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

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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 Vα24$^+$ Vß11$^+$ 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, Vα24$^+$ 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$^+$, Vα24$^+$, Vß11$^+$ lymphocyte-gated cells were sorted on a MoFlo cell sorter (DAKO).

Stimulation of TCR Vα24$^+$ Vß11$^+$ 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

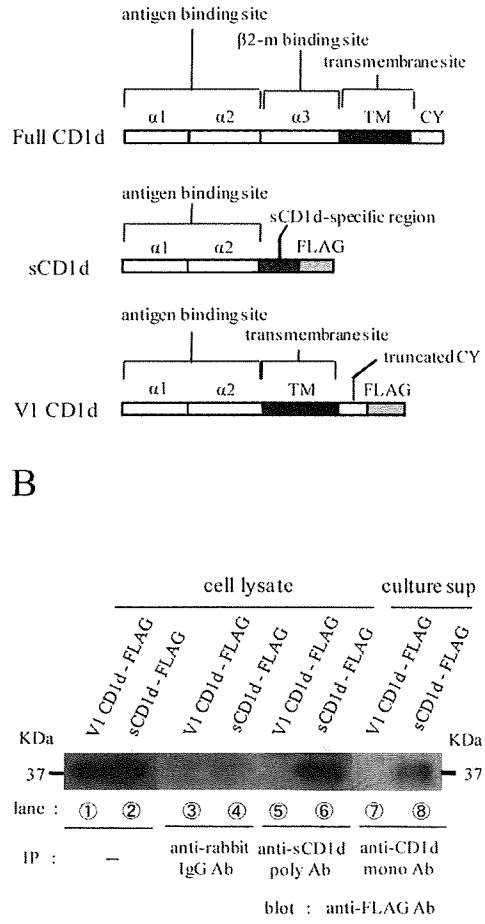

Figure 1. Expression of recombinant variant CD1d proteins. (A) Recombinant proteins of sCD1d and V1 CD1d were cloned into expression vectors, with FLAG tags expressed at the C-terminus for purification and detection. (B) Cos-7 cells were transfected to express V1 CD1d and sCD1d. After incubation for 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*.

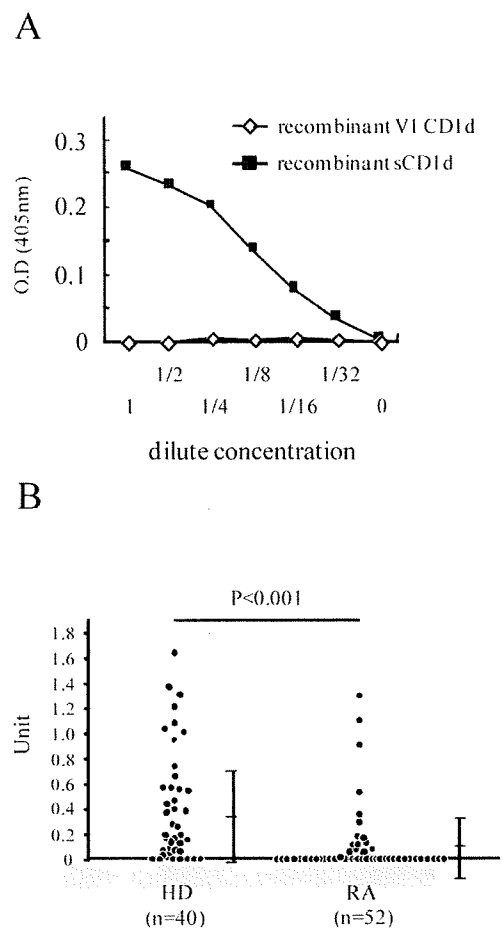

Figure 2. Establishment of sCD1d-specific ELISA using anti-CD1d monoclonal and anti-sCD1d polyclonal antibodies to reveal low sCD1d proteins in RA patients. (A) Purified recombinant V1 CD1d and sCD1d proteins as specifically measured by the developed ELISA system. (B) The levels of sCD1d proteins in 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.

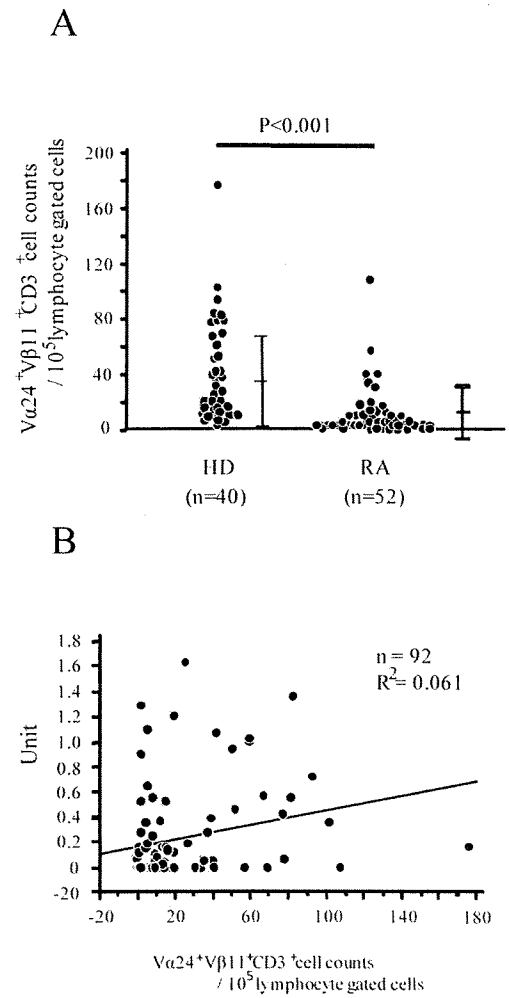

Figure 3. Correlation between sCD1d protein in plasma and NKT cells in PBMCs. (A) NKT cells among PBMCs from healthy donors (n=40) and RA patients (n=52) were stained with monoclonal antibodies, FITC-labeled anti-TCR Vα24 mAb, PE-labeled anti-TCR Vß11 mAb, and APC-labeled anti-CD3 mAb. The number of 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+ Vß11+ 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+ Vß11+ 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

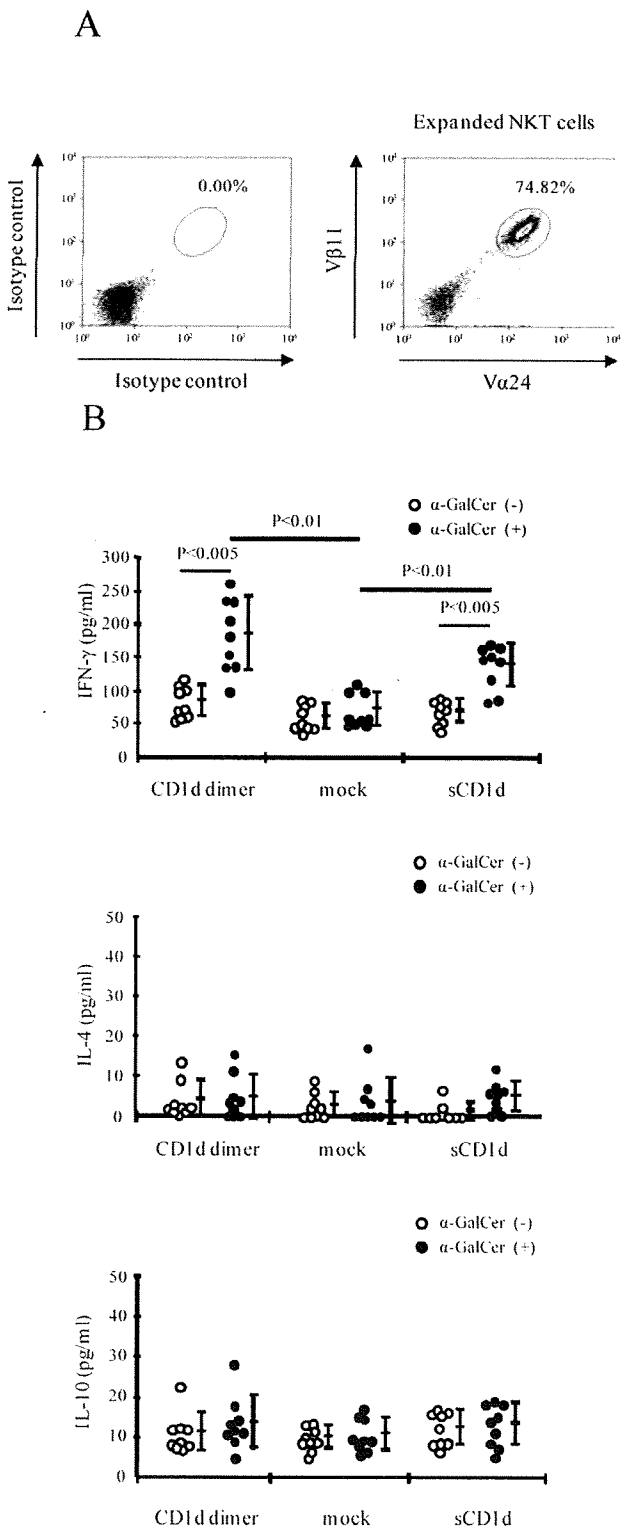

Figure 4. Stimulation of Vα24+ Vß11+ NKT cells by sCD1d protein. (A) NKT cells from healthy donors were expanded as described in Materials and methods. Expanded NKT cells were stained with FITC-labeled anti-TCR Vα24 mAb, PE-labeled anti-TCR Vß11 mAb, APC-labeled anti-CD3 mAb, and FITC + PE-labeled isotype 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.

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ORIGINAL ARTICLE

Efficacy of mizoribine pulse therapy in patients with rheumatoid arthritis who show a reduced or insufficient response to infliximab

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Abstract The efficacy of infliximab, a chimeric antibody against tumor necrosis factor-α used to treat patients with rheumatoid arthritis (RA), tends to decrease as patients develop human antichimeric antibody against infliximab (HACA). The clinical study reported here was designed to evaluate the efficacy of mizoribine (MZR) pulse therapy in patients who show a reduced or insufficient response to infliximab. Ten RA patients who had active arthritis despite infliximab therapy were treated with MZR pulse therapy at a dose of 100 mg MZR and methotrexate (MTX) and the disease activity assessed at baseline and at weeks 4–8, 12–16, and 20–24. The dose was increased to 150 mg in those patients who showed an insufficient response to MZR. The mean 28-joint disease activity score (DAS28) at weeks 12–16 and 20–24 of therapy was significantly lower than that at baseline. A moderate or good European League against Rheumatism (EULAR) response was achieved in seven patients (70%) at weeks 12–16 and in five patients (50%) at weeks 20–24. The dose of 150 mg MZR was effective in one of the three patients who showed an insufficient response to pulse therapy with 100 mg MZR. Based on these results, we propose that MZR pulse therapy should be attempted before the patient is switched to other biologics.

Keywords Infliximab · Mizoribine · Rheumatoid arthritis

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Introduction

Infliximab is a chimeric anti-tumor necrosis factor-alpha (TNF-α) monoclonal antibody that has proven effective in patients with rheumatoid arthritis (RA) [1–3]. In Japan, mizoribine (MZR; 4-carbamoyl-1-b-D-ribofuranosylimidazolium) is used as an immunosuppressive agent in patients undergoing renal transplantation and receiving treatment for RA and lupus nephritis in Japan. This drug inhibits inosine monophosphate dehydrogenase, a rate-limiting enzyme in the de novo pathway of nucleic acid synthesis, thereby inhibiting lymphocyte proliferation [4, 5]. Mizoribine, which is usually used at a daily dose of 75–150 mg administered in three separate doses, is known for its low rate of side effects [6], but it has been considered comparatively less effective than other disease-modifying antirheumatic drugs (DMARDs) in patients with RA. A correlation between the peak MZR blood concentration and clinical response to the therapy has been observed in patients with lupus nephritis [7]. It has also been recently shown that a drug therapeutic regimen consisting of 100–150 mg MZR once daily is more effective than the three divided doses because the achieved blood concentration was higher with the former [8]. Mizoribine pulse therapy has also been found to be effective in patients with RA who show an inadequate effect of methotrexate (MTX) [9–11]. In the case of MZR pulse therapy, patients receive MZR on one or two days of the week combined with MTX. The basis of this combination therapy is that MZR inhibits the synthesis of purines and MTX inhibits primarily the synthesis of pyrimidines; consequently, both drugs together inhibit the de novo pathway of nuclei acid synthesis. As such, the combined use of these two drugs is considered to inhibit lymphocyte proliferation more effectively than either used solely (monotherapy) [12].

Tokuda et al. [9] reported the efficacy of MZR pulse therapy as additional therapy for nine patients who showed an insufficient effect of MTX. Five of the nine patients responded to the combined MZR pulse + MTX drug therapeutic regimen within 20 weeks. Kohriyama et al. [10] and Murai et al. [11] also reported the efficacy of MZR pulse therapy as additional therapy in patients showing an inadequate effect of MTX.

An important problem associated with the use of infliximab in therapeutic drug regimens is that its efficacy often decreases during prolonged treatment. In Japan, the approved dose of infliximab is up to 3 mg/kg, or 200 mg/body, and that of MTX is up to 8 mg/week. Insufficient doses of these drugs may contribute to a decrease in the clinical efficacy of infliximab. The objective of this clinical study was to evaluate the efficacy of MZR pulse therapy in patients who show reduced or insufficient response to infliximab.

Patients and methods

Background of the patients

Ten RA patients treated with infliximab between 2005 and 2008 at Tsukuba University Hospital were enrolled in this study. Of these, eight showed a reduced response to infliximab (=high disease activity despite the clinical response to infliximab by week 30 of treatment), and two showed an insufficient response to infliximab (=no clinical response to infliximab during the 30-week treatment period). All ten patients fulfilled the American College of Rheumatology criteria for RA revised in 1987 [13]. They all had active arthritis as defined by the 28-joint disease activity score (DAS28) >3.2 at study entry, with the exception of one patient who wanted to receive MZR therapy despite a DAS28 of 3.0. The mean ± standard deviation (SD) age of the patients was 50.3 ± 12.8 years, the mean ± SD disease duration was 6.5 ± 6.2 years, and the mean ± SD DAS28 using erythrocyte sedimentation rate (ESR; DAS28-ESR) was 5.0 ± 1.5.

All patients were concomitantly receiving MTX, 6–8 mg/week. The doses of concomitant prednisolone and DMARDs, including MTX, were not increased during the last 3 months prior to entry in the study.

Study protocol and clinical response

This study was approved by the ethical committee of our hospital. Informed consent was obtained before the study. Patients first received 100 mg of MZR together with MTX. The patients received 300 mg MZR on the first two days of the week: 200 mg MZR on the first day in two divided doses and 100 mg MZR in one dose on the second day (Fig. 1). At the time of each infusion of infliximab, the swollen joint count (SJC), tender joint count (TJC), visual analogue score (VAS), ESR, and DAS28 were recorded. Five of the ten patients were started on MZR pulse therapy between the infusions of infliximab. We therefore we assessed the patients' DAS28 at baseline and after 12–16 weeks, 20–24 weeks and, thereafter, every 8 weeks.

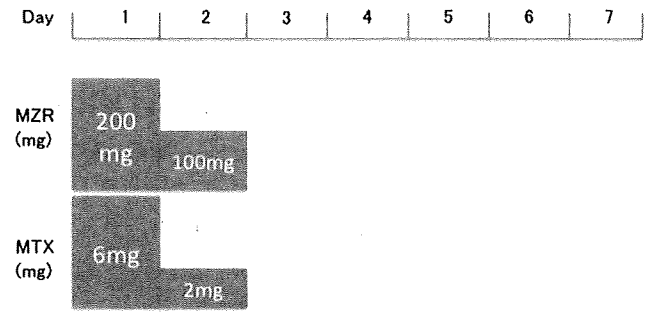

Fig. 1 The schedule of mizoribine (*MZR*) pulse therapy, consisting of combination drug therapy with 100 mg methotrexate (*MTX*). Total drug therapeutic program was 8 mg/week of MTX

Statistical analysis

We analyzed data using the Student's t test to assess whether the changes in DAS28 and laboratory data from baseline during the course of the treatment were significant.

Results

The clinical socio-demographic and clinical characteristics of the patients, including previously administered DMARDs (as well as those drugs continued during the study), response to MZR pulse therapy [according to the EULAR (European League Against Rheumatism) response criteria at weeks 12–16, and weeks 20–24], response to infliximab (according to the EULAR response criteria at week 30), and change in the dose of prednisolone (PSL) between baseline and week 24, are shown in Table 1.

All of the patients were followed for more than 24 weeks. The MZR pulse therapy was well tolerated, and none of the patients discontinued the therapy. Seven patients (70%) had achieved a moderate or good EULAR response at weeks 12–16, and five patients (50%) had achieved a moderate or good EULAR response at weeks 20–24,.

The mean DAS28 decreased from 5.0 at baseline to 3.9 ($P = 0.047$) at week 16, and 4.1 ($P = 0.043$) at week 24 (Fig. 2). The mean C-reactive protein (CRP) and ESR levels decreased, although not significantly during MZR pulse therapy.

Table 1 Clinical and socio-demographic characteristics of the patient cohort

Case no.	Sex	Age (years)	Duration of RA (years)	Stage[a]	Previous DMARDs[b]	Response to IFX[c]	Baseline DAS28[d]	Response to MZR[e]/ DAS28 (weeks 12–16)	Response to MZR/ DAS28 (weeks 20–24)	Dose of predonisolone
1	M	64	8	II	SASP BC	Moderate	7.9	Good 3.0	Moderate 4.5	No change
2	F	48	4	III	SASP BC	Good	4.0	Good 2.5	Good 2.5	No change
3	F	32	2.5	II	SASP BC	Good	4.8	Moderate 4.2	No 4.7	No change
4	F	41	1.5	I	SASP BC	Moderate	6.9	No 7.3	No 7.3	8 → 3 mg
5	F	55	2.5	II	SASP BC	No	5.2	Moderate 4.2	Moderate 4.3	8 → 7 mg
6	M	29	20	IV	SASP BC D-PC	No	4.1	Moderate 3.4	No 4.2	No change
7	F	65	2	I	SASP BC	Moderate	3.0	No 3.1	No 2.8	No change
8	M	59	1	I	SASP	Good	3.8	Moderate 2.9	No 3.3	No change
9	F	50	11	IV	SASP BC GST	Good	4.2	No 3.9	Good 2.7	10 → 8 mg
10	M	60	12	II	BC	Moderate	5.6	Moderate 4.3	Moderate 5.0	No change

RA rheumatoid arthritis, *IFX* infliximab, *MZR* mizoribine

[a] Steinbrocker stage of radiographs

[b] Disease-modifying antirheumatic drugs, including drugs continued during the study. *SASP* salazosulfapyridine, *BC* bucillamine, *D-PC* D-penicillamine, *GST* gold sodium thiomalate

[c] EULAR (European League Against Rheumatism) response criteria, at week 30

[d] DAS28-ESR, 28-joint disease activity score based on erythrocyte sedimentation rate

[e] EULAR response criteria

Three patients showed insufficient or reduced response to MZR pulse therapy after 24 weeks; we therefore increased the dose of MZR up to 150 mg in these patients. One of these patients showed a favorable response to the higher dose (case 2). None of the patients had an adverse reaction to the higher dose, not even a minor infection, nor were there any abnormalities in the laboratory data. A complete blood count, including white blood cells, neutrophils, lymphocytes, hemoglobin, and platelet counts, demonstrated the absence of any significant changes that could be related to MZR pulse therapy (Table 2).

Two successful cases of MZR pulse therapy are described below in detail.

Case 1 was a 48-year-old woman who had been successfully treated with 10 mg/kg of infliximab during a clinical trial for 54 weeks. Her DAS28 had been less than 2.6 during the trial, but infliximab therapy was stopped after the eighth infusion because the trial was finished. Thereafter, her disease activity increased, (DAS28 3.7), and infliximab therapy was restarted at a dose of 2.6 mg/kg (the maximum approved dose is 200 mg and her body weight was 77 kg; therefore, she was administered 2.6 mg/kg infliximab). However, her disease activity did not decrease despite three additional infusions of infliximab. We therefore considered that 2.6 mg/kg infliximab had limited efficacy in this patient and added MZR pulse therapy at a dose of 100 mg together with MTX. By 4 weeks after the iniation of the MZR pulse thereapy, her DAS28 had decreased to 2.4. At week 20 on MZR pulse therapy, she achieved a good EULAR response (Fig. 3).

Case 2 was a 64-year-old man whose disease had been successfully controlled with infliximab, but who showed an increase of the disease activity while still on this drug. We therefore added MZR pulse therapy at a dose of 100 mg together with MTX 4 weeks before the 19th infusion of infliximab. Twenty weeks later, his DAS28 had decreased to 3.0 ,and he had achieved a good EULAR response. Thereafter, his disease activity was under control for over 24 weeks. At the time of the 25th infusion of infliximab, his DAS28 was 5.4, and his disease activity had increased again. We then increased the dose of MZR to 150 mg. Eight weeks later, his DAS28 had decreased to 3.5 (Fig. 4).

This second case suggests that increasing the dose of MZR may be effective. The clinical response to MZR pulse therapy was most clearly observed in cases 1 and 2, probably because infliximab showed some degree of efficacy in these patients. In case 4, although the patient's DAS28 did not decrease until week 24 of treatment, MZR pulse therapy was considered to be clinically effective because we were able to decrease the dose of PSL from 8 to 3 mg.

Discussion

In Japan, infliximab has been used to treat RA patients since 2003. Although it efficacy in Japanese RA patients was demonstrated in the RECONFIRM study [14], the results of this study also indicated that the clinical response to infliximab may decline after 30 weeks of drug therapy.

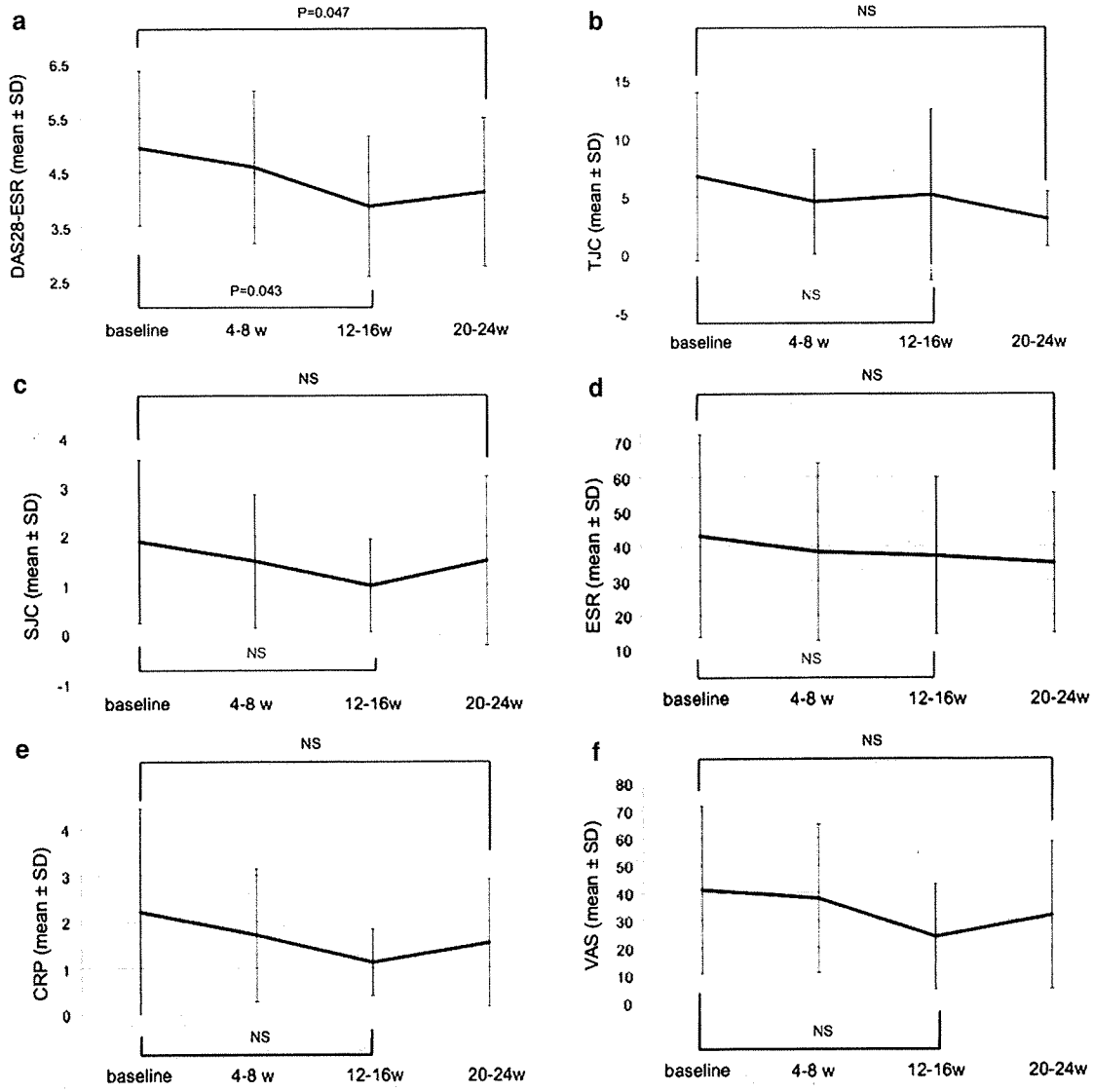

Fig. 2 **a** Changes in the 28-joint disease activity score (DAS28) from baseline and during the MZR pulse therapy regimen, at weeks 4–8, 12–16, and 20–24. The DAS28 significantly decreased at 12–16 weeks ($P = 0.047$) and at 20–24 weeks ($P = 0.043$). **b** Change in the tender joint count (*TJC*) at baseline and during therapy, at 4–8, 12–16, and 20–24 weeks. **c** Change in the swollen joint count (*SJC*) at baseline and during therapy, at 4–8, 12–16, and 20–24 weeks. **d** Change in the erythrocyte sedimentation rate (*ESR*) at baseline and during therapy, at 4–8, 12–16, and 20–24 weeks. **e** Change in the C-reactive protein (*CRP*) at baseline and during therapy, at 4–8, 12–16, and 20–24 weeks. **f** Change in the visual analog scale (*VAS*) at baseline and during therapy, at 4–8, 12–16, and 20–24 weeks. *NS* Not significant

This reduced effect of infliximab therapy in relation to the development of human antichimeric antibody against infliximab (HACA) has been reported in several studies [15, 16]. An increase of the dose of infliximab beyond 3 mg/kg (e.g., 5, 10 mg/kg) or the shortening of the interval between infliximab infusions (e.g., every 6 weeks) has proven to be effective in such cases [2, 17, 18]. However, these methods are not approved by the Japanese Ministry of Health, Labor and Welfare. Etanercept is another biological product available in Japan. Alternating anti-TNF therapies, such as switching between etanercept and infliximab, has been reported to be effective in patients who do not respond to their first anti-TNF drug [19, 20]. However, such switching of anti-TNF therapy was strictly limited in Japan because only two biologics were available when we started this study. Tocilizumab and adalimumab were approved in Japan in April 2008, thereby doubling the number of biologics that can be used to treat patients with RA (four); however, the choice of biologics is still limited because some patients refuse self-injection. In our opinion,

Table 2 Results of a complete blood count among the patient cohort at baseline and at 20–24 weeks after the initiation of MZR pulse therapy

Laboratory blood tests	Baseline	At weeks 20–24	*P*
WBC (/μl)	7410 ± 2100	7000 ± 1510	NS
Lymphocyte (/μl)	2050 ± 1690	1750 ± 1110	NS
Neutrocyte (/μl)	5030 ± 1870	4470 ± 940	NS
Hemoglobin (g/dl)	12.7 ± 1.1	12.6 ± 1.5	NS
Platelet count ($\times 10^4$/μl)	32.0 ± 8.0	33.3 ± 9.9	NS

Values are given as the mean ± standard deviation of white blood cell (WBC), lymphocyte, neutrocyte, hemoglobin, and platelet counts before (baseline) and 20–24 weeks after the initiation of MZR pulse therapy

NS not significant

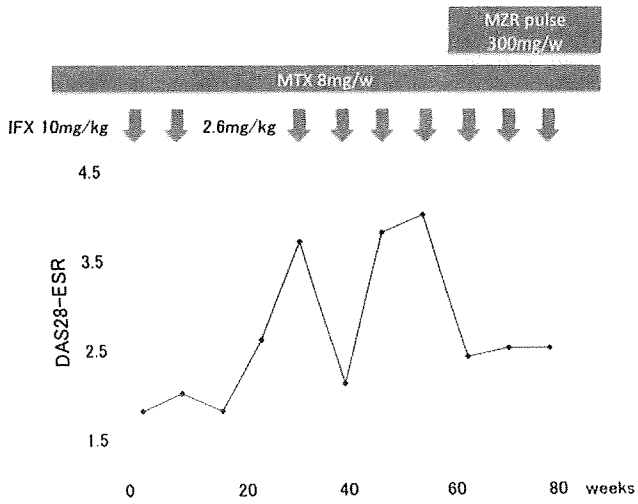

Fig. 3 Response to therapy by patient 1 (case 1). *IFX* Infliximab

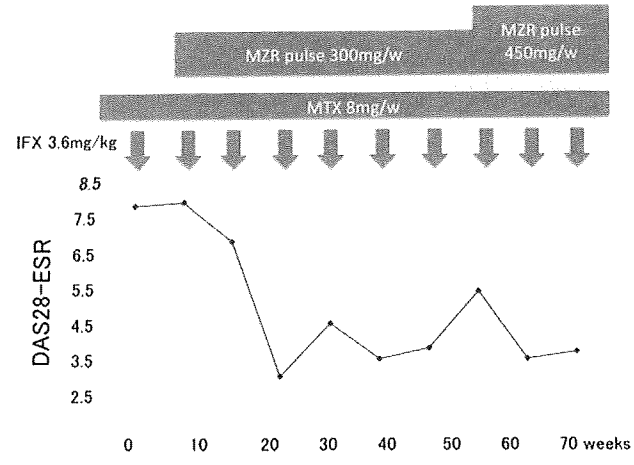

Fig. 4 Response to therapy by patient 2 (case 2)

it is better to use one biological agent as long as possible—and not a combination—because it is still unclear whether the other biologics decrease the effect.

The objective of this study was to evaluate the efficacy and safety of MZR pulse therapy in patients who showed a reduced or insufficient response to infliximab. We observed significant efficacy at weeks 12–16 and at weeks 20–24. The decrease in the number of patients who responded to the therapy at weeks 20–24 (relative to weeks 12–16) may suggest a decline in the response of our patients to MZR pulse therapy. In this situation, a higher dose of MZR combined with MTX may be effective, as shown in case 1. Further studies are needed to confirm the efficacy of the higher dose of MZR.

The response rate using MZR pulse therapy that we obtained in this study in patients who showed a reduced response to infliximab (70%; 7/10 patients with a moderate or good EULAR score) was higher than that reported in previous studies using MZR pulse therapy (16–50%) in patients who showed inadequate response to MTX (without infliximab) [9–11]. This difference in response rate suggests that MZR pulse therapy may have some additional effect other than that as a DMARD. Although we could not measure anti-infliximab antibody levels, it would appear that MZR pulse therapy administered concomitantly with infliximab, in addition to its effect as a DMARD, also inhibits HACA.

In conclusion, in our small study cohort of patients with RA, MZR pulse therapy proved to be effective in patients who showed a reduced response to infliximab. We suggest that, in cases where infliximab is ineffective, MZR pulse therapy should be attempted before the patient is switched to another biologic.

Conflict of interest statement None.

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Overexpression of T-bet in T cells accelerates autoimmune glomerulonephritis in mice with a dominant Th1 background

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ABSTRACT

***Background*: An imbalance of T helper 1 (Th1) / Th2 is thought to contribute to the pathogenesis of autoimmune diseases. The differentiation of T cells into Th1 or Th2 subtypes is under the regulation of several transcription factors. Among these, transcription factor T-bet has been demonstrated to play an important role in Th1 cell differentiation.**

***Methods*: To examine how Th1/Th2 imbalance affects the development of autoimmune disease, we overexpressed T-bet in the T lymphocytes of C57BL/6 x BXSB/MpJ-*Yaa* F1 (Yaa) mice.**

***Results*: Yaa mice developed autoimmune nephritis similarly to BXSB/MpJ-*Yaa* mice, which are commonly used as a model for the Th1-dominant murine lupus. T-bet overexpression in T cells aggravated the 50% mortality of T-bet transgenic Yaa mice relative to Yaa mice, and increased proteinuria, the severity of glomerulonephritis in T-bet transgenic Yaa mice. T-bet overexpression in Yaa mice led simultaneously to an elevated ratio of Th1/Th2 immunoglobulin (IgG2a/IgG1). Intracellular cytokine analysis showed that IL-4 and IL-5 was suppressed, and IFN-γ was induced in stimulated T cells from the T-bet transgenic Yaa mice.**

***Conclusions*: These results indicate that T-bet stimulated the differentiation of Th1 cells and accelerated the disease severity in Yaa mice. These results suggest that Th1/Th2 imbalance contributes to the glomerulonephritis severity in Yaa mice, and Th1/Th2 transcriptional regulation is important for autoimmune glomerulonephritis.**

Key words: *Autoimmunity, Mice, Th1 cells, Th2 cells, Transcription factors*

INTRODUCTION

$CD4^+$ T helper (Th) cells have an essential role in the immune system to respond to antigen stimulations and to secrete concentrated cytokines. In 1986, Mosman et al proposed that helper T cells comprise 2 subsets: Th1 cells and Th2 cells (1). Th1 cells mainly produce IL-2 and IFN-γ, whereas Th2 cells produce IL-4, IL-5, IL-6, IL-10 and IL-13. An imbalance in the relative levels of Th1 cells and Th2 cells is thought to be involved in the pathogenesis of certain diseases (2-6). In general, a Th1-dominant condition is thought to be responsible for the pathogenesis of organ-specific autoimmune diseases. On the other hand, a Th2-dominant condition is associated with allergic disease.

The transcription factor T-bet, a member of the T-box family, is thought to be a key regulator of Th1 cell differentiation (7), whereas GATA-3 is thought to be the master regulator of Th2 cell differentiation (8-14). T-bet–deficient mice exhibit impaired IFN-γ production by $CD4^+$ T cells and fail to mount Th1 immune responses (7), and retroviral expression of T-bet in polarized Th2 cells promotes IFN-γ production and represses IL-4 and IL-5 production (15). These results showed that T-bet plays a critical role in Th1 differentiation.

BXSB/MpJ-*Yaa* mice develop autoimmune glomerulonephritis that is accelerated by the *Yaa* (Y chromosome-linked autoimmune acceleration) gene (16, 17). Glomerulonephritis in BXSB/MpJ-*Yaa* mice is characterized by production of autoantibodies and immune complex deposition in glomeruli, and a skewing of the Th1 response is thought to be a factor contributing to the pathogenesis in BXSB/MpJ-*Yaa* mice (16-18).

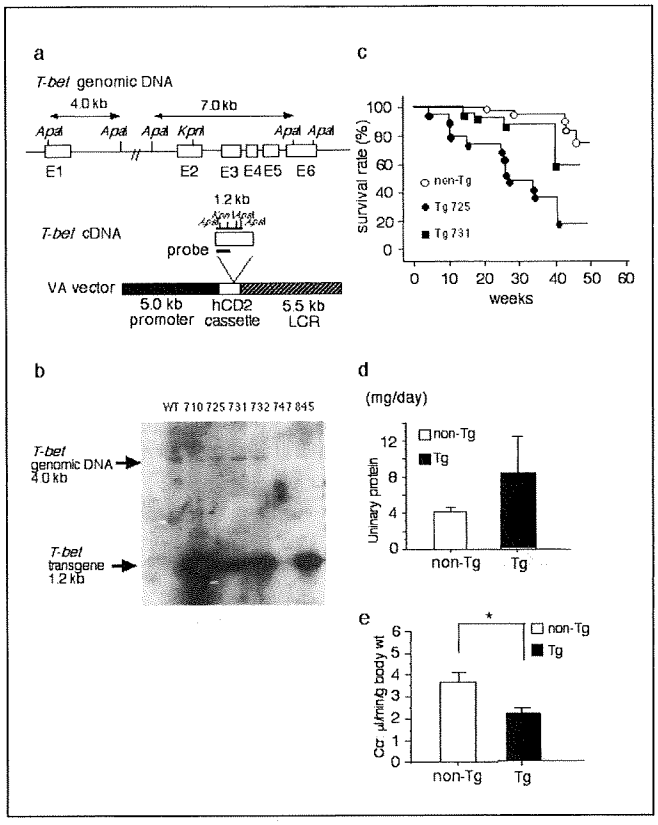

Fig. 1 - Characteristics of T-bet transgenic mice. a) Schematic diagram of the T-bet Tg construct. T-bet Tg was inserted into a VA vector containing a human CD2 cassette. b) Southern blot analysis of the T-bet genomic DNA and T-bet transgene. In this study, lines 725 and 731 were used for analysis. c) Survival rate of T-bet transgenic Yaa (Tg) mice. There was a significant difference between Tg 725 and non-Tg mice ($p<0.01$). d) Urinary protein excretion (mg/day) in Tg mice (n=7) and non-Tg mice (n=12). e) Creatinine clearance (μL/min per g body weight) in Tg mice (n=7) and non-Tg mice (n=12). Tg mice showed a significant decrease; *$p<0.05$. Each column represents the mean + SEM.

In this study, we generated T-bet–overexpressing BXSB/MpJ-*Yaa* F_1 transgenic mice (T-bet transgenic Yaa mice) to investigate the contribution of a Th1-dominant condition to the pathogenesis of BXSB/MpJ-*Yaa* mice. We show exacerbation of the autoimmune glomerulonephritis in these transgenic mice.

SUBJECTS AND METHODS

Generation of T-bet transgenic mice

The generation of T-bet transgenic mice has been previously described (19). The construct of T-bet transgene and Southern blot analysis are shown in Figure 1a,b. We generated 6 T-bet transgenic lines, but the transgenes were only stably transmitted to progeny in line 725 and line 731. Estimated copy numbers of transgenes for line 725 and line 731 were 12 and 8 copies, respectively. These mice were intercrossed with BXSB/MpJ-*Yaa* mice. Only F1 male mice were used for this study. Mice were maintained in specific pathogen-free conditions in a laboratory animal resource center. All experiments were performed according to the guide for the care and use of laboratory animals at the University of Tsukuba, and the study was approved by the institutional review board.

Measurement of urinary protein and creatinine clearance

The urine of each mouse at 30 weeks of age was collected in an individual metabolic cage over a 24-hour period. Proteinuria was assessed by measuring the turbidity obtained with 3% sulphosalicylic acid. The concentrations of serum and urinary creatinine were measured by an automated analyzer for routine laboratory test (Synchron CX3; Beckman Coulter, Fullerton, CA, USA).

Histopathological analyses of renal tissues

Organs were fixed with 10% formalin in 0.01 M phosphate buffer (pH 7.2) and embedded in paraffin. Sections (3 mm) were stained with hematoxylin and eosin for histopathological examinations under light microscopy. For semi-quantitative histological analysis, 20 glomeruli from 3 kidney sections were examined based on the severity and extent of changes estimated on a 0 to 3 scale. The scale is described in Figure 2d (20). Sections frozen for immunofluorescence analyses were stained with fluorescein isothiocyanate (FITC)-labeled anti-mouse immunoglobulin (ICN Pharmaceuticals, Cleveland, OH, USA).

Enzyme-linked immunosorbent assay (ELISA)

Nunc-Immunoplates (Nunc A/S, Roskilde, Denmark) were coated with goat anti-mouse immunoglobulin (ICN Pharmaceuticals, Aurora, OH, USA). The plates were kept at room temperature for 1 hour and then washed with 0.1 mol/L phosphate-buffered saline (PBS). After washing, the plates were blocked with 0.5% bovine serum albumin (BSA) in PBS solution. Serial dilutions of test serum sam-

ples were applied and incubated at room temperature for 1 hour. After washing with PBS, the plates were treated with alkaline phosphatase–conjugated goat anti-mouse IgG, IgG1 or IgG2a (Sigma) at room temperature for 1 hour. After additional washes, alkaline phosphatase substrate (Sigma) solution was added and allowed to develop. Absorption at 405 nm was measured with an immuno-plate reader (BenchMark; Bio-Rad, Hercules, CA, USA). IgG3 was measured by using the Mouse IgG3 ELISA Quantitation Kit (Bethyl Laboratories, Montgomery, TX, USA).

Culture medium, cytokines and antibodies

RPMI 1640 medium supplemented with 10% fetal calf serum, 2-mercaptoethanol (0.05 mM), L-glutamine (2 mM), penicillin (100 U/mL), streptomycin (100 mg/mL), HEPES buffer (10 mM) and sodium pyruvate (1 mM) was used as culture medium. Recombinant mouse cytokines were IL-2 (Genzyme-Techne, Minneapolis, MN, USA), IL-4 (BD PharMingen, San Diego, CA, USA) and IL-12 (BD PharMingen). Purified rat anti-mouse IL-4 (11B11), IL-12 (C17.8), CD3e (145-2C11) and CD28 (37.51) mAb, phycoerythrin-conjugated anti-mouse IL-4 (11B11), phycoerythrin-conjugated anti-mouse IL-5 (TRFK5) and FITC-conjugated anti-mouse IFN-γ (XMG1.2) were purchased from BD PharMingen.

Flow cytometric analysis of intracellular cytokines synthesis

$CD4^+$ T cells were prepared from each mouse spleen and were enriched by positive selection, using a magnetically activated cell sorting system with anti-CD4 mAb (Miltenyi Biotec, Bergisch Gladbach, Germany). Primary stimulations of $CD4^+$ T cells (2.5×10^5 cells/well) were performed with crosslinked anti-CD3ε (1 μg/mL) and anti-CD28 (10 μg/mL) plus IL-2 (10 ng/mL), in a total volume of 2 mL, in 24-well plates. In addition, some cultures received cytokines (10 ng/mL IL-4 or 10 ng/mL IL-12) or mAb to block endogenous cytokines (10 μg/mL anti-IL-4 or 10 μg/mL anti-IL-12). T cells were expanded and maintained under constant culture conditions. After a week of culture, cells were stimulated with phorbol myristate acetate (50 ng/mL) plus ionomycin (500 ng/mL). Two hours before cell harvesting, brefeldin A was added at 10 μg/mL, using a stock solution of 1 mg/mL in ethanol (100%). After fixation for 20 minutes at room temperature, cells were stained for cytokines. For intracellular staining, all reagents and washes contained 1% BSA and 0.5% saponin, and all incubations were performed at room temperature. Cells were washed and preincubated for 10 minutes in PBS/BSA/saponin and then incubated with phycoerythrin-conjugated anti-mouse IL-4 (5 μg/mL), allophycocyanin-conjugated anti-mouse IL-5 (5 μg/mL) and anti-mouse IFN-γ (5 μg/mL), or isotype-matched control antibodies (10 μg/mL), for 30 minutes. After 20 minutes, cells were washed with PBS/BSA/saponin and were then washed with PBS/BSA without saponin, to allow membrane closure. Samples were analyzed with a FACS Calibur flow cytometer (Becton Dickinson, Mountain View, CA, USA). Results were analyzed using CellQuest software.

Statistical analyses

All data are expressed as means ± SEM. Multiple data comparisons were performed by using 1-way ANOVA. A p value of <0.05 was considered statistically significant. Comparisons of survival rates were performed with the Kaplan-Meier method, with differences in survival curves being evaluated with log-rank sum testing.

RESULTS

Shorter life span of Yaa mice overexpressing T-bet

BSXB/MpJ-*Yaa* mice developed autoimmune glomerulonephritis and died due to renal failure. First, we compared the survival rate of T-bet transgenic mice and their transgene-negative (non-Tg) littermates by a Kaplan-Meier plot with a subsequent log-rank test. T-bet transgenic Yaa mice Tg 725 and Tg 731 demonstrated a shorter life span than non-Tg mice (Fig. 1c). In our previous report (19), we showed that Tg 725 had greater overexpression of T-bet than Tg 731 mice. In particular, the Tg 725 T-bet–transgenic group had a significantly lower survival rate than non-Tg mice ($p<0.001$). Tg 725 mice were used in the following studies.

T-bet transgenic Yaa mice demonstrated more severe deterioration of renal function

To investigate the reason behind the acceleration of mortality, we measured urinary protein excretion and creatinine clearance. The level of urinary protein excretion in T-bet transgenic Yaa mice was higher than that in non-Tg mice, although this difference did not reach statistical significance (Fig. 1d). The creatinine clearance in T-bet trans-

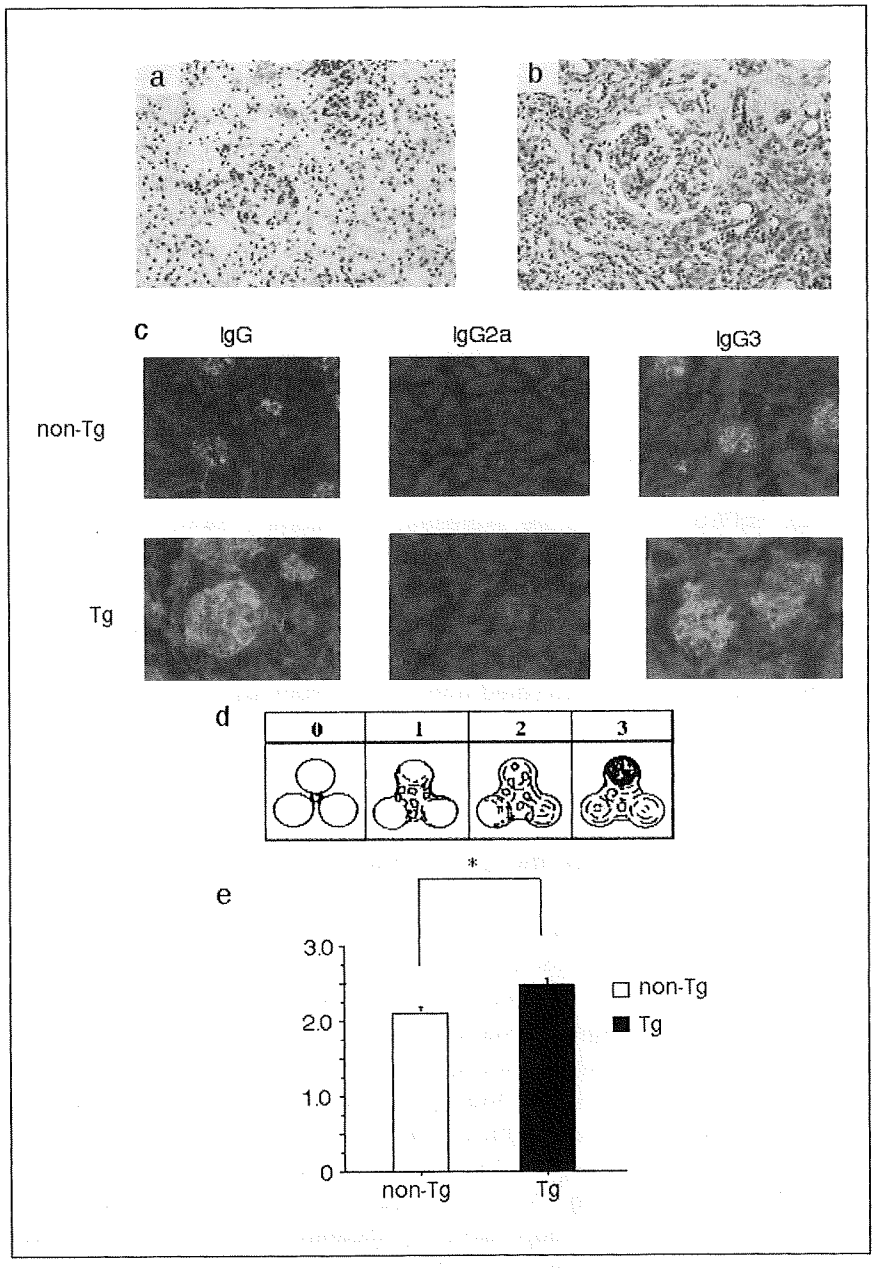

Fig. 2 - Histopathological analysis of renal tissue in T-bet transgenic Yaa (Tg) mice. a) Appearance of glomeruli from 30-week-old non-Tg mice with the periodic acid–Schiff (PAS) stain reaction; magnification x200. b) Appearance of glomeruli from 30-week-old T-bet transgenic Yaa mice with the PAS stain reaction; magnification x200. More severe cellular proliferation, mesangial matrix expansion and increased glomerular size were observed. c) Immunofluorescence staining of IgG, IgG2a and IgG3 in non-Tg and T-bet transgenic Yaa mice. IgG, IgG2a and IgG3 deposits were prominent in T-bet transgenic Yaa mice compared with non-Tg mice. d) Scale for measuring glomerular lesions: degree of severity was calculated on a 0 to 3 scale. e) Semiquantitative analysis of glomerular lesions of T-bet Tg mice and non-Tg mice. T-bet Tg mice showed significantly more severe glomerular lesions than non-Tg mice; values represent means + SEM; *p<0.05.

genic Yaa mice was significantly lower than that in non-Tg mice at 30 weeks of age ($p<0.05$) (Fig. 1e). These results indicated that deterioration of renal function had occurred in T-bet transgenic Yaa mice.

Exacerbation of glomerulonephritis in T-bet transgenic Yaa mice

Next, we analyzed the histopathological changes in the glomerulonephritis of both T-bet transgenic Yaa mice and non-Tg mice. As shown in Figure 2a,b, transgenic mice showed a significantly more severe phenotype, and increased cellular proliferation, mesangial matrix accumulation and lobular formation than that observed in the non-Tg mice at 30 weeks of age (Fig. 2a, b, e). Immunofluorescence staining revealed IgG and IgG3 deposits in the mesangial regions of both T-bet transgenic mice and non-Tg mice, but more prominent deposits were observed in T-bet transgenic mice (Fig. 2c).

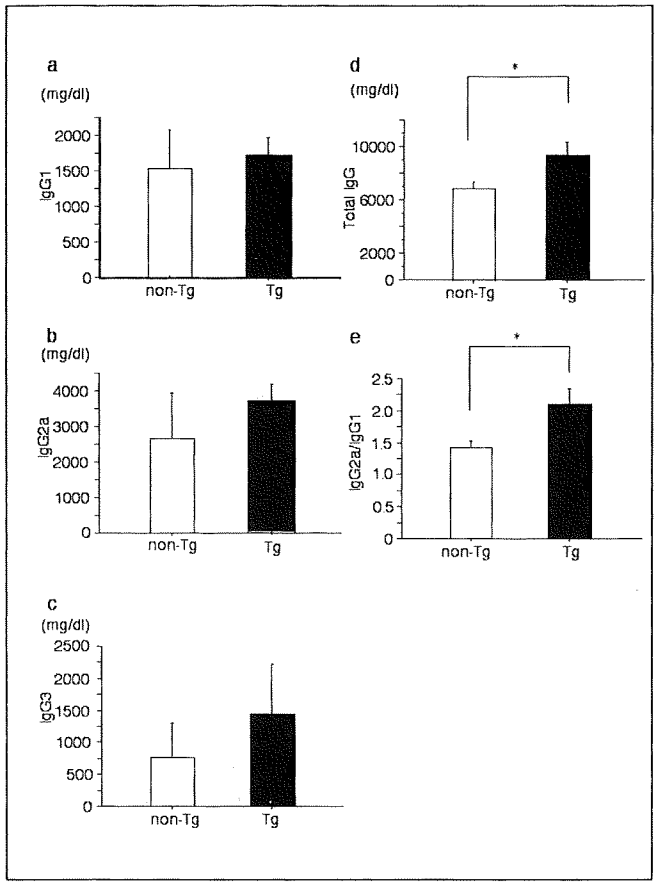

Fig. 3 - Serum immunoglobulin levels in T-bet transgenic Yaa (Tg) mice. a-d) Serum IgG1, IgG2a, IgG3 and total IgG levels were measured in non-Tg mice (n=7) and T-bet transgenic Yaa mice (n=8) at 30 weeks of age. The total IgG level of T-bet transgenic Yaa mice was significantly higher than non-Tg mice. e) The IgG2a/IgG1 ratio was significantly higher in T-bet transgenic Yaa mice than in non-Tg mice. Values represent means + SEM; *p<0.05.

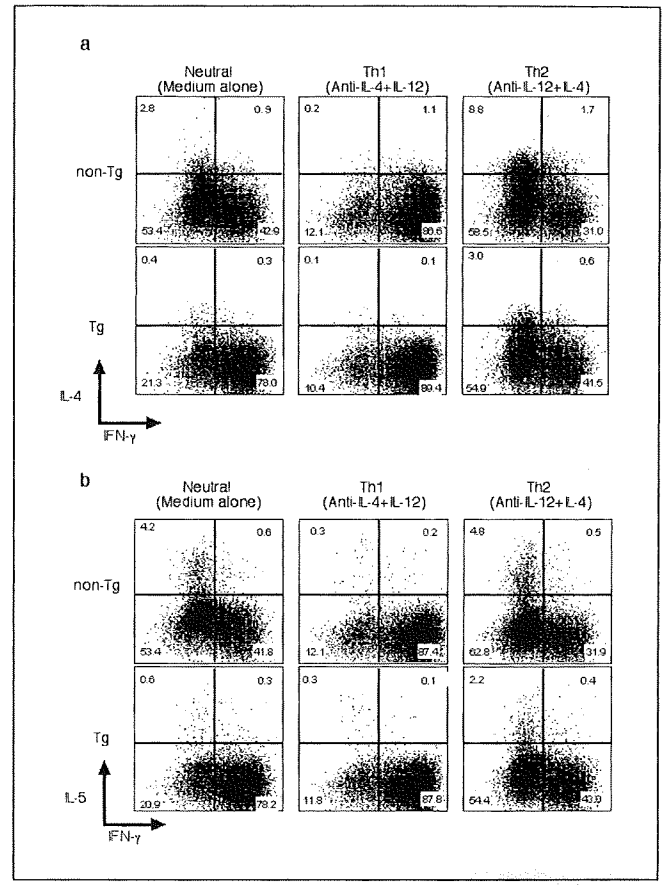

Fig. 4 - Intracellular cytokine analysis of CD4+ T cells in T-bet transgenic (Tg) Yaa mice. a) Intracellular synthesis of IFN-γ and IL-4 was analyzed with flow cytometry under 3 different conditions: medium alone, anti-IL-4 plus IL-12 (Th1 differentiation condition) and anti-IL-12 plus IL-4 (Th2 differentiation condition). b) Intracellular synthesis of IFN-γ and IL-5 was analyzed with flow cytometry under 3 different conditions: medium alone, Th1 differentiation condition and Th2 differentiation condition. Results are representative of 3 independent experiments.

Significantly elevated IgG2a/IgG1 ratio in T-bet transgenic Yaa mice

Because Th1/Th2 cytokines contribute to controlling immunoglobulin subtype production, we next analyzed serum IgG1, IgG2a and IgG3. Th1 cells support macrophage activation, delayed-typed hypersensitivity responses and immunoglobulin isotype switching to IgG2a. In contrast, Th2 cells provide efficient help for B-cell activation and class switching to IgG1 (4, 21). We measured the serum immunoglobulin levels in these mice to evaluate further the mechanism behind the accelerated autoimmune glomerulonephritis in T-bet transgenic Yaa mice. Although there was no significant difference in the levels of IgG1 and IgG2a between T-bet transgenic Yaa mice and non-Tg mice (Fig. 3a, b), T-bet transgenic Yaa mice showed significantly higher total IgG levels and IgG2a/IgG1 ratio than those observed in the non-Tg mice ($p<0.05$) (Fig. 3d, e). IgG3 levels (Fig. 3c) and IgG3/IgG1 ratio (data not shown) of T-bet Tg mice were also higher than those of non-Tg mice, but not significantly. The production of IgG2a is considered to be associated with a Th1 response, and IgG2a is also thought to contribute to the pathogenesis of glomerulonephritis in Yaa mice (22). The higher IgG2a/IgG1 ratio in T-bet transgenic Yaa mice demonstrated the promotion of a Th1 response in these transgenic mice, and this Th1-

dominant shift may have contributed to the accelerated autoimmune glomerulonephritis.

Increased synthesis of IFN-γ in T-bet transgenic Yaa mice

To determine cytokine levels, we first analyzed serum by enzyme-linked immunosorbent assay (ELISA). Serum IL-4 levels were below the level of detection. Furthermore, there was no significant difference between T-bet transgenic Yaa mice and non-Tg mice in the IFN-γ analysis (data not shown). To confirm the observed differences in cytokine production at the single-cell level, we studied the intracellular synthesis of cytokines in T-bet transgenic Yaa mice and non-Tg mice with flow cytometry (Fig. 4). $CD4^+$ T cells from T-bet transgenic Yaa mice demonstrated higher levels of IFN-γ than did those from non-Tg mice with medium alone (Fig. 4a). In contrast, IL-4 and IL-5 levels in non-Tg mice were higher than those in T-bet transgenic Yaa mice under the conditions with medium alone and Th2 differentiation (Fig. 4a, b). These results indicated that T cells in T-bet transgenic Yaa mice differentiated via a more Th1-dominant pattern than did the cells of non-Tg mice.

Discussion

BXSB/MpJ-*Yaa* mice developed autoimmune glomerulonephritis and died due to renal failure. In this study, we generated T-bet transgenic Yaa mice overexpressing T-bet in T cells and demonstrated that these mice exhibited a shorter life span than their transgenic negative littermates. T-bet transgenic Yaa mice showed a more severe impairment of renal function and the prominent deposition of IgG antibodies, especially IgG3, in their glomeruli. In MRL/lpr mice, which represent an animal model of autoimmune glomerulonephritis, IgG3 production is thought to be responsible for the development of nephritis (22). Based on these results, we speculated that the main reason underlying the shorter life span of T-bet transgenic Yaa mice is an acceleration of the autoimmune glomerulonephritis.

In general, a shift in Th1 condition is thought to be a contributing factor of the autoimmune glomerulonephritis in BXSB/MpJ-*Yaa* mice (16-18). Our results showed that T-bet transgenic Yaa mice exhibited a greater number of IFN-g–producing $CD4^+$ T cells under neutral conditions, whereas an analysis of intracellular cytokines revealed fewer IL-4– and IL-5–producing $CD4^+$ T cells. These results suggest that the activation of Th1 cells and suppression of Th2 cells occurred in T-bet transgenic Yaa mice. Moreover, T-bet transgenic Yaa mice exhibited an increased ratio of serum IgG2a (Th1) / IgG1 (Th2). Th1 cells switch their immunoglobulin isotype to IgG2a and IgG3, whereas Th2 cells switch their immunoglobulin production to IgG1 and IgE (4, 21). Based on these findings, we conclude that the overexpressed T-bet gene promotes a Th1 shift in Yaa mice and consequently contributes to the exacerbation of autoimmune glomerulonephritis.

It is well known that the zinc finger type transcription factor, GATA-3, is a master regulator of Th2 differentiation. Previously we reported that transgenic overexpression of GATA-3 in T lymphocytes improved autoimmune glomerulonephritis in Yaa mice (23). GATA-3–overexpressing Yaa mice demonstrated diminished production of IFN-γ and exhibited a decreased IgG2a/IgG1 ratio. These results suggested that GATA-3 overexpression contributed to the improvement of the Th1/Th2 imbalance and consequently ameliorated the glomerulonephritis in Yaa mice.

In conclusion, overexpression of the transcription factor T-bet in T cells induced a more severe Th1 condition in Yaa mice and accelerated the autoimmune glomerulonephritis. This study suggests that Th1/Th2 transcriptional regulation is important for autoimmune glomerulonephritis, and the transcriptional regulation of the Th1/Th2 balance may provide new therapies for the treatment of autoimmune glomerulonephritis.

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