

Mechanism of Inflammation in Murine Eosinophilic Myocarditis Produced by Adoptive Transfer with Ovalbumin Challenge

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Key Words

CC chemokine receptor 3 · Interleukin 5 · Murine eosinophilic myocarditis · Ovalbumin challenge · RANTES

Abstract

Background: Interleukin (IL)-5, RANTES and CC chemokine receptor 3 (CCR3) are essential for induction of eosinophil recruitment in organs, but the precise pathogenesis of eosinophilic myocarditis is still unclear. We investigated the relationships between these cytokines and receptors in the development of inflammation in murine myocarditis produced by adoptive transfer, with reference to eosinophil infiltration and signal transduction. **Methods:** The splenocytes from male donor DBA/2 mice were separated after ovalbumin (OVA) sensitization. These cells had a CD4/CD8 ratio of approximately 3.0. Cells (2.0×10^7) were individually transfused to recipient adoptive male DBA/2 mice, and OVA challenge was performed serially. The heart and spleen of the recipient were analyzed to determine the kinetics of IL-5, RANTES, CCR3 and eosinophil production with simultaneous determination of Janus kinase 3 (JAK3) mRNA. **Results:** Approximately 85% of recipient mice developed myocarditis; 35% had recognizable cell infiltration in the left ventricular endocardium, an effect which was absent in control mice. Eosinophilic myocarditis was usually associated with animals having several degenerative changes in myocardial cells, and IL-5, RANTES and CCR3 expressions were usually present in these eosinophils ($p < 0.05$). CCR3 and

JAK3 mRNAs were detected in the spleens and hearts of recipient animals providing histological evidence for kinetics related to eosinophil infiltration. **Conclusion:** The murine model of adoptive transferred myocarditis is suitable for studying the mechanism of eosinophilic myocarditis. A unique pathogenesis of this disorder may be controlled by the synergism of CD4 dominance in the donor and JAK-STAT signaling in the recipient, which may cause recruitment of eosinophils into heart lesions.

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Introduction

Human eosinophilic and hypersensitive myocarditis may be induced by several initiators [1–3], including the side effect of drugs [1], the chief clinical feature of Churg-Strauss syndrome [2], or accompanying symptoms due to parasite infection [3]. These disorders are usually intractable [4–6] and the exact pathogenesis is still unclear, although a role for eosinophilic cationic protein in myocardiocyte injury has been proposed [7–9]. Generally, CD4+ T cells and related cytokines are considered to be important for eosinophil migration into inflammatory lesions [10, 11], and allergic reactions in some organs may be caused by specific environmental conditions [12, 13]. Such a type 1 allergy is also attributed to specific cytokines such as interleukin (IL)-5, RANTES, eotaxin and other CD4-related cytokines [14–17].

In animal models, allergic inflammation in the heart occurs less easily than in organs such as the skin, conjunctiva, nasal and bronchial mucosa, which are often exposed to specific and nonspecific antigens. Immunological treatments such as ovalbumin (OVA) challenge and/or adoptive transfer [18–20] have been used to induce immunological tolerance and type 1 allergy in several organs in animal models, and adoptive transfer is particularly useful for establishing a hypersensitive state in the host before allergen challenge. On the other hand, some strains of inbred mice develop inflammatory changes in the heart tissue: the DBA/2 mouse is one such inbred strain, and spontaneous myocarditis [21–24] with calcinosis [21–23] occurs at the right ventricular site in these mice [21, 22]. This strain is also useful for the investigation of immunological mechanisms of eosinophil infiltration in both spontaneous and OVA-challenged myocarditis, as shown in our previous studies [25–29].

In the current study, we investigated the mechanisms underlying murine eosinophilic myocarditis by serial adoptive transfer and OVA challenge in DBA/2 mice to determine the pathogenesis of human heart disease associated with intractable allergic disorders. Simultaneously, we also examined the relationship between local eosinophil infiltration and the kinetics of CD4-related cytokines and CC chemokine receptor 3 (CCR3). Additionally, we examined the roles of signal transduction in the reticular organ and the cardiovascular system, given the relationships between the above cytokines and eosinophils in this adoptive transferred eosinophilic myocarditis.

Material and Methods

Mice

Thirty-six male DBA/2 mice aged 21 days were obtained from the Shizuoka Agricultural Cooperative Association (SLC, Shizuoka, Japan). The mice were of the major histocompatibility complex H-2d haplotype [27] and were fed a standard maintenance diet and water under specific pathogen-free conditions [26, 27]. The mice were divided into donor (n = 6) and recipient (n = 30) groups in our experimental protocol.

Treatment of Donor Mice before Spleen Resection

Donor DBA/2 mice (n = 6) were immunized by intraperitoneal injection of a mixture of 0.1 mg/mouse OVA, chicken egg grade 5 (Sigma, Calif., USA) and 10 mg/mouse aluminum hydroxide (Sigma) at 5 and 7 weeks of age. At 9 weeks of age, the mice were euthanized with an intraperitoneal injection of 30 mg/kg pentobarbital sodium, and their spleens were removed under sterile conditions.

Preparation and Characterization of Donor Spleen Cells

Six spleens excised from each donor mouse (n = 6) were collected. These spleens were entirely teased and purified in sterilized RPMI-1640 (Sigma, St. Louis, Mo., USA), containing 20 mM HEPES and L-glutamine. After centrifugation, washing and excluding erythrocytes, total spleen cells were obtained in 2 ml of phosphate-buffered saline (PBS). The total number of splenocytes was determined using light microscopy as 4.7×10^8 cells/2 ml PBS. The CD4+ and CD8+ subsets of these splenocytes were also characterized by FACS analysis with Guava PCATM Express (Guava Technologies, Calif., USA), using rat anti-mouse CD4 (L3T4, BD Biosciences, USA) and CD8 (Ly-2, BD Bioscience) antibodies. Ultimately, 2.0×10^7 cells/mouse were used for adoptive transfer, and these cells included progenitor cells of CD4+ and CD8+ T cells, naïve T cells and mature CD4+ and CD8+ T cells.

Adoptive Transfer to the Recipient

As a pretreatment, we first immunized the recipient mice (n = 30) before adoptive transfer at 5 weeks of age. That is, 30 DBA/2 mice (5 weeks old) were immunized by intraperitoneal injection of a mixture of 0.1 mg/mouse OVA, chicken egg grade 5 (Sigma) and 10 mg/mouse of aluminum hydroxide (Sigma). After pretreatment, recipient mice were divided into two groups: the adoptive transfer group (n = 20) and a control group that did not undergo cell transfer (n = 10). Two days after pretreatment, 2.0×10^7 spleen cells were individually transfused to the recipient mice in the adoptive transfer group using intravenous injection. PBS was injected intravenously in the control group. Two days after adoptive transfer, OVA immunization was repeated, and 7 days after adoptive transfer, OVA challenge was performed with intraperitoneal injection of high-concentration OVA solution (1.0% OVA in 0.4 ml saline) for both the adoptive transfer and control groups (fig. 1A). Mice were euthanized with an intraperitoneal injection of 30 mg/kg pentobarbital sodium 23 and 48 h and 4 and 5 days after serial treatment (in the transfer group, n = 5 each; in the control group, n = 3, n = 2, n = 3 and n = 2, respectively). Heart tissue, spleen and peripheral blood serum were analyzed using the methods described below. For histological examination, the hearts and spleens were divided into several blocks for light microscopic, immunohistochemical and immunofluorescence studies.

Light Microscopy with Hematoxylin-Eosin and Luna Staining

Light microscopy was performed using the basal portion of the ventricles of the heart. Heart blocks were fixed in 10% formalin solution and embedded in paraffin. Sections were cut transversely and stained with hematoxylin and eosin. The spleen was fixed and stained in the same way. These organs were observed using light microscopy, focusing on the location of eosinophil infiltration and myocardiocyte injury. For further specific detection of eosinophilic granules, Luna's procedure was performed using respective staining with hematoxylin-Biebrich Scarlet, 1% acid alcohol and lithium carbonate solutions.

Immunohistochemical Analysis of the Heart and Spleen

Qualitative analysis of CD4+ T cells, IL-5, RANTES and CCR3 in heart tissue was performed first. The labeled streptavidin biotin method in paraffin blocks was used for screening of cytokines, using the apical portion of the heart beginning at the midventricular level. Specimens were fixed in 10% formalin solution and

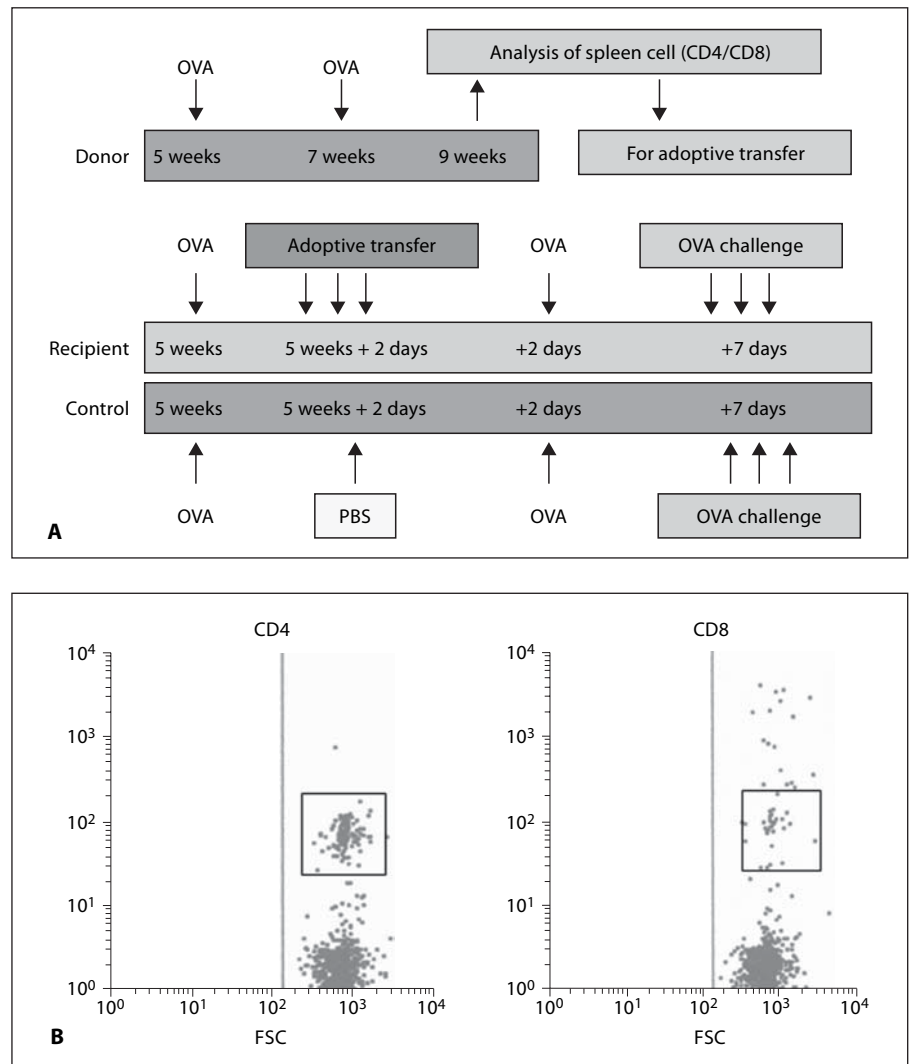


Fig. 1. Experimental protocols and immunological characteristics of the donor animals. **A** Methods of adoptive transfer. Both donor and recipients are DBA/2 mice. Spleen cell analysis of donors was performed before transfer (**B**). Recipient mice were classified into an adoptive transfer group ($n = 20$) and controls without cell infusion ($n = 10$). **B** Characterization of the CD4/CD8 ratio of donor splenocytes by FACS analysis. Approximately 15% of CD4+ and 5% of CD8+ T cells were detected among the purified splenocytes, providing a CD4/CD8 ratio of about 3.0.

embedded in paraffin. Sections of 5.0–8.0 μm thickness were cut from the paraffin section and then incubated at 4°C overnight with rat anti-mouse CD4 (RM 4-5, Pharmingen, San Diego, Calif., USA), goat anti-mouse IL-5 and RANTES (Dako, Carpinteria, Calif., USA) and CCR3 (Santa Cruz Biotechnology, Santa Cruz, Calif., USA) antibodies at approximate dilutions of 1:10 to 1:20. Biotinylated monoclonal rabbit anti-rat (Dako) and polyclonal anti-goat immunoglobulin (Nichirei Cooperation, Tokyo, Japan) were applied at 1:250 dilutions as secondary antibodies for CD4, IL-5, RANTES and CCR3. Sections were incubated with streptavidin-conjugated horseradish peroxidase complexes for 10 min, followed by reaction with imidazole hydrochloride-diluted 3,3'-diaminobenzidine for 5 min. Sections were counterstained with hematoxylin. Lastly, CD4+ T cells, IL-5, RANTES and CCR3 were observed in the heart tissue in labeled streptavidin biotin-incubated sections, as above.

Simultaneously, fluorescence immunohistochemistry was performed to obtain a more detailed distribution of CD4+ T cells, IL-5, RANTES, CCR3 and eotaxin in the heart and spleen. The

method until monoclonal antibody incubation was similar to that described above, and goat anti-mouse eotaxin (Santa Cruz Biotechnology) antibody was used at a dilution of 1:50. After washing with Tris-buffered saline, anti-goat Alexa Fluor 488 (CosmoBio, Tokyo, Japan) at a 1:250 dilution was applied for 30 min as a fluorescent secondary antibody. After washing with distilled water, sections were counterstained with 1:100 diluted propidium iodides for 10 min. CD4+ T cells, IL-5, RANTES, CCR3 and eotaxin were then observed with confocal laser scanning microscopy (CLSM) using a Radiance 2000 MP microscope (Bio-Rad, Tokyo, Japan).

Quantitative Immunohistochemical Analysis of the Relationship between CD4+ T Cells, IL-5, RANTES, CCR3 and Eosinophils in Heart Tissue

The local infiltrative eosinophils and total white blood cells (WBC) were counted in arbitrary 0.1-mm² areas in the heart using an MCID image analyzer (Fuji Film, Tokyo, Japan), and eosinophil/WBC ratios were calculated. Simultaneously, the num-

ber of CD4+ T cells and the IL-5, RANTES and CCR3 levels were determined in the same 0.1-mm² areas using previously reported methods [26, 27]. The eosinophil/WBC ratio, CD4+ T cell count and the IL-5, RANTES and CCR3 levels were examined statistically using SPSS software (see below).

Time course data for CD4+ T cells, IL-5, RANTES, CCR3 and eosinophil/WBC were assessed through measurements at 23 and 48 h and 4 and 5 days after challenge to determine the kinetics associated with eosinophilic myocarditis. The differences in CD4+ T cell counts in heart lesions in adoptive transfer and non-transfer animals were determined. Correlations between IL-5, RANTES and CCR3 levels and the eosinophil/WBC ratio were examined. These analyses were performed to determine the role of these factors in the pathogenesis of eosinophilic myocarditis.

Reverse Transcription Polymerase Chain Reaction of CCR3 from Recipient Heart and Spleen

Heart and spleen specimens (70–100 mg) were homogenized in Trizol reagent (Invitrogen Japan, Tokyo, Japan). After precipitation using chloroform with isopropanol, the total RNA solutions were quantified based on their absorbance at 260 nm. They were then used as templates with a random hexamer primer (Roche Diagnostic Co., Tokyo, Japan) for reverse transcription (RT) of first-strand cDNA. A polymerase chain reaction (PCR) was performed using specific primers for CCR3, i.e. 5'-TTTCCTGCAGTCCTCGCTAT-3' (forward) and 5'-TGCCCACTAAAACACCACA-3' (reverse), and for GAPDH (as control), i.e. 5'-GGTGCTGAGTATGTCGTGGAGTCTACTG-3' (forward) and 5'-TTGCTGTTGAAGTCGCAGGAGACAACCT-3' (reverse). PCR conditions were based on a computerized thermal cycle using the Gene Amp[®] PCR system 2700 (Applied Biosystems, Tokyo, Japan). Amplified PCR products of the CCR3 and GAPDH genes were obtained, and these products were analyzed by 2.0% agarose gel electrophoresis over 30 min. The double-stranded DNA was photographed under ultraviolet irradiation after gel staining with 5 µg/ml ethidium bromide.

Detection of Janus Kinase 3 Signaling in the Heart and Spleen, with Detection of Signal Transducers and Activators of Transcription 6 in the Heart

The presence of Janus kinase 3 (JAK3) signaling in the spleen and heart tissue was examined to study the relationship of cell signaling with the hypersensitivity of these tissues. Fluorescence immunohistochemistry and RT-PCR were performed using similar methods to those described above. For fluorescence immunohistochemistry, goat anti-mouse JAK3 (Santa Cruz Biotechnology) was used at a 1:10 dilution as the specific monoclonal antibody. In RT-PCR, the primers 5'-ACTGGCGGGACTTATCACAC-3' (forward) and 5'-TCCCCGGTACTTGACGATGA-3' (reverse) were used for detection and amplification of JAK3 with GAPDH control primers, as above.

Detection of signal transducers and activators of transcription 6 (STAT6) was simultaneously examined to determine if the JAK-STAT signal transduction system is correctly induced by activation of JAK3 in the heart tissue. RT-PCR was performed using similar methods to those described above with the primers 5'-GGGTCTGCAGTTTCTAGGGA-3' (forward) and 5'-CACTGAGGCTGTTGTCGTTG-3' (reverse) used for the detection and amplification of STAT6, and the GAPDH control primers as above.

Statistical Analysis

All data are expressed as means $\pm$ SEM. Comparison of CD4+ T cell counts between adoptive transfer and nontransfer mice was assessed by unpaired t test. The time courses of CD4+ T cell, IL-5, RANTES, CCR3 and eosinophil/WBC were assessed with one-factor ANOVA with a Bonferroni/Dunn comparison test, and correlations between IL-5, RANTES, CCR3 and eosinophil/WBC were assessed using Pearson's correlation test. A p value <0.05 was considered significant.

Results

Characterization of CD4+ and CD8+ T Cells and CD4/CD8 in Donor Splenocytes

FACS analysis showed that 14.7% of CD4+ and 5.0% of CD8+ T cells (a CD4/CD8 ratio of about 3.0) were present in the total number of purified splenocytes (fig. 1B). These cells were used for adoptive transfer. Spleen CD4/CD8 ratios in an inbred mouse model have been determined under several environmental conditions [30], and the CD4/CD8 ratio of 3.0 obtained in our FACS analysis is higher than the normal ratio.

Morphological Features of Eosinophilic Myocarditis in Recipient Mice

Epicardial inflammation with infiltrations of mononuclear cells and eosinophils is usually seen in the DBA/2 mouse strain [26, 27], and these characteristics remained after adoptive cell transfer; eosinophilic infiltration could be seen in the epicardium and subepicardium at the right ventricular site. Eosinophils were also observed in the myocardial cell layer, and cell infiltration to the endocardium was seen occasionally. These findings are somewhat similar to those associated with cardiovascular problems in human hypereosinophilic syndrome (fig. 2A–D). Furthermore, eosinophils were occasionally conglomerated in other types of inflammatory WBC, and Luna staining showed eosinophil-specific granules in inflamed heart lesions where myocardiocyte injury had been recognized (fig. 2E).

The morbidity of myocarditis, with cell infiltration in the interstitium and/or myocardial cell degeneration, reached 80% in all mice, and 85% morbidity occurred in adoptive transfer animals. Eosinophilic myocarditis was found in approximately 65% of these latter mice, and endocardial involvement was found in 35% of the adoptive transfer animals, but in none of the control animals (table 1).

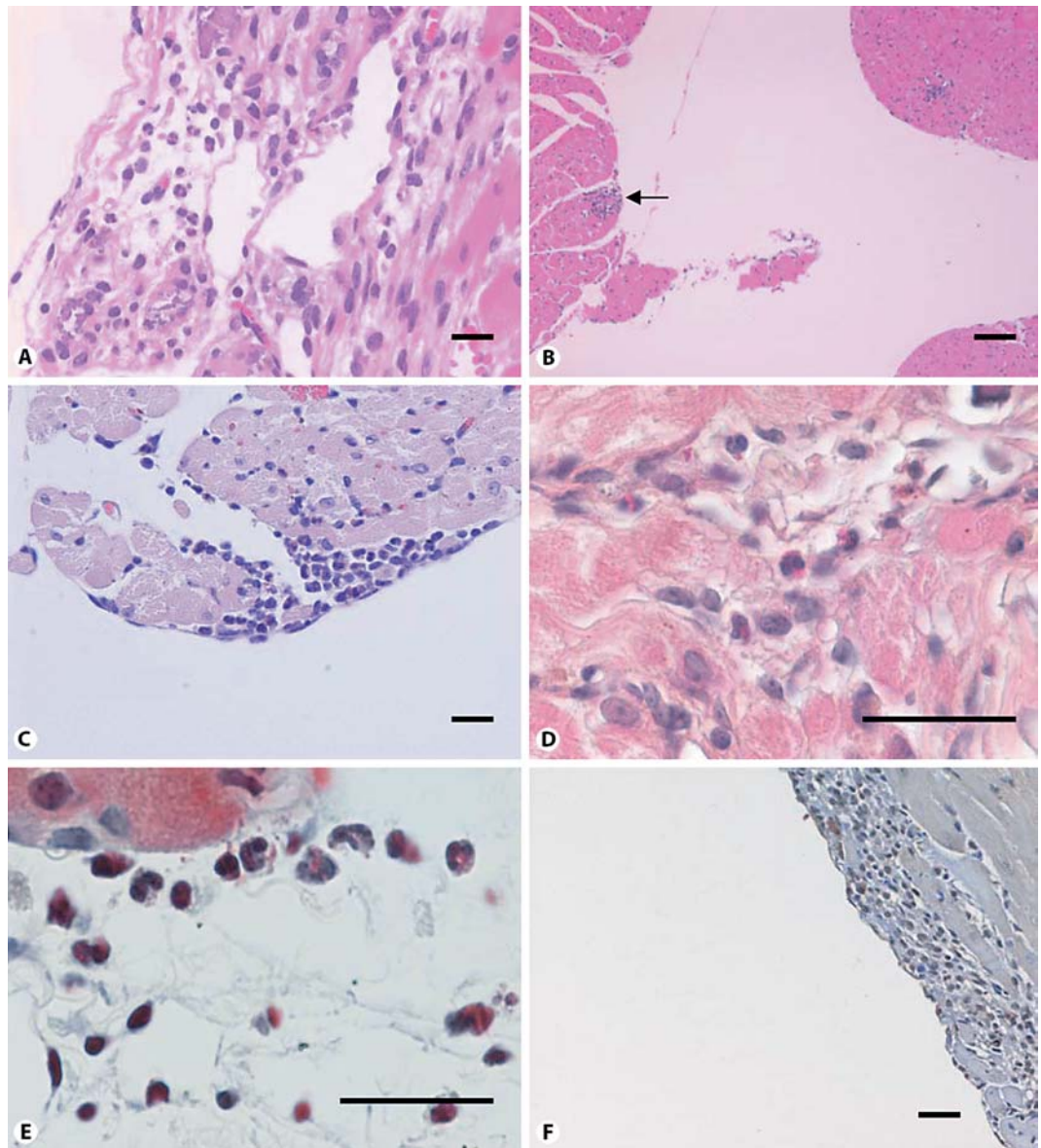


Fig. 2. Characterization of the type of myocarditis. Histological findings of eosinophilic myocarditis with endocardial involvement, and immunological relationships among CD4, IL-5, RANTES, CCR3, JAK3 and eotaxin expression are shown. **A** Eosinophil infiltration at the right ventricular site. **B** Endomyocardial involvement in inflammation on the left ventricular side (arrow). **C** Higher magnification showing endomyocardial infiltration of inflammatory cells on the left ventricular side. **D** Eosinophil infiltration in the myocardial cell layer. **E** A Luna stain showing the eosinophils and cytoplasmic granules more clearly. IL-5, RANTES, CCR3, JAK3 and eotaxin were usually detected in these lesions. **F** Expression of RANTES in pericardial inflammatory lesions. **G** IL-5 was observed in inflammatory white blood cells in endocardial lesions. **H** Expression of CCR3 in endocardial inflammatory lesions in CLSM analysis (arrow). **I** CCR3 was also detectable in pericardial sites (arrow). **J** Eotaxin was also detectable in pericardial sites (arrow) where CCR3 could be observed. **K** JAK3 expression was simultaneously detected in CCR3-positive lesions. **L** CD4+ T cells congregated in inflammatory white blood cells and damaged myocardium (arrow). Scale bars = 50 μm (**A**, **C-E**, **G-L**) and 100 μm (**B**, **F**).

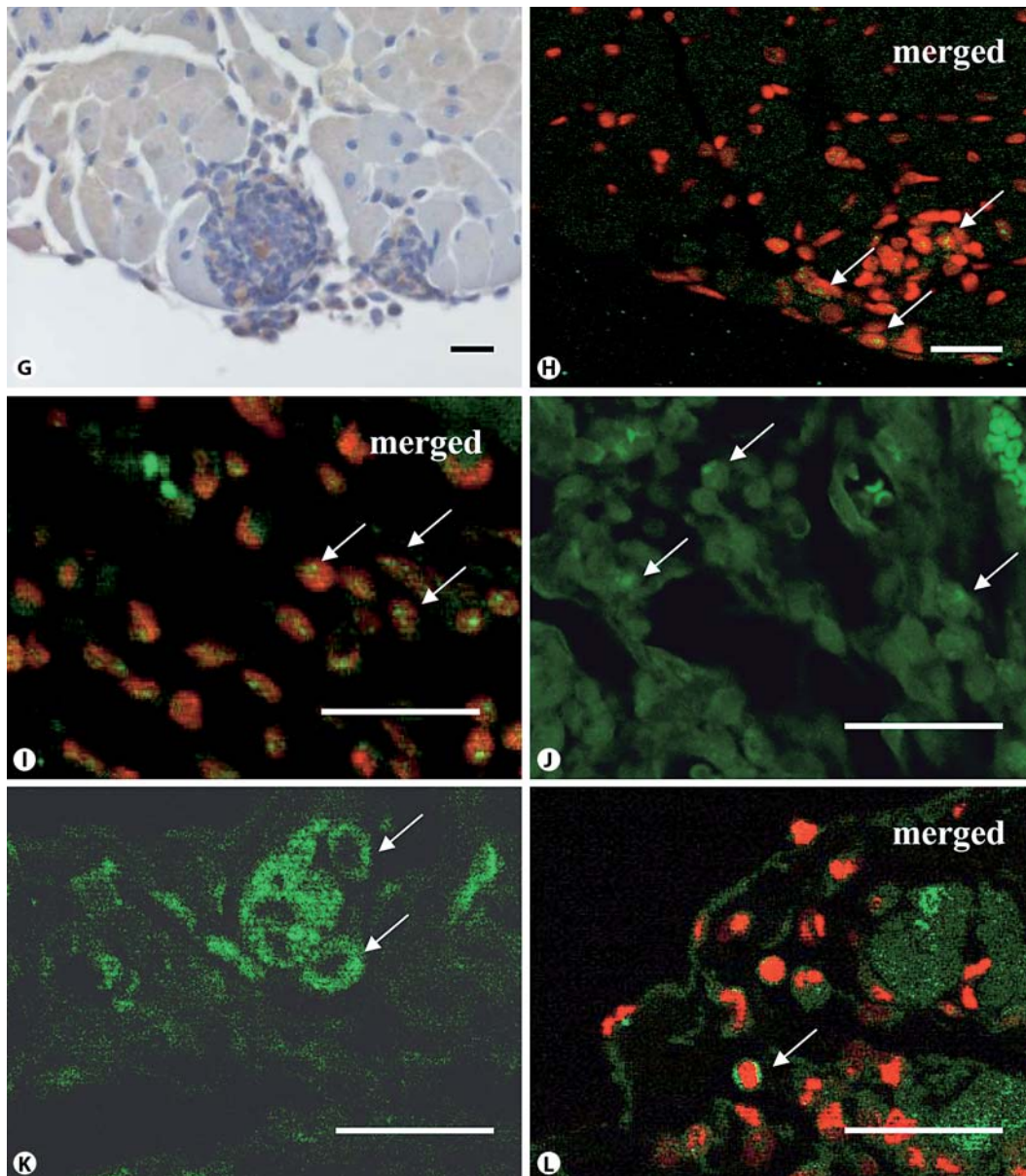


Table 1. Prevalence of histologically demonstrable myocarditis, calcium deposition, eosinophilic myocarditis and endocardial involvement in adoptive transfer animals and nontransfer controls

	Transfer cases (n = 20)	Nontransfer cases (n = 10)	Total (n = 30)
Myocarditis	85.0% (17/20)	70.0% (7/10)	80.0% (24/30)
Calcium deposition in the myocardium	65.0% (13/20)	80.0% (8/10)	70.0% (21/30)
Eosinophil infiltration (eosinophil/WBC >10.0%) ¹	64.7% (11/17)	42.9% (3/7)	58.3% (14/24)
Endocardial involvement	35.0% (7/20)	0.0% (0/10)	23.3% (7/30)

¹ Eosinophil to total WBC ratio (%) per 0.1 mm² of inflammatory lesion.

Table 2. Time courses of CD4+ T cell, IL-5, RANTES, CCR3 and eosinophil/WBC in heart lesions in adoptive transferred myocarditis

Parameters	Time courses			
	24 h (n = 5)	48 h (n = 5)	4 days (n = 5)	5 days (n = 5)
CD4+ T cell	22.8 ± 7.1	30.4 ± 2.2	14.2 ± 5.7	17.6 ± 12.0
IL-5	11.4 ± 1.9	14.8 ± 2.9	3.8 ± 1.3	5.2 ± 2.2
			* *	
RANTES	10.2 ± 3.6	17.0 ± 3.4	2.2 ± 1.6	12.0 ± 8.0
CCR3	20.8 ± 6.6	27.4 ± 5.0	7.8 ± 4.1	12.0 ± 9.8
Eosinophil/WBC	11.2 ± 1.2	13.6 ± 2.6	6.5 ± 2.2	8.1 ± 4.6

Figures are n/0.1 mm², except for eosinophil/WBC, which is given as % per 0.1 mm².

* p < 0.05.

Detection of IL-5, RANTES, CCR3 and Eotaxin in Spleen and Heart Tissue

IL-5, RANTES and CCR3 were detected in inflammatory heart lesions in which eosinophils and CD4+ T cells were found (fig. 2F–L). Many of these lesions were detected in the right ventricular pericardium and myocardium, but cell infiltrations in the left ventricular endocardium were found in 35% of adoptive transfer animals as mentioned above. In particular, expression of CCR3 in endocardial inflammatory lesions was observed in CLSM analysis, with simultaneous detection of IL-5 in lesions of the left ventricular endocardium (fig. 2G, H). Expression of eotaxin was also observed in subepicardial inflammatory lesions (fig. 2J). Hence, these molecules are considered to play pivotal roles in eosinophil infiltration in the pathogenesis of eosinophilic and hypersensitive myocarditis. Simultaneously, expressions of RANTES and CCR3 were observed in white pulp on the spleen cells of recipients (fig. 5A). Therefore, RANTES and CCR3 in spleen may also be primary initiators that support eosinophil infiltration into heart lesions.

Quantitative Immunohistochemistry of IL-5, RANTES, CCR3 and Eosinophils in Inflammatory Heart Lesions

Cell quantification was used to clarify the conclusions drawn from qualitative conventional and fluorescence immunohistochemistry. The eosinophil/WBC ratio after adoptive transfer plus OVA challenge remained high for 48 h and then decreased in the chronic post-48-hour phase (table 2). Time courses for the CD4+ T cell count

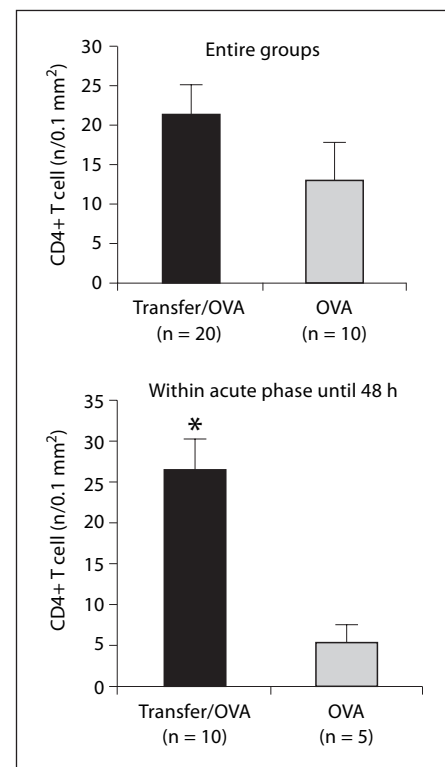


Fig. 3. Effects of adoptive transfer on the recipient heart compared with simple OVA-challenged controls, based on the number of CD4+ T cells in inflammatory lesions. More CD4+ T cells were observed in adoptive transfer mice than in controls, and the difference between the two groups was particularly significant (unpaired t test, * p < 0.05) in the acute phase.

and the IL-5, RANTES and CCR3 levels showed the same tendency as the eosinophil/WBC ratio (table 2). These data were consistent with eosinophil infiltration. IL-5, in particular, showed significant statistical differences after 48 h ($14.8 \pm 2.9/0.1 \text{ mm}^2$) and after 4 days ($3.8 \pm 1.3/0.1 \text{ mm}^2$; p < 0.05) and 5 days ($5.2 \pm 2.2/0.1 \text{ mm}^2$; p < 0.05) (table 2). Increased numbers of CD4+ T cells were observed in adoptive transfer mice compared with controls; this was especially true in the acute phase within 48 h, in which the difference in the CD4+ T cell count between the two groups was significant (fig. 3). Furthermore, in individual mice, the IL-5, RANTES and CCR3 levels in heart lesions were directly proportional to the eosinophil/WBC ratio. Equations relating the levels of these molecules to the eosinophil/WBC ratio are shown in figure 4A–C.

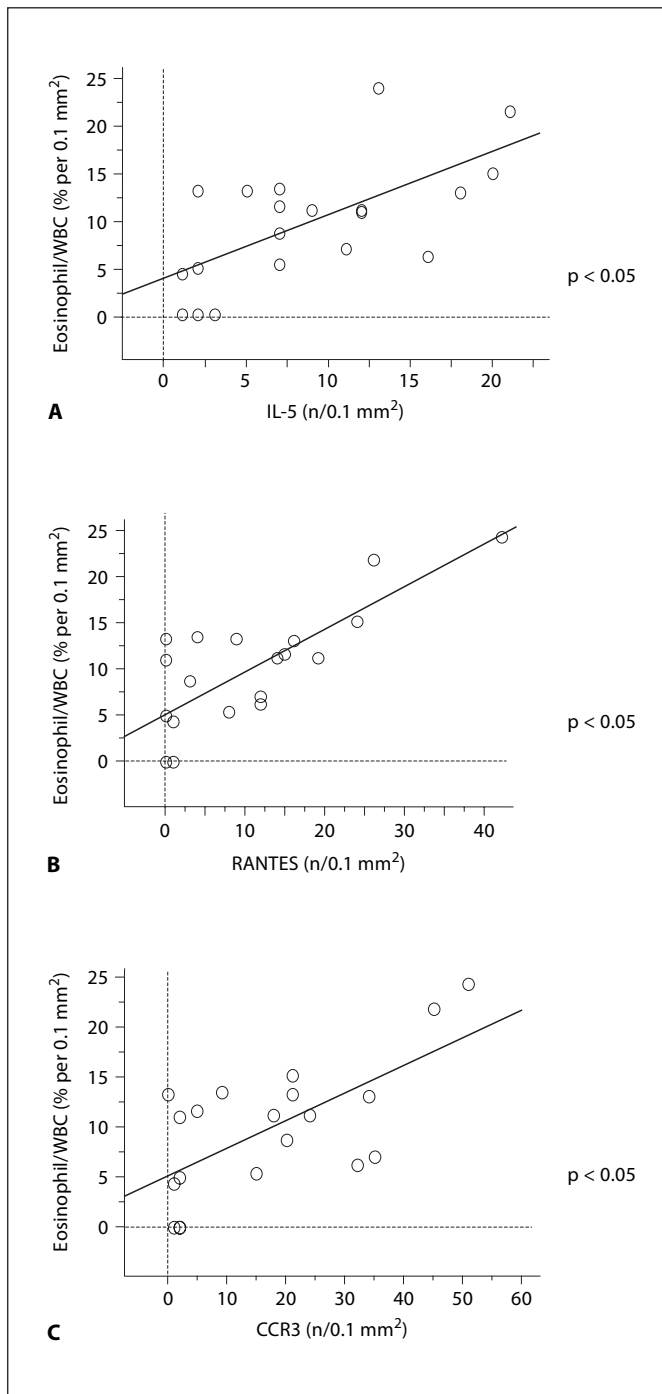


Fig. 4. Lesions exhibiting positive correlations between eosinophil/WBC and IL-5, RANTES and CCR3 levels, with the following regression equations. **A** IL-5: eosinophil/WBC = $3.975 + 0.667x$ ($n = 20$, $r = 0.644$, $p < 0.05$). **B** RANTES: eosinophil/WBC = $5.101 + 0.458x$ ($n = 20$, $r = 0.774$, $p < 0.05$). **C** CCR3: eosinophil/WBC = $5.146 + 0.276x$ ($n = 20$, $r = 0.663$, $p < 0.05$).

Detection of CCR3 mRNA in the Spleen and Heart

As described above, RANTES and CCR3 were detected in the spleen by fluorescence immunohistochemistry (fig. 5A), and CCR3 mRNA was detected in the recipient spleen. CCR3 was present in the early phase of myocarditis, whereas CCR3 mRNA was not found in the spleen of control mice (fig. 5A). Therefore, CCR3 mRNA in the reticular organ may be related to the pathogenesis of the cardiovascular lesions. In the heart, CCR3 expression was somewhat uniquely distributed in the qualitative and quantitative immunohistochemistry, as described above (fig. 2H, I, 4A–C). A 534-nucleotide CCR3 mRNA was expressed in the heart during the acute and chronic phases (fig. 5B), suggesting the importance of CCR3-mediated cell signaling in the reticular organ and the heart in this form of adoptive transferred eosinophilic myocarditis.

Molecular Detection of JAK3 and STAT6 mRNAs in the Spleen and Heart

Expression of the signal transduction factor JAK3 was examined to study its involvement in molecular mechanisms associated with heart disease. JAK3 was detected in the spleen and heart using fluorescence immunohistochemistry (fig. 2K, 5A), and lesions containing JAK3 were similar to those in which CD4-related cytokines, CCR3 and eosinophils were occasionally found. Molecular detection of JAK3 mRNA was performed in specimens of the spleen from adoptive transfer and control mice. These specimens corresponded to those chosen for detection of CCR3 mRNA. Interestingly, JAK3 mRNA was detected in the recipient spleen of controls, but at low fluorescence densities compared with adoptive transfer animals (fig. 5A). The JAK3 expression pattern differed from that of CCR3 mRNA (fig. 5A). JAK3 mRNA (412 nucleotides) was also detected in the heart during acute and chronic phases (fig. 5B, C), but JAK3 expression in the acute phase was higher than in the chronic phase. This JAK3 expression pattern was somewhat similar to the time course kinetics of CD4-related cytokines, CCR3 and eosinophil/WBC ratio in the heart lesions (table 2).

Expression of STAT6, a signal transduction factor related to JAK3, was simultaneously observed in heart tissue, and STAT6 mRNA (575 nucleotides) was detected in the heart during the acute and chronic phases, showing parallel expression to that of JAK3 (fig. 5C). This suggests that STAT6 plays a role in eosinophil recruitment into heart tissues. Overall, the pattern of JAK3 and STAT6 expression may reflect the severity of inflammatory heart disease.

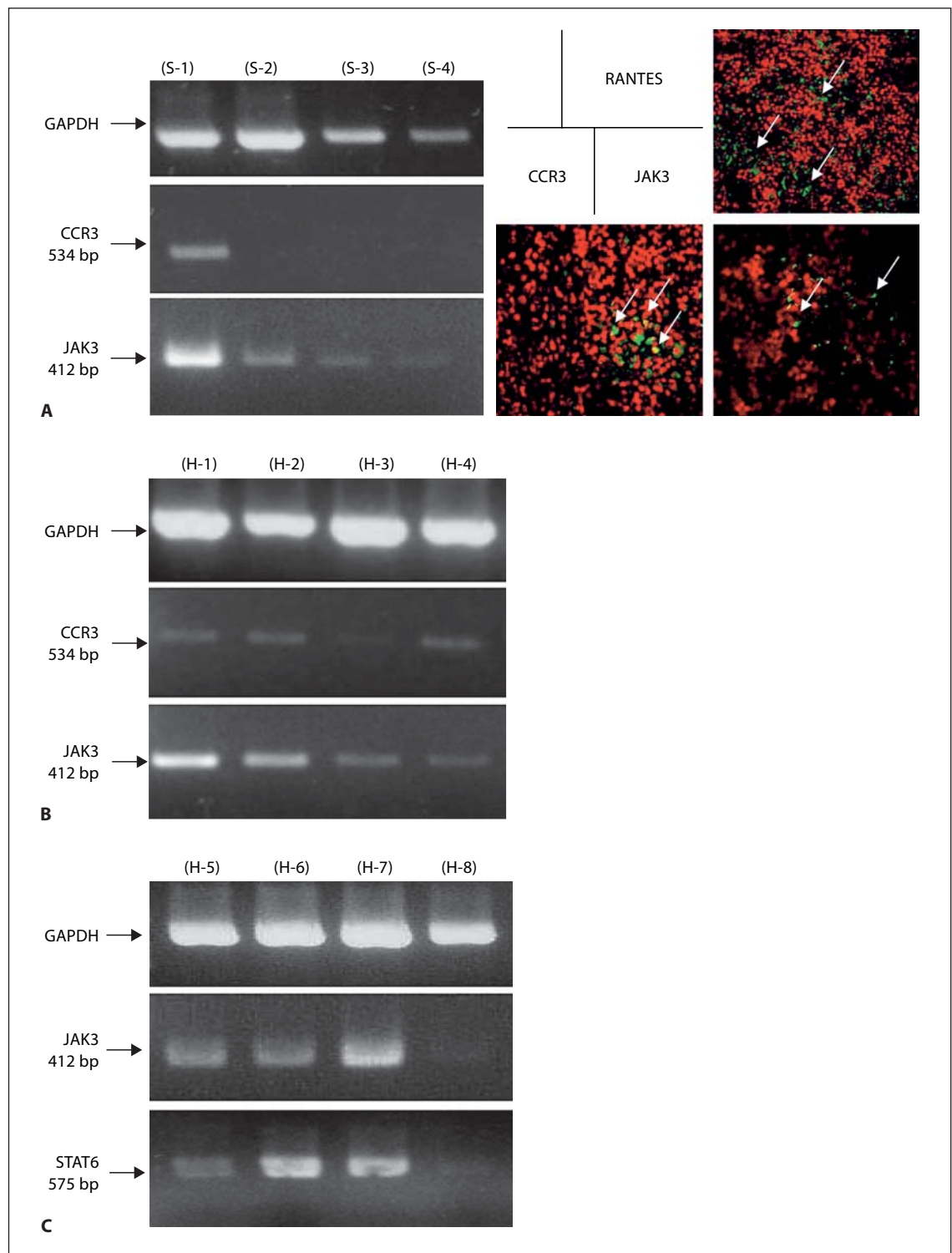


Fig. 5. Representative RT-PCR analyses for CCR3, JAK3 and STAT6 kinetics in the spleen and heart. **A** In the recipient spleen, CCR3 was detected in the acute phase of myocarditis only, while JAK3 was detected during acute and chronic phases. Histologically, RANTES, CCR3 and JAK3 were seen in the spleen by CLSM analysis. (S-1) = Acute phase; (S-2) = acute phase in control;

(S-3) = subacute phase in control; (S-4) = chronic phase in control. Arrows indicate the RANTES, CCR3 and JAK3, respectively. **B** In heart tissue, CCR3 and JAK3 were detected during acute and chronic phases, and therefore, the JAK-STAT signaling pathway may play a role in the pathogenesis of this myocarditis. (H-1) = Acute phase; (H-2) = acute phase in control; (H-3) = sub-

Discussion

This study was designed to investigate the relationships among IL-5, RANTES and CCR3 in the pathogenesis of eosinophil infiltration into heart lesions in an animal model of adoptive transferred myocarditis, with particular reference to JAK3 kinetics. The results showed that the levels of IL-5, RANTES, CCR3 and JAK3 were mutually correlated with eosinophil infiltration in this animal model.

As described above, we used the DBA/2 mouse strain, which is classified into the major histocompatibility complex H-2d haplotype group [10, 27]. Compared with other mouse strains, H-2d strains (including DBA/2) are known to be more susceptible to eosinophilic disorders with CD4 dominance under several pathogenic conditions [10, 11, 27]. DBA/2 mice develop a unique type of myocarditis with calcium deposition, which is located in the epicardium and subepicardium of the right ventricle [21–25]. This spontaneous myocarditis in DBA/2 mice has been studied as a function of various factors [21–24]. We proposed that eosinophils may be among the inflammatory cells in the heart lesion [25, 26] and that these cells often invaded necrotic cardiocytes. Therefore, we suggested that local eosinophils play a partial role in the pathogenesis of myocarditis in DBA/2 mice, based on morphological [26] and immunological [27] characteristics.

OVA challenge has been shown to be an appropriate and standard procedure for developing a local allergic reaction in several organs in animal models. In particular, this technique is often used in studies of airway responsiveness [11–13, 15] associated with eosinophil recruitment, and these studies indicate that the CD4⁺ T cell count has a direct role in antigen-induced eosinophil recruitment into the tissue [12]. Therefore, OVA may be an important agent for causing eosinophilic disorder in the experimental model through systemic and/or local Th2 responses [11–14, 18]. In order to examine the mechanism of OVA challenge in the cardiovascular disorder, we have previously studied the histological and serological kinetics of IL-5, RANTES and CD4⁺ T cell levels in eo-

sinophilic myocarditis after serial treatment using OVA sensitization and challenge [28, 29].

Eosinophil recruitment is observed under certain environmental conditions, and IL-5, CC chemokines and CCR3 are likely to become the first initiators of eosinophilic inflammatory lesions [28, 29]. In relation to this, the induction of immunological tolerance and type 1 allergy by adoptive transfer has recently been attempted in several organs [31, 32]. For example, adoptive transfer has been used as an immunological method for establishing animal models of inflammatory bowel disease (IBD), such that the role of donor CD4⁺ T cells in the pathogenesis of IBD could be explored [31]. Additionally, in the field of cardiovascular disorders, the influence of innate immunity on CD8⁺ T cells in heart tissue was investigated in an interesting study of the immune mechanisms in heart disease after adoptive transfer [32]. The responsible T cell subsets differed between IBD and allograft myocarditis; however, we speculate that some type of innate immunity must be evoked, and eosinophil and/or neutrophil infiltration may occur in the organs after adoptive T cell infusion. Therefore, adoptive transfer may be appropriate for establishing a hypersensitive status *in vivo* through an immunomodulation mechanism.

We first attempted to establish hypersensitive conditions by means of OVA pretreatment of the donor since cells from normal donor mice are unlikely to result in an immunomodulated environment in the recipient. The donor splenocytes showed CD4 dominance in FACS analysis (fig. 1B), and these CD4-dominant cells from immunized mice may be able to generate an immunologically tolerant state in the recipient. Eosinophilic myocarditis (fig. 2A–L) may occur with high prevalence, and endocardial involvement may also be easily seen in cell transfer animals (table 1). Calcium deposition in heart tissue occurred more frequently in nontransfer cases (table 1), and this degenerative change in heart tissue has usually been considered as the postinflammatory chronic stage in DBA/2 mice [21, 22, 26]. In contrast, CD4⁺ T cell counts in inflammatory heart lesions in adoptive transfer animals were significantly higher than in controls in the acute stage of myocarditis (fig. 3). These findings may indicate that adoptive transfer is especially suitable for examination of pathological changes in the early stage of heart disease in mice.

The underlying mechanism of the CD4-dependent pathogenesis is still unclear; however, we speculate that RANTES and/or CCR3 may have a role in activation of CD4⁺ T cells. In fact, Forssmann et al. [33] described the significance of the relationship between CC chemokines

acute phase; (H-4) = chronic phase. **C** In heart tissue, JAK3 and STAT6 were generally detected during acute and chronic phases, but in 1 animal in the chronic phase, JAK3 and STAT6 were not detectable. (H-5) = Acute phase; (H-6) = subacute phase; (H-7) = subacute phase; (H-8) = chronic phase.

and CCR3 for the induction of local eosinophil infiltration in monkey skin and proposed a specific role for eotaxin 2 in selective linkage to CCR3, while Moser et al. [34] proposed that RANTES and CCR3 are involved in innate and adaptive immunity in functional subfamilies. Our results suggest that CD4⁺ T cells, IL-5, RANTES, CCR3 and eotaxin played fundamental roles in eosinophil recruitment into inflammatory lesion of the heart (fig. 2F–L). The quantitative immunohistochemical studies support these histological findings (fig. 4A–C). RANTES and CCR3 were also recognized in the spleen (fig. 5A), supporting the hypothesis of a CCR3-dependent mechanism for eosinophil recruitment in our adoptive transfer model.

Moreover, detection of CCR3 and JAK3 mRNA confirms the above histological findings (fig. 5A–C). In the heart, CCR3 and JAK3 mRNAs were observed in the adoptive transfer mice (fig. 5B, C). However, CCR3 mRNA was not detected in the spleen of controls, whereas JAK3 mRNA was found in the control spleen (fig. 5A). Hence, while there are some CCR3 mRNA-negative cases in the spleen, the JAK-STAT signaling pathway may play a partial role in eosinophil recruitment via certain cytokine/chemokine receptors.

An immunotherapy study of cytotoxic T-cell-resistant tumors by Th2 has provided interesting information about signaling factors [35]. In this study, clearance of lung metastasis by Th2 cells was found to be dependent on the eosinophil chemokine, eotaxin and partially on STAT6 in degranulating eosinophils within the tumors [35]. STAT6 plays a partial role in the JAK-STAT signaling pathway and works as an activator for several physiological phenomena under the control of JAK. In turn, JAK3 is associated with signaling via the IL-2R γ chain, a component of multiple cytokine receptors [36]. If certain signaling factors act positively in the pathogenesis of adoptive transferred myocarditis, transduction from JAK3 to STAT families may become one of the significant pathways for eosinophil recruitment. In fact, JAK3 and STAT6 mRNAs show parallel expression in heart tissue in our model (fig. 5C), supporting the hypothesis that JAK3 to STAT6 signaling plays a role in the pathogenesis of adoptive transferred eosinophilic myocarditis in our model. Considering these mechanisms, the importance of precise comprehension of the signaling pathways in inflamed heart tissue is very important, especially for the application of regeneration therapy for eosinophilic and viral myocarditis, or dilated cardiomyopathy.

In summary, the current study shows the importance of CCR3 and JAK-STAT signaling in the pathogenesis of

an animal model of adoptive transferred eosinophilic myocarditis. Recipient mice were made susceptible to allergic reaction by spleen cell transfusion, allowing signaling factors including JAK3 to be delivered and communicate with STAT family proteins. Such systems may become initiators for activation of CD4-related cytokines and the CCR3/eotaxin cascades, which enhance RANTES production and promote IL-5 secretion. These mechanisms assist in the recruitment of granulocytes and eosinophils in the inflamed area with myocardiocyte injury. However, the signaling network of most importance in the relationship between induction of CCR3 and eosinophil infiltration remains unknown, and an understanding of this specific mechanism will require further studies. Overall, these findings may be of particular importance, since we anticipate that regeneration therapy in the field of intractable human cardiovascular disorders will become increasingly important in the near future.

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