IMMUNOPATHOLOGY AND INFECTIOUS DISEASES

Type II Natural Killer T Cells that Recognize Sterol Carrier Protein 2 Are Implicated in Vascular Inflammation in the Rat Model of Systemic Connective Tissue Diseases

Yusuke Nishioka,* Madoka Yamaguchi,* Ai Kawakami,* Maya Munehiro,† Sakiko Masuda,† Utano Tomaru,‡ and Akihiro Ishizu‡

From the Graduate School of Health Sciences,* the Undergraduate School of Health Sciences,† and the Faculty of Health Sciences,‡ Hokkaido University, Sapporo; and the Department of Pathology,§ Hokkaido University Graduate School of Medicine, Sapporo, Japan

Accepted for publication September 13, 2016.
Address correspondence to Akihiro Ishizu, M.D., Ph.D., Faculty of Health Sciences, Hokkaido University, Kita-12, Nishi-5, Kita-ku, Sapporo 0600812, Japan. E-mail: aishizu@med.hokudai.ac.jp.

We previously generated a rat model that developed systemic connective tissue diseases, including synovitis, myositis, and small-vessel vasculitis (SVV). In this model, a predominant infiltration of mononuclear cells was observed in the synovial and cardiac tissues and skeletal muscles and around small vessels in the systemic organs. An autoimmune mechanism could be involved in the pathogenesis because several autoantibodies, such as anti-nuclear and anti-DNA antibodies, were detected in the serum. The presence of hyperreactivity of peripheral T cells, disordered differentiation of T cells in the thymus, and functional impairment of regulatory T cells had been found in the model. In addition, we recently established a T-cell clone reactive with autologous vascular endothelial cells from the rat model and designated the clone as VASC-1. VASC-1 was determined to be a clone of natural killer T (NKT) cells because this clone recognized a certain antigen presented by CD1d.

NKT cells belong to a unique subset of T cells that share surface markers and function with natural killer (NK) cells and play important roles in the immune response. The hallmark of NKT cells is their capacity to recognize antigens presented by CD1d.7 The development of SVV was significantly accelerated in the rat model immunized with rSCP2518-532.8 Furthermore, the rat model with CD1d-knockout VASC-1 clones did not develop SVV, indicating that CD1d-dependent recognition of antigen is essential for the development of SVV.

Supported by grant-in-aid 26293082 from the Ministry of Education, Culture, Sports, Science and Technology of Japan (A.I.), a grant for Research on Rare and Intractable Vasculitis from the Ministry of Health, Labor and Welfare of Japan (A.I.), and grant 15ek0109121 from the Japan Agency for Medical Research and Development (A.I.).

Y.N., M.Y., and A.K. contributed equally to this work.

Disclosures: None declared.

Copyright © 2017 American Society for Investigative Pathology. Published by Elsevier Inc.
This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).
The NKT cell clone, VASC-1, established from the rat model of systemic connective tissue diseases, exhibited a TCR use other than the type I invariant TCR \(\alpha\)-chain and did not bind to \(\alpha\)-GalCer—loaded CD1d; therefore, it was regarded as a type II NKT cell clone. Because VASC-1 produced proinflammatory cytokines after an interaction with autologous vascular endothelial cells, a functional impairment of the immunoregulatory autoreactive type II NKT cells could be present in the rat model. In this study, we attempted to identify the antigen recognized by VASC-1 and discovered an intracellular lipid transfer molecule, sterol carrier protein 2 (SCP2), as one of the autoantigens.

### Materials and Methods

#### Rats

The rat model of systemic connective tissue diseases established in our laboratory and maintained under specific pathogen-free condition was used. These rats were transgenic for the \(env-pX\) gene of human T-cell leukemia virus type I. The transgene was expressed ubiquitously in the systemic organs, including hematopoietic cells. Because the transgene coded the transcription factor p40tax, which could disturb the ordinary transcription in the cells but did not code other viral

### Table 1

<table>
<thead>
<tr>
<th>Name</th>
<th>Amino acid sequence</th>
<th>Hydropathy index</th>
</tr>
</thead>
<tbody>
<tr>
<td>rSCP2508-522</td>
<td>MTGKMNPQSAFFQGK</td>
<td>-0.023</td>
</tr>
<tr>
<td>rSCP2509-523</td>
<td>TGMNPQSAFFQGKL</td>
<td>0.005</td>
</tr>
<tr>
<td>rSCP2510-524</td>
<td>GKMNPQSAFFQGKL</td>
<td>-0.092</td>
</tr>
<tr>
<td>rSCP2511-525</td>
<td>KMNPQSAFFQGKLK</td>
<td>-0.032</td>
</tr>
<tr>
<td>rSCP2512-526</td>
<td>MNPQSAFFQGKLIA</td>
<td>0.109</td>
</tr>
<tr>
<td>rSCP2513-527</td>
<td>NPQSAFFQGKLIA</td>
<td>0.099</td>
</tr>
<tr>
<td>rSCP2514-528</td>
<td>PQSAFFQGKLIAAGN</td>
<td>0.133</td>
</tr>
<tr>
<td>rSCP2515-529</td>
<td>SSAFFQGKLIAAGNM</td>
<td>0.222</td>
</tr>
<tr>
<td>rSCP2516-530</td>
<td>AFFQGKLIAAGNMGL</td>
<td>0.305</td>
</tr>
<tr>
<td>rSCP2517-531</td>
<td>FFFQGKLIAAGNMGLA</td>
<td>0.305</td>
</tr>
<tr>
<td>rSCP2518-532</td>
<td>FFQGKLIAAGNMGLAM</td>
<td>0.268</td>
</tr>
<tr>
<td>rSCP2519-533</td>
<td>QGKLIAAGNMGLAMLK</td>
<td>0.089</td>
</tr>
<tr>
<td>rSCP2520-534</td>
<td>GKLKIAAGNMGLAMLK</td>
<td>0.216</td>
</tr>
<tr>
<td>rSCP2521-535</td>
<td>KLIKIAAGNMGLAMLKQ</td>
<td>0.127</td>
</tr>
<tr>
<td>rSCP2522-536</td>
<td>LKIAAGNMGLAMLKQS</td>
<td>0.215</td>
</tr>
<tr>
<td>rSCP2523-537</td>
<td>KIAGNMGLAMLKQLS</td>
<td>0.215</td>
</tr>
<tr>
<td>rSCP2524-538</td>
<td>IAGNMGLAMLKQLSL</td>
<td>0.259</td>
</tr>
<tr>
<td>rSCP2525-539</td>
<td>AGNMGLAMLKQLSLQL</td>
<td>0.237</td>
</tr>
<tr>
<td>rSCP2526-540</td>
<td>GNMGLAMLKQLSLQL</td>
<td>0.139</td>
</tr>
<tr>
<td>rSCP2527-541</td>
<td>NMGMLAMLKQLSLQLQP</td>
<td>0.115</td>
</tr>
<tr>
<td>rSCP2528-542</td>
<td>MLMAMLKQLSLQLQPD</td>
<td>0.107</td>
</tr>
<tr>
<td>rSCP2529-543</td>
<td>GLMAMLKQLSLQLQPDKA</td>
<td>-0.035</td>
</tr>
<tr>
<td>rSCP2530-544</td>
<td>LAMLKQLSLQLQPDKA</td>
<td>-0.026</td>
</tr>
<tr>
<td>rSCP2531-545</td>
<td>AMMLKQLSLQLQPDKAK</td>
<td>-0.197</td>
</tr>
<tr>
<td>rSCP2532-546</td>
<td>MKLQLSLQLQPDKAK</td>
<td>-0.167</td>
</tr>
<tr>
<td>rSCP2533-547</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*The boldface peptides represent those with a hydropathy index >0.200.*

*The inserted region in 4D2.*
constructive proteins, these rats were considered as models with abnormal gene transcription rather than simple models of human T-cell leukemia virus type I infection. Experiments were performed in accordance with the Guidelines for the Care and Use of Laboratory Animals in Hokkaido University (permission No. 10-0029, 15-0034).

Cells

The rat inferior vena cava–derived vascular endothelial cells (RECs) and REC-reactive type II NKT cell clone, VASC-1, were established in our laboratory.5,17 RECs were maintained in RPMI 1640 medium containing 10% fetal bovine serum (FBS) and 5 × 10^{-5} mol/L 2-mercaptoethanol (2-ME). VASC-1 was maintained in RPMI 1640 medium containing 20% FBS, 5 × 10^{-5} mol/L 2-ME, and 0.01 μg/mL recombinant rat IL-2 (R&D Systems, Minneapolis, MN). The monkey kidney–derived fibroblastic cell line, COS-7, was maintained in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% FBS.

Co-Culture of VASC-1 with RECs or COS-7

RECs or COS-7 was grown subconfluently in a well of six-well plates (1 × 10^5 per well) at 37°C. VASC-1 (2 × 10^5 per well) was co-cultured with the cells in RPMI 1640 medium containing 20% FBS, 5 × 10^{-5} mol/L 2-ME, and 0.01 μg/mL recombinant rat IL-2 overnight at 37°C. On the next day, after the removal of the supernatants, the remaining cells were washed with the co-culture medium three times and then observed under a phase-contrast microscope.

Knockdown of Rat Genes of RECs

RECs were seeded in 6-cm dishes (2 × 10^5/dish) and incubated overnight at 37°C. On the next day, the culture medium was replaced with serum-free 2-ME–free RPMI 1640 medium. Thereafter, siRNA for rat CD1d or rat SCP2 or negative control siRNA (Life Technologies, Tokyo, Japan) was transfected into RECs using siGENE (Life Technologies) in accordance with the manufacturer’s instruction. Forty-eight hours after the siRNA transfection, total RNA was extracted from RECs using an RNeasy Mini kit (Qiagen, Alameda, CA). RNA underwent reverse transcription to cDNA using transcriptase and oligo-dT primers (Promega, Tokyo, Japan). The primers for rat genes used in this study were CD1d sense: 5’-TAGAACGGAGAAGCCAGACC-3’, CD1d antisense: 5’-TCCGCAATTTGCGAGGATGC-3’, SCP2 sense: 5’-CTTCACGATTGCTTCTCTACC-3’, SCP2 antisense: 5’-CAGTGCTCACCTGTCTCC-3’, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) sense: 5’-ATGGGAGTTGCTGTTGAAGTCA-3’, and GAPDH antisense: 5’-CCGAGGCCCCACTAAAGG-3’. Real-time PCR was run as follows: after denaturation at 94°C for 2 minutes, 40 cycles of reaction at 94°C for 15 seconds and at 62°C for 60 seconds were performed using the GoTaq 2-Step RT-qPCR System (Promega).

Figure 1: Involvement of CD1d on rat inferior vena cava–derived vascular endothelial cells (RECs) in vascular endothelial cell–reactive T-cell clone (VASC-1) binding. A and B: Representative images of co-cultured VASC-1 with RECs. Knockdown of CD1d of RECs by siRNA at the levels of mRNA (C) and cell surface protein (D). Experiments were performed in triplicate. Decrease in VASC-1 binding to RECs by the inhibition of CD1d using antibody and knockdown of CD1d using siRNA (E and F). Data are expressed as means ± SD (E and F). n = 3 (F). *P < 0.05, **P < 0.01 (t-test). Scale bars: 10 μm (A); 50 μm (E). TCR, T-cell receptor.
Flow Cytometry

Seventy-two hours after the siRNA transfection, RECs were detached from the dishes and then allowed to react with the anti-mouse/rat CD1d antibody WTH-1 (Abcam, Cambridge, UK) or mouse IgG2a (BD Biosciences, San Jose, CA) was the isotype control on ice for 20 minutes (1 μg per 10^6 cells). After washing, the cells were then made to react with 1:2000 diluted phycoerythrin-conjugated donkey anti-mouse IgG antibody (eBioscience, San Diego, CA) on ice for 30 minutes. Flow cytometry was conducted using FACS Canto II (BD Biosciences), and the data were analyzed using CellQuest Pro software version 6.0 (BD Biosciences).

VASC-1 Binding to RECs or REC Knockdown of CD1d

Twenty-four hours before this experiment, the medium of VASC-1 was replaced with a fresh one without IL-2. RECs or REC knockdown of CD1d was reseeded in two-well slide chambers (1 × 10^5 per well) and incubated overnight at 37°C. On the next day, carboxyfluorescein succinimidyl ester—labeled VASC-1 (2 × 10^5 per well) was co-cultured with the cells in RPMI 1640 medium containing 20% FBS and 5 × 10^{-5} mol/L 2-ME for 2 hours at 37°C. For reference, a similar co-culture was conducted in the presence of 1 μg/mL of the anti-CD1d antibody WTH-1. After the removal of the supernatants, the remaining cells were washed with PBS once and then fixed by acetone for 5 minutes at room temperature. The chambers were removed, and then the slide glasses were mounted with VECTASHIELD (Vector Laboratories, Burlingame, CA), which contains DAPI.

Comparison of Amino Acid Sequences of Monkey CD1d and Rat CD1d

The alignment analysis of amino acid sequences of monkey CD1d (XP_007974824) and rat CD1d (NP_058775) was conducted using Basic Local Alignment Search Tool (BLAST).

Transfection of Rat Genes into COS-7

COS-7 was grown subconfluently in wells of a six-well plate (1 × 10^5 per well) at 37°C. Rat cDNA library derived from the lungs of Sprague-Dawley strain (TKR9543, Takara Bio, Otsu, Japan) was transfected into COS-7 cells using the transfection reagent.
FuGENE (Roche Diagnostics, Tokyo, Japan) in accordance with the manufacturer’s instruction. Because the lungs were the most susceptible organs of SVV induced by an i.v. injection of VASC-1,5 we used the cDNA library derived from the lungs. This library contains more than $1 \times 10^6$ pAP3 neoplasms carrying diverse rat cDNA fragments in its multiple cloning sites that lie between the T3 and T7 promoters. Seventy-two hours after the transfection, the cells were exposed to 650 μg/mL of G418 in DMEM and cultured further for a week at 37°C. Live cells were collected, reseeded sparsely (200 cells per 9-cm dish), and cultured in DMEM containing 325 μg/mL of G418 at 37°C. Consequently, 98 COS-7 transfectants were established. These cells were stored at −150°C until use.

Verification of Rat Gene Insertion in COS-7 Transfectants

DNA was extracted from the aforementioned 98 COS-7 transfectants using a DNeasy Blood and Tissue Mini Kit (Qiagen). The insertion of rat genes was detected by PCR using the T3 and T7 promoter primers, which lie at either end of the multiple cloning sites for rat genes. PCR was run using AmpliTaq Gold 360 Master Mix (Applied Biosystems, Yokohama, Kanagawa) as follows: after denaturation at 94°C for 2 minutes, 35 cycles of reaction at 94°C for 30 seconds, at 55°C for 30 seconds, and at 72°C for 60 seconds. Electrophoresis of the PCR products through 1% agarose gel revealed that a single rat gene fragment was inserted in 45 COS-7 transfectants and more than two fragments were inserted in 42 COS-7 transfectants, although no insertion of the rat gene was evident in 11 COS-7 transfectants.

Co-Culture of VASC-1 and COS-7 Transfectants

Twenty-four hours before this experiment, the medium of VASC-1 was replaced with a fresh one without IL-2. Each COS-7 transfectant that carried a single rat gene fragment ($n = 45$) was subconfluently grown at 37°C. VASC-1 ($2 \times 10^5$ per well) was co-cultured with the cells in RPMI 1640 medium containing 20% FBS and 5 × 10⁻⁵ mol/L 2-ME overnight at 37°C. On the next day, after the removal of the supernatants, the remaining cells were washed with the co-culture medium three times and then observed under a phase-contrast microscope. A COS-7 transfectant, 4D2, was found to bind VASC-1.

Detection of Cytokine Expression in VASC-1 Co-Cultured with COS-7 Transfectants

After an overnight co-culture with the COS-7 transfectant, 4D2, according to the same protocol as above, total RNA was extracted from the aforementioned 98 COS-7 transfectants using a DNeasy Blood and Tissue Mini Kit (Qiagen). The expression of cytokines was detected by PCR using the T3 and T7 promoter primers, which lie at either end of the multiple cloning sites for rat genes. PCR was run using AmpliTaq Gold 360 Master Mix (Applied Biosystems, Yokohama, Kanagawa) as follows: after denaturation at 94°C for 2 minutes, 35 cycles of reaction at 94°C for 30 seconds, at 55°C for 30 seconds, and at 72°C for 60 seconds. Electrophoresis of the PCR products through 1% agarose gel revealed that a single rat gene fragment was inserted in 45 COS-7 transfectants and more than two fragments were inserted in 42 COS-7 transfectants, although no insertion of the rat gene was evident in 11 COS-7 transfectants.

Figure 3  Involvement of sterol carrier protein 2 (SCP2) of rat inferior vena cava–derived vascular endothelial cells (RECs) in vascular endothelial cell–reactive T-cell clone (VASC-1) binding. A: Rat SCP2 cDNA. The yellow box indicates the open reading frame. The 3' flanking fragment in red letters had been introduced into 4D2. Effects of SCP2 knockdown of RECs on SCP2 mRNA expression (B) and VASC-1 binding (C). Data are expressed as means ± SD (B and C). $n = 3$ (C). *$P < 0.05$, **$P < 0.01$ (t-test). GAPDH, glyceraldehyde-3-phosphate dehydrogenase; TCR, T-cell receptor.
was extracted from VASC-1 using an RNeasy Mini kit. RNA underwent reverse transcription to cDNA using transcriptase and oligo-dT primers. The primers for rat genes used in this study were IL-5 sense: 5'-GACGAGCAAT-GAGACGATGAGGCT-3', IL-5 antisense: 5'-ACAGTGCC-CCCCTCGGACAGTT-3', and the former GAPDH sense and GAPDH antisense. PCR was run using AmpliTaq Gold 360 Master Mix as follows: after denaturation at 94°C for 30 seconds, 35 cycles of reaction at 58°C for 30 seconds and at 72°C for 30 seconds. For the positive and negative controls, VASC-1 was co-cultured with RECs and with another COS-7 transfectant, 6A4, respectively.

Identification of the Rat Gene Transfected into 4D2

DNA was extracted from the COS-7 transfectant, 4D2, which could bind to VASC-1, using the DNeasy Blood and Tissue Mini Kit (Qiagen). The transfected rat gene via the pAP3 neoplasmid was amplified by PCR using the T3 and T7 promoter primers, and then the PCR product was sequenced directly using GenomeLab Dye Terminator Cycle Sequencing with Quick Start Kit (Beckman Coulter, Tokyo, Japan). Consequently, the rat gene transfected into 4D2 was identified to code SCP2.

VASC-1 Binding to RECs or REC Knockdown of SCP2

This assay was performed similarly to the assay for VASC-1 binding to REC knockdown of CD1d.

Transduction of SCP2 Peptides into COS-7

According to the sequence transfected into 4D2, 26 series of SCP2 peptides were designed. The amino acid sequences and expected hydropathy indexes are listed in Table 1. The greater the index is, the more hydrophobic the amino acid becomes. Because CD1d expressed preferably hydrophobic peptides, we requested Thermo Fisher Scientific (Osaka, Japan) to generate the nine peptides with a hydropathy index >0.200 (Table 1). The BioPORTER Protein Delivery Reagent (Genlantis, Tokyo, Japan) was applied for the intracellular delivery of SCP2 peptides according to the manufacturer’s instruction. In brief, COS-7 was seeded in two-well slide chambers (1 × 10⁵ per well) and incubated overnight at 37°C. SCP2 peptides were diluted in PBS (4 μg/40 μL), and then BioPORTER-coated tubes were hydrated with the SCP2 peptide solution and diluted to 500 μL with serum-free DMEM. The total volume of BioPORTER/SCP2 peptide complexes was transferred onto

---

**Figure 4** Screening of sterol carrier protein 2 (SCP2) peptide recognized by vascular endothelial cell-reactive T-cell clone (VASC-1). A: Screening of nine SCP2 peptides for the induction of VASC-1 binding to COS-7. B and C: Adherent VASC-1 per 100 COS-7 values were calculated from six random fields of view. VASC-1 binding was increased by the transduction of rSCP2518-532, but not rSCP2526-540, into COS-7. Data are expressed as means ± SEM (A and C). n = 3 (C). *P < 0.05 (t-test). Scale bar = 50 μm (B). PBS, phosphate-buffered saline.
COS-7 cells after being washed with serum-free DMEM, and then these cells were incubated in the culture medium for 5 hours at 37°C.

**VASC-1 Binding to COS-7 or COS-7 Transduced SCP2 Peptides**

This assay was performed similarly to the assay for VASC-1 binding to RECs.

**VASC-1 Proliferation Assay**

Twenty-four hours before this experiment, the culture medium of VASC-1 was replaced with RPMI 1640 medium devoid of FBS, 2-ME, and rat IL-2. The knockdown for the SCP2 gene of RECs was conducted as aforementioned. In some wells of REC knockdown of SCP2, SCP2 peptides (rSCP2518-532 and rSCP2526-540) were transduced into the cells similarly to the transduction into COS-7. Thereafter, these cells were washed with PBS twice. VASC-1 (4 × 10^5 per well) was co-cultured with the cells for 2 hours at 37°C. Finally, the supernatants (90 μL) were transferred into wells of a 96-well plate. Ten microliters of AlamarBlue (Thermo Fisher Scientific) were added to each well and incubated for 2 hours at 37°C. Thereafter, the absorbance at 570 nm was measured, and VASC-1 proliferation was calculated as follows: Proliferation (%) = (Sample Well − Blank Well) × 100/(Control Well − Blank Well).

**Immunization of Rats**

SCP2 peptide solution (rSCP2518-532 or rSCP2526-540, 400 μg/mL in dimethyl sulfoxide) was mixed with an equal volume of Freund’s complete adjuvant. The emulsion was injected into the skin on the back of premorbid young disease-prone rats (5-week-old male, 100 μL per site × 5 sites per rat). Ten days later, the emulsion that contained SCP2 peptide and Freund’s incomplete adjuvant was prepared and then injected similarly as the first immunization. Four days later, all rats were euthanized for histologic examination. Six rats were used for rSCP2518-532 immunization, and seven rats were used for rSCP2526-540 immunization.

**Histologic Examination**

The systemic organs of rats, including the cerebrum, cerebellum, thymus, heart, lungs, liver, spleen, pancreas, kidneys, adrenal glands, testis, skin, muscle, salivary glands, and lymph nodes, were processed for tissue sections with hematoxylin and eosin staining, Masson trichrome staining, and immunohistochemistry for α-smooth muscle actin (α-SMA). Immunohistochemistry for α-SMA was performed using the anti-human α-SMA antibody (1A4) and LSAB2 kit, universal (Dako, Tokyo, Japan). Briefly, after antigen retrieval by heat mediation (121°C, 15 minutes), consumption of the endogenous peroxidase activity by exposure to 3% H2O2, and inhibition of nonspecific binding by exposure to the blocking buffer in the kit, the sections were incubated with 1:100 diluted 1A4 for 30 minutes at room temperature. After removal of unbound antibodies, the sections were next allowed to react with the biotin-conjugated secondary antibody in the kit for 1 hour at room temperature, followed by development of color according to the kit protocol. The histologic score of SVV was determined according to the number of organs with vasculitic lesions (0 for no organ with

---

**Figure 5** Identification of rSCP2518-532 as the epitope recognized by vascular endothelial cell—reactive T-cell clone (VASC-1). The proliferation of VASC-1 that interacted with rat inferior vena cava—derived vascular endothelial cells (RECS) was decreased when sterol carrier protein 2 (SCP2) of RECs had been knocked down. However, the decrease was canceled by the transduction of rSCP2518-532, but not rSCP2526-540, into REC knockdown of SCP2. Data are expressed as means ± SD. n = 3. *P < 0.05 (t-test).
lesions; 1, 2, and 3 for one, two, and three organs with lesions; and 4 for four or more organs with lesions).

Statistical Analysis

The t-test was applied for comparison of the mean values between the two in vitro groups. The Mann-Whitney U-test was applied for comparison of the two in vivo groups. $P < 0.05$ was regarded as statistically significant.

Results

Involvement of CD1d on RECs in VASC-1 Binding

When VASC-1 was co-cultured with RECs, VASC-1 became bound to RECs (Figure 2A). The results of our previous study suggest that RECs present a certain auto-antigen on CD1d, and VASC-1 recognizes the autoantigen via TCR (Figure 1B). To confirm the involvement of CD1d on RECs in VASC-1 binding, we attempted to knock down CD1d of RECs by siRNA. At first, we determined whether siRNA could exactly knock down CD1d of RECs using real-time RT-PCR and flow cytometry. As a result, both mRNA and cell surface expression of CD1d were markedly reduced in RECs transfected with siRNA for CD1d (Figure 1, C and D). Next, VASC-1 was co-cultured with RECs in the presence of anti-CD1d antibody or with REC knockdown of CD1d. As a result, the quantity of VASC-1 bound to RECs was significantly reduced by both the inhibition and knockdown of CD1d (Figure 1, E and F). These findings confirmed the involvement of CD1d on RECs in VASC-1 binding.

VASC-1 Binding to COS-7 Transfected with the Rat Gene

Naturally, VASC-1 does not bind to monkey-derived COS-7 (Figure 2A). It seems likely that the TCR of VASC-1 cannot recognize monkey antigens presented by monkey CD1d (Figure 2B). However, it is known that CD1d molecules are well conserved among species. For instance, Arg78 and Asp79 in the $\alpha_2$ helix, which are critical for antigen presentation to type I NKT cells, are identical between monkey CD1d and rat CD1d (Figure 2C). Therefore, we hypothesized that VASC-1 could bind to COS-7 if the monkey CD1d would present rat antigens (Figure 2D). To verify the hypothesis, rat cDNA library ($1 \times 10^6$ diversity) was transfected into COS-7, and consequently, 98 COS-7 transfectants carrying various rat genes were established. By screening for VASC-1 binding under the co-culture condition, a COS-7 transfectant, named 4D2, was found to bind to VASC-1 (Figure 2E). Furthermore, VASC-1 co-cultured with the COS-7 transfectant 4D2, which could bind to VASC-1, was able to express IL-5; this was also the case when VASC-1 was co-cultured with RECs (Figure 2F). On the contrary, no expression of IL-5 was observed in VASC-1 that was co-cultured with another COS-7 transfectant, 6A4. Because IL-5 expression was mediated by CD1d-dependent stimulation via TCR, the collective findings suggested that the TCR of VASC-1 could cross-react with monkey CD1d when an appropriate rat antigen was presented.

Identification of the Rat Gene Transfected into 4D2

DNA sequencing identified that the rat gene transfected into 4D2 was a 3’-flanking fragment of the gene coding an intracellular lipid transfer molecule, SCP2 (Figure 3A). The C-terminal open reading frame of SCP2 was included in the fragment.

Involvement of SCP2 in VASC-1 Binding to RECs

To determine whether SCP2 could really be involved in VASC-1 binding to RECs, SCP2 of RECs was knocked down by siRNA. When we examined the mRNA expression of...
SCP2 of RECs transfected with siRNA for SCP2, the expression was markedly reduced (Figure 3B). Correspondingly, VASC-1 binding to RECs was decreased significantly by the knockdown of SCP2 (Figure 3C). These findings suggest the involvement of SCP2 in VASC-1 binding to RECs.

Identification of SCP2 Epitope Recognized by VASC-1

On the basis of the SCP2 gene sequence that had been transfected into 4D2, we designed 26 series of SCP2 peptides (Table 1). Because CD1d expressed preferably hydrophobic peptides, the nine peptides with a hydropathy index >0.200 (Table 1) were selected to be generated. To identify the epitope of SCP2 recognized by VASC-1, the nine peptides were transduced into COS-7, and then VASC-1 binding was screened. It is conceivable that cell surface CD1d molecules have been occupied by putative antigens. Thus, we used the transfection procedure to express the SCP2 peptides on CD1d. We also determined that the addition of SCP2 peptides in the culture medium could not increase the VASC-1 proliferation co-cultured with REC knockdown of SCP2 (data not shown). As a result, transduction of rSCP2518-532 induced the highest binding of VASC-1 to COS-7 (Figure 4A). On the contrary, transduction of rSCP2526-540 did not promote VASC-1 binding to COS-7 at all. Repeated experiments using rSCP2518-532 and rSCP2526-540 reproduced similar results (Figure 4, B and C).

Moreover, we found that REC-dependent proliferation of VASC-1 was decreased significantly when SCP2 of RECs had been knocked down by siRNA and that the decrease could be canceled by the transduction of rSCP2518-532 but not rSCP2526-540 (Figure 5). The collective findings suggest that rSCP2518-532 includes the epitope recognized by VASC-1.

Accelerated Development of SVV by Immunization with rSCP2518-532

Next, we determined whether immunization with rSCP2518-532 could promote the development of SVV. For this purpose, premorbid young disease-prone rats were used. Although SVV usually develops in rats >2 months old,1,20 all rats immunized with rSCP2518-532 (n = 6/6) developed SVV at 7 weeks old (Figure 6, A–D). The histologic scores...
according to the number of organs with vasculitic lesions were significantly higher in rats immunized with rSCP2518-532 than in those immunized with the control peptide, rSCP2526-540 (Figure 6, E and F). These results indicate that rSCP2518-532-reactive cells, including type II NKT cells, are critically implicated in the development of vascular inflammation in the rat model.

**Discussion**

In the present study, we found that the rat type II NKT cell clone, VASC-1, can recognize SCP2 peptides presented by CD1d. CD1d is known as a class I major histocompatibility complex—like antigen-presenting molecule that prefers to present hydrophobic antigens, including bacterial glycolipids. Although recent studies have revealed that sulfatides and peptides can be also presented by CD1d, the hydrophobicity of CD1d is a primary characteristic based on the molecular structure.

SCP2 is a 13-kDa protein that is implicated in intracellular lipid transfer. The N-terminal 32 amino acids of SCP2 form an amphipathic α-helix domain, which functions as a lipid carrier and a ligand-binding region simultaneously. On the contrary, the hydrophobic surface of the α helix constitutes a hollow structure with β-strands in the SCP2 molecule. This hollow structure binds to phospholipids.

The SCP2 gene fragment that had been transduced in the COS-7 transfectant with VASC-1 binding, 4D2, lacked the translation initiation codon for the complete SCP2 molecule. However, it contained some in-frame codons coding methionine, which could initiate the subsequent translation into certain fragmented proteins, including a part of the β-strands of the SCP2 molecule. It is conceivable that the SCP2 fragments expressed in 4D2 are hydrophobic in terms of the expected binding to phospholipids. Therefore, it is reasonable to consider that the SCP2 peptides can be presented by CD1d in 4D2. Moreover, this study has revealed that the SCP2 epitope recognized by VASC-1 is present in rSCP2518-532. This peptide is the most hydrophobic among the 26 candidate peptides; hence, it is consistent with the hydrophobicity of CD1d.

More recently, Girardi et al. reviewed that peptides, which included the [FW]-X-X-[ILM]-X-X-W motif, could bind to mouse CD1d. Although rSCP2518-532 does not include the CD1d-binding motif, another CD1d-binding peptide, mCII707-721, does not include the motif either. We considered that diverse antigens could be presented by CD1d.

We do not have any evidence to conclude that the SCP2 peptide is the sole autologous antigen presented by CD1d. Because the CD1d molecule can present diverse kinds of antigens, such as glycolipids, sulfatides, and peptides, regardless of the presence of the [FW]-X-X-[ILM]-X-X-W motif, it seems likely that several peptides derived from intracellular proteins, if hydrophobic, can be presented by CD1d. We were probably able to isolate an NKT cell clone that recognized the SCP2 peptide by chance.

The expression of SCP2 is distributed widely in the lipid metabolism–related organs and cells, including the liver and vascular endothelial cells. Although SCP2 is associated with the pathogenesis of arteriosclerosis, the association between SCP2 and vasculitides remains unrevealed. Further studies are needed to clarify the role of SCP2 in the pathogenesis of vasculitides.

Accumulated studies have revealed the immunoregulatory properties of type II NKT cells in the immune reaction. Liu et al. found that NKT cells reactive with autologous peptides (suggestive of type II phenotype) exhibited an immunosuppressive property in healthy mice. Correspondingly, the activation of type II NKT cells ameliorated the murine experimental hepatitis. Furthermore, Terabe et al. found that type II NKT cells were essential for the down-regulation of tumor immunosurveillance in wild-type mice. On the contrary, the association of the disorder of type II NKT cells with inflammatory diseases is also reported. Liao et al. found that the disordered regulation of type II NKT cells could cause a spontaneous development of colitis in CD1d transgenic mice. In summary, type II NKT cells belong to the immunoregulatory T cells, and the disorder of these cells can be implicated in immune-related inflammatory diseases. In our previous study, the type II NKT cell clone, VASC-1, had proinflammatory but not anti-inflammatory properties when made to react with autologous vascular endothelial cells.

In conclusion, the collective evidence suggests that a disordered immunoregulatory function of autoreactive type II NKT cells can be implicated in the development of vascular inflammation in the rat model. The impairment of vascular endothelial cell-reactive type II NKT cells with an immunoregulatory property could be implicated in the pathogenic modification of diverse human vasculitides, in which vascular endothelial cells are injured by inflammation (Figure 7). Although a full comprehension of the pathogenesis of vasculitides remains far off, this study can be a stream of light that illuminates the pathway.

**References**


The American Journal of Pathology ■ ajp.amjpathol.org


