Chemokines are a group of small, structurally related proteins with four conserved cysteine forming two essential disulphide bonds. Based on NH₂-terminal cysteine motifs, chemokines are classified into four major subfamilies: CC, CXC, C, and CX3C. Although increasing evidence has demonstrated multiple functions of chemokines, including lymphocyte differentiation and angiogenesis, the main function of chemokines is lymphocyte traffic control. Chemokine gradients mediate chemotaxis by providing directional cues for lymphocyte motility. Chemokines also play a key role in lymphocyte/endothelial-cell recognition. Chemokines presented on endothelial cells trigger rapid activation of integrins, resulting in arrest and firm adhesion of lymphocytes.

Based on functional characteristics, chemokines are classified into homeostatic and inflammatory chemokines. Homeostatic chemokines are produced constitutively and are involved in the proper development and homeostasis of the immune system. Inflammatory chemokines are upregulated under inflammatory conditions and recruit effector cells, including monocytes, granulocytes, and effector T cells, through integrin activation.

SR-PSOX/CXCL16 is a CXC chemokine and exists in a transmembrane form. SR-PSOX/CXCL16 is expressed in dendritic cells in lymphoid organs and attracted activated T cells in vitro, suggesting that it may be involved in T cell-antigen presenting cell interactions in initiation of immune responses. Moreover, upregulation of SR-PSOX/CXCL16 by inflammatory stimuli indicated that SR-PSOX/CXCL16 may be involved in efficient recruitment of effector T cells into inflammatory lesions. Although the expression of SR-PSOX/CXCL16 has been described in adult lymphoid tissues, its distribution pattern outside the immune system is unknown. Therefore, in this study, we first examined the
spatial and temporal expression pattern of SR-PSOX/CXCL16 during murine development. Interestingly, we found that SR-PSOX/CXCL16 was specifically expressed in endothelial cells of the cardiac valves. Histological examination of human cardiac valves and in vitro studies demonstrated that the chemokine might play an important role in CD8+ T cell recruitment and interferon-γ (IFN-γ) production during inflammatory valvular heart disease.

**Methods**

**Mouse Tissue Samples, In Situ Hybridization, and Immunohistochemistry**

 Noon on the day of mating was referred to as E0.5 (day 0.5 postcoitus). Paraffin sections (5 μm) of embryonic and adult tissues from C57BL/6 mice were fixed with 4% paraformaldehyde, treated with 0.1 mol/L HCl, acetylated in 0.23% acetic anhydride in 0.1 mol/L triethanolamine, and hybridized with 3'-S-CTP--labeled riboprobe at 55°C overnight in 50% formaldehyde, 0.3 mol/L sodium chloride, 20 mmol/L Tris-HCl, 5 mmol/L EDTA, 10 mmol/L sodium pyrophosphate, 1XDenhardt, 10% dextan sulfate, and 0.5 mg/mL yeast RNA. After hybridization, they were treated with RNase-A, washed and dehydrated through graded ethanol, and emulsion autoradiography was performed. The SR-PSOX/CXCL16 riboprobe comprised a full-length cDNA. Hybridization with a sense probe did not give any signals.

For immunohistochemistry, paraffin sections were deparaffinized in xylene and rehydrated in graded alcohol and incubated with anti-mouse CXCL16 antibody (R&D Systems, Minneapolis, MN) or mouse anti-platelet--endothelial cell adhesion molecule-1 (PECAM-1) antibody (Pharmingen, San Diego, CA) overnight at 4°C. After washing, sections were incubated with biotinylated goat anti-mouse IgG and horseradish peroxidase-conjugated streptavidin (Vector Laboratories, Burlingame, CA), and staining was detected with 3,3′-diaminobenzidine (DAB).

**Human Tissue Samples and Immunohistochemistry**

Paraffin sections (5 μm) were prepared from human valve specimens from 14 patients who underwent valve replacement. The diagnosis of rheumatic valve disease was based on a history of rheumatic fever and characteristic echocardiographic features. All tissue samples were fixed in 10% neutral formalin and embedded in paraffin wax. Sections were deparaffinized in xylene, rehydrated in graded alcohol, and autoclaved for 10 minutes at 121°C in 10 nM EDTA (pH 8.0). The tissue sections were then incubated with anti-SR-PSOX/CXCL16, anti-CDS, anti-CD4, anti-CD68, anti-CD29, or anti-VCAM-1 antibody overnight at 4°C. Anti-human SR-PSOX/CXCL16 monoclonal antibody was generated by immunizing mice with recombinant mouse SR-PSOX/CXCL16 cDNA. After selection with 800 μg/mL of G418 for 2 weeks, drug-resistant clones were isolated, and clones expressing SR-PSOX/CXCL16 were identified by Western blotting. Activated CD8+ T cells at a concentration of 1 × 10^6/mL were co-cultured with control CHO cells or CHO cells stably expressing SR-PSOX/CXCL16 in 0.1% BSA-RPMI for 24 hours in 96-well plates. After incubation, 100 μL of supernatants were collected and assayed for IFN-γ production using an ELISA kit (Bio Source International, Camarillo, CA) according to the manufacturer’s protocol. The detectable range of the ELISA kit was 1 to 500 pg/mL and the optical density of individual wells was determined at 450 nm using a microplate reader. Anti-mouse SR-PSOX/CXCL16 monoclonal antibody was generated by immunizing mice with recombinant mouse SR-PSOX/CXCL16 protein. This antibody gave a single band on Western blotting (R.Y. and M.T., unpublished data) and inhibited chemotactic activity of human SR-PSOX/CXCL16 on human CXCR6-expressing cells.15 Monoclonal anti-human CD8+ and CD4+ antibodies were purchased from Medical & Biological Laboratories (Nagoya, Japan), and monoclonal anti-human CD29, CD34, and VCAM-1 antibodies were from Novocastra Laboratories (Newcastle, UK). After washing, sections were incubated with biotinylated goat anti-mouse IgG and horseradish peroxidase-conjugated streptavidin, and staining was detected with DAB.

**Preparation of Cell Suspension From Spleen**

Lymphocytes from spleen were prepared as previously described.16,17 CD8+ T cells were prepared from spleen of C57BL/6 mice by positive selection using MACS immunobeads (Miltenyi Biotec, Bergisch Gladbach, Germany). For in vitro activation, CD8+ T cells were incubated with anti-CD28 (10 μg/mL; Pharmingen, San Diego, CA) in RPMI1640 medium supplemented with 10% fetal calf serum, 24 mmol/L NaHCO3, 50 μmol/L 2-mercaptoethanol, and IL-2 (4 ng/mL) in 96-well plates coated with anti-CD3 (1 μg/mL; Becton Dickinson, San Jose, CA) for 5 days. The activated T cells were then rested in the aforementioned medium supplemented with 2 ng/mL of IL-2 for 4 days. Recombinant human IL-2 was kindly provided by Takeda Pharmaceutical Company (Osaka, Japan).

**Cell Adhesion Assay**

Adhesion assay was performed as described.18–20 Briefly, polystyrene 96-well microtiter plates were coated with soluble VCAM-1 (2.5 μg/mL) and blocked by heat-denatured BSA. Activated CD8+ T cells suspended at a concentration of 1.5 × 10^6/mL in 0.1% BSA-RPMI were incubated for the indicated times in a CO2 incubator at 37°C in the presence or absence of soluble SR-PSOX/CXCL16 (R&D Systems, Minneapolis, MN). After incubation, nonadherent cells were removed by centrifugation (topside down) at 48g for 5 minutes. Attached cells were fixed with 5% glutaraldehyde, stained with 0.1% crystal violet in 200 mM MES (pH 6.0), and solubilized with 10% acetic acid. The absorbance of each well at 595 nm was measured using a multiscan enzyme-linked immunosorbent assay reader (TECAN, Männedorf, Switzerland). Anti-mouse α4-integrin antibody was purchased from Pharmingen.

**Cell Stimulation and Measurement of IFN-γ Production**

CHO cell lines stably expressing mouse SR-PSOX/CXCL16 were established by transfection with a plasmid (pME18S) expressing full-length mouse SR-PSOX/CXCL16 cDNA. After selection with 800 μg/mL of G418 for 2 weeks, drug-resistant clones were isolated, and clones expressing SR-PSOX/CXCL16 were identified by Western blotting. Activated CD8+ T cells at a concentration of 1 × 10^6/mL were co-cultured with control CHO cells or CHO cells stably expressing SR-PSOX/CXCL16 in 0.1% BSA-RPMI for 24 hours in 96-well plates. After incubation, 100 μL of supernatants were collected and assayed for IFN-γ production using an ELISA kit (Bio Source International, Camarillo, CA) according to the manufacturer’s protocol. The measurable range of the ELISA kit was 1 to 500 pg/mL and the optical density of individual wells was determined at 450 nm using a microplate reader. Anti-mouse SR-PSOX/CXCL16 monoclonal antibody was generated by immunizing mice with recombinant mouse SR-PSOX/CXCL16 protein. This antibody gave a single band on Western blotting (R.Y. and M.T., unpublished data) and blocked chemotaxis of mouse CXCR6-expressing cells to mouse SR-PSOX/CXCL16 (T.S. and S.Y., unpublished data).

**Statistical Analysis**

Data were analyzed by one-factor ANOVA followed by Fisher PLSD as a post hoc test. P<0.01 was considered to be statistically significant.

**Results**

**SR-PSOX/CXCL16 Is Expressed in Cushion and Valve Endothelium During Development**

Initial expression of SR-PSOX/CXCL16 was detected in the heart at E11.5. SR-PSOX/CXCL16 mRNA was exclusively expressed in endothelial cells covering endocardial cushions (Figure 1A through 1D). Mesenchymal cells transformed from these cushion endothelial cells lost SR-PSOX/CXCL16 expression (Figure 1A through 1D). From mid-gestation to adult, SR-PSOX/CXCL16 expression was confined to endothelial cells of the cardiac valves at mRNA (Figure 1E through 1H) and protein (Figure 1I) levels. Expression of SR-PSOX/CXCL16 protein in valvular endothelium was confirmed by positive PECAM-1 staining (Figure 1J).
Upregulation of SR-PSOX/CXCL16 in Inflammatory Valvular Disease

The specific expression of SR-PSOX/CXCL16 in valvular endothelium suggested that this chemokine may play a role in the pathogenesis of valvular heart disease. We thus examined expression of SR-PSOX/CXCL16 protein in human cardiac valves from patients who underwent valve replacement (Table). In noninflammatory valvular disease (prolapse of mitral valve), SR-PSOX/CXCL16 protein was undetectable (Figure 2A). In contrast, dramatic upregulation of SR-PSOX/CXCL16 protein was observed in valvular endothelium in patients with infective endocarditis (Figure 2C, 2F, and 2H). SR-PSOX/CXCL16 protein expression in valvular endothelial cells was confirmed by positive CD34 staining (Figure 2D) and control IgG was substituted for the primary antibody in negative control experiments (Figure 2E). Macrophages (open arrowheads in Figure 2F), as revealed by positive CD68 staining (Figure 2G), also expressed SR-PSOX/CXCL16 protein. Interestingly, endothelial cells of neocapillaries within cardiac valves were also positive for SR-PSOX/CXCL16 (Figure 2J). In patients with rheumatic and atherosclerotic valvular disease, SR-PSOX/CXCL16 protein expression was observed in neocapillary endothelial cells within cardiac valves (Figure 2C and 2E), whereas no expression was detected in valvular endothelium (Figure 3A).

Because it was previously reported that SR-PSOX/CXCL16 attracted activated CD8+ T lymphocytes in vitro, we next examined whether CD8+ T cells existed in these lesions. As expected, we detected abundant CD8+ T cells underneath valvular endothelial cells in patients with infective endocarditis (Figure 2I). CD8+ T cells were also detected around neocapillaries within cardiac valves in patients with endocarditis (Figure 2L). Although SR-PSOX/CXCL16 was also shown to attract activated CD4+ T cells in vitro,12 we did not observe any CD4+ T cells in cardiac valves in patients with inflammatory valvular heart disease (Figure 2L).

SR-PSOX/CXCL16-Mediated Adhesion of Activated CD8+ T Lymphocytes to VCAM-1

The close association of SR-PSOX/CXCL16 expression with CD8+ T cells suggested that SR-PSOX/CXCL16 may be involved in CD8+ T cell recruitment. To find out if SR-PSOX/CXCL16 mediates adhesion of CD8+ T lymphocytes, we performed adhesion assays. We examined adhesion of activated CD8+ T cells to VCAM-1 by functional blocking of the 4-subunit of integrins abolished the effect of SR-PSOX/CXCL16 (Figure 4B), indicating that the adhesion mediated by SR-PSOX/CXCL16 was VLA-4-dependent.

Figure 1. Expression of SR-PSOX/CXCL16 in the heart is confined to valvular endothelium. In situ hybridization and immunohistochemistry of murine samples. In situ hybridization was performed on paraffin sections of mouse embryonic and adult tissues using 35S-labeled mouse SR-PSOX/CXCL16 riboprobe. A–D, Sagittal sections of day 11.5 postcoitus (A, B) and day 13.5 postcoitus (C, D) embryos. SR-PSOX/CXCL16 was expressed in endothelial cells covering the endocardial cushion (EC). RV indicates right ventricle; LV, left ventricle. E, Sagittal section of a day 13.5 postcoitus embryo, showing SR-PSOX/CXCL16 expression in the forming mitral valve (MV). AS indicates atrial septum. F, Sagittal section of a neonate, showing hybridization signals in the aortic valve (AoV). G and H, Sagittal section of an adult heart. SR-PSOX/CXCL16 was expressed in valvular endothelium. LA indicates left atrium. I and J, Immuno-histochemical staining of mitral valve. SR-PSOX/CXCL16 was expressed in valvular endothelium (l), as confirmed by positive PECAM-1 staining in a serial section (j). Scales bars = 200 μm (C, F, G), 100 μm (A, D, H), 50 μm (B, E), and 10 μm (I, J).
We thus examined expression of VCAM-1 and VLA-4 in cardiac valves in patients with infective endocarditis. Interestingly, neocapillary endothelial cells expressing SR-PSOX/CXCL16 were positive for VCAM-1 (Figure 2N), and CD8+ T cells within neocapillary lumens (Figure 2O) expressed VLA-4 (Figure 2P).

**Stimulation of IFN-γ Production in Activated CD8+ T Lymphocytes by SR-PSOX/CXCL16**

To assess the involvement of SR-PSOX/CXCL16 in inflammatory processes, we examined whether SR-PSOX/CXCL16 could activate IFN-γ production by CD8+ T cells. As shown in Figure 4C, co-culture with CHO cells stably expressing SR-PSOX/CXCL16 significantly enhanced IFN-γ production by activated CD8+ T cells. IFN-γ production by naive CD8+ T cells was undetectable either in the presence or in the absence of SR-PSOX/CXCL16 (data not shown). The enhancement of IFN-γ production by SR-PSOX/CXCL16 in activated CD8+ T cells was completely blocked by anti-SR-PSOX/CXCL16 antibody (Figure 4C). Either control CHO cells or CHO cells with stable expression of SR-PSOX/CXCL16 did not produce IFN-γ (data not shown).

**Discussion**

SR-PSOX/CXCL16 was detected in the cushion endothelium at a time coincident with endothelial–mesenchymal transformation. The endothelium covering the endocardial cushion is considered to be a specific population of endothelial cells that has acquired competence to receive transformation signals from the myocardium.21 To our knowledge, SR-PSOX/CXCL16 is the first molecular marker specific for the cushion endothelium, demonstrating at a molecular level that endothelial cells covering the cushion are distinct from other endocardial cells. Furthermore, SR-PSOX/CXCL16 was later expressed in endothelial cells of the cardiac valves, indicating that valve endothelium is derived from endothelial cells covering endocardial cushions at earlier stages.

In human cardiac valves, SR-PSOX/CXCL16 expression was undetectable in the absence of valvular inflammation. The difference in the expression pattern between human and mice may be caused by species difference. Interestingly, SR-PSOX/CXCL16 expression was upregulated during inflammation and CD8+ T cells were closely associated with SR-PSOX/CXCL16-positive endothelial cells. Adhesion assays demonstrated that SR-PSOX/CXCL16 mediated adhesion of activated CD8+ T lymphocytes to VCAM-1, suggesting that SR-PSOX/CXCL16 may, at least in part, play a role in CD8+ T cell recruitment in inflammatory valvular disease. However, it is possible that other inflammatory chemokines may be also involved in this process. Lymphocyte firm adhesion generally requires activation of integrins by chemokines.7,22 The effect of SR-PSOX/CXCL16 on lymphocyte recruitment may be associated with the expression of chemokine receptors.

## Table: Expression of CXCL16 in Valvular and Neocapillary Endothelium

<table>
<thead>
<tr>
<th>Case</th>
<th>Valve</th>
<th>Sex/Age</th>
<th>Diagnosis</th>
<th>CXCL16 (Valvar Surface Endothelium)</th>
<th>CXCL16 (Capillary Endothelium)</th>
<th>CD8+ T cell</th>
<th>CRP (×10^9)</th>
<th>WBC (×10^9)</th>
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<tbody>
<tr>
<td>1</td>
<td>Mitral</td>
<td>M/61</td>
<td>IE</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<td>14.5</td>
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<td>M/48</td>
<td>IE</td>
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<td>+</td>
<td>+</td>
<td>2.9</td>
<td>7.3</td>
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<tr>
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<td>M/74</td>
<td>IE</td>
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<td>+</td>
<td>1.3</td>
<td>7.2</td>
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<td>10.5</td>
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<tr>
<td>6</td>
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<td>M/50</td>
<td>IE</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>8.8</td>
<td>7.8</td>
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<tr>
<td>7</td>
<td>Mitral</td>
<td>F/73</td>
<td>MR (MVP)</td>
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<td>–</td>
<td>–</td>
<td>0</td>
<td>4.2</td>
</tr>
<tr>
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<td>M/46</td>
<td>MR (MVP)</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>0.1</td>
<td>6.9</td>
</tr>
<tr>
<td>9</td>
<td>Mitral</td>
<td>F/73</td>
<td>MSR (rheumatic)</td>
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<td>+</td>
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<td>4.7</td>
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<tr>
<td>10</td>
<td>Mitral</td>
<td>F/64</td>
<td>AR+MSR (rheumatic)</td>
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<td>+</td>
<td>+</td>
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<td>4.6</td>
</tr>
<tr>
<td>11</td>
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<td>+</td>
<td>0.1</td>
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<tr>
<td>12</td>
<td>Aortic</td>
<td>F/74</td>
<td>AS (atherosclerotic)</td>
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<td>+</td>
<td>+</td>
<td>3.9</td>
<td></td>
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<td>M/59</td>
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<td>–</td>
<td>+</td>
<td>+</td>
<td>0</td>
<td>6.8</td>
</tr>
</tbody>
</table>

IE indicates infective endocarditis; MR, mitral regurgitation; AS, aortic stenosis; AR, aortic regurgitation; MSR, mitral stenosis and regurgitation; MVP, mitral valve prolapse; WBC, white blood cells.
remodeling and destruction of inflamed valves. Moreover, IFN-γ increased expression of VCAM-1 on endothelial cells. The upregulation of VCAM-1 by IFN-γ may further enhance CD8⁺ T cell trafficking mediated by SR-PSOX/CXCL16.

Expression of SR-PSOX/CXCL16 in valvular endothelium was only observed in patients with infective endocarditis. What is the pathological significance for the different expression pattern between patients with infective endocarditis and rheumatic/atherosclerotic valvular disease? First, expression of SR-PSOX/CXCL16 in valvular endothelium as well as in neocapillary endothelium may further enhance recruitment of CD8⁺ T cells into inflamed tissues. Second, expression of SR-PSOX/CXCL16 on valvular surface may mediate bacterial adhesion to valvular tissues. Recently, it was reported that SR-PSOX/CXCL16 mediated adhesion and phagocytosis of bacteria by antigen presenting cells. SR-PSOX/CXCL16 may be involved in the attachment of bacteria to valvular endothelial cells, which is the first step in the development of infective endocarditis.

Does inflammation still persist in the chronic phase of rheumatic valve disease? It was recently reported that plasma high-sensitivity C-reactive protein (hsCRP) level was significantly higher in patients with chronic rheumatic valve disease than in patients with prosthetic valves or in healthy subjects. Moreover, patients with multiple valve disease had significantly higher plasma hsCRP levels than those with single valve involvement. These results suggested the persistence of inflammation in rheumatic valves at chronic stages. Expression of SR-PSOX/CXCL16 in neocapillary endothelial cells and invasion of CD8⁺ T cells in rheumatic valves may support their findings.

In summary, cardiac expression of SR-PSOX/CXCL16 was restricted to valvular endothelium and the expression in valvular endothelium was dramatically upregulated during infective endocarditis. Moreover, SR-PSOX/CXCL16 expression was detected in neocapillaries within cardiac valves in chronic inflammatory valvular disease. The result of the present study suggested that SR-PSOX/CXCL16 might be involved in the recruitment of activated CD8⁺ T lymphocytes and enhancement of IFN-γ production during inflammatory valvular heart disease.
We thank Noboru Ashida for his help in cell adhesion assay and Tomohiro Watanabe for his help in isolation of CD8⁺ T cells. This work was supported by research grants from the MEXT of Japan (grants 13045019, 13832003, 15590738, 12CE2006, and 13307034) and by a grant provided by the Ichiro Kanehara Foundation.

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