 (n=6) (chronic phase).

2.3. Isolation of cells, flow cytometric analysis and cell purification

Cardiomyocytes and non-cardiomyocytes in hearts of normal and myocarditis rats on days 18 and 90 were isolated after collagenase perfusion treatment for 15-20 minutes using a Langendorff apparatus as reported previously [8, 9]. Isolated cells in an isotonic buffer were separated serially through a 38μm stainless steel sieve twice and a 20μm stainless steel sieve twice. Cells larger than 38μm and smaller than 20μm were considered as cardiomyocytes and non-cardiomyocytes, respectively (Fig. 1). The cells fraction between 20μm to 38μm consisted of both cell types, and was therefore discarded. The number of non-cardiomyocytes recovered from normal hearts was very small but the number from myocarditis hearts on day 18 was sufficient for statistically significant analysis. Phenotype was analyzed in both samples, and cell

purification was performed only in samples obtained from myocarditis hearts on day 18.

Phycoerythrin (PE)-conjugated mouse monoclonal antibodies against rat CD8 (clone OX-8) and CD25 (OX-39) were purchased from Immunotech, Marseille, France. PE-conjugated anti-CD3 (G4.18), CD4 (OX-35), TCRαβ (R73), CD161 (10/78), CD11b (OX-42), and biotinylated anti-TCRγ/δ were purchased from Pharmingen, San Diego, CA. Biotinylated ED1 and PE-conjugated streptavidin (SA-PE) were purchased from Serotec, Oxford, UK, and Becton Dickinson, San Jose, CA, respectively. Cells suspended in 50% rat serum in a buffer were directly stained with a PE-conjugated monoclonal antibody, or serially stained with a biotinylated monoclonal antibody and SA-PE, then analyzed using a FACScan flow cytometer (Becton Dickinson).

TCRα/β$^+$ T cells, TCRα/β$^-$/CD11b$^+$ cells, and TCRα/β$^-$/CD11b$^-$ cells (NCNI cells) were sorted using PE-conjugated monoclonal antibodies, anti-PE micro beads (Miltenyi Biotec, Bergisch Gladbach, Germany) and a MACS magnetic cell sorting system (Miltenyi Biotech) [10]. In brief, cells were serially labeled with PE-conjugated anti-TCRα/β and anti-PE micro beads, and separated with a MS column in a magnetic field. The positive fraction was further purified using another MS column (purity, 99.4±0.5 %, n = 3). Contaminated cells in the negative fraction were removed using an LD column, and utilized for the succeeding sorting. The TCRα/β$^-$ cells were serially labeled with PE-conjugated anti-CD11b and anti-PE micro beads, then separated with a MS column in a magnetic field. The positive fraction was further purified using another MS column (purity, 97.0±2.3 %, n = 3). Contaminated cells in the negative fraction were removed using an LD column (final contamination of positive cells, 2.2±1.1 %, n = 3, Fig. 2).

2.4. Immunostaining

Cytospin preparations of purified cells fractions were made and slides were

stained with May-Giemsa stain or naphthol AS-D chloroacetate esterase and α -naphthylbutyrate esterase stain. Cytospin slides prepared from NCNI cell fraction were immunostained wirh mouse monoclonal anti-α smooth muscle actin antibodies (SIGMA, Saint Louis, MO), rabbit anti-Factor VIII related antigen antibodies (Zymed Laboratories, San Francisco, CA) and rabbit anti rat collagen III antibodies (Monosan, Uden Netherland). Sections were incubated for 60 min at 37℃ in a humidified chamber with these antibody. The slides were washed in TBS three times. Immunodetection was performed using biotinylated anti-rabbit and anti-mouse immunoglobulins followed by alkaline phosphatase conjugated streptavidin and a Fast Red chromogen (kit LSAB2; DAKO Corp., Carpinteria, CA) for red staining. The sections were lightly counterstained with Mayer's hematoxylin. Negative control slides were incubated with either mouse IgG2a or normal rabbit serum instead of the primary antibody. Sections of EAM heart were stained with hematoxylin and eosin stain. For immunostaining of OPN, paraffin sections of EAM heart on day 18 were cut at 6μm, deparaffinized with xylene, hydrated with decreasing concentrations of ethanol and heated in a hot water bath for 40 min at 95℃ in ChemMate Target retrieval solution (Daco corp). The sections were probed with rabbit anti-human OPN polyclonal antibody (1:50 dilution; IBL, Gunma, Japan) for 60 min in a humidified chamber. After several washings with TBS, immunodetection was performed using biotinylated anti-rabbit and anti-mouse immunoglobulins followed by alkaline phosphatase conjugated streptavidin and a Fast Red chromogen. The sections were lightly counterstained with Mayer's hematoxylin.

2.5. RNA Isolation from Heart and Reverse Transcription

We prepared 1 to 5 x 10^6 purified cells and total RNA was isolated from each purified cell fraction (cardiomyocytes; n=5, $CD11b^+$ cells; n=5, αβT cells; n=5 and

NCNI cells; n=6) of EAM hearts on day 18 (acute phase) after immunization and from each cardiomyocytes fraction of normal hearts (n=5) and EAM hearts on day 90 (chronic phase n=6) using Trizol (LifeTechnologies, Tokyo, Japan) [5]. To confirm that gene expression of cardiomyocytes after collagenase preparation is similar to native cardiomyocytes, we prepared purified cardiomyocytes (n=4) and whole heart (n=4), which are homogenated immediately in Trizol, on day 0, 14, 21 and 28 and then total RNA was isolated from each sample. cDNA was synthesized from 2-5μg of total RNA with random primers and murine Moloney leukemia virus reverse transcriptase in a final volume of 20μl.

2.6. Plasmid Construction as Standard Sample for Real Time RT-PCR

Immunologic molecules and specific marker cDNAs were amplified with AmpliTaq polymerase (TOYOBO, Osaka, Japan), for each primer (Table 1) from 1μl of cDNA according to the following amplification protocol: 35 cycles at 94℃ for 60 seconds, 58℃ for 90 seconds, and 73℃ for 120 seconds. Amplified cDNAs were directly inserted into the pGEM-T easy vector and the recombinant plasmids were isolated after transforming with *Escherichia coli* JM109 competent cells using the MagExtractor plasmid Kit (TOYOBO, Osaka, Japan). The plasmids were diluted with DNAse-free water in a siliconised tube including 10 ng/μl MS2 RNA (Roche, Indianapolis, IN) to prevent adherence to the tube wall.

2.7. Quantitative RT-PCR

cDNA was diluted 100-fold with DNAse-free water in a siliconised tube (including 10 ng/μl MS2 RNA to prevent adherence to the tube wall), and 5μl was then

used for real-time PCR. cDNA and diluted plasmid were amplified with the same primer used for making the plasmid and LightCycler-FastStart DNA Master SYBR Green I (Roche, Indianapolis, IN). RNAs without reverse transcription were used as a negative control. After an initial denaturation step of 10 min at 95℃, a three-step cycle procedure was used (denaturation 95℃, 10 sec, annealing 62-65℃, 10 sec and extension 72℃, 13 sec) for 40 cycles. The LightCycler Software calculated a standard curve using five plasmid standards. The standard curve was created by plotting the cycle numbers at which the fluorescent signals entered the log-linear phase versus the concentrations of the standards. The absolute copy numbers of all the samples were calculated by the LightCycler software using this standard curve [5].

2.8. Non-cardiomyocytic cell culture with TNF-α

On day 18, non-cardiomyocytic (NC) cells were isolated from the hearts of EAM rats via collagenase preparation and were cultured for three weeks on 35mm-well dishes in 2ml of RPMI medium supplemented with 10% FCS. These cultivated NC cells were suggested to contain mainly fibroblasts, smooth muscle cells, endothelial cells and $CD11b^+$ cells and to express enough mRNA of TNF-α receptor, as determined by gene expression analysis (Table 2). After reaching confluency, NC cells were stimulated by adding TNF-α (Pepro Tech, London, England) (no TNF-α group, n=6; 80ng/ml of TNF-α group, n=6; 160ng/ml of TNF-α group, n=6). After culture for 24 hours at 37℃, NC cells were collected and total RNA was isolated as described above. The absolute copy numbers of γ-actin, fractalkine, monocyte chemoattractant protein (MCP)-1 and osteopontin mRNA were measured by quantitative real-time PCR.

2.9. Statistical Analysis

Data obtained from quantitative RT-PCR are expressed as mean±standard error of the mean (SEM). Data obtained from flow cytometric analysis are expressed as mean±standard deviation (SD). Differences between groups of purified cells were determined by one-way ANOVA and Bonferroni's multiple comparison test. A value of $P \leq 0.05$ was considered statistically significant.

3. Results

3.1. Flow cytometry of Non-Cardiomyocytes

The number of $CD4^+\alpha\beta$T cells and $CD11b^+$ cells increased on day 18 as compared with the control. On day 18, 65% of non-cardiomyocytes were $CD11b^+$ cells and ED-1^+ cells (macrophages/ granulocytes), and 13% of them were $CD3^+$ T cells which were almost all $TCR\alpha\beta^+$ and $CD4^+$. $CD161^+$ NK cells were hardly found in the non-cardiomyocytes (Table 3).

3.2. Staining of purified cell

Images of cells stained with May-Giemsa stain or naphthol AS-D chloroacetate esterase and α-naphthylbutyrate esterase stain in αβT cell and $CD11b^+$ cell fractions were compatible with typical images of T cells or macrophage/ granulocytes. However, images of cells in a NCNI cell fraction differed from those of cardiomyocytes, T cells or macrophage/ granulocytes. Cells in a NCNI cell fraction showed positive staining for α smooth muscle actin, Factor VIII related antigen or collagen III (Fig. 3).

3.3. Gene expression of immunological molecule in separated cells from EAM hearts

Gene expression of α-cardiac myosin, which should be found only in cardiomyocytes, was in fact detected only in a cardiomyocyte fraction and not in the other fractions (Table 4). T cell specific marker CD3 was detected in a αβT cell fraction but CD3 gene expression in the other fractions was under 5% presumably due to αβ T cells contaminating those fractions and a few γδT cells in a NCNI cell fraction. CD11b, which should be found in macrophages and granulocytes, was expressed in a $CD11b^+$ cell fraction, but its gene expression in a αβT cell fraction was under 8% and that in the other fractions was under 3%, again presumed to be due to contaminating $CD11b^+$ cells. The fibroblast-specific marker (collagen type III) was detected in a NCNI cell fraction but its expression in the other fractions was under 1%. The endothelial cell-specific marker (von Willebrand factor) was detected in a NCNI cell fraction but its expression in the other fractions was under 10%. Because gene expression in smooth muscle cells is similar to that in cardiomyocytes and macrophages, it is difficult to find a specific marker for smooth muscle cells. However, generally speaking, because calponin [11] and caldesmo [12], which smooth muscle cells contain, were detected in a NCNI cell franction, most smooth muscle cells were presumed to be in that fraction.

Gene expression of cardiac myosin isoform and ANP was examined in purified cardiomyocytes after collagenase preparation and native cardiomyocytes, namely whole hearts immediately homogenated in Trizol, on day 0, 14, 21 and 28 to confirm that gene expression in cardiomyocytes wasn't influenced by collagenase preparation. Their ratios of copy numbers at each day to copy numbers at day 0 were almost equivalent for purified cardiomyocytes and native cardiomyocytes (Fig. 4).

Gene expression of immunologic molecules was examined in fractions of purified cells (cardiomyocytes, $CD11b^+$ cells, αβT cells and NCNI cells) from day 18

EAM hearts (Table 5). Interleukine (IL)-2 and Interferon (IFN)-γ were detected in a αβT cells fraction alone, and IFN-γ receptor was detected in $CD11b^+$ cell, αβT cells, NCNI cell fractions and, to a lesser degree, in a cardiomyocyte fraction. IL-10 (Fig. 5A) and MCP-1 (Fig. 5B) in a NCNI cell fraction were significantly more than the other fractions, and IL-10 receptor was detected in $CD11b^+$ cell andαβT cell fractions. TNF-α was detected in a $CD11b^+$ cell fraction and TNF-α receptor was detected in a NCNI cell fraction and, to a lesser degree, in $CD11b^+$ cell and cardiomyocyte fractions. Osteopontin was detected in $CD11b^+$ cell and NCNI cell fractions. One of its ligands, CD44, was found mainly in a αβT cell fraction, smaller amounts were found in $CD11b^+$ cell and NCNI cell fractions, and very little was found in a cardiomyocyte fraction. Most of programmed death-1 (PD-1) was detected in a αβT cell fraction, and PD-1 ligand mainly in a $CD11b^+$ cell fraction. Fractalkine was detected in NCNI cell and cardiomyocyte fractions (Fig. 5C), and its receptor CX3CR1 in αβT cell and $CD11b^+$ cell fractions (Fig. 5D). Major histocompatibility complex (MHC) Class I and MHC Class II molecules were detected mostly in αβT cell and $CD11b^+$ cell fractions respectively, but both were detected at all fractions.

Expression of the genes for fractalkine, IFN-γ receptor, TNF-α receptor, CD44, MHC Class I and MHC Class II, thought to be present in cardiomyocytes, was examined in cardiomyocyte fractions of normal and day 18 and 90 EAM hearts (Table 6). Fractalkine, TNFα receptor, CD44, MHC Class I and MHC Class II expression significantly increased in a fraction of cardiomyocytes of acute myocarditis hearts.

3.4. Effect of TNF-α on gene expression of immunologic molecules in cultivated NC cells from EAM hearts

Because NCNI cells strongly expressed TNF-α receptor (Table 2), we examined the effect of TNF-α on fractalkine, MCP-1 and osteopontin which they expressed. In cultivated NC cells containing fibroblasts, smooth muscle cells, endothelial cells and $CD11b^+$ cells, fractalkine (160ng/ml of TNF-α group, 20.0±4.8-fold, $p<0.001$) and MCP-1 (160ng/ml of TNF-α group, 63.2±8.0-fold, $p<0.0001$) were significantly upregulated by TNF-α (Fig. 6A and B). However, we couldn't find the significant influence of gene expression of osteopontin by TNF-α in cultivated NC cells (Fig. 6C).

3.5 Immunostaining of osteopontin

Osteopontin expression was observed in some mononuclear cells infiltrating into EAM hearts on day 18 (Fig 7B). However, osteopontin expression was hardly detected in normal heart (Fig 7C).

4. Discussion

In this study, gene expression of some immunologic molecules (fractalkine, IL-10, MCP-1, CD44, IFN-γ receptor, TNFα receptor et al.) was found in non-inflammatory cells. This suggests that they may be mediators not only for inflammatory cell activity but also for originally constitutive cells in the heart – cardiomyocyte and NCNI cells.

Fractalkine has been identified as a novel chemokine that exhibits cell adhesion and chemoattractive properties in the central nervous system [13]. It is interesting that fractalkine mRNA is found in purified cardiomyocytes and NCNI cells. This result supports previous reports that endothelial cells of the coronary vasculature and endocardium were the principal source of fractalkine and some fractalkine immunoreactivity was also found on the myocytes [14]. Normal cardiomyocytes express some fractalkine, but during the acute phase of inflammation expression increases. TNF-α produced by $CD11b^+$ cells in EAM hearts strongly upregulated fractalkine mRNA in cultivated NC cells. It has been reported that fractalkine expression is markedly induced by inflammatory cytokines, TNF-α, IL-1 and IFN-γ in primary cultured endothelial cells [15,16]. Because cytokines as TNF-α, IL-1β or IFN-γ increase in EAM hearts [5], fractalkine mRNA in cardiomyocytes of EAM hearts may be upregulated. CX3CR1, receptor of fractalkine was found in $CD11b^+$ cells and αβT cells. This suggests that cytokines produced by inflammatory cells increase fractalkine on residential cells and then it attracts and activates inflammatory cells in EAM hearts. Recently, it was reported that fractalkine was secreted by central nervous system neurons and astrocytes [17]. Neuronal fractalkine expression in virus encephalitis plays important roles in macrophage recruitment and neuroprotection in the central nervous system [18]. Fractalkine may plays a similar role in the heart.

IL-10 was expressed unexpectedly in NCNI cells. Before this study, we thought that cells expressing IL-10 were Th2 cytokine-secreting T cells. We could detect IL-2 and IFN-γ gene expression in αβT cells, but IL-10 gene expression was barely detected in them. Gene expressions of IL-4 and IL-13 (Th2 cytokines), which are produced only by hemopoietic cells, were not found in EAM whole hearts on day 18 and 28 by using quantitative RT-PCR [data not shown]. Therefore, it is thought that α

βT cells in acute EAM hearts secrete Th1 but not Th2 cytokine. Previously, we reported that IL-10 producing cells in peripheral blood increased on day 28 in EAM, however, on day 28, most parts of the infiltrating cells disappeared and the fibrosis remained in myocardium [19]. In rheumatoid arthritis, IL-10 is released by fibroblast-like synoviocytes [20]. It may be that, in EAM hearts, fibroblasts, not T cells, secrete most of IL-10 and it modulate $CD11b^+$ and αβT cells expressing IL-10 receptors by paracrine mechanisms. It has been reported that IL-10 gene therapy ameliorated EAM and other models of autoimmune inflammatory disease [21], therefore NCNI cells producing IL-10 may play an important role for EAM.

It has been reported that MCP-1 is secreted by activated lymphocytes, macrophages, endothelial cells, smooth muscle cells and platelet-derived growth factor-activated fibroblasts [22-24]. Our previous immunohistochemistry analysis in EAM showed that large monocytes were stained by anti-MCP-1 antibody [25]. This study suggests that they are not macrophages but NCNI cells, which attract macrophages into myocardits hearts by paracrine mechanisms. It has been reported that MCP-1 secretion was regulated by the proinflammatory cytokines, IL-1 and TNF-α in colonic subepithelial myofibroblasts [26]. In this study, we also showed that MCP-1 mRNA in cultivated NC cells from EAM hearts were upregulated by TNF-α. Increase of TNF-α and IL-1β produced by macrophages in EAM hearts [5], upregulates expression of MCP-1 gene in NCNI cells and it may attract macrophage.

We previously reported that large amounts of osteopontin mRNA are expressed in early EAM [5]. Osteopontin protein was detected in EAM hearts by immunohistochemistry. Osteopontin is an extracellular matrix protein as well as a cytokine that contributes to the development of Th1 immunity [27]. Osteopontin is secreted by $CD11b^+$ cells and NCNI cells. It may activate T cells expressing CD44 as one of ligands for osteopontin [28]. It may also modulate $CD11b^+$ cells, NCNI cells and to a lesser degree probably cardiomyocytes in myocarditis. Osteopontin is also a potentially important mediator of AII regulation of cardiac fibroblast behavior in the

cardiac remodeling process [29].

PD-1 deficiency causes a variety of autoimmune diseases [30] and dilated cardiomyopathy with severely impaired contraction and death by congestive heart failure [31]. It has been reported that the parenchymal cells of heart, lung and kidney constitutive express PD-1 ligand [30]. Cytokine such as IFN-γ or other inflammatory stimuli induces PD-1 ligand expression [32]. In this study, $CD11b^+$ cells in EAM hearts expressed both IFN-γ receptor and PD-1 ligand. $CD11b^+$ cells stimulated by IFN-γ may regulate αβT cells activation in EAM by PD-1-PD1 ligand binding. However, more evidence is needed to support this concept.

$CD4^+$T cells play important roles in EAM. MHC class II expressing cells that can bind CD4 are mainly $CD11b^+$ cells. Some papers reported that MHC class I and MHC class II expression increases in myocarditis hearts or DCM [33-35]. This suggest that their expression in cardiomyocytes in acute myocarditis increased, which may mean that lymphocytes have contact with cardiomyocytes in acute myocarditis more closely than in normal heart or in chronic myocarditis.

This method of isolating and purifying the subgroups of cells from myocarditis hearts by using stainless-steel sieves at 4℃ is thought not to damage cells in contrast to purification by centrifuge in Percoll [36]. Actually, the gene expression of cardiac myosin isoform and ANP [5], which is found only in cardiomyocytes, was the same in purified cardiomyocytes as in unpurifed whole hearts. Therefore we assume that the gene expression in purified cell fractions is practically unchanged by these preparations.

It was reported that immunologic factors might be of greater prognostic importance than the more conventional assessments of the hemodynamic and clinical status [7]. It is clinically significant to define the mechanisms by which these factors influence cardiac function and remodeling. Our study indicated that inflammatory cells and non-inflammatory cells influenced each other. It is to be hoped that a clarification of the mechanism lead to therapies for myocarditis and DCM by regulation of immunologic factors.

We present here a possible crosstalk by immunologic molecules among constitutive cells in EAM hearts. They play important roles not only among inflammatory cells but also among non-inflammatory cells containing cardiomyocytes, fibroblasts, endothelial cells and smooth muscle cells.

Acknowledgments

This study was supported in part by grants for scientific research from the Ministry of Education, Science and Culture of Japan (numbers 14570645).

References

1. Kodama M, Matsumoto Y, Fujiwara M, Masani F, Izumi T, Shibata A. A novel experimental model of giant cell myocarditis induced in rats by immunization with cardiac myosin fraction. Clin Immunol Immunopathol 1990;57:250-62.

2. Kodama M, Hanawa H, Saeki M, Hosono H, Inomata T, Suzuki K, et al. Rat dilated cardiomyopathy after autoimmune giant cell myocarditis. Circ Res 1994;75:278-84.

3. Hanawa H, Tsuchida M, Matsumoto Y, Watanabe H, Abo T, Sekikawa H, et al. Characterization of T cells infiltrating the heart in rats with experimental autoimmune myocarditis. Their similarity to extrathymic T cells in mice and the site of proliferation. J Immunol 1993;150:5682-95.

4. Kodama M, Zhang S, Hanawa H, Shibata A. Immunohistochemical characterization of infiltrating mononuclear cells in the rat heart with experimental autoimmune giant cell myocarditis. Clin Exp Immunol 1992;90:330-5.

5. Hanawa H, Abe S, Hayashi M, Yoshida T, Yoshida K, Shiono T, et al. Time course of gene expression in rat experimental autoimmune myocarditis. Clin Sci 2002;103:623-632.

6. Okura Y, Yamamoto T, Goto S, Inomata T, Hirono S, Hanawa H, et al. Characterization of cytokine and iNOS mRNA expression in situ during the course of experimental autoimmune myocarditis in rats. J Mol Cell Cardiol 1997;29:491-502.

7. Rauchhaus M, Doehner W, Francis DP, Davos C, Kemp M, Liebenthal C, et al. Plasma cytokine parameters and mortality in patients with chronic heart failure. Circulation 2000;102:3060-7.

8. Hwang TC, Horie M, Nairn AC, Gadsby DC. Role of GTP-binding proteins in the regulation of mammalian cardiac chloride conductance. J Gen Physiol 1992;99:465-89.

9. Isenberg G, Klockner U. Calcium tolerant ventricular myocytes prepared by preincubation in a "KB medium". Pflugers Arch 1982;395:6-18.

10. Toba K, Hanawa H, Fuse I, Sakaue M, Watanabe K, Uesugi Y, et al. Difference in CD22 molecules in human B cells and basophils. Exp Hematol 2002;30:205-11.

11. Ya J, Markman MW, Wagenaar GT, Blommaart PJ, Moorman AF, Lamers WH. Expression of the smooth-muscle proteins alpha-smooth-muscle actin and

calponin, and of the intermediate filament protein desmin are parameters of cardiomyocyte maturation in the prenatal rat heart. Anat Rec 1997;249:495-505.

12. Hayashi K, Yano H, Hashida T, Takeuchi R, Takeda O, Asada K, et al. Genomic structure of the human caldesmon gene. Proc Natl Acad Sci U S A 1992;89:12122-6.

13. Bazan JF, Bacon KB, Hardiman G, Wang W, Soo K, Rossi D, et al. A new class of membrane-bound chemokine with a CX3C motif. Nature 1997;385:640-4.

14. Harrison JK, Jiang Y, Wees EA, Salafranca MN, Liang HX, Feng L, et al. Inflammatory agents regulate in vivo expression of fractalkine in endothelial cells of the rat heart. J Leukoc Biol 1999;66:937-44.

15. Fraticelli P, Sironi M, Bianchi G, D'Ambrosio D, Albanesi C, Stoppacciaro A, et al. Fractalkine (CX3CL1) as an amplification circuit of polarized Th1 responses. J Clin Invest 2001;107:1173-81.

16. Garcia GE, Xia Y, Chen S, Wang Y, Ye RD, Harrison JK, et al. NF-kappaB-dependent fractalkine induction in rat aortic endothelial cells stimulated by IL-1beta, TNF-alpha, and LPS. J Leukoc Biol 2000;67:577-84.

17. Hatori K, Nagai A, Heisel R, Ryu JK, Kim SU. Fractalkine and fractalkine receptors in human neurons and glial cells. J Neurosci Res 2002;69:418-26.

18. Tong N, Perry SW, Zhang Q, James HJ, Guo H, Brooks A, et al. Neuronal fractalkine expression in HIV-1 encephalitis: roles for macrophage recruitment and neuroprotection in the central nervous system. J Immunol 2000;164:1333-9.

19. Fuse K, Kodama M, Ito M, Okura Y, Kato K, Hanawa H, et al. Polarity of helper T cell subsets represents disease nature and clinical course of experimental autoimmune myocarditis in rats. Clin Exp Immunol 2003;134:403-8.

20. Ritchlin C, Haas-Smith SA. Expression of interleukin 10 mRNA and protein by synovial fibroblastoid cells. J Rheumatol 2001;28:698-705.

21. Watanabe K, Nakazawa M, Fuse K, Hanawa H, Kodama M, Aizawa Y, et al. Protection against autoimmune myocarditis by gene transfer of interleukin-10 by electroporation. Circulation 2001;104:1098-100.

22. Antoniades HN, Neville-Golden J, Galanopoulos T, Kradin RL, Valente AJ, Graves DT. Expression of monocyte chemoattractant protein 1 mRNA in human idiopathic pulmonary fibrosis. Proc Natl Acad Sci U S A 1992;89:5371-5.

23. Ozaki K, Hanazawa S, Takeshita A, Chen Y, Watanabe A, Nishida K, et al.

Interleukin-1 beta and tumor necrosis factor-alpha stimulate synergistically the expression of monocyte chemoattractant protein-1 in fibroblastic cells derived from human periodontal ligament. Oral Microbiol Immunol 1996;11:109-14.

24. Yoshimura T, Leonard EJ. Secretion by human fibroblasts of monocyte chemoattractant protein-1, the product of gene JE. J Immunol 1990;144:2377-83.

25. Fuse K, Kodama M, Hanawa H, Okura Y, Ito M, Shiono T, et al. Enhanced expression and production of monocyte chemoattractant protein-1 in myocarditis. Clin Exp Immunol 2001;124:346-52.

26. Okuno T, Andoh A, Bamba S, Araki Y, Fujiyama Y, Fujiyama M, et al. Interleukin-1beta and tumor necrosis factor-alpha induce chemokine and matrix metalloproteinase gene expression in human colonic subepithelial myofibroblasts. Scand J Gastroenterol 2002;37:317-24.

27. Ashkar S, Weber GF, Panoutsakopoulou V, Sanchirico ME, Jansson M, Zawaideh S, et al. Eta-1 (osteopontin): an early component of type-1 (cell-mediated) immunity. Science 2000;287:860-4.

28. Weber GF, Ashkar S, Glimcher MJ, Cantor H. Receptor-ligand interaction between CD44 and osteopontin (Eta-1). Science 1996;271:509-12.

29. Ashizawa N, Graf K, Do YS, Nunohiro T, Giachelli CM, Meehan WP, et al. Osteopontin is produced by rat cardiac fibroblasts and mediates A(II)-induced DNA synthesis and collagen gel contraction. J Clin Invest 1996;98:2218-27.

30. Nishimura H, Honjo T. PD-1: an inhibitory immunoreceptor involved in peripheral tolerance. Trends Immunol 2001;22:265-8.

31. Nishimura H, Okazaki T, Tanaka Y, Nakatani K, Hara M, Matsumori A, et al. Autoimmune dilated cardiomyopathy in PD-1 receptor-deficient mice. Science 2001;291:319-22.

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 of lymphocyte activation. J Exp Med 2000;192:1027-34.

33. Herskowitz A, Ahmed-Ansari A, Neumann DA, Beschorner WE, Rose NR, Soule LM, et al. Induction of major histocompatibility complex antigens within the myocardium of patients with active myocarditis: a nonhistologic marker of myocarditis. J Am Coll Cardiol 1990;15:624-32.

34. Noutsias M, Seeberg B, Schultheiss HP, Kuhl U. Expression of cell adhesion

molecules in dilated cardiomyopathy: evidence for endothelial activation in inflammatory cardiomyopathy. Circulation 1999;99:2124-31.

35. Wojnicz R, Nowalany-Kozielska E, Wojciechowska C, Glanowska G, Wilczewski P, Niklewski T, et al. Randomized, placebo-controlled study for immunosuppressive treatment of inflammatory dilated cardiomyopathy: two-year follow-up results. Circulation 2001;104:39-45.

36. Harada M, Saito Y, Kuwahara K, Ogawa E, Ishikawa M, Nakagawa O, et al. Interaction of myocytes and nonmyocytes is necessary for mechanical stretch to induce ANP/BNP production in cardiocyte culture. J Cardiovasc Pharmacol 1998;31:S357-9.

Figure Legends

Fig. 1.

Microscopic findings. (A) Cardiomyocytes which were not passed through 38μm stainless-steel sieve twice. (B) non-cardiomyocytes which were passed through 20μm stainless-steel sieve twice. May-Giemsa-staining. Bar represents 10μm.

Fig. 2.

Purification quality of Non-Cardiomyocytes. (A, B) Before sorting. (C, D, E) Sorted cells of TCRα/β$^+$, TCRα/β$^-$/CD11b$^+$, and TCRα/β$^-$/CD11b$^-$, respectively. Vertical axis and horizontal axis, side scattergram and PE-fluorescence, respectively. The results are representative of studies with three separate myocarditis rats on day 18.

Fig. 3.

Purified cells on cytospin slides. (A) CD11b$^+$ cell fraction cells were stained with May-Giemsa stain. (B) CD11b$^+$ cell fraction cells were stained with naphthol AS-D chloroacetate esterase and α-naphthylbutyrate esterase stain. Macrophages were visualized in brown and neutrophilic granulocytes in blue. (C) αβT cell fraction cells were stained with May-Giemsa stain. (D, E, F) NCNI cell fraction cells were stained with May-Giemsa stain. (G, H) NCNI cell fraction cells were stained with anti-Factor VIII related antigen antibodies. (I) NCNI cell fraction cells were stained with anti-α smooth muscle actin antibodies. (J, K) NCNI cell fraction cells were stained with anti rat collagen III antibodies. Bar represents 10μm. Arrows indicate positive staining.

Fig. 4.

mRNA copy numbers of α cardiac myosin, β cardiac myosin and ANP at each day/copy numbers at day 0. (○) ANP from purified cardiomyocytes. (●) ANP from whole heart. (△) α cardiac myosin from purified cardiomyocytes. (▲) α cardiac myosin from whole hearts. (◇) β cardiac myosin from purified cardiomyocytes. (◆) β cardiac myosin from whole hearts. Data obtained from quantitative RT-PCR were presented as mean±SEM.

Fig. 5

Absolute copy numbers of immunologic molecule mRNA /μg of total RNA in Day 18 EAM hearts. Data obtained from quantitative RT-PCR were presented as mean±SEM. (A) IL-10. (B) MCP-1. (C) Fractalkine. (D) CX3CR1.

*P<0.05, †P<0.01, ‡P<0.001, ††P<0.0001.

Fig. 6

Copy numbers of immunologic molecule mRNA / copy numbers of γ-actin mRNA in cultivated NC cells from EAM hearts with TNF-α. Data obtained from quantitative RT-PCR were presented as mean±SEM. (A) Fractalkine. (B) MCP-1. (C) Osteopontin. Fractalkine and MCP-1 were significantly increased by TNF-α. No significant increase of osteopontin by TNF-α was observed

†P<0.01, ‡P<0.001, ††P<0.0001.

Fig. 7

(A) A section of EAM heart on day 18 was stained with hematoxylin and eosin stain. (B) The same section as A was stained with anti-osteopontin antibodies. The immunoreactivity was observed in some mononucleated cells. Arrow heads indicate positive staining. (C) A section of normal heart was stained with anti-osteopontin antibodies. The immunoreactivity was hardly observed. Bar represents 10μm.

Figure(s)

Fig. 1

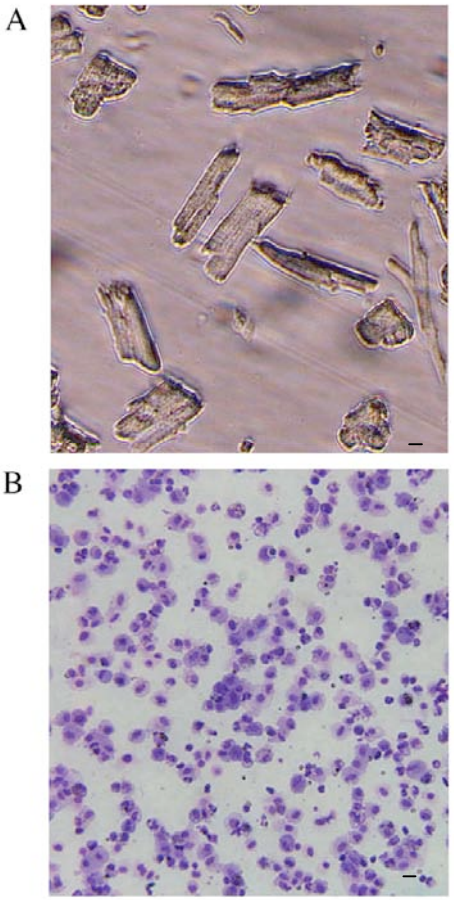


Fig. 2

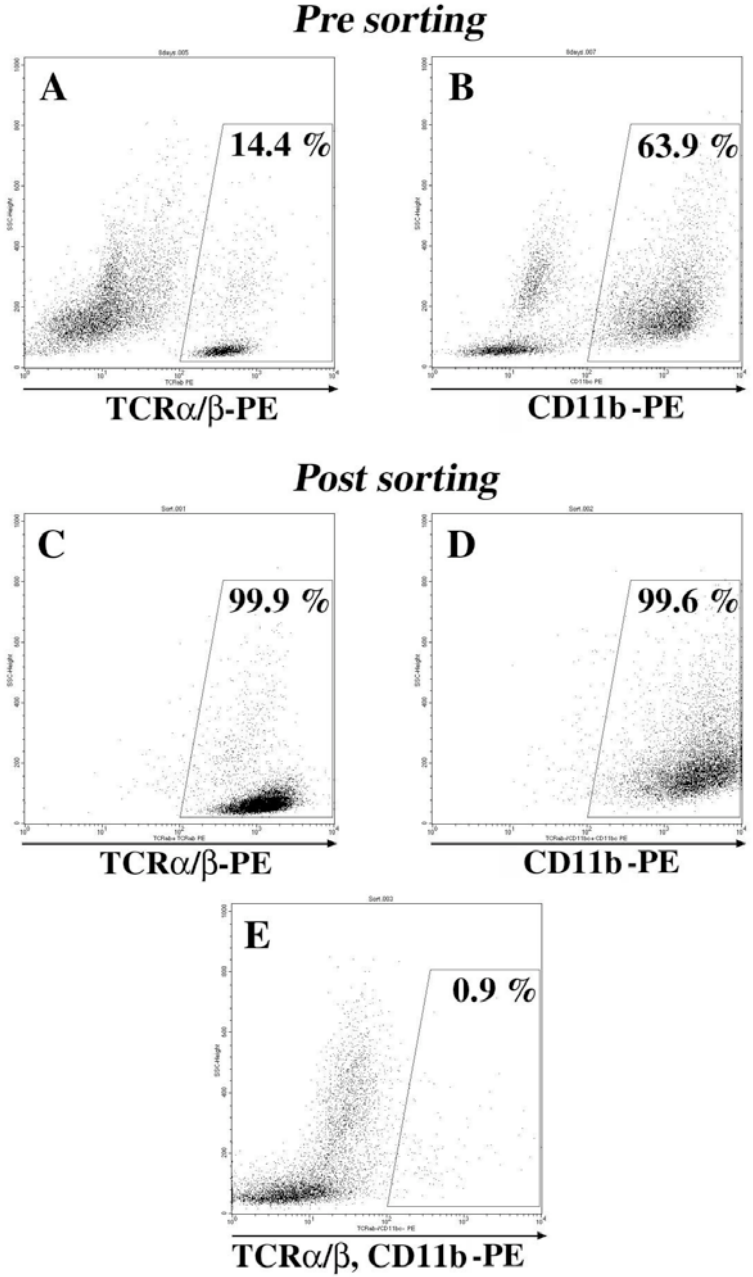

Pre sorting
A
14.4 %
TCRα/β-PE
B
63.9 %
CD11b-PE
Post sorting
C
99.9 %
TCRα/β-PE
D
99.6 %
CD11b-PE
E
0.9 %
TCRα/β, CD11b-PE

Fig. 3

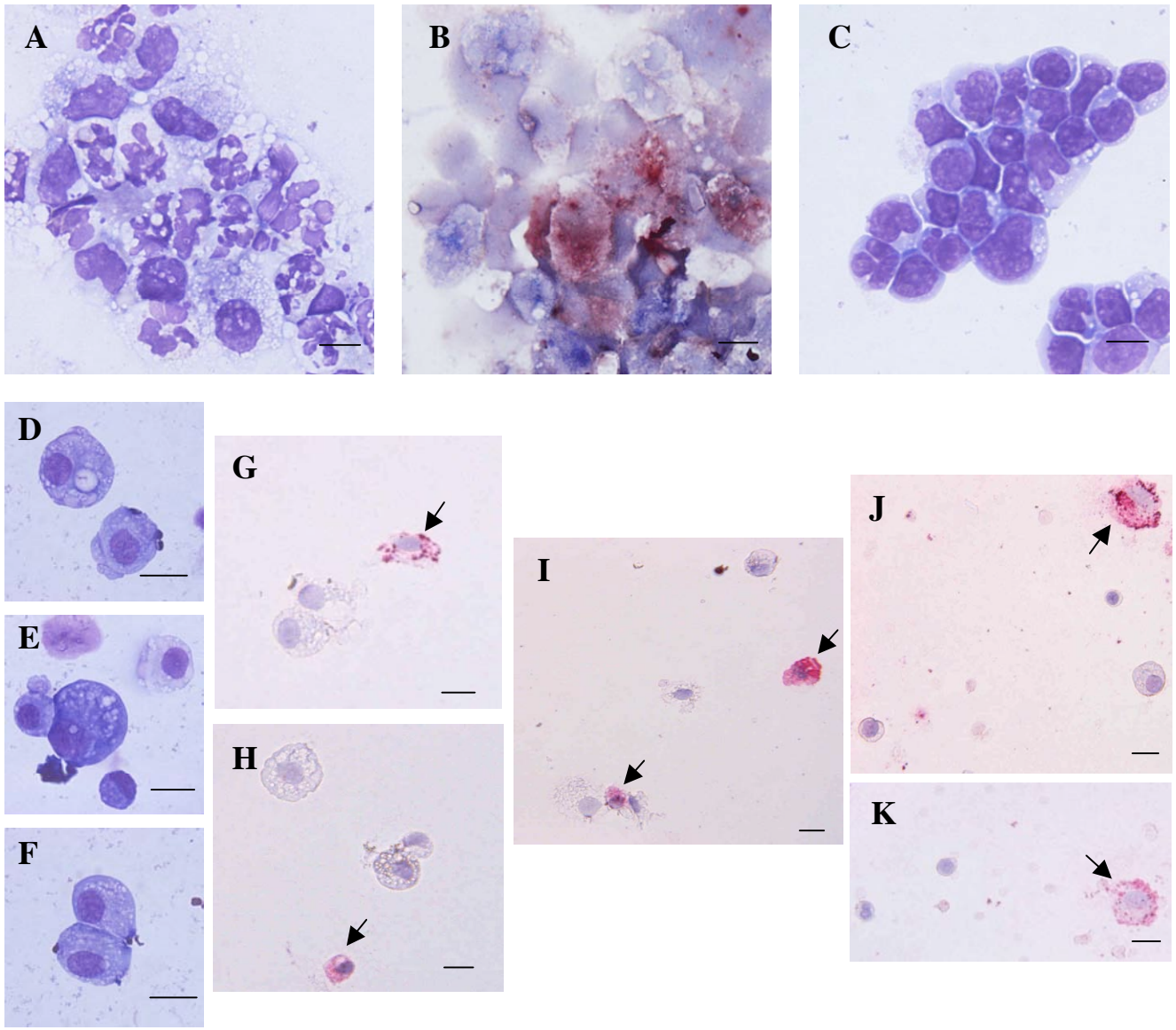


Fig. 4

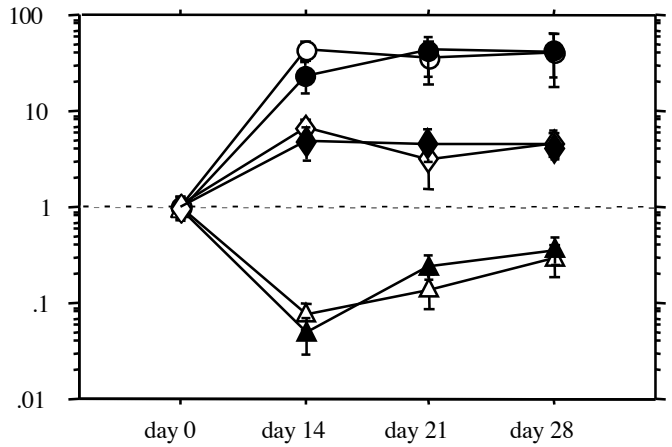


Fig. 5

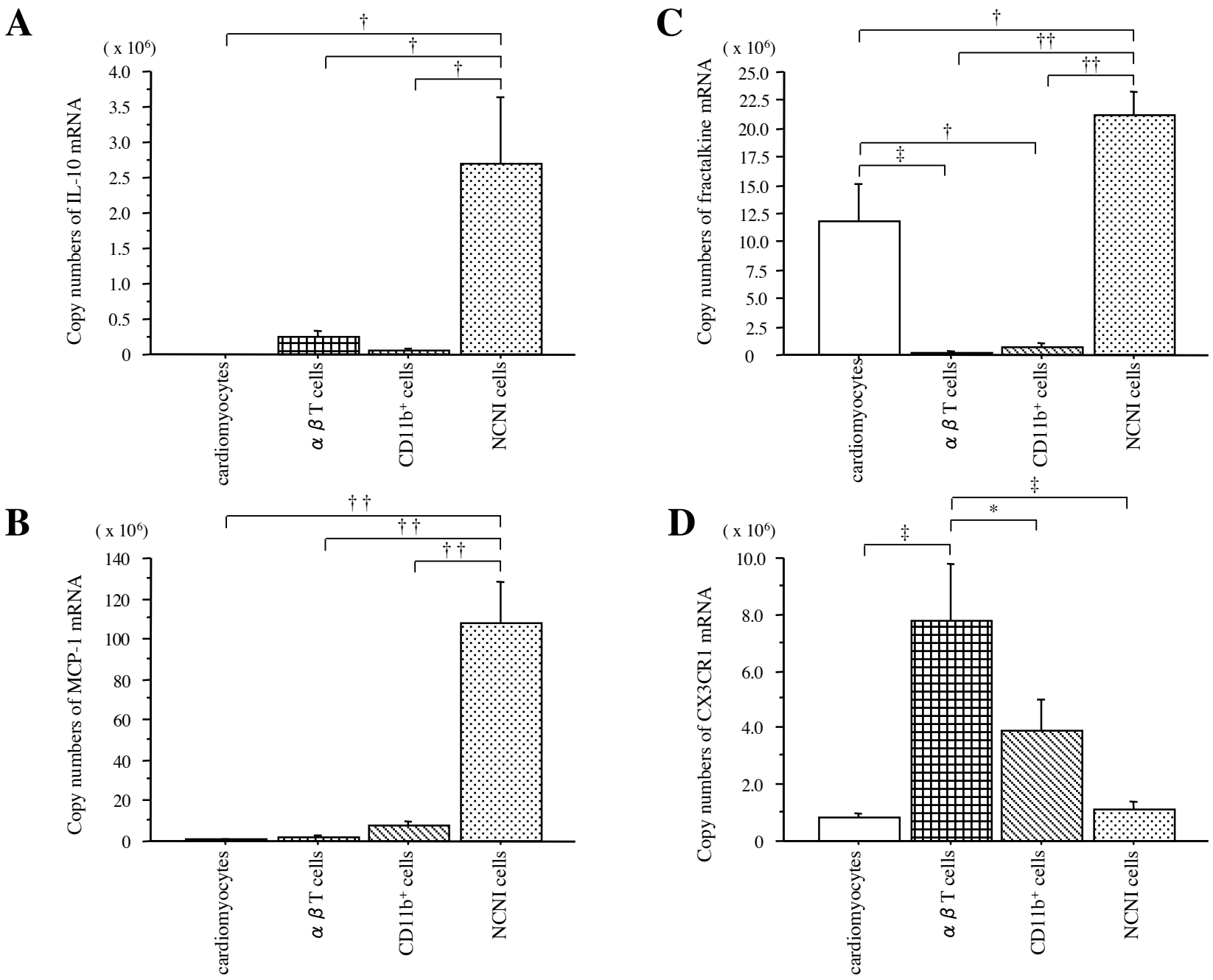

A
(x 10⁶)
Copy numbers of IL-10 mRNA
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0
†
†
†
cardiomyocytes
α β T cells
CD11b⁺ cells
NCNI cells
B
(x 10⁶)
Copy numbers of MCP-1 mRNA
140
120
100
80
60
40
20
0
† †
† †
† †
cardiomyocytes
α β T cells
CD11b⁺ cells
NCNI cells
C
(x 10⁶)
Copy numbers of fractalkine mRNA
25.0
22.5
20.0
17.5
15.0
12.5
10.0
7.5
5.0
2.5
0
†
††
††
†
‡
cardiomyocytes
α β T cells
CD11b⁺ cells
NCNI cells
D
(x 10⁶)
Copy numbers of CX3CR1 mRNA
10.0
8.0
6.0
4.0
2.0
0
‡
*
‡
cardiomyocytes
α β T cells
CD11b⁺ cells
NCNI cells

Fig. 6

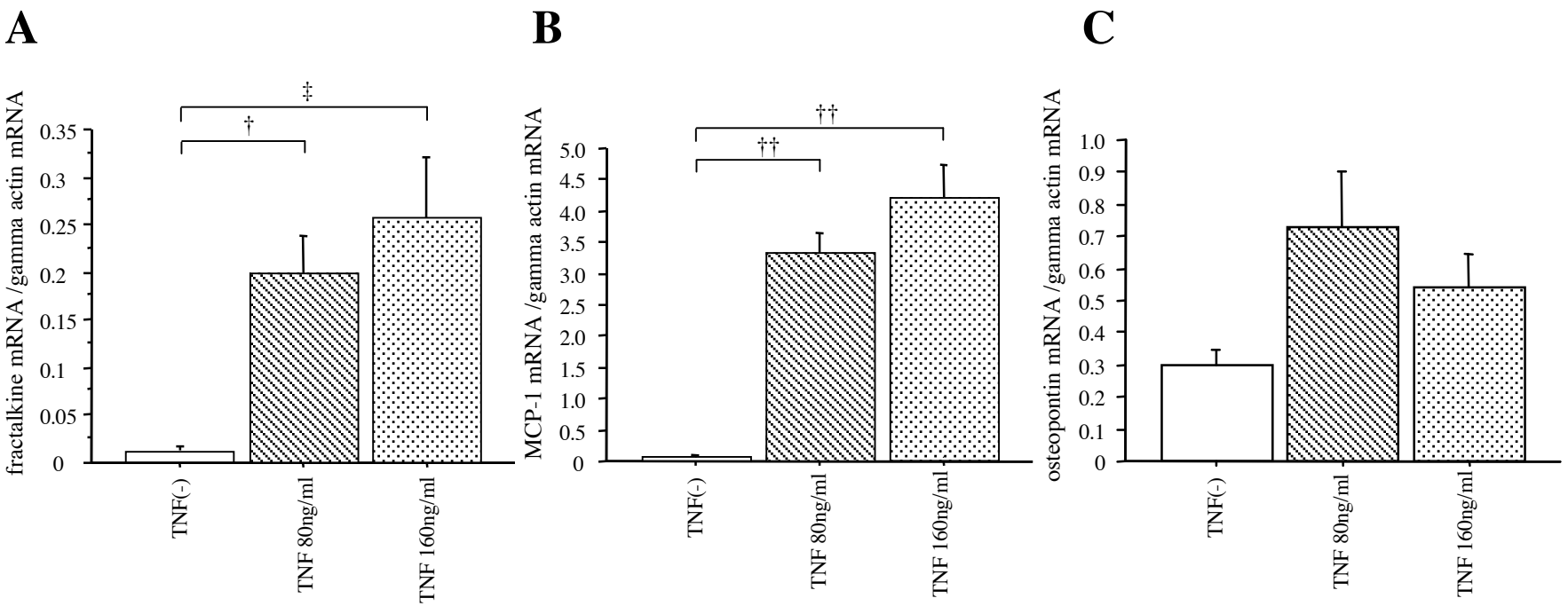
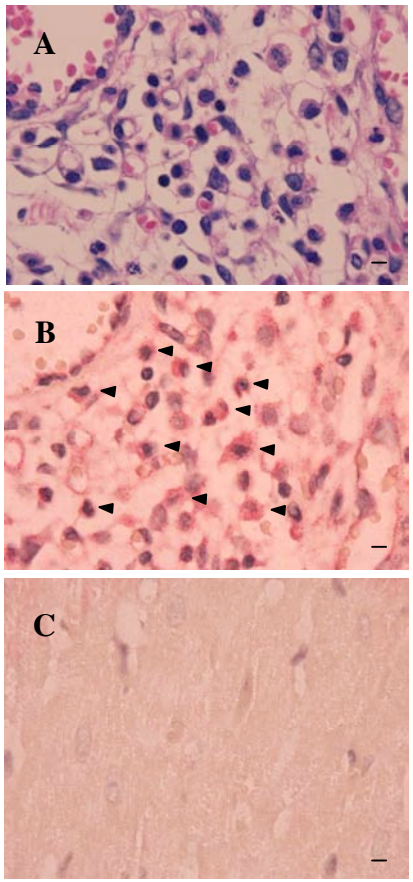

A
fractalkine mRNA /gamma actin mRNA
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
†
‡
TNF(-)
TNF 80ng/ml
TNF 160ng/ml
B
MCP-1 mRNA /gamma actin mRNA
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0
††
††
TNF(-)
TNF 80ng/ml
TNF 160ng/ml
C
osteopontin mRNA /gamma actin mRNA
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
TNF(-)
TNF 80ng/ml
TNF 160ng/ml

Fig. 7



Table(s)

Table 1 List of Primers

	Sense Primer	Antisense Primer
α cardiac myosin	5'-acaaggttaaaaacctgacagagg-3'	5'-tactgttctgctgactgatgtcaa-3'
β cardiac myosin	5'-cactccagaagagaagaactccat-3'	5'-ccagttgaacatcttctcgtacac-3'
ANP	5'-atggatttcaagaacctgctagac-3'	5'-gctccaatcctgtcaatcctac-3'
CD3	5'-gatcccaaactctgctatatgcta-3'	5'-ctttcatgccaatctcactgtag-3'
CD11b	5'-gggatccgtaaagtagtgagaa-3'	5'-aaaggagctggtacttcctgtct-3'
collagen type 3	5'-cgcaattgcagagacctgaa-3'	5'-acagtcatgggactggcatttat-3'
von Willebrand factor	5'-agaggctacacatctctcagaagc-3'	5'-gaccttcttcttcttgaaaccttg-3'
calponin	5'-aacataggaaatttcatcaaagcc-3'	5'-gtagactgatagttgcctgatcca-3'
caldesmon	5'-atggaagaaacagaagtggtcatc-3'	5'-cctcagctctctctctctcttctg-3'
IL-2	5'-ctgagagggatcgataattacaaga-3'	5'-attggcactcaaatttgttttcag-3'
IFN-γ	5'-atctggaggaactggcaaaaggacg-3'	5'-ccttaggctagattctggtgacagc-3'
IFN-γ receptor	5'-aagagtttattatgtgccgaaagg-3'	5'-tgtattaacttgccagaaagacga-3'
IL-10	5'-actgctatgttgcctgctcttact-3'	5'-gaattcaaatgctccttgatttct-3'
IL-10 receptor	5'-tgacagtgacctagtccagaactc-3'	5'-ttgattccactgtctacttggtgt-3'
TNF-α	5'-atgggctccctctcatcagt-3'	5'-actccagctgctcctctgct-3'
TNF-α receptor	5'-aaaggaacctacttggtgagtgac-3'	5'-gttacacacggtgttctgtttctc-3'
MCP-1	5'-ctgtctcagccagatgcagttaat-3'	5'-tatgggtcaagttcacattcaaag-3'
fractalkine	5'-ctcgccaatcccagtgaccttgctc-3'	5'-gattggtagacagcagaactcggccaaatg-3'
CX3CR1	5'-agctgctcaggacctcaccat-3'	5'-gttgtggaggccctcatggctgat-3'
MHC Class I	5'-ctcttgggaaggagcagaattac-3'	5'-tttacaatctgggagagacacatc-3'
MHC Class II	5'-aaatacttcaggacagttgaaccc-3'	5'-ccgactccactcatcttcttattt-3'
PD-1	5'-cagcaaccagactgaaaaacag-3'	5'-acaatgaccaagccttgaaact-3'
PD-1L	5'-gcagattcccagtagaacagaaat-3'	5'-acattagttcatgctcagaagtgg-3'
osteopontin	5'-aacggatgactttaagcaagaaac-3'	5'-tactgttcatcagaaacagggaaa-3'
CD44	5'-aacatgcaggtatgggttcatag-3'	5'-tcatctataatgtttgaggcatcg-3'
γ-actin	5'-agccttccttcctgggcatggagt-3'	5'-tggaggggcctgactcgtcatact-3'

Table 2. Absolute copy numbers of mRNA/μg of total RNA

	Cultivated NC cells (n=6)
α cardiac myosin	N.D.
CD3	N.D.
CD11b	2,470,000±1,460,000
von Willebrand factor	531,000±161,000
collagen typeIII	470,000,000±158,000,000
calponin	13,500,000±8,380,000
TNF-α receptor	66,000,000±40,5000,000

Results are expressed as the mean±SEM. N.D.; not detected.

Table 3 Subpopulations of Non-Cardiomyocytes in Normal (day 0) and Myocarditis (day 18) Rat Hearts

	day 0 (n=3)	day 18 (n=5)
CD3 (%)	7.9±2.4	12.6±2.6
TCR α/β (%)	7.7±2.0	14.1±2.9
TCR γ/δ (%)	0.4±0.1	0.1±0.1
CD161 (%)	11.0±4.3	1.9±0.4
CD4 (%)	4.4±2.8	12.2±2.1
CD8 (%)	8.5±2.1	1.5±0.6
CD25 (%)	0.3±0.2	0.6±0.2
CD11b (%)	43.4±12.4	64.3±5.3
ED1 (%)	30.6±8.2	65.0±4.5

Results are expressed as the mean±SD.

Table 4 Absolute Copy Numbers of Specific Cell Marker mRNA in Day 18 EAM Hearts

	copy numbers of mRNA/μg of total RNA (copy numbers of mRNA/the most copy numbers of mRNA)							
	cardiomyocytes (n=5)		αβ T cells (n=5)		$CD11b^+$ cells (n=5)		NCNI cells (n=6)	
αcardiac myosin	62,000,000±27,500,000†	(100±44.4%)	N.D.		N.D.		N.D.	
CD3	65,700±27,500	(0.25±0.10%)	26,300,000±8,840,000†	(100±33.6%)	93,100±28,400	(0.35±0.11%)	1,120,000±346,000	(4.3±1.3%)
CD11b	785,000±219,000	(2.8±0.8%)	2,020,000±957,000	(7.2±3.4%)	28,000,000±10,800,000†	(100±38.6%)	154,000±85,000	(0.55±0.30%)
von Willebrand factor	487,000±98,800	(9.9±2.0%)	416,000±217,000	(8.5±4.4%)	171,000±28,400	(3.5%±0.58%)	4,890,000±1,160,000†	(100±23.7%)
collagen typeIII	19,800,000±1,810,000	(0.54±0.05%)	24,200,000±11,400,000	(0.66±0.31%)	16,400,000±4,200,000	(0.45±0.11%)	3,660,000,000±472,000,000†	(100±12.9%)
calponin	1,000,000±285,000	(42.4±12.1%)	N.D.		104,000±86,200	(4.4±3.7%)	2,360,000±594,000*	(100±25.2%)
caldesmon	21,800±4,940	(11.3±2.6%)	24,400±9,670	(12.6±5.0%)	28,500±7,790	(14.8±4.0%)	193,000±41,300†	(100±21.4%)

Results are expressed as the mean±SEM. N.D.; not detected. *P<0.05 vs any other group. †P<0.01 vs any other group.

Table 5 Absolute Copy Numbers of Immunologic Molecule mRNA in Day 18 EAM Hearts

	copy numbers of mRNA/ μ g of total RNA (copy numbers of mRNA/the most copy numbers of mRNA)							
	cardiomyocytes (n=5)		α β T cells (n=5)		CD11b+ cells (n=5)		NCNI cells (n=6)	
IL-2	N.D.		329,000±103,000†	(100±31.3%)	N.D.		N.D.	
IFN- γ	N.D.		6,240,000±1,820000†	(100±29.2%)	N.D.		N.D.	
IFN- γ receptor	3,760,000±944,000	(15.4±1.4%)	24,400,000±4,070,000*	(100±16.7%)	15,800,000±2,870,000	(64.8±11.8%)	21,900,000±1,950,000	(89.8±8.0%)
IL-10	9,090±4,590	(0.34±0.17%)	240,000±96,200	(8.92±3.58%)	53,300±17,000	(1.98±0.63%)	2,690,000±951,000†	(100±35.4%)
IL-10 receptor	406,000±81,900	(5.29±1.07%)	6,050,000±2,170,000	(78.8±28.3%)	7,680,000±2,380,000	(100±31.0%)	1,490,000±376,000	(19.4±4.90%)
TNF- α	247,000±69,200	(1.35±0.38%)	2,560,000±699,000	(14.0±3.83%)	18,200,000±6,580,000†	(100±36.1%)	1,410,000±282,000	(7.73±1.55%)
TNF- α receptor	4,440,000±1,260,000	(11.7±3.33%)	2,380,000±448,000	(6.29±1.18%)	12,900,000±3,250,000	(34.1±8.59%)	37,800,000±4,860,000†	(100±11.2%)
MCP-1	754,000±122,000	(0.70±0.11%)	1,870,000±999,000	(1.73±0.92%)	7,590,000±2,510,000	(7.03±2.32%)	108,000,000±20,600,000†	(100±19.0%)
fractalkine	11,900,000±3,270,000	(56.1±15.4%)	236,000±107,000	(1.11±0.50%)	680,000±346,900	(3.21±1.64%)	21,200,000±1,990,000†	(100±9.39%)
CX3CR1	812,000±151,000	(10.4±1.93%)	7,810,000±1,950,000*	(100±25.0%)	3,920,000±1,098,000	(50.2±14.1%)	1,100,000±262,000	(14.1±3.35%)
MHC Class I	41,700,000±2,480,000	(20.1±2.00%)	207,000,000±83,200,000*	(100±40.2%)	39,700,000±11,900,000	(19.2±5.76%)	63,700,000±8,920,000	(30.8±4.32%)
MHC Class II	319,000±46,000	(17.3±2.50%)	654,000±211,000	(35.6±11.5%)	1,840,000±535,000†	(100±29.1%)	358,000±70,700	(19.5±3.84%)
PD-1	8,380±5,300	(0.13±0.05%)	16,500,000±5,180,000†	(100±31.4%)	26,300±17,300	(0.39±0.16%)	30,300±14,200	(0.18±0.09%)
PD-1L	198,000±80,300	(1.21±0.49%)	5,360,000±1,570,000	(32.9±9.62%)	16,300,000±3,470,000†	(100±21.3%)	1,580,000±304,000	(9.69±1.86%)
osteopontin	3,140,000±791,000	(7.87±1.98%)	2,860,000±1,240,000	(7.17±3.11%)	39,900,000±21,700,000	(100±54.3%)	24,400,000±4,360,000	(61.2±10.9%)
CD44	11,300,000±1,430,000	(8.74±1.11%)	129,000,000±34,600,000*	(100±26.8%)	53,700,000±5,870,000	(41.5±4.54%)	48,300,000±14,300,000	(37.4±11.1%)

Results are expressed as the mean±SEM. N.D.; not detected. *P<0.05 vs any other group. †P<0.01 vs any other group.

Table 6 Absolute Copy Numbers of mRNA in Cardiomyocytes of EAM Hearts

	copy numbers of mRNA/μg of total RNA		
	normal (n=5)	day18 (n=5)	day90 (n=6)
fractalkine	4,880,000±998,000	11,900,000±3,270,000*	4,420,000±667,000
IFN-γ receptor	4,440,000±639,000	3,760,000±944,000	2,690,000±382,000
TNF-α receptor	576,000±90,100	5,130,000±1,610,000†	703,000±204,000
CD44	1,060,000±140,000	11,300,000±1,430,000†	2,540,000±149,000
MHC Class I	3,150,000±901,000	41,700,000±2,480,000†	5,450,000±1,150,000
MHC Class II	N.D.	319,000±46,000†	7,160±2,870

Results are expressed as the mean±SEM. N.D.; not detected. *P<0.05 vs any other group.
†P<0.01 vs any other group.