Low levels of soluble CD1d protein alters NKT cell function in patients with rheumatoid arthritis

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Received April 6, 2009; Accepted May 15, 2009

DOI: 10.3892/ijmm_00000256

Abstract. CD1d molecules on the cell surface play a critical role in the presentation of glycolipid antigens to natural killer T (NKT) cells. We previously showed that the human CD1d gene has 8 splice variants, one of which is a soluble form lacking the ß2-m and transmembrane domains. This study focused on soluble CD1d (sCD1d) by generating recombinant sCD1d proteins and assaying them in plasma using a newly established ELISA method. The amount of sCD1d proteins in plasma was significantly decreased in rheumatoid arthritis (RA) patients (55.2±13.3 years, mean ±SD) compared with healthy donors (31.2±7.4 years). Plasma sCD1d protein levels correlated with the number of NKT cells (TCR Vα24$^+$ Vß11$^+$ CD3$^+$) in peripheral blood mononuclear cells (r^2=0.061). Furthermore, sCD1d proteins induced IFN-γ production from NKT cells, but neither IL-4 nor IL-10. These findings suggest that the low plasma levels of sCD1d protein in RA patients reduce the number and thus activation of peripheral NKT cells. It is therefore hypothesized that sCD1d stimulates NKT cells and low plasma sCD1d levels in RA reflect a pathogenic mechanism associated with a decrease in NKT cells.

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Abbreviations: APC, antigen-presenting cell; α-GalCer, α-galactosylceramide; CIA, collagen-induced arthritis; ELISA, enzyme-linked immunosorbent assay; FBS, fetal bovine serum; IFN, interferon; IL, interleukin; mAb, monoclonal antibody; MHC, major histocompatibility complex; NKT, natural killer T; PBS, phosphate-buffered saline; RA, rheumatoid arthritis; rh, recombinant human; sCD1d, soluble CD1d; SD, standard deviation; TCR, T cell receptor; Th, T helper; ß2-m, ß2-microglobulin

Key words: natural killer T cells, autoimmune disease, rheumatoid arthritis, CD1d, soluble CD1d, rheumatoid arthritis

Introduction

The CD1 family of molecules comprises nonpolymorphic major histocompatibility complex (MHC) class I-like proteins (1-3), characterized by a 43-49-kDa heavy chain in noncovalent association with a 12-kDa ß2-microglobulin (ß2-m) light chain. CD1 genes map to chromosome 1q22-23 (4) and are classified into two groups based on sequence homology, group 1 (CD1a, 1b, 1c, and 1e) and group 2 (CD1d) (5,6). Group 1 CD1 molecules mainly present lipid antigens to clonally diverse T cells to mediate adaptive immunity against a vast range of microbial lipid antigens. In contrast, CD1d (group 2) molecules are expressed on the surface of cortical thymocytes (7), B cells (8), dendritic cells (9-11), Langerhan's cells in the skin (12), and gastrointestinal epithelial cells (9,11). The soluble form of CD1d (sCD1d) presents glycolipid antigens to natural killer T (NKT) cells.

NKT cells express the surface marker, NKR-P1A (CD161), and a highly restricted T-cell antigen receptor (TCR) comprised of an invariant TCRα chain with a single rearrangement (TCR Vα14-Jα18 in mice, and TCR Vα24-Jα18 in humans) (13) coupled with TCRß chains with limited heterogeneity due to marked skewing of TCR Vß gene usage (mostly TCR Vß8.2 in mice and TCR Vß11 in humans) (14). NKT cells recognize glycosphingolipid α-galactosylceramide (α-GalCer), bind to CD1d, and respond by secreting a variety of cytokines, including Th1 cytokines such as IL-2 and IFN-γ, and Th2 cytokines such as IL-4 and IL-10 in humans or IL-17 in mice (15). This ability to potently modulate adaptive immunity upon stimulation of a restricted set of antigen-specific receptors, together with a lack of immunological memory, closely resembles the properties of cell types belonging to the innate immune system (16).

Human NKT cells are believed to regulate immune tolerance or autoimmunity (17). Indeed, the NKT cell number is selectively decreased in human autoimmune diseases, such as rheumatoid arthritis (RA), systemic sclerosis, systemic lupus erythematosus, Sjögren's syndrome, and type I diabetes mellitus (18-20).

We previously identified alternatively spliced variants of human CD1d mRNA in peripheral blood mononuclear cells (PBMCs) (21). Two of these, V1 and V2, were considered functional due to complete conservation of the antigen-binding site. V1 lacks exon 4 (ß2-m binding domain) of the CD1d gene, resulting in unstable antigen presentation, while V2 lacks both

exon 4 and 5 (transmembrane domain), resulting in sCD1d. The expression levels of sCD1d mRNA were significantly lower in RA patients than healthy donors, although there was no significant difference in the number of intact $CD1d^+$ cells in peripheral blood (22). The functional relevance of sCD1d protein, which is present in plasma, remains unclear. The present study was designed to determine the plasma sCD1d levels by developing a new two-sites enzyme-linked immunosorbent assay (ELISA) detection system. Preliminary testing with this method showed significantly low plasma sCD1d protein and sCD1d mRNA levels in PBMCs of RA patients compared with those of healthy donors. In addition, plasma levels of sCD1d protein correlated with the proportion of NKT cells among PBMCs. These findings implicate a role for sCD1d in stimulating NKT cell production. The relevant effects in RA are also discussed.

Materials and methods

Patients and healthy donors. We examined 52 patients with RA (age 55.2±13.3 years, mean ±SD) diagnosed according to the criteria of the American College of Rheumatology (ACR; formerly, the American Rheumatism Association). All patients and 40 disease-free healthy donors (31.2±7.4 years of age) were referred to the University of Tsukuba Hospital and gave their written consent for this study. The study was approved by the ethics committee of the university of Tsukuba Hospital.

Plasma and PBMC samples. The PBMCs of patients and healthy donors were isolated using Ficoll-Paque (GE Healthcare UK, Little Chalfont, UK) density-gradient centrifugation. The supernatant was recovered as plasma, and the pelleted PBMC fraction was ready for use after washing twice with phosphate-buffered saline (PBS).

Polyclonal antibody specific for sCD1d. Rabbits were injected every 2 weeks for a total of 5 times with sCD1d-specific C-terminal 14-mer (QDLWTSGSQDFSPG) peptides linked to a carrier protein (KLH). Whole blood was collected and the serum obtained.

Constructs and reagents. V1 CD1d and sCD1d cDNA were obtained from PBMCs as described previously (22). The PCR-products were subsequently digested with *Hind*III-*Not*I and cloned into pcDNA3.1 (Invitrogen, San Diego, CA), resulting in pcDNA3.1-sCD1d, -V1 CD1d, and -mock as a control. The cloned PCR-fragments were sequenced in both directions according to a standard protocol (ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit) and analyzed using an ABI PRISM 310 Genetic Analyzer (Applied Biosystems, Framingham, PA).

Production and purification of soluble CD1d proteins. Cos-7 cells ($5x10^5$) were grown on 10-cm tissue culture dishes (TPP) in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (BioWest, FL) and 1% penicillin/streptomycin (Invitrogen) at 37˚C with 5% CO_2. Plasmid DNA was transfected into Cos-7 cells using FuGeneHD transfection reagents (Roche, Basel, Switzerland), and the cells were cultured for 24 h before rinsing in PBS and lysis in the following buffer, 50 mM Na_2PO_4, 300 mM NaCl, 0.5% NP-40, and 2 mM phenylmethylsulfonyl fluoride (PMSF), pH 7.4. The supernatant obtained after centrifugation at 10,000 x g was used as purification sample.

Proteins were then purified using HiTrap NHS-activated HP affinity columns (GE Healthcare) according to the instructions provided by the manufacturer. The FLAG column prepared above was used for immunoaffinity purification of FLAG M2 antibody (Sigma, St. Louis, MO). The column was washed sequentially with start buffer (10 mM Tris-HC1, pH 7.5) and elution buffer (100 mM glycine, pH 2.5), and was finally equilibrated with start buffer. Purified samples were loaded onto the column. Bound proteins were eluted with 0.1 M glycine, pH 2.5, and the pH was brought to neutral by adding 0.1 volume of neutralizing buffer (1 M Tris-HC1, pH 8.0). After elution, the samples were concentrated and dialyzed against PBS using an Amicon Ultra with a 10-kDa cut-off (Millipore, Billerica, MA).

Immunoprecipitation and immunoblotting. sCD1d-transfected cells were lysed and subjected to immunoblotting (blot). Aliquots of the lysates were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to membranes (Bio-Rad, Hercules, CA). The membranes were subjected to blotting with anti-FLAG antibodies. The culture supernatants of sCD1d-transfected cells were subjected to immunoprecipitation (IP) with anti-CD1d monoclonal antibodies followed by adsorption to protein G Sepharose (Pharmacia Biotech, Uppsala, Sweden). The precipitates were immunoblotted with anti-FLAG antibodies.

Specific ELISA assay systems for soluble CD1d. A two-sites ELISA assay was established to detect and measure sCD1d. Anti-human CD1d monoclonal antibody (mAb; 0.1 μg/ml, Santa Cruz Biotechnology, Santa Cruz, CA) in PBS was added to wells of a plate (Nunc, Roskilde, Denmark) and incubated overnight at 4˚C. The wells were washed three times with a wash buffer (0.05% Tween-20 in PBS), and then blocked with commercial blocking buffer (Dainippon Sumitomo Seiyaku, Osaka, Japan) for 2 h at 37˚C. We then added 100 μl of a plasma sample diluted 1:5 in PBS and incubated overnight at room temperature. After washing three times with wash buffer, 100 μl of anti-sCD1d polyclonal antibody (prepared in-house) diluted to 1:1,000 in PBS was added to each well and incubated for 4 h at 37˚C. After washing three times with wash buffer, 100 μl of horseradish peroxidase (HRP)-labeled anti-rabbit antibody (Santa Cruz Biotechnology) diluted 1:3,000 in PBS, was added to each well, and incubated for 2 h at 37˚C. After final washing (6 times) with the wash buffer, 100 μl of substrate (Pierce, Rockford, IL) was added to each well, and left for 90 min. The optical density (OD) of each well was measured at 405 nm using a microplate reader (Bio-Rad).

Staining and flow cytometry. Fluorescein isothiocyanate (FITC)-labeled anti-TCR Vα24 and phycoerythrin (PE)-labeled anti-TCR Vß11 monoclonal antibodies were purchased from Beckmann Coulter (Fullerton, CA). The allophycocyanin (APC)-conjugated anti-CD3 monoclonal antibody (mAb) was obtained from BioLegend (San Diego, CA). The frequency of invariant NKT cells was estimated using three-color anti-TCR

Vα24/anti-TCR Vß11/anti-CD3 staining. The stained cells were analyzed on a CyAn ADP (DAKO, Glostrup, Denmark) and data were processed using Summit4.3 (DAKO).

Preparation of antigen presenting cells. PBMCs were isolated using Ficoll-Paque (GE Bioscience) density gradient centrifugation, and $CD14^+$ monocytes were harvested from the PBMCs using a MACS system (Miltenyi Biotec, Bergisch Gladbach, Germany). The $CD14^+$ monocytes were cultured for 6 days in complete RPMI 1640 (supplemented with 10% heat-inactivated fetal bovine serum (BioWest), 1% penicillin/streptomycin, 10 mM N-2-hydroxyetylpiperazine-N'-ethane-sulphonic acid (HEPES)-NaOH, 0.1 mM minimum essential medium (MEM) nonessential amino acids, 1 mM sodium pyruvate, and 5.5 μM 2-mercaptoethanol (Invitrogen) in the presence of 50 ng/ml recombinant human granulocyte-monocyte colony-stimulating factor (GM-CSF) and 100 ng/ml recombinant human IL-4 (R&D Systems, Minneapolis, IL) to obtain human monocyte-derived dendritic cells (Mo-DCs).

Expansion and sorting of TCR $V\alpha24^+$ $V\beta11^+$ NKT cells. PBMCs were isolated using Ficoll-Paque (GE Bioscience) density gradient centrifugation and cultured with 100 ng/ml α-galactosylceramide (α-GalCer, Krin Brewery, Gunma, Japan) and 100 ng/ml recombinant human IL-2 (rhIL-2, MBL, Woburn, MA) at a density of 10^6 cells/ml complete RPMI 1640. After 7 days, cells were restimulated with α-GalCer-pulsed Mo-DCs and co-cultured with 100 ng/ml rhIL-2. On day 7 after restimulation, $V\alpha24^+$ cells were isolated using a MACS system (Miltenyi Biotec). These isolated cells were then again restimulated with α-GalCer pulsed Mo-DCs and co-cultured with 100 ng/ml rhIL-2. The expanded NKT cells were stained with FITC-labeled anti-TCR Vα24 mAb, PE-labeled anti-TCR Vß11 mAb and APC-labeled anti-CD3 mAb. The $CD3^+$, $V\alpha24^+$, $V\beta11^+$ lymphocyte-gated cells were sorted on a MoFlo cell sorter (DAKO).

Stimulation of TCR $V\alpha24^+$ $V\beta11^+$ NKT cells by plate-bound CD1d dimer or sCD1d protein. Multiwell Plates were coated with CD1d dimer XI (1 μg in 100 μl PBS/well), purified sCD1d protein, or mock protein for 16 h. After washing with PBS, α-GalCer was added (0.1 ng/μl in PBS/well) and incubated at 37˚C for another 24 h. NKT cells were then added to the wells and cytokine production analyzed after a further 72 h.

Measurement of cytokines. The cytokine levels in the culture supernatants were evaluated by ELISA (R&D Systems).

Statistical analysis. Data are expressed as a median and mean ±SD. Data were analyzed using a statistical software package (Stat View 5.0, SAS Institute, NC). Differences between groups were examined for statistical significance using the Mann-Whitney U-test and Spearman's rank correlation. A P-value <0.05 denoted the presence of a statistically significant difference.

Results

Soluble CD1ds are expressed intra- and extracellularly. We reported previously the expression of sCD1d mRNA in PBMCs

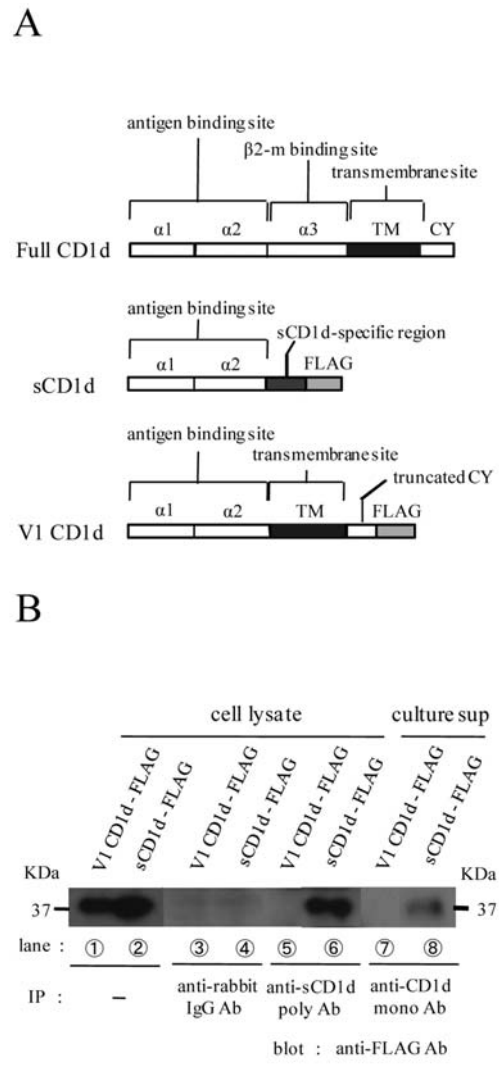

Figure 1. Expression of recombinant variant CD1d proteins. (A) Recombinant proteins of sCD1d and V1 CD1d were cloned into expression vectors, with FLAG tags expressed at the C-terminus for purification and detection. (B) Cos-7 cells were transfected to express V1 CD1d and sCD1d. After incubation for 24 h, cells were harvested to analyze the expression using anti-sCD1d polyclonal antibodies. We performed immunoprecipitation (IP) and immunoblotting (blot) analysis as indicated. Lanes 1-2, positive control; lanes 3-4, negative control; lanes 5-6, anti-sCD1d polyclonal antibodies specific for sCD1d protein; lanes 7-8. The secreted recombinant sCD1d was also detected in the Cos-7 cell culture supernatant. Only sCD1d molecules were detected and not other CD1d variant (V1) proteins.

(21). sCD1ds are characterized by defective alignment of the ß2-m binding and transmembrane domains, compared to other family members (21) (Fig. 1A). To examine whether sCD1d proteins are secreted, we expressed recombinant FLAG-tagged sCD1d in Cos-7 cells. Culture supernatants were immunoprecipitated using anti-CD1d monoclonal antibodies, and bound proteins detected by immunoblotting with anti-FLAG antibodies (Fig. 1B). The sCD1d protein was present in culture supernatants, although the V1 was not detected. V1 CD1d is considered insoluble, because the transmembrane domain unique to CD1d is completely conserved (Fig. 1A). These results indicate that sCD1d protein is secreted extracellularly and it is likely that the same mechanism applies *in vivo*.

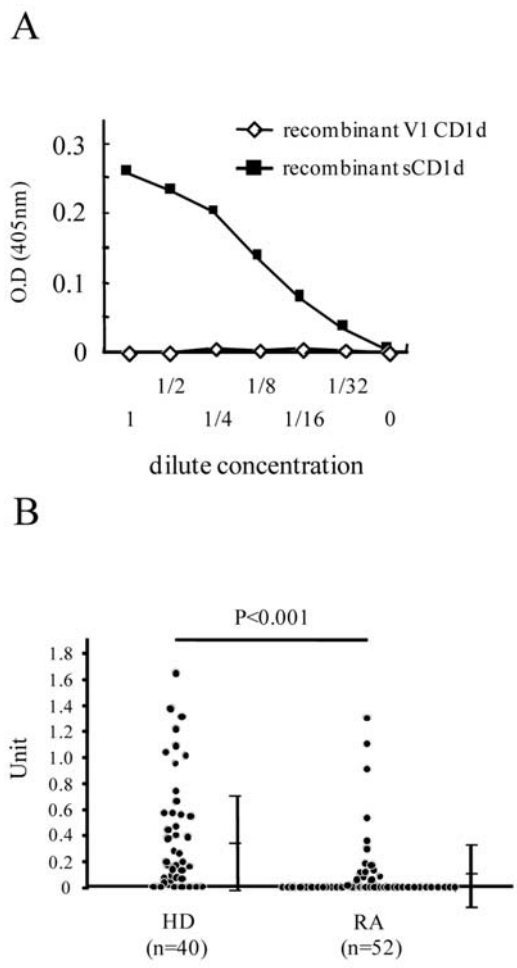

Figure 2. Establishment of sCD1d-specific ELISA using anti-CD1d monoclonal and anti-sCD1d polyclonal antibodies to reveal low sCD1d proteins in RA patients. (A) Purified recombinant V1 CD1d and sCD1d proteins as specifically measured by the developed ELISA system. (B) The levels of sCD1d proteins in plasma of healthy donors (n=40) and RA patients (n=52) determined by the sCD1d-specific ELISA. The level of secreted sCD1d protein in RA patients (0.10±0.29 U/ml) was significantly lower than in healthy donors (0.39±0.42 U/ml). Each point represents the sCD1d ratio (unit) from a specific healthy control. Comparison of median between different groups was performed using the Mann-Whitney U test.

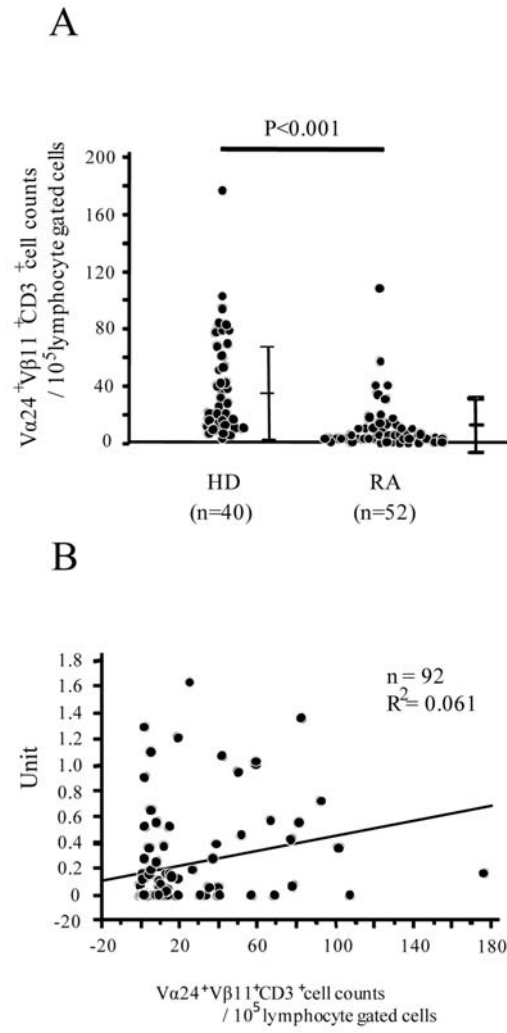

Figure 3. Correlation between sCD1d protein in plasma and NKT cells in PBMCs. (A) NKT cells among PBMCs from healthy donors (n=40) and RA patients (n=52) were stained with monoclonal antibodies, FITC-labeled anti-TCR Vα24 mAb, PE-labeled anti-TCR Vß11 mAb, and APC-labeled anti-CD3 mAb. The number of NKT cells in 10^5 PBMCs was counted. NKT cells were significantly fewer in number in RA patients (10.6±18.2 cells) compared with healthy donors (40.5±36.1 cells). Comparison of median values between different groups was performed using the Mann-Whitney U test. (B) Plasma sCD1d protein levels correlated with the number of NKT cells in PBMCs (r^2=0.061). Comparison of median values between different groups was performed using Spearman's rank correlation.

Patients with RA have low plasma levels of sCD1d. To examine the levels of sCD1d proteins in the peripheral blood of patients with RA, we established the sCD1d-specific ELISA. The specificity of sCD1d binding was confirmed by recombinant sCD1d and V1 CD1d proteins (Fig. 2A). Preliminary experiments indicated that detection of sCD1d protein was more sensitive in plasma compared with serum (data not shown). Therefore, plasma samples from 52 RA patients and 40 healthy donors were tested. The sCD1d protein concentrations in plasma samples of RA patients (0.10±0.29 U/ml) were significantly lower than those of healthy controls (0.39±0.42 U/ml) (Fig. 2B). Though there was a significant difference in age between healthy donors and RA patients, we confirmed age had no influence on sCD1d expression in plasma (data not shown).

Plasma levels of sCD1d protein correlated with number of TCR Vα24⁺ Vβ11⁺ NKT cells. We also determined the proportion of NKT cells in PBMCs from the same set of plasma samples. RA patients had significantly fewer NKT cells (10.6±18.2 cells) than healthy controls (40.5±36.1 cells) (Fig. 3A). Interestingly, the plasma levels of sCD1d protein correlated significantly with the number of NKT cells in peripheral blood, as we reported previously (20) (Fig. 3B). This result suggests that sCD1d stimulates and activates NKT cells.

α-GalCer-bound sCD1d protein stimulates TCR Vα24⁺ Vβ11⁺ NKT cells. To determine the functional significance of sCD1d, NKT cells were incubated with sCD1d and cytokine production was measured. NKT cells from healthy donors were expanded using α-GalCer (Fig. 4A), and then sorted to isolate TCR Vα24⁺ Vß11⁺ NKT. These cells were then cultured in the presence of recombinant sCD1d protein plus α-GalCer, or plate-bound CD1d dimer XI plus α-GalCer as a control. After incubation for 72 h, secreted IFN-γ, IL-4 and IL-10 were

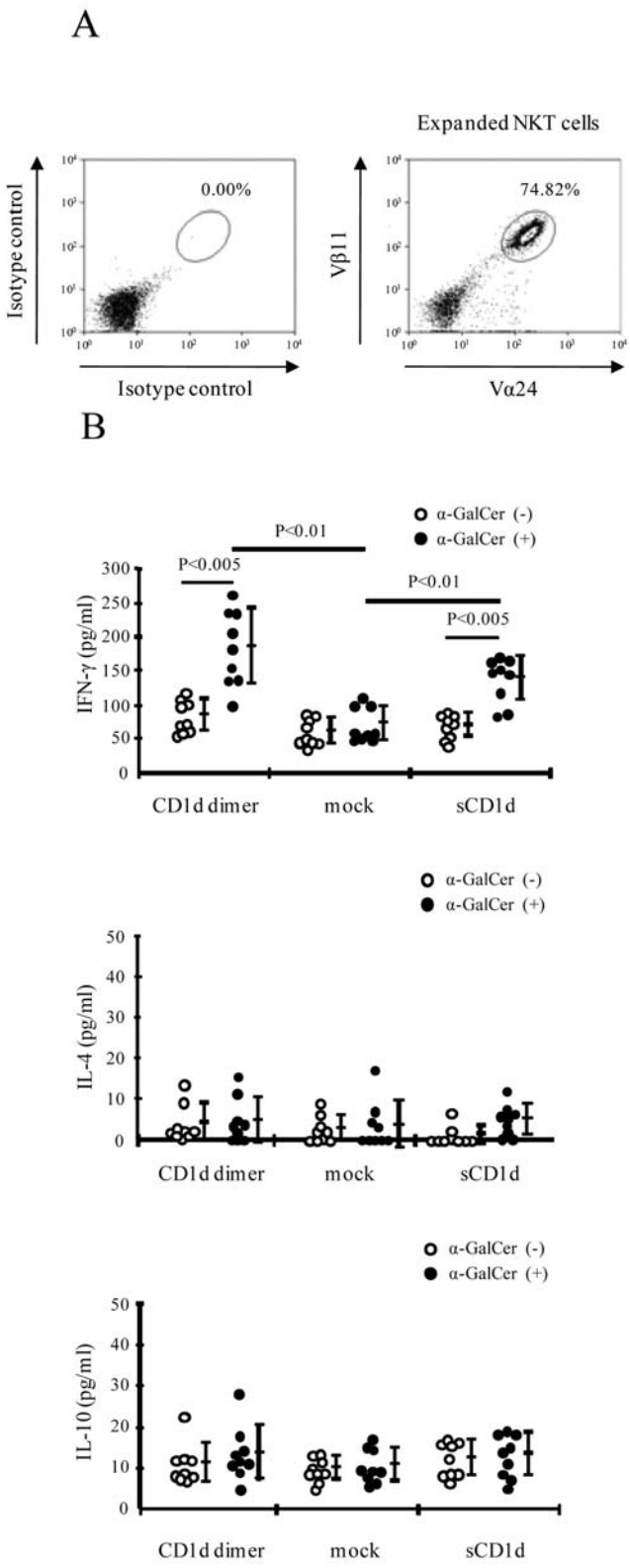

Figure 4. Stimulation of Vα24+ Vß11+ NKT cells by sCD1d protein. (A) NKT cells from healthy donors were expanded as described in Materials and methods. Expanded NKT cells were stained with FITC-labeled anti-TCR Vα24 mAb, PE-labeled anti-TCR Vß11 mAb, APC-labeled anti-CD3 mAb, and FITC + PE-labeled isotype control Ab. Values are the percentage of cells relative to the gated CD3+ cell population. (B) NKT cells from healthy donors (n=9) were expanded and sorted (CD3+Vα24+ Vß11+). Sorted NKT cells were stimulated by plate-bound CD1d dimer XI, mock proteins, or sCD1d proteins with (•) and without (○) α-GalCer. IFN-γ, IL-4 and IL-10 were assayed in culture supernatants after 72 h. The secretion of IFN-γ was increased in the presence of sCD1d plus α-GalCer. Comparison of median values between different groups was performed using Student's t-test.

measured by ELISA. Secretion of IFN-γ from NKT cells increased with sCD1d plus α-GalCer (138.5±33.1 pg/ml) compared with mock protein plus α-GalCer (73.53±17.36 pg/ml) ($p<0.01$) (Fig. 4B), whereas that of IL-4 and IL-10 did not (Fig. 4B).

Discussion

Human NKT cells are thought to regulate immune tolerance or autoimmunity (17), with autoimmune disease patients showing significantly fewer NKT cells than healthy controls (18-20). However, the mechanisms by which these cell numbers are reduced remain unknown. The current study reveals significantly less sCD1d protein in the plasma of a group of RA patients compared with healthy donors. Considering the demonstrated relationship between sCD1d proteins and NKT cell numbers, these findings implicate a role for sCD1d in NKT cell activation.

Overexpression experiments demonstrated that the sCD1d protein was indeed secreted into the extracellular medium, even if without the ß2-m binding domain. In support of this, others have shown CD1d expression on intestinal epithelial cells in ß2-m-deficient mice (23).

The results obtained here also imply that sCD1d stimulates NKT cells, by combining NKT cells with recombinant sCD1d proteins and measuring cytokine production. NKT cells were stimulated to produce IFN-γ in the presence of sCD1d mixed with lipids antigens (α-GalCer). Previous studies (24,25) demonstrated soluble HLA class I molecules (sHLAs) in sera from patients with RA, SLE, and multiple sclerosis. These soluble HLAs acted by binding to TCR on alloreactive T cells (26). It is therefore possible that sCD1d binds to TCR on NKT cells *in vivo*, stimulated by a natural ligand, and thus activates the NKT cells.

In an arthritis mouse model, Chiba *et al* (27) showed that *in vivo* activation of NKT cells using α-GalCer inhibited collagen-induced arthritis (CIA). Another study (28) also demonstrated NKT cell activation in α-GalCer-aggravated joint inflammation. The amount of lipid antigens present in these experiments were insufficient to suppress inflammations in the models used. The current study, however, strongly supports that the lipid antigens activated NKT cells not only via intact CD1d, but also via sCD1d *in vivo*. We speculate that the RA patients had decreased sCD1d protein secreted, resulting in reduced NKT cell numbers and thus activation.

Acknowledgements

We thank Dr F.G. Issa for a critical reading of the manuscript. This study was supported in part by the Grant-in-Aid for Scientific Research by the Japan Society for the Promotion of Science and the Japanese Ministry of Health, Labour and Welfare.

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