HOKKAIDO UNIVERSITY

Title	Establishment of vascular endothelial cell-reactive type II NKT cell clone from rat model of autoimmune vasculitis
Author(s)	Iinuma, C.; Waki, M.; Kawakami, A. et al.
Citation	International Immunology, 27(2), 105-114 https://doi.org/10.1093/intimm/dxu088
Issue Date	2015-02
Doc URL	https://hdl.handle.net/2115/59840
Rights	This is a pre-copy-editing, author-produced PDF of an article accepted for publication in International Immunology following peer review. The definitive publisher-authenticated version International Immunology is available online at: http://dx.doi.org/10.1093/intimm/dxu088
Type	journal article
File Information	Accepted Manuscript_Iinuma et al_Int Immunol-2.pdf

Hokkaido University Collection of Scholarly and Academic Papers : HUSCAP

Establishment of vascular endothelial cell-reactive type II NKT cell clone from rat model of autoimmune vasculitis

Running title: Autoreactive vasculitogenic type II NKT cell clone

Chihiro Iinuma,[1*] Masashi Waki,[1*] Ai Kawakami,[1*] Madoka Yamaguchi,[1] Utano Tomaru,[2] Naomi Sasaki,[2] Sakiko Masuda,[1] Yuki Matsui,[1] Sari Iwasaki,[2] Tomohisa Baba,[2**] Masanori Kasahara,[2] Takashi Yoshiki,[2] Daniel Paletta,[3] Thomas Herrmann,[3] and Akihiro Ishizu[4]

[1]Graduate School of Health Sciences, Hokkaido University, Sapporo, Japan

[2]Department of Pathology, Hokkaido University Graduate School of Medicine, Sapporo, Japan

[3]Institut für Virologie und Immunbiologie, Julius-Maximilians-Universität Würzburg, Würzburg, Germany

[4]Faculty of Health Sciences, Hokkaido University, Sapporo, Japan

[*] These authors contributed equally to this work.

[**]Current address: Division of Molecular Bioregulation, Cancer Research Institute, Kanazawa University, Kanazawa, Japan

Correspondence: Akihiro Ishizu, Faculty of Health Sciences, Hokkaido University. Kita-12, Nishi-5, Kita-ku, Sapporo 060-0812, Japan. FAX: +81-11-706-4916, E-mail: aishizu@med.hokudai.ac.jp

Total number of pages: 24; Total number of figures: 6

Abstract

We previously generated a rat model that spontaneously developed small vessel vasculitis (SVV). In this study, a T cell clone reactive with rat vascular endothelial cells (REC) was established and named VASC-1. Intravenous injection of VASC-1 induced SVV in normal recipients. VASC-1 was a TCRαβ/CD3-positive CD4/CD8 double-negative T cell clone with expression of NKG2D. The cytokine mRNA profile under unstimulated condition was positive for IL-4 and IFN-γ but negative for IL-2 and IL-10. After interaction with REC, the mRNA expressions of IL-2, IL-5, and IL-6 were induced in VASC-1, which were inhibited by blocking of CD1d on the REC surface. Although the protein levels of these cytokines seemed to be lower than the detection limit in the culture medium, IFN-γ was detectable. The production of IFN-γ from the VASC-1 stimulated with LPS-pretreated REC was inhibited by the CD1d blockade on the REC. These findings indicated VASC-1 as an NKT cell clone. NKT cell pool includes two major subsets, namely type I and type II. Type I NKT cells are characterized by expression of semi-invariant TCRs and potential to bind to marine sponge-derived α-galactosylceramide (α-GalCer) loaded on CD1d; whereas, type II NKT cells do not manifest the characteristics. VASC-1 exhibited a usage of TCR other than the type I invariant TCR α chain and did not bind to α-GalCer-loaded CD1d; therefore, it was determined as a type II NKT cell clone. The collective evidence suggested that REC-reactive type II NKT cells could be involved in the pathogenesis of SVV in rats.

Key words: small vessel vasculitis, type II NKT cell, vascular endothelial cell

Introduction

Systemic vasculitides is classified into 3 categories, including large vessel vasculitis (LVV), medium vessel vasculitis (MVV), and small vessel vasculitis (SVV) (1). However, caliber of the affected vessels sometimes overlaps between MVV and SVV. This could be interpreted by the presence of common pathogenesis, at least in part, in MVV and SVV. Although anti-neutrophil cytoplasmic Ab (ANCA)-associated mechanism and immune complex-mediated mechanism are considered as such common pathogeneses, other mechanisms remain unrevealed.

Although animal models are required to investigate into the unrevealed pathogenesis, suitable models have not been established (2). We earlier generated a rat model that spontaneously developed MVV and SVV (3). Fibrinoid necrosis was frequent in the affected medium-sized arteries, while predominant infiltration of mononuclear cells with some eosinophils was seen around the affected small vessels. Fibrinoid necrosis was absent in the affected small vessels. Characteristically, renal glomeruli were intact in these rats. Since anti-nuclear and anti-DNA Abs were present in the serum, autoimmune mechanism was considered to be involved in the pathogenesis. However, ANCA was not detected in the serum. In addition, deposition of immunoglobulin was not detected in the affected vessels. Therefore, the vasculitides in these rats was regarded as a model of MVV and SVV different from ANCA-associated or immune complex-mediated vasculitis in humans. Since both MVV and SVV were transferred into normal recipients by peripheral T cells from the vasculitis-prone rats, autoreactive T cell-mediated vascular injury could be involved commonly in the pathogenesis of MVV and SVV (4). In the present study, a T cell clone reactive with vascular endothelial cells and pathogenic for SVV was established from the rat model. Characterization of the phenotype identified the clone as an NKT cell clone.

NKT cells belong to a subset of T cells that share surface markers and function with NK cells and play important roles in physiological and pathological immune responses (5). A hallmark of NKT cells is their capacity to recognize Ags presented by class I MHC-like CD1d (6, 7). Among the NKT cells, two major subsets, namely type I and type II, have been noted (7, 8). Type I NKT cells are characterized by expression of a conserved TCR α chain (Vα24-Jα18 in humans and Vα14-Jα18 in rodents) that pairs with a limited repertoire of β chain (Vβ11 in humans, Vβ8.2, 7, and 2 in mice, and Vβ8 family members in rats) (9-11; Herrmann and Paletta, unpublished data). These cells, also called invariant NKT cells, bind to marine sponge-derived glycolipid, α-galactosylceramide (α-GalCer), presented by CD1d. On the other hand, type II NKT cells express a more variable TCR repertoire and do not respond to stimulation with α-GalCer. Thus, type II NKT cells are considered to exhibit Ag specificity different from type I NKT cells.

The NKT cell clone established from the rat model of autoimmune vasculitis exhibited a TCR usage other than the type I invariant TCR α chain and did not bind to α-GalCer-loaded CD1d; therefore, it was regarded as a type II NKT cell clone. The data presented in this study suggested that vascular endothelial cell-reactive type II NKT cells could be involved in the pathogenesis of SVV in rats.

Methods

Rats

The vasculitis-prone rats established in our laboratory and maintained under specific pathogen-free condition were used (2). These rats are transgenic for the *env-pX* gene of human T cell leukemia virus type I (HTLV-I). The transgene was expressed ubiquitously in systemic organs, including hematopoietic cells. Since the transgene coded the transcription factor p40tax, which could disturb ordinary transcription in the cells, but did not code other viral constructive proteins, these rats were considered as models with abnormal gene transcription rather than simple models of HTLV-I infection. The vasculitis-prone rats and the wild-type WKAH/Hkm rats (male, 6 weeks old) were used in this study. WKAH/Hkm rats were purchased from Sankyo Laboratories (Sapporo, Japan). Experiments were done in accordance with the guidelines for the care and use of laboratory animals in Hokkaido University.

Rat cell lines

WKAH/Hkm rat-derived vascular endothelial cells (REC) and synovial fibroblastic cells (RFC) were used (12, 13). REC was isolated from the inferior vena cava (IVC).

Immunization of rats

REC (5×10^6/500 µl PBS) was mixed with an equal volume of Freund's complete adjuvant. The emulsion was injected into the skin on the back of 6-week-old vasculitis-prone rats (100 µl/site × 10 sites). Ten days later, injection of emulsion containing REC (5×10^6/500 µl PBS) and Freund's incomplete adjuvant (500 µl) was employed similar to the first immunization.

Extraction of T cells reactive with REC from vasculitis-prone rats

For *in vitro* cultivation of T cells reactive with REC, 6-week-old vasculitis-prone rats were immunized with REC as described above. Four days after the second immunization, cervical lymph nodes were extirpated. The lymph nodes were homogenized and filtered in order to separate the lymph node cells from the connective tissues. Then, the lymph node cells were cultured in RPMI 1640 medium supplemented with 20 % FBS and 2×10^{-8} M 2-ME in 9 cm dishes (1×10^{8}/dish) at 37 °C. After incubation for 30 min, these cells were divided into dish-adherent cells and non-adherent cells. The adherent cells were treated with 50 μg/ml of mitomycin C (MMC) at 37 °C for 30 min and then served as APC. REC was similarly treated with MMC. The non-adherent cells (2×10^{7}) were mixed with the MMC-treated APC (5×10^{6}) and REC (5×10^{6}) in the medium added with 0.1 μg/ml of recombinant rat IL-2 (R&D Systems, Minneapolis, MN). The mixed cells were repeatedly stimulated by MMC-treated APC (dish-adherent lymph node cells derived from REC-immunized WKAH/Hkm rats) and REC every 2 weeks. One month later, cell clusters were observed in the dish.

Cloning of T cells

To obtain a T cell clone, the clusters were initially picked up from the mixed cell culture. In brief, the mixed cells were re-suspended sparsely in the medium (1×10^{2}/10 ml) and cultured in a 9 cm dish. After incubation for 4 days, grown clusters were picked up and transferred into wells of the 24-well plate (one cluster/well). The transferred clusters were incubated in the culture medium added with 0.1 μg/ml of recombinant rat IL-2. Among them, one cluster grew continuously. Next, limiting dilution of the grown cluster was carried out. In brief, the cells were re-suspended sparsely in the medium and cultured in wells of the 96-well plates without feeder cells (0.3/well). Recombinant rat

IL-2 (0.1 μg/ml) was added into the wells, in which a single cell was microscopically verified. Finally, a T cell clone, named VASC-1, was established.

Maintenance of T cell clone in vitro

VASC-1 was maintained in RPMI 1640 medium containing 20% FBS, 2×10^{-8} M 2-ME, and 0.01 μg/ml recombinant rat IL-2.

RNA extraction and reverse transcription to cDNA

Total RNA was extracted from cultured cells using RNeasy Mini kit (Qiagen, Alameda, CA). The RNA underwent reverse transcription to cDNA using reverse transcriptase and oligo-dT primers.

PCR and real-time PCR for rat cytokines

Primers for rat genes, including IL-2, IL-4, IL-5, IL-6, IL-10, IFN-γ, TGF-β, and GAPDH, are listed in Table S1. PCR was run as follows; after denaturation at 95 ºC for 10 min, 35 cycles of reaction at 94 ºC for 30 sec, at 60 ºC for 30 sec, and at 72 ºC for 30 sec were carried out. Real-time PCR was performed using Go Taq® 2-step RT-qPCR System (Promega, Tokyo, Japan).

Abs

Mouse anti-rat monoclonal Abs used were anti-TCRαβ, anti-CD3, anti-CD4, anti-CD8, anti-CD25, anti-CD62L (BD Biosciences, Franklin Lakes, NJ), anti-CD68 (AbD Serotec, Raleigh, NC), anti-NKG2D (eBioscience, San Diego, CA), anti-MHC class I and class II (AbD Serotec), and anti-CD1d (14).

Injection of VASC-1 into rats

VASC-1 (1×10^7/1 ml PBS) was intravenously injected into 6-week-old WKAH/Hkm rats (n=10) via the tail vein. For controls, lymph node cells from the vasculitis-prone rats (1×10^7/1 ml PBS) were similarly injected (n=6). Five days later, these rats were killed for histological examination.

Immunohistochemistry

The formaldehyde-fixed paraffin-embedded sections were made to react with each Ab based on the manufacturers' recommended dilution at 4°C overnight. To detect the reaction, the labeled-streptavidin biotin method was applied.

Proliferation assay

Both REC and RFC were treated with 50 μg/ml of MMC for 30 min at 37 °C. VASC-1 (1×10^3) was cultured with the MMC-treated REC (5×10^2) in wells of the 96-well plate for 3 days. For controls, VASC-1 (1×10^3) was cultured alone or with the MMC-treated RFC (5×10^2). Live cell numbers were counted every 24 hours using Cell counting kit-8 (Dojindo, Tokyo, Japan).

Survival assay

VASC-1 (2×10^6) was cultured with or without REC or RFC (1×10^6) for 24 h at 37 °C in IL-2-free culture medium and then subjected to FCM using FACS Calibur (Becton Dickinson, Tokyo, Japan).

Co-culture assay

VASC-1 (2×10^6) was cultured with or without REC or RFC (1×10^6) for 24 h at 37 °C in IL-2-free culture medium and then subjected to FCM, RT-PCR, and real-time RT-PCR.

Blocking assay

Prior to this experiment, REC (1×10^6) was treated with or without 1 μg/ml of LPS for 1 h at 37 ºC. Thereafter, REC (1×10^6) was allowed to react with 5 μg of anti-MHC class I Ab (OX18), anti-MHC class II Ab (OX6), anti-CD1d Ab (WTH1), or isotype-matched controls for 30 min at room temperature. VASC-1 (2×10^6) was then cultured with the pre-treated REC (1×10^6) for 24 h at 37 ºC in IL-2-free culture medium. Total RNA was extracted from VASC-1 cultured with the pre-treated REC and then subjected to RT-PCR. Culture supernatants were subjected to Luminex® analysis.

Luminex® analysis

The concentrations of rat cytokines in the culture supernatants were measured by Luminex® at Genetic Lab Co. Ltd. (Sapporo, Japan).

PCR for invariant TCR α chain of rat type I NKT cells

PCR for the invariant TCR α chain (Vα14-Jα18) of rat type I NKT cells was carried out as previously described (11).

CD1d binding assay

Dimers consisting of the extracellular domain of rat CD1d linked with a mouse IgG heavy chain (11) were used. For negative and positive controls of α-GalCer-loaded CD1d binding, BWr/mCD28 (mouse hybridoma 58C transduced with rat/mouse CD28) and BWr/mCD28 with TCR (BWr/mCD28 transduced with rat TCR Vα14 and Vβ8.2 genes) were used, respectively (15). These cells and VASC-1 (1×10^6) were allowed to react with 1 μg of rat CD1d dimers loaded with or without α-GalCer for 30 min at room temperature followed by reaction to PE-labeled $F(ab')_2$ donkey anti-mouse IgG (H+L)

(eBioscience). After blocking the nonspecific binding of the secondary Ab by addition of mouse IgG in the samples, the cells were subjected to FCM.

Statistics

Student *t*-test was applied for comparison of mean values between 2 groups. A *p*-value below 0.05 was regarded statistically significant.

Results

Establishment of T cell clone from rats with autoimmune vasculitis

We had previously established a rat model that developed MVV and SVV (3). Our earlier studies suggested that T cells reactive with self-vasculature were critically involved in the development of both MVV and SVV (4). According to the hypothesis that the pathogenic T cells would react with vascular endothelial cells, a T cell clone reactive with REC was established from the rat model and was named VASC-1.

Pathogenicity of VASC-1

In order to determine the pathogenicity of VASC-1, WKAH/Hkm rats were intravenously injected with VASC-1 (1×10^7/rat). Since VASC-1 was derived from the transgenic rats carrying the viral gene, rejection was possible 1-2 weeks after the injection in immunocompetent WKAH/Hkm rats. Therefore, acute events were evaluated within 1 week after the cell transfer. Histological examination revealed that vasculitic lesions were induced in all rats injected with VASC-1 (n=10). Inflammatory cells, including mononuclear cells and eosinophils, infiltrated around the affected small vessels (Figures 1A and 1B). Large vessels, such as the aorta and its branches, were not affected. In addition, MVV with fibrinoid necrosis in the vascular wall was not observed. Vasculitic lesions were seen mainly in the lungs but also in the systemic connective tissues. Immunohistochemistry revealed that $CD3^+$ cells (Figure 1C), $CD8^+$ cells (Figure 1D), and $CD68^+$ cells (Figure 1E) were present in the vasculitic lesions. On the contrary, $CD4^+$ cells and B cells were hardly detected. Vasculitis was never induced in WKAH/Hkm rats administered with control lymphocytes (Figure 1F). These findings demonstrated the pathogenicity of VASC-1 and suggested that VASC-1 reacted with vascular endothelial cells and then recruited host inflammatory cells into the vasculitic

lesions.

Phenotype of VASC-1

The VASC-1 cultured alone for at least 2 weeks from the last stimulation with REC was subjected to characterization. FCM demonstrated that VASC-1 was a TCRαβ/CD3-positive CD4/CD8 double-negative T cell clone with expression of NKG2D and CD25 (Figure 2A). The cytokine mRNA profile under unstimulated condition was positive for IL-4, IFN-γ, and TGF-β but negative for IL-2 and IL-10 (Figure 2B). The concentration of IFN-γ in culture supernatants (2×10^6 VASC-1/200 μl medium, after 24 h incubation) was 271.9±24.5 pg/ml, while that of IL-4 was below the detection limit at 3.2 pg/ml.

Interaction of VASC-1 with REC

In order to determine the reactivity of VASC-1 with REC, proliferation of VASC-1 co-cultured with REC was examined. Compared to the effect of RFC, co-cultivation with REC induced a significant augmentation of proliferation of VASC-1 (Figure 3A). Next, VASC-1 was cultured alone or with REC or RFC in IL-2-free culture medium (Figure 3B). When IL-2 was removed from the culture medium, the majority of VASC-1 underwent apoptosis within a day (entered into R1 gate, 80.2 % in left panel). However, VASC-1 survived in the cultivation with REC (entered into R2 gate, 88.8 % in middle panel), which effect was greater than when VASC-1 was co-cultured with RFC (38.4 % of VASC-1 entered into R2 gate in right panel). These findings suggested that REC could supply the survival factors for VASC-1.

Subsequently, we examined the dynamics of activation markers on VASC-1 co-cultured with REC or RFC. Since CD25, which is the most popular activation marker of T cells, was highly expressed on VASC-1 even under unstimulated condition

(Figure 2A), we focused on the expression of CD62L, which was known to be shed off from the cell surface by activation (16). The cell surface expression of CD62L on VASC-1 was decreased by adherence to the REC but not to the RFC (Figure 4A). This finding indicated that VASC-1 was activated by binding with REC but not with RFC. Correspondingly, the mRNA expression of several cytokines, including IL-2, IL-5, and IL-6, was induced in VASC-1 co-cultured with REC but not with RFC (Figure 4B). On the contrary, the mRNA expression of anti-inflammatory TGF-β was significantly decreased by interaction with REC (Figure 4C). The collective findings demonstrated the interaction of VASC-1 with REC and suggested that the REC-reacted VASC-1 exhibited a pro-inflammatory phenotype.

Involvement of CD1d on REC in Ag recognition by VASC-1

The characteristic cell surface phenotype and cytokine mRNA profile suggested the possibility that VASC-1 was an NKT cell clone. The identity of NKT cells is to recognize Ags presented by CD1d (6, 7). In order to identify VASC-1 as an NKT cell clone, the involvement of CD1d on the REC surface in the Ag recognition by VASC-1 was examined. First, FCM demonstrated that REC expressed CD1d on the surface (Figure 5A). Next, we examined if the mRNA induction of cytokines in VASC-1, which reacted with REC, would be inhibited by anti-CD1d Abs. The anti-CD1d Ab used in this study has been shown to block TCR-mediated activation of NKT cell hybridomas and transductants as well as of primary invariant NKT cells (14). When REC was treated with the anti-CD1d Ab but not with the anti-MHC class I and anti-MHC class II Abs, the mRNA induction of IL-2, IL-5, and IL-6 in VASC-1 was inhibited (Figure 5B).

Although the concentrations of these cytokines in the supernatants did not reach up to the detection limit (IL-2: 5.4 pg/ml; IL-5: 7.4 pg/ml; IL-6: 30.7 pg/ml), IFN-γ could be measured (detection limit: 6.2 pg/ml). Since VASC-1 produced IFN-γ

originally and the expression of IFN-γ was not increased significantly by the REC-stimulation alone, we considered stimulation of REC prior to the co-culture. It has been shown that the quantities of CD1d-bound Ags were markedly increased by LPS (17); therefore, LPS was employed to enhance the effects of REC-stimulation in this experiment. Under this condition, the production of IFN-γ from VASC-1 that reacted with the LPS-treated REC was significantly decreased by the CD1d blocking (Figure 5C). The collective findings suggested that VASC-1 could recognize Ags presented by CD1d on the REC surface; hence, VASC-1 was identified as an NKT cell clone.

No usage of invariant TCR α chain of type I NKT cells and no binding to α-GalCer-loaded CD1d dimers of VASC-1

NKT cell pool includes two major subsets, termed type I and type II NKT cells (8). Type I rat NKT cells are characterized by the expression of a conserved TCR α chain (Vα14-Jα18) that pairs with a limited repertoire of β chain (Vβ8 family members) and the potential to bind to α-GalCer loaded on CD1d; whereas, type II NKT cells do not manifest the said characteristics (9, 11). VASC-1 did not express the type I invariant TCR α chain (Figure 6A). In addition, it did not bind to α-GalCer-loaded CD1d dimers (Figure 6B). These findings suggested VASC-1 as a type II NKT cell clone.

Discussion

NKT cell pool includes two major subsets, type I and type II NKT cells (8). Although both NKT cells recognize Ags presented by CD1d, these subsets are distinct in terms of TCR usage and antigen specificity (6, 7). Type I NKT cells express semi-invariant TCRs composed of a conserved α chain (Vα24-Jα18 in humans and Vα14-Jα18 in rodents) and a limited repertoire of β chain (Vβ11 in humans, Vβ8.2, 7, and 2 in mice, and Vβ8 family members in rats) (9-11; Herrmann and Paletta, unpublished data). These so-called invariant NKT cells bind to marine sponge-derived α-GalCer presented by CD1d. On the other hand, type II NKT cells express a more variable TCR repertoire and do not bind to α-GalCer presented by CD1d. Therefore, type II NKT cells are considered to exhibit antigen specificity different from type I invariant NKT cells. Although the differences in TCR usage and antigen specificity between type I and type II subsets have been well characterized, their functional diversity remains ambiguous.

The physiological roles of type I NKT cells could be evaluated directly by using Jα18$^{-/-}$ mice (18). Since Jα18$^{-/-}$ type I NKT cell-deficient mice demonstrated an increased disease severity in various experimental autoimmune models, such as encephalomyelitis (EAE) (19) and uveitis (EAU) (20), type I NKT cells could be regarded as the suppressors for autoimmune diseases. On the other hand, due to the lack of a direct evaluation strategy, the physiological roles of type II NKT cells remain elusive. Currently, it can be speculated from the difference between CD1d$^{-/-}$ mice, which lack both type I and type II NKT cells, and Jα18$^{-/-}$ mice, which lack exclusively type I NKT cells. However, this is somewhat problematic because the effect of type II NKT cell-deficiency could be masked by the type I NKT cell-deficient effect in CD1d$^{-/-}$ mice. Therefore, further investigations are required concerning the type II NKT cell biology.

In the present study, a REC-reactive T cell clone, VASC-1, was established

from the rats with autoimmune vasculitis. VASC-1 was identified as an NKT cell clone based on the reactivity to CD1d on the REC surface. The characteristic cell surface phenotype and cytokine mRNA profile also supported VASC-1 as an NKT cell clone. Furthermore, the TCR analysis and CD1d binding assay using α-GalCer suggested VASC-1 as a type II NKT cell clone. As far as we know, VASC-1 is the first established autoreactive type II NKT cell clone. Since small vessel lesions were induced in normal recipients by the adoptive transfer of VASC-1, it was considered that vascular endothelial cell-reactive type II NKT cells could be involved in the pathogenesis of SVV in rats.

The histological findings of VASC-1-induced SVV suggested that vascular endothelial cell-reactive type II NKT cells recruited host inflammatory cells, including eosinophils, $CD8^+$ T cells, and $CD68^+$ macrophages, into the vasculitic lesions. The potential of VASC-1 to produce multiple cytokines, such as IL-2, IL-5, and IL-6, appeared to be compatible with the histological findings. Although the concentrations of these cytokines did not reach up to the detection limit in culture supernatants, they appeared to function in a small amount in the microenvironment. The decreased mRNA expression of anti-inflammatory TGF-β in VASC-1, which reacted with REC, could also be related to the continuous inflammation in the vasculitic lesions. The collective findings suggested that autoreactive type II NKT cells could play as disease accelerators in autoimmune and/or inflammatory diseases. Recently, Satoh *et al*. demonstrated that type II NKT cells induced adipose tissue inflammation and consequent obesity-related steatohepatitis and insulin resistance in mice treated with high fat diet (21). In addition, Liao *et al*. showed that aberrant type II NKT cell responses contributed to intestinal inflammation in mice (22). Thus, these reports are consistent with our results.

Vasculitides in the rat model exhibited various pathological characteristics, such as necrotizing vasculitis of medium-sized arteries and SVV without fibrinoid

necrosis (3). In this study, vascular endothelial cell-reactive type II NKT cells could be involved in the pathogenesis of SVV. However, it remains elusive whether these cells are also related to the development of MVV. Because of the possible rejection of VASC-1 in the immunocompetent normal recipients, only a short course effect at 5 days after the adoptive transfer of VASC-1 could be evaluated in this study. This seems to be a critical limitation of this study. Longer period of observation is needed to determine if VASC-1 would be involved in the pathogenesis of MVV, as well as SVV. Another possibility of the limited relation between VASC-1 and SVV is also considered. In this case, the difference in the caliber of target vessels could be dependent on the diversity of Ags recognized by autoreactive T cells, including NKT cells.

Currently, the REC Ags recognized by VASC-1 have not been identified. Although the importance of lipid recognition by NKT cells is stressed, it has been pointed out that NKT cells possibly recognize sulfatides and peptides, including autoantigens (23). Further studies are needed to clarify which type of autoantigens presented by CD1d on the REC surface, namely glycolipids or sulfatides/peptides, are recognized by VASC-1. This important issue should be revealed in our future studies. Identification of the VASC-1 TCR sequence would bring helpful information to discover the antigens.

In summary, we established an autoreactive and pathogenic type II NKT cell clone from the autoimmune disease model. This clone could be a useful tool for understanding the roles of type II NKT cells in the immune response and autoimmune diseases, including SVV.

Conflict of interest statement

The authors declare no conflicts of interest.

Funding

This work was supported by grants-in-aid from the Ministry of Education, Culture, Sports, Science and Technology of Japan, the Ministry of Health, Labour and Welfare of Japan (Research on Rare and Intractable Diseases), Japan Rheumatism Foundation, the Akiyama Life Science Foundation, the Suhara Memorial Foundation, and Deutsche Forschungsgemeinschaft (DFG) HE 2346/6-1.

References

1. Jennette JC, Falk RJ, Bacon PA, *et al.* 2013. 2012 revised international Chapel Hill consensus conference nomenclature of vasculitides. *Arthritis Rheum* 65:1-11.
2. Korkmaz B, Jenne DE, and Gauthier F. 2013. Relevance of the mouse model as a therapeutic approach for neutrophil proteinase 3-associated human diseases. *Int Immunopharmacol* 17:1198-205.
3. Yamazaki H, Ikeda H, Ishizu A, *et al.* 1997. A wide spectrum of collagen vascular and autoimmune diseases in transgenic rats carrying the env-pX gene of human T lymphocyte virus type I. *Int Immunol* 9:339-46.
4. Fugo K, Ishizu A, Ikeda H, *et al.* 2002. The role of the thymus in development of necrotizing arteritis in transgenic rats carrying the env-pX gene of human T-cell leukemia virus type-I. *Am J Pathol* 161:755-61.
5. Wu L, Gabriel CL, Parekh VV, *et al.* 2009. Invariant natural killer T cells: Innate-like T cells with potent immunomodulatory activities. *Tissue Antigens* 73:535-45.
6. Girardi E, and Zajonc DM. 2012. Molecular basis of lipid antigen presentation by CD1d and recognition by natural killer T cells. *Immunol Rev* 250:167-79.
7. Rossjohn J, Pellicci DG, Patel O, *et al*. 2012. Recognition of CD1d-restricted antigens by natural killer T cells. *Nat Rev Immunol* 12:845-57.
8. Godfrey DI, MacDonald HR, Kronenberg M, *et al*. 2004. NKT cells: What's in a name? *Nat Rev Immunol* 4:231-7.
9. Matsuura A, Kinebuchi M, Chen HZ, *et al.* 2000. NKT cells in the rat: Organ-specific distribution of NKT cells expressing distinct Vα14 chains. *J Immunol* 164:3140-8.
10. Godfrey DI, Stankovic S, and Baxter AG. 2010. Raising the NKT cell family. *Nat*

Immunol 11:197-206.

11. Monzon-Casanova E, Paletta D, Starick L, *et al.* 2013. Direct identification of rat iNKT cells reveals remarkable similarities to human iNKT cells and a profound deficiency in LEW rats. *Eur J Immunol* 43:404-15.
12. Ishizu A, Ishikura H, Nakamaru Y, *et al.* 1995. Thy-1 induced on rat endothelium regulates vascular permeability at sites of inflammation. *Int Immunol* 7:1939-47.
13. Ishizu A, Tsuji T, Abe A, *et al.* 2003. Transduction of dominant negative ATF-1 suppresses the pX gene expression in joint fibroblastic cells derived from HTLV-I transgenic rats. *Exp Mol Pathol* 74:309-13.
14. Monzon-Casanova E, Steiniger B, Schweigle S, *et al.* 2010. CD1d expression in paneth cells and rat exocrine pancreas revealed by novel monoclonal antibodies which differentially affect NKT cell activation. *PLoS One* 5,e13089.
15. Pyz E, Naidenko O, Miyake S, *et al.* 2006. The complementarity determining region 2 of BV8S2 (Vβ8.2) contributes to antigen recognition by rat invariant NKT cell TCR. *J Immunol* 176:7447-55.
16. Yang S, Liu F, Wang QJ, *et al*. 2011. The shedding of CD62L (L-selectin) regulates the acquisition of lytic activity in human tumor reactive T lymphocytes. *PLoS One* 6:e22560.
17. Muindi K, Cernadas M, Watts GFM, *et al*. 2010. Activation state and intracellular trafficking contribute to the repertoire of endogenous glycosphingolipids presented by CD1d. *Proc Natl Acad Sci USA* 107:3052-7.
18. Cui J, Shin T, Kawano T, *et al*. 1997. Requirement for Vα14 NKT cells in IL-12-mediated rejection of tumors. *Science* 278:1623-6.
19. Oh SJ, and Chung DH. 2011. Invariant NKT cells producing IL-4 or IL-10, but not IFN-γ, inhibit the Th1 response in experimental autoimmune encephalomyelitis, whereas none of these cells inhibits the Th17 response. *J Immunol* 186:6815-21.

20. Oh K, Byoun OJ, Ham DI, *et al*. 2011. Invariant NKT cells regulate experimental autoimmune uveitis through inhibition of Th17 differentiation. *Eur J Immunol* 41:392-402.
21. Satoh M, Andoh Y, Clingan CS, *et al*. 2012. Type II NKT cells stimulate diet-induced obesity by mediating adipose tissue inflammation, steatohepatitis and insulin resistance. *PLoS One* 7:e30568.
22. Liao CM, Zimmer MI, Shanmuganad S, *et al*. 2012. Dysregulation of CD1d-restricted type II natural killer T cells leads to spontaneous development of colitis in mice. *Gastroenterology* 142:326-34.
23. Liu Y, Teige A, Mondoc E, *et al*. 2011. Endogenous collagen peptide activation of CD1d-restricted NKT cells ameliorates tissue-specific inflammation in mice. *J Clin Invest* 121:249-64.

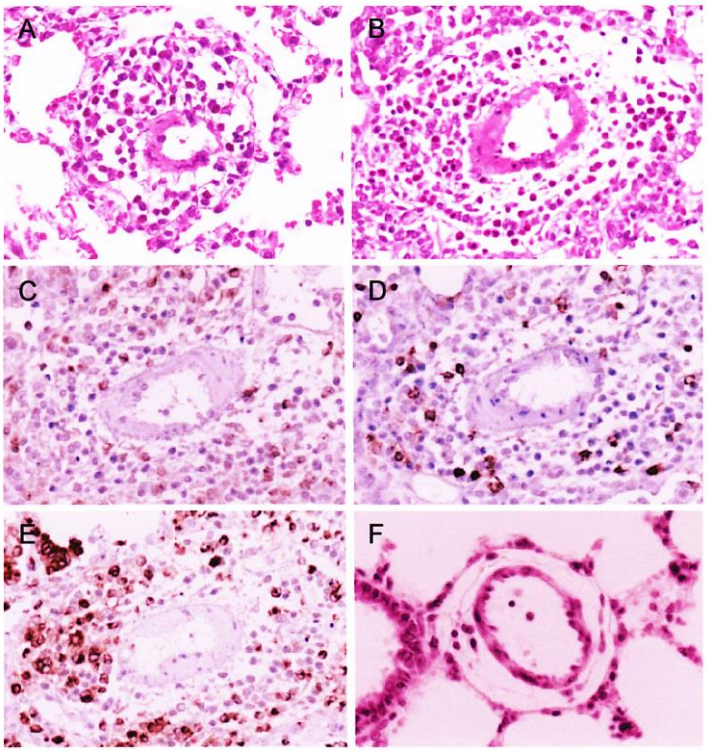


Figure 1. Vasculitic lesions induced by intravenous injection of VASC-1

(A-E) Histology of WKAH/Hkm rats given intravenous injection of VASC-1. Vasculitic lesions observed in the lungs (A and B, H&E staining). Immunohistochemistry for CD3 (C), CD8 (D), and CD68 (E). No vasculitis in WKAH/Hkm rats administered with control lymphocytes (cervical lymph node cells derived from a premorbid vasculitis-prone rat) (F). The representative figures are shown (original magnification: ×400).

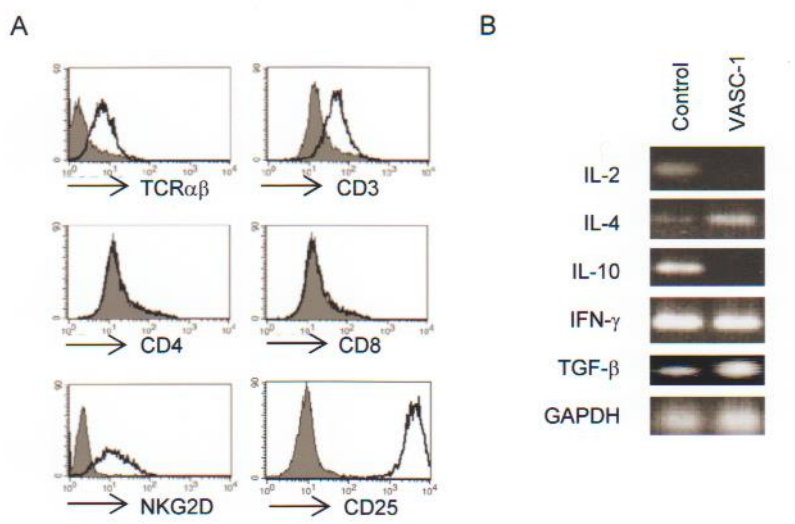


Figure 2. Phenotype of VASC-1

(A) Cell surface markers of VASC-1 examined by FCM. The VASC-1 cultured alone for at least 2 weeks from the last stimulation with REC was subjected to this assay. Filled histograms represent the background staining of isotype-matched controls. (B) Cytokine mRNA profile of VASC-1 examined by RT-PCR. The VASC-1 cultured alone for at least 2 weeks from the last stimulation with REC was subjected to this assay. Control: Cervical lymph node cells derived from a morbid vasculitis-prone rat.

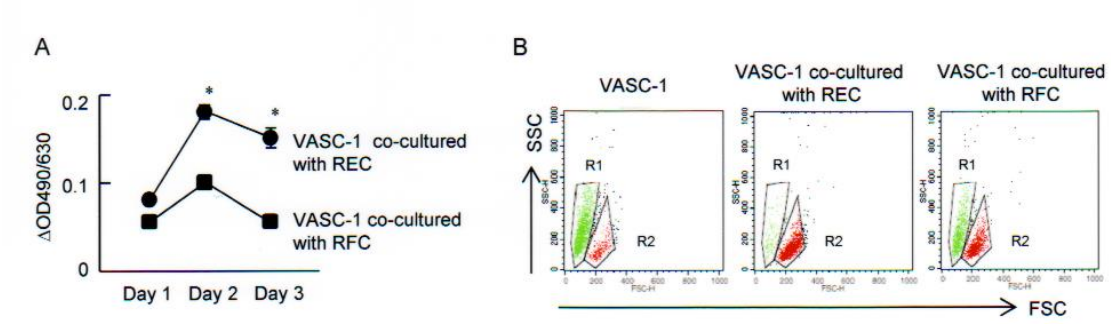


Figure 3. Interaction of VASC-1 with REC

(A) Proliferation of VASC-1 co-cultured with REC. VASC-1 (1×10^3) was co-cultured with MMC-treated REC (5×10^2) for 3 days. For controls, VASC-1 (1×10^3) was co-cultured with MMC-treated RFC (5×10^2) or cultured alone. Live cell numbers were counted every 24 h using the cell counting kit. Experiments were done in triplicate. Data are presented as mean ± SD of ΔOD (OD values of co-cultured VASC-1 – mean OD value of VASC-1 cultured alone). *$p<0.05$. (B) Survival of VASC-1 in IL-2-free culture medium. VASC-1 (2×10^6) was cultured with REC or RFC (1×10^6) or cultured alone for 24 h at 37 °C in IL-2-free culture medium and then subjected to FCM. Cells in R1 and R2 gates represent apoptotic cells and live cells, respectively. Experiments were repeated 3 times and similar results were reproduced. The representative results are shown.

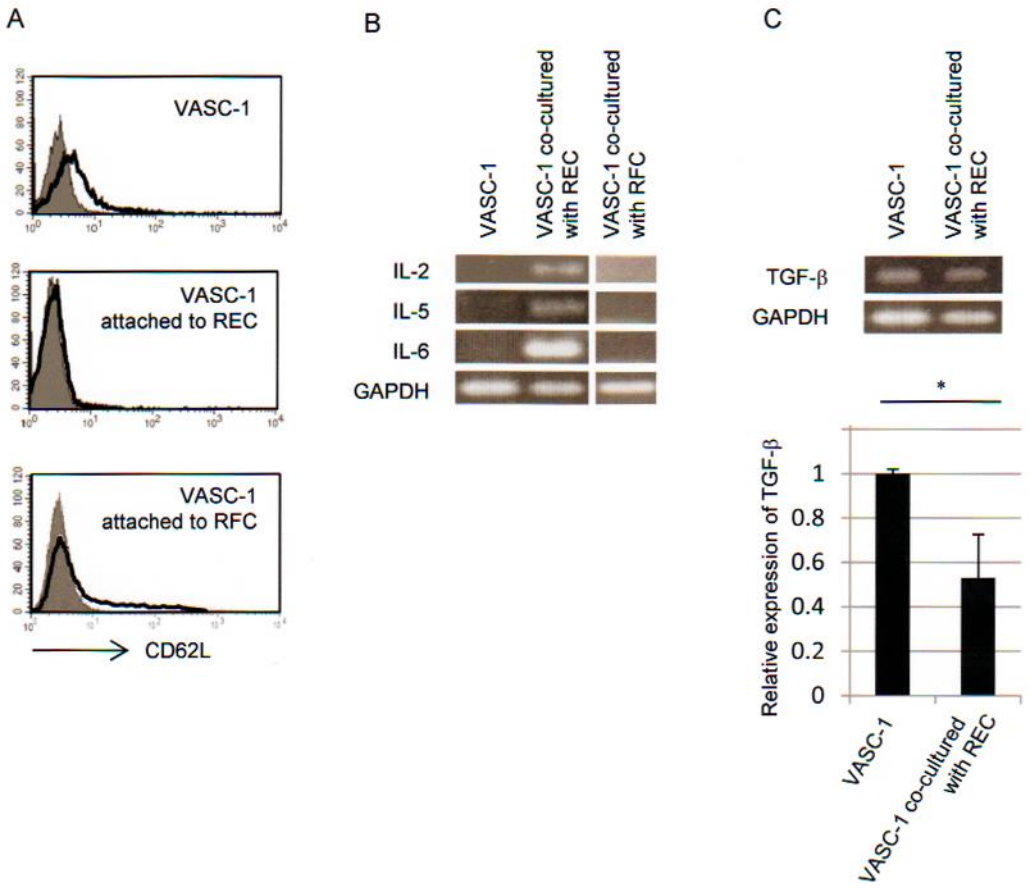


Figure 4. Activation of VASC-1 after interaction with REC

(A) VASC-1 (2×10^6) was co-cultured with REC or RFC (1×10^6) in IL-2-free culture medium. After incubation for 24 h at 37 ºC, adherent cells, including REC/RFC and VASC-1 attached to the REC/RFC, were subjected to examination of cell surface expression of CD62L by FCM. VASC-1 attached to REC/RFC was distinguished from the REC/RFC by the size of cells. Filled histograms represent the background staining of isotype-matched control. (B) Subsequently, total RNA was extracted from VASC-1 co-cultured with REC or RFC for examination of the mRNA expression of IL-2, IL-5, and IL-6 by RT-PCR. GAPDH was applied for quality control of RNA samples. (C) The alteration of TGF-β mRNA expression in VASC-1 after interaction with REC was determined by RT-PCR and real-time RT-PCR. GAPDH was applied for quality control of RNA samples. The expression in VASC-1 without interaction with REC was set as 1. *$p<0.05$.

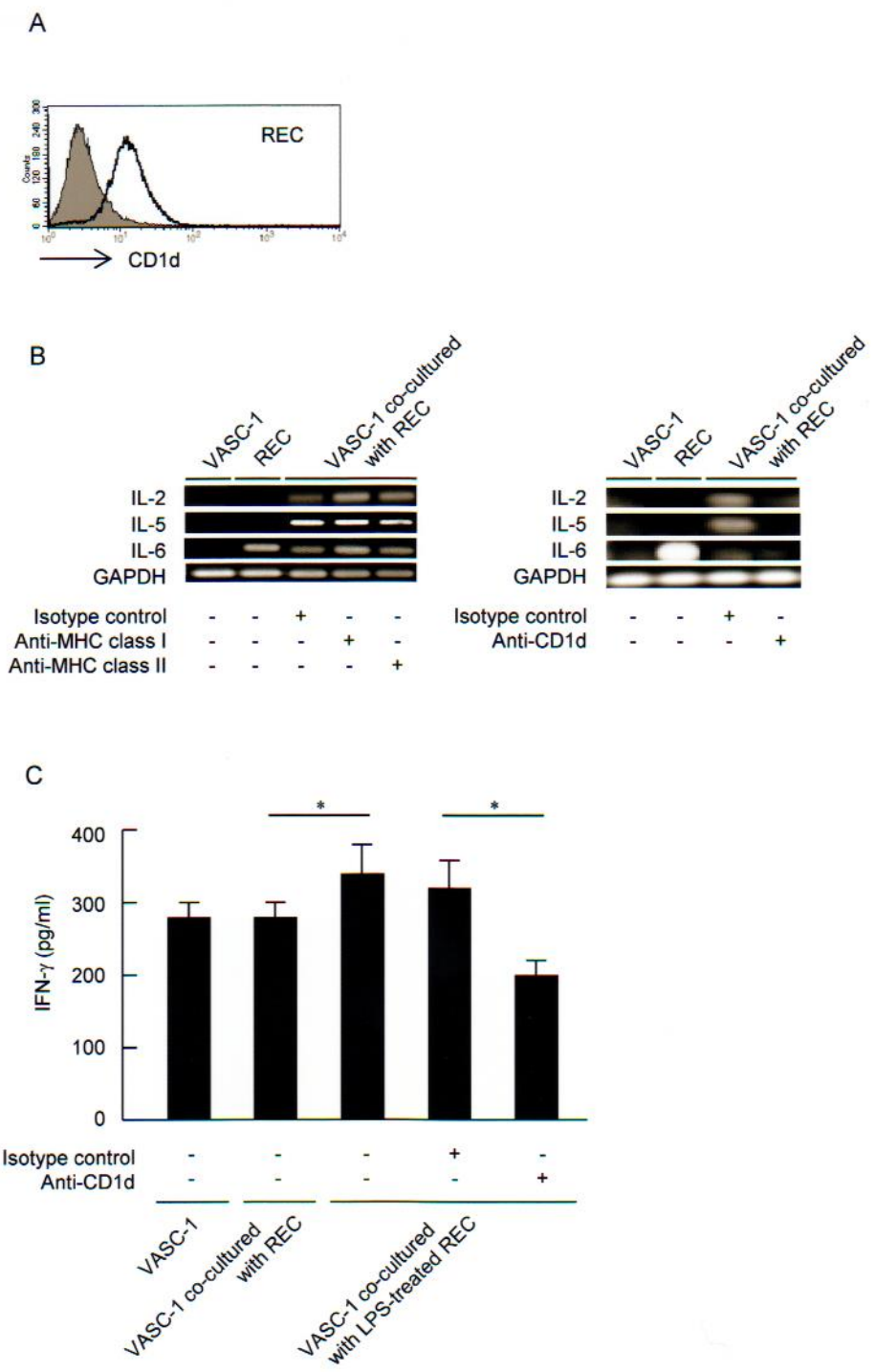


Figure 5. Involvement of CD1d on REC in antigen recognition by VASC-1

(A) Cell surface expression of CD1d on REC. Filled histogram represents the background staining of isotype-matched control. (B, C) Blocking assay. (B) The mRNA expression of IL-2, IL-5, and IL-6 examined by RT-PCR. REC (1×10^6) was treated with 5 μg of anti-MHC class I (OX18), anti-MHC class II (OX6), anti-CD1d Ab

(WTH1), or isotype-matched controls for 30 min at room temperature. VASC-1 (2×10^6) was then cultured with the pre-treated REC (1×10^6) for 24 h at 37 ºC in IL-2-free culture medium. Total RNA was extracted from VASC-1 co-cultured with the pre-treated REC, and then subjected to RT-PCR. For controls, each sample of VASC-1 and REC cultured alone was used. GAPDH was applied for quality control of RNA samples. (C) Concentrations of IFN-γ in culture supernatants. Prior to this experiment, REC (1×10^6) was treated with or without 1 μg/ml of LPS for 1 h at 37 ºC. Thereafter, REC (1×10^6) was allowed to react with 5 μg of anti-CD1d Ab (WTH1) or isotype-matched control for 30 min at room temperature. VASC-1 (2×10^6) was then cultured with the pre-treated REC (1×10^6) for 24 h at 37 ºC in IL-2-free culture medium. Culture supernatants were collected and then subjected to Luminex® analysis. Experiments were done in triplicate. Data are presented as mean ± SD. *$p<0.05$.

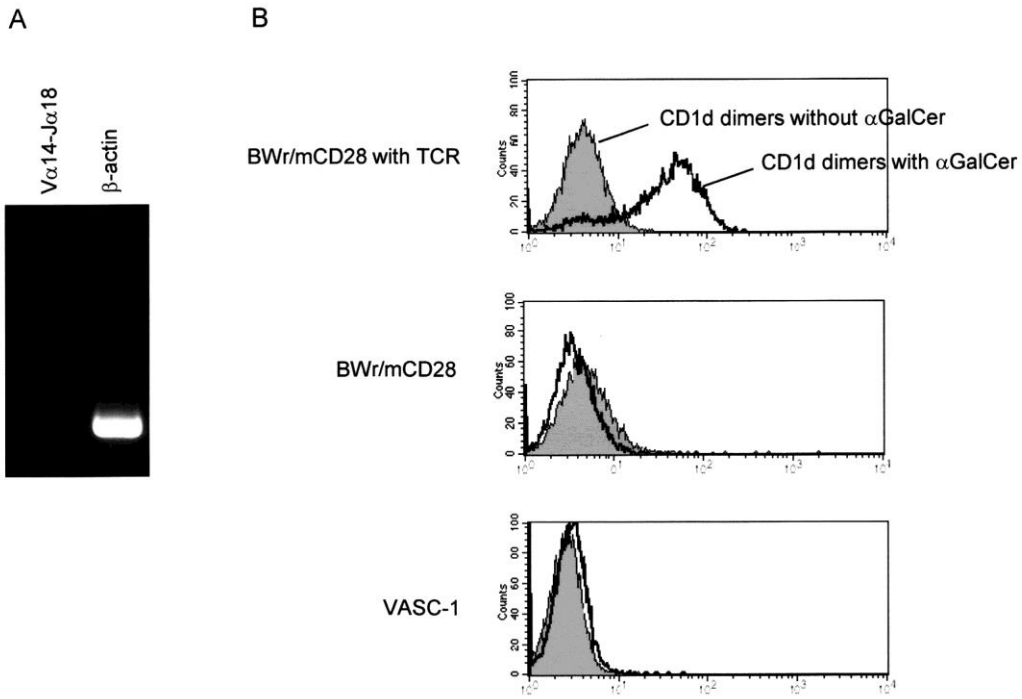


Figure 6. No usage of invariant TCR α chain of type I NKT cells and no binding of α-GalCer-loaded CD1d dimers of VASC-1

(A) Expression of the type I invariant TCR α chain (Vα14-Jα18) of VASC-1 examined by RT-PCR. β-actin was applied for quality control of RNA samples. (B) CD1d binding assay. Rat CD1d dimers connected with mouse IgG were used. Cells (1×10^6) were allowed to react with 1 μg of rat CD1d dimers loaded with or without α-GalCer for 30 min at room temperature followed by reaction to PE-labeled donkey anti-mouse IgG (H+L) $F(ab')_2$ fragment. After blocking the nonspecific binding of the secondary Ab by addition of mouse IgG in the samples, the cells were subjected to FCM. Filled histograms represent the background binding of CD1d dimers without α-GalCer.