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Immunopathology and Infectious Diseases

Increased Foxp3$^+$ CD4$^+$ Regulatory T Cells with Intact Suppressive Activity but Altered Cellular Localization in Murine Lupus

Jun Abe,* Satoshi Ueha,* Jun Suzuki,*† Yoshiaki Tokano,† Kouji Matsushima,* and Sho Ishikawa*

From the Department of Molecular Preventive Medicine, Graduate School of Medicine, University of Tokyo, Tokyo; and the Department of Rheumatology,† School of Medicine, Juntendo University, Tokyo, Japan*

Foxp3$^+$ CD4$^+$ regulatory T (T_{reg}) cells play a pivotal role in the maintenance of dominant self tolerance. Understanding how the failures of immune control by T_{reg} cells are involved in autoimmune diseases is important for the development of effective immunotherapies. In the present study, we analyzed the characteristics of endogenous T_{reg} cells in (NZB × NZW) F1 (BWF1) mice, a murine model of systemic lupus erythematosus. Unexpectedly, T_{reg} number and frequency in aged BWF1 mice with developing lupus nephritis were increased, not decreased, and *in vitro* suppressive activity in lymphoid organs was intact. In addition, T_{reg} cells trafficked to target organs because cells were present in the kidney and lung. T_{reg} cells of aged BWF1 mice exhibited altered localization within lymph organs, however, and an altered phenotype, with higher expression levels of chemokine receptors and activation markers, suggesting a highly activated cellular state. Notably, the expression levels of costimulatory molecules were also markedly enhanced in the T_{reg} cells of aged BWF1 mice. Furthermore, T_{reg} cells of BWF1 mice did not show any suppressive effects on antibody production *in vitro*. Taken together, we conclude that T_{reg} cells in BWF1 mice are not predisposed to functional incompetence but rather are present in a highly activated state. *(Am J Pathol 2008, 173:1682–1692; DOI: 10.2353/ajpath.2008.080314)*

Systemic lupus erythematosus (SLE) is an autoimmune disease of unknown etiology characterized by a massive production of autoantibodies against various nuclear antigens. The deposit of immune complexes in the target organs, ie, skin, kidney, lung, joints, and central nervous system, is thought to cause fatal dysfunction of the body system. (NZB × NZW) F1 (BWF1) is a mouse strain that has been widely used as a model for SLE since the 1960s. These mice spontaneously develop severe autoimmune disease highly resembling human SLE in terms of serological and hematological abnormalities, and severe nephritis accompanying massive production of antinuclear antibodies.[1]

Reconstitution of SCID (severe combined immunodeficiency) mice with cultured pre-B cells of BWF1 mice recapitulates many symptoms of the disease of BWF1 mice. Cultured pre-B cells alone, however, are not sufficient to fully reproduce the disease.[2] These data suggest that cellular subset(s) in addition to B cells are necessary for the development of the lupus-like syndrome of BWF1 mice, although abnormalities of the immune system predominantly lie within B cells. One of the possible candidates is CD4$^+$ T cells, because depletion of CD4$^+$ T cells with anti-CD4 antibody from 5 months old, slightly before the disease onset, prevents the development of the disease.[3,4] CD4$^+$ T cells are, therefore, also required for the development of the disease in BWF1 mice, possibly by providing help for the production of high-affinity autoantibodies.

Studies in this decade have clearly shown the key roles of naturally occurring regulatory T (T_{reg}) cells in the maintenance of dominant self tolerance of the immune system.[5] T_{reg} cells in normal mice are mostly of thymic origin and are considered to be autoreactive T-cell clones that have bypassed negative selection by unknown mechanism(s).[6] There also exists T_{reg} cells of extra-thymic origin induced from conventional T cells during immune responses,[7] although the underlying mechanisms of this

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Address reprint requests to Sho Ishikawa, 7-3-1, Hongo, Bunkyo-ku, Tokyo, 113-0033, Japan. E-mail: yamasho@m.u-tokyo.ac.jp.

process are still unclear. Foxp3, a member of forkhead-box family of transcription factors, is specifically expressed in the whole life of T_{reg} cells and programs their functional properties.[8–10] In contrast to the previously used marker CD25 or combination of CD25 and CD62L, expression of Foxp3 is specific for T_{reg} cells, and thus can be used for the definitive identification of these cells.[11] Immunoregulatory function of T_{reg} cells is dependent on Foxp3 and genetic deficiency of *Foxp3* causes fatal organ-specific autoimmune disease because of the lack of functional T_{reg} cells.[12–14] Furthermore, many groups have reported the reduced number and/or function of T_{reg} cells in both organ-specific and systemic autoimmune diseases.[15]

A recent study has shown that the decreased frequency of T_{reg} cells in the peripheral blood was associated with disease activity in SLE patients.[16] Frequency of T_{reg} cells identified as $CD25^+$ $CD62L^{hi}$ $CD4^+$ T cells in the spleen was also decreased in aged BWF1 mice.[17] Accordingly, adoptive transfer of *in vitro*-expanded T_{reg} cells, or administration of histone-derived peptides or peptides derived from the complementarity-determining region 1 of anti-double-strand DNA immunoglobulin has been shown to ameliorate the disease in BWF1 mice by a mechanism involving T_{reg} cells.[17–20] These studies suggest that the function of endogenous T_{reg} cells is, at least partially, abrogated by unidentified mechanisms in BWF1 mice.

Despite the effort to develop therapeutic methods involving T_{reg} cells, their nature in BWF1 mice remains unclear. Here we performed a detailed characterization of T_{reg} cells in BWF1 mice using Foxp3 as their marker. Our results demonstrated that aged BWF1 mice had increased frequency and number of T_{reg} cells with apparently normal function, but with an activated phenotype including enhanced expression of co-stimulatory molecules and altered localization.

Materials and Methods

Mice

Female 6- to 8-week-old BWF1 and BALB/c mice were purchased from Japan SLC (Shizuoka, Japan) and were kept under specific pathogen-free conditions in the animal facility of our laboratory until analysis. Mice were used at 6 to 10 or 32 to 40 weeks of age as young or aged, respectively. All animal experiments were approved by the animal care committee of The University of Tokyo.

Antibodies

Monoclonal anti-mouse CD4 (clone RM4-5), CD5 (55-7.3), CD8α (53-6.7), CD11b (M1/70), CD16/32 (2.4G2), CD19 (1D3), CD23 (B3B4), CD25 (7D4), CD43 (S7), CD44 (IM7), CD45 (30-F11), CD45R (RA3-6B2), CD62L (MEL-14), CD69 (H1.2F3), CD90.2 (53-2.1), CD103 (M290), OX40 (OX-86), CXCR4 (2B11/CXCR4), CCR5 (C34-3448), NK1.1 (PK136), TER-119 (TER-119), and streptavidin were purchased from BD Biosciences (San Diego, CA); monoclonal anti-mouse 4-1BB (17B5), ICOS (7E.17G9), F4/80 (BM8), CCR7 (4B12), and Foxp3 (FJK-16s) were purchased from eBioscience (San Diego, CA); monoclonal anti-mouse CXCR3 (220803) was purchased from R&D Systems (Minneapolis, MN). Antiserum raised against mouse type IV collagen was purchased from LSL (Tokyo, Japan). Details of monoclonal anti-mouse CCR4 antibody (clone 2G11) will be described elsewhere by Nagakubo and colleagues.[21,22]

Cell Isolation

Single cell suspensions of the thymus, spleen, and lymph nodes were prepared by passing the tissue through a cell strainer (BD Bioscience). Single cell suspension of the kidney and lung were prepared by dissociating the tissue with collagenase D (Roche, Basel, Switzerland). Mononuclear cells in the kidney and lung were isolated from the single cell suspension by Percoll (Invitrogen, Carlsbad, CA) gradient centrifugation. $CD25^+$ $CD4^+$ T cells were isolated from the single cell suspension of various organs by magnetic enrichment of $CD25^+$ cells followed by fluorescence-activated cell sorting with the Epics Altra cell sorter (Beckman Coulter, Fullerton, CA). $CD25^-$ $CD4^+$ T cells were isolated from the single cell suspension of spleen by magnetic depletion of the cells positive for CD8α, CD11b, CD25, B220, CD138, NK1.1, or TER-119. B1 cells were isolated from peritoneal lavage cells by magnetic depletion of the cells positive for CD23, Thy-1.2, or F4/80. B2 cells were isolated from spleen by magnetic depletion of the cells positive for CD43, Thy-1.2, or TER-119. All procedures involving magnetic isolation were performed with an autoMACS system (Miltenyi Biotec, Bergisch Gladbach, Germany).

Flow Cytometry

Cells were incubated with fluorochrome- or biotin-labeled antibodies for 20 minutes at 4°C, following the blockade of FcγRII/III with unlabeled anti-CD16/32 for 10 minutes at 4°C; for the staining with biotin-labeled anti-CCR7, incubation after the blockade of Fc receptors was performed at 37°C. Biotin-labeled antibodies were visualized by further incubating with phycoerythrin-conjugated streptavidin for 15 minutes at 4°C. Staining of Foxp3 was performed according to the manufacturer's instructions. Data were collected using BD LSR II (BD Biosciences) and analyzed with FlowJo software (Tree Star, Ashland, OR).

Immunofluorescent Staining

Explanted tissues embedded in OCT compound were snap-frozen in liquid nitrogen and stored at −80°C until use. Six-μm-thick sections of frozen tissues were fixed with cold acetone for 10 minutes and rehydrated with phosphate-buffered saline (PBS) for 10 minutes at room temperature. Rehydrated sections were blocked for nonspecific binding of proteins with Blocking One (Nacalai Tesque, Kyoto, Japan) for 20 minutes at room temperature and incubated with unlabeled or biotinylated anti-

Table 1. Primers and Probes for Real-Time PCR

Gene	Sense	Probe	Antisense
CCL19	5′-GAAAGCCTTCCGCTACCTTCT-3′	5′-CCCATCCCGGCAATCCTGTTCTTA-3′	5′-CCCTTAGTGTGGTGAACACAA-CA-3′
CCL21	5′-GGCTATAGGAAGCAAGAACCAA-GT-3′	5′-TTACTTCTACCGACGTCCCACGGA-3′	5′-TCAGGCTTAGAGTGCTTCCG-3′
CXCL9	5′-TGATAAGGAATGCACGATGCTC-3′	5′-AGCCGAGGCACGATCCACTACAAA-TC-3′	5′-TTCCTTGAACGACGACGACTTT-3′
CXCL10	5′-CGTCATTTTCTGCCTCATCCT-3′	5′-AAGCTTGAAATCATCCCTGCGAG-CC-3′	5′-TGGTCTTAGATTCCGGATTCAG-3′
CXCL12	5′-GCTCTGCATCAGTGACGGTAA-3′	5′-ATCGCCAGAGCCAACGTCAAGCAT-CT-3′	5′-AGCCGTGCAACAATCTGAAG-3′
GAPDH	5′-AGTATGACTCCACTCACGGCAA-3′	5′-AACGGCACAGTCAAGGCCGAGAAT-3′	5′-TCTCGCTCCTGGAAGATGGT-3′

bodies, or antisera for 60 minutes at room temperature. Sections were then incubated with Alexa Fluor-labeled anti-Ig secondary antibodies or streptavidin (Invitrogen) for 30 minutes at room temperature. After the staining, sections were fixed with phosphate-buffered 4% paraformaldehyde for 10 minutes at room temperature and were mounted with Prolong Gold Antifade Reagent (Invitrogen). Specimens were observed under IX70 confocal laser-scanning microscopy (Olympus, Tokyo, Japan).

Quantification of Histological Analysis

Images obtained from confocal microscopic observation were processed with Win ROOF software (Mitani Corporation, Fukui, Japan), and the number of the signals was counted manually or automatically using Win ROOF software.

In Vitro *Proliferation and Suppression Assay*

2×10^4 cells/well of purified $CD25^-$ $CD4^+$ T cells were stimulated with 2 μg/ml of concanavalin A (Sigma-Aldrich, St. Louis, MO) and 8×10^4 cells/well of mitomycin-C (Sigma-Aldrich)-treated $Thy1.2^-$ splenocytes with or without titrated number of $CD25^+$ $CD4^+$ T cells were incubated in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 10 mmol/L HEPES, 55 μmol/L 2-mercaptoethanol, 100 U/mL penicillin, and 100 μg/mL streptomycin in a round-bottom 96-well plate for 72 hours at 37°C. $CD25^+$ $CD4^+$ T cells were cultured under the same conditions to measure their proliferative capacity in the absence of $CD25^-$ $CD4^+$ T cells. Cells were pulsed with 1 μCi/well [^{3}H-methyl]-thymidine (GE Health Care, Buckinghamshire, UK) for the last 6 to 8 hours of the culture, and proliferation was measured by cpm value of the harvested cells. Suppressive activity of $CD25^+$ $CD4^+$ T cells was expressed as percent suppression[23] calculated as following: 100 × [cpm (responder) − cpm($CD25^+$ + $CD25^-$)]/cpm(responder).

In Vitro *Antibody Production Assay*

In vitro antibody production by B cells was analyzed as previously described[24] with several modifications. Briefly, 2×10^5 B1 or B2 cells isolated from young or aged BWF1 mice and equal numbers of $CD25^-$ $CD4^+$ T cells isolated from the spleen of young or aged BWF1 mice were cultured with or without 1×10^5 $CD25^+$ $CD4^+$ T cells in supplemented RPMI 1640 medium for 5 days at 37°C. The concentration of IgG antibody in the culture supernatant was measured by enzyme-linked immunosorbent assay using a Mouse IgG quantitation kit (Bethyl, Montgomery, TX).

Preparation of cDNA and Real-Time Polymerase Chain Reaction (PCR)

Mice were perfused with 30 mL of PBS, and spleen, lymph nodes, kidney, and lung were excised. Tissues were homogenated with TRIzol reagent (Invitrogen), and purified total RNA was reverse-transcribed with a high-capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA). Real-time PCR was performed with an ABI Prism 7500 (Applied Biosystems) using primers and Taq Man probes listed in Table 1.

Statistical Analysis

Statistical significance of the difference between data sets was analyzed by Welch's unpaired *t*-test for the comparison of two groups or by one-way analysis of variance with Bonferroni's multiple comparison test for more than three groups. $P < 0.05$ was considered to be statistically significant.

Results

Increased Number and Frequency of T_{reg} Cells in Aged BWF1 Mice

Suppressive activity of T_{reg} cells is strongly correlated with the expression of Foxp3.[11] To clarify whether an increase or decrease in the frequency and/or number of T_{reg} cells exists, we analyzed the population of $Foxp3^+$ $CD4^+$ T cells by flow cytometry. We found that aged BWF1 mice had substantially increased frequency (Figure 1A) and number (Figure 1B) of $Foxp3^+$ $CD4^+$ T cells in the lymphoid organs compared with young BWF1 mice. A recent study has shown an age-dependent increase in $CD25^-$ $Foxp3^+$ $CD4^+$ T cells in 24-month-old normal mice,[25] but increased $Foxp3^+$ $CD4^+$ T cells in aged BWF1 mice was not merely an age-dependent event be-

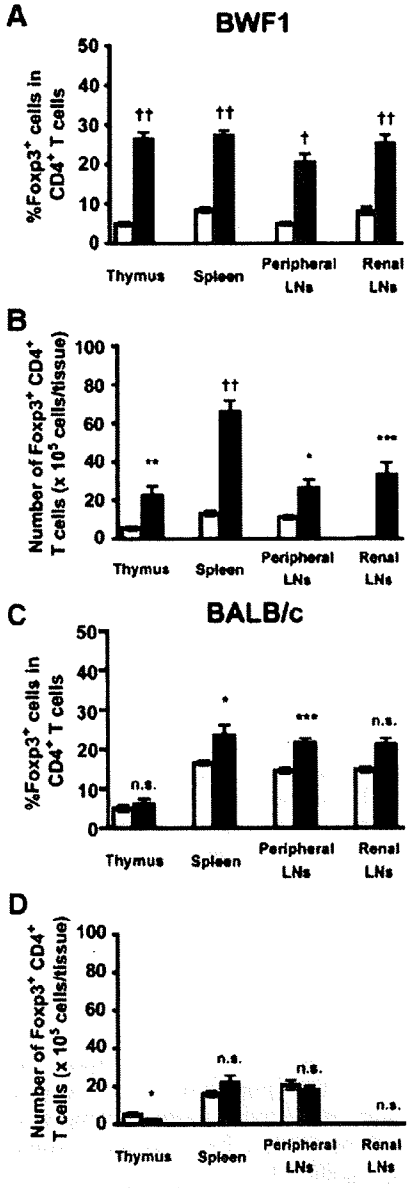

Figure 1. Increased Foxp3$^+$ CD4$^+$ T_{reg} cells in aged BWF1 mice. Frequency (**A**, **C**) and number (**B**, **D**) of Foxp3$^+$ CD4$^+$ T cells within thymus, spleen, peripheral LNs (inguinal, axillary, brachial, submandibular, and cervical), and renal lymph node of BWF1 (**A**, **B**) or BALB/c (**C**, **D**) mice were analyzed by flow cytometry. Data were presented as mean ± SEM (n = 4 to 9 for each group). Open bar, young; filled bar, aged. Statistical significance of the difference between young and aged mice was analyzed by Welch's unpaired t-test. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.005$, $^{\dagger}P < 0.0005$, $^{\dagger\dagger}P < 0.0001$.

cause age-matched BALB/c mice did not show a marked increase in Foxp3$^+$ CD4$^+$ T cells (Figure 1, C and D).

CD25$^+$ CD4$^+$ T Cells Showed Normal Suppressive Activity Both in Young and Aged BWF1 Mice

Valencia and colleagues[16] reported a decreased suppressive activity of CD25$^+$ CD4$^+$ T cells in SLE patients, possibly because of the lower proportion of Foxp3$^+$ cells among CD25$^+$ CD4$^+$ T cells. This result, however, does not exclude the possibility that a functional defect intrinsic to T_{reg} cells exists as well. To test the functional competency of T_{reg} cells of BWF1 mice, we performed an *in vitro* suppression assay. Because Foxp3 expression could be detected only in permeabilized cells, we used CD25$^+$ CD4$^+$ T cells as a surrogate for Foxp3$^+$ CD4$^+$ T cells. Concurrent with the high proportion of Foxp3$^+$ cells among CD25$^+$ CD4$^+$ T cells even after disease onset (Figure 2A), CD25$^+$ CD4$^+$ T cells isolated from the spleen and lymph nodes of both young and aged BWF1 mice did not proliferate on stimulation (Figure 2B) and showed suppressive activity (Figure 2C). Furthermore, CD25$^+$ CD4$^+$ T cells isolated from the kidney (Figure 2C) and lung (data not shown), ie, the target organs, of aged BWF1 mice also showed suppressive activity comparable to those from the spleen and lymph nodes. CD25$^+$ CD4$^+$ T cells of thymus or lymph nodes showed similar suppressive activity (data not shown). We did not note a significant difference in the suppressive activity between young and aged, or lymphoid and nonlymphoid CD25$^+$ CD4$^+$ T cells in BWF1 mice at any dose of CD25$^+$ CD4$^+$ T cells. Taken together, our data suggest that aged BWF1 mice have an expanded pool size of T_{reg} cells with intact suppressive activity.

T_{reg} Cells Infiltrated into the Target Organs

Defective migration into the site of inflammation is known to impair the *in vivo* suppressive activity of T_{reg} cells even if they were functionally competent *in vitro*.[26] Because our data indicated that T_{reg} cells of BWF1 mice have intact suppressive activity *in vitro*, we asked whether T_{reg} cells in aged BWF1 mice infiltrated into the target organs, ie, kidney and lung. Flow cytometric analysis of mononuclear cells within the target organs revealed that Foxp3$^+$ as well as Foxp3$^-$ CD4$^+$ T cells infiltrated into these organs, and that the frequency of Foxp3$^+$ cells in CD4$^+$ T cells was comparable to that in the lymph nodes of normal mice[11] (18.76 ± 3.79% in the kidney and 14.08 ± 2.50% in the lung). Foxp3$^+$ CD4$^+$ T cells infiltrated into the glomeruli, interstitium, and perivascular region of the kidney along with Foxp3$^-$ CD4$^+$ T cells (Figure 3B). Young BWF1 mice and nonautoimmune control mice did not show the infiltration of inflammatory cells (data not shown). Moreover, both Foxp3$^+$ and Foxp3$^-$ CD4$^+$ T cells were apparently distributed evenly within the infiltrating site of the target organs (Figure 3, A and C), indicating that clustering of these cells that were essential for T_{reg} cell-mediated suppression[26,27] would take place in the target organs as well as in the lymphoid organs.

Medullary Localization of T_{reg} Cells within the Thymus

The thymus, another target organ of the disease in BWF1 mice, is the major site of the development of T_{reg} cells.[6] Disruption of the architecture of the thymic medulla where development of T_{reg} cells occurs is known to impair that process.[28] To determine whether T_{reg} cells are properly

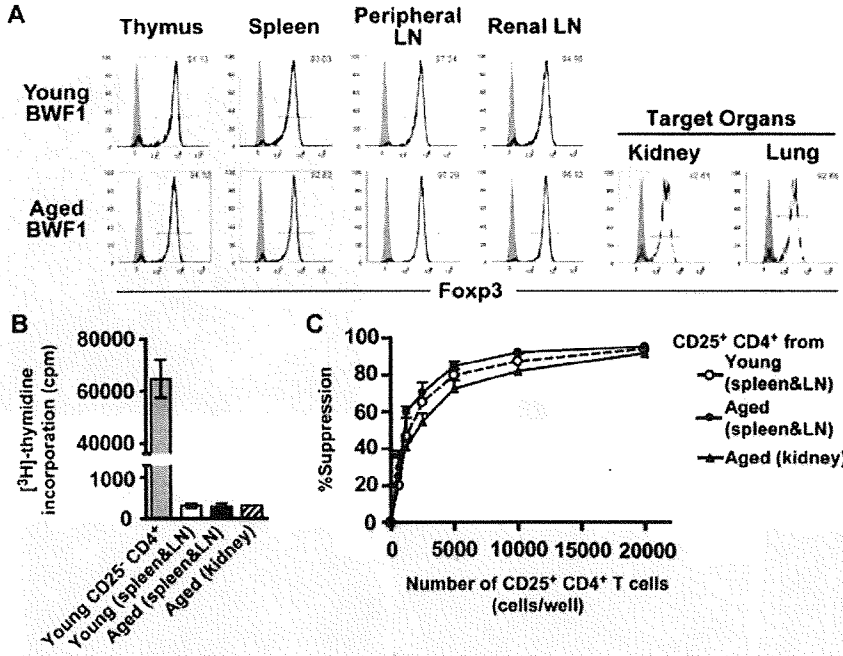

Figure 2. Suppressive activity of $CD25^+$ $CD4^+$ T cells. **A:** Representative profile of Foxp3 expression in $CD25^+$ $CD4^+$ T cells of young or aged BWF1 mice used for suppression assay (n = 3 for each group). Numbers in the histograms indicate the frequency of $Foxp3^+$ cells. Shaded histogram indicates isotype control. Note that $CD25^+$ $CD4^+$ T cells are highly enriched for $Foxp3^+$ T_{reg} cells. **B:** Proliferation of $CD25^+$ $CD4^+$ T cells isolated from the spleen and lymph nodes of young or aged BWF1 mice or from the target organs of aged BWF1 mice. Data are presented as mean ± SEM. **C:** *In vitro* suppressive activity of $CD25^+$ $CD4^+$ T cells isolated from the spleen and lymph nodes of young or aged BWF1 mice or from the kidney of aged BWF1 mice. Data were presented as mean + SEM. Differences between the three groups presented in the graph were not significant when analyzed by one-way analysis of variance with Bonferroni's multiple comparison test. A representative of three independent experiments that gave similar results is shown.

located within the thymus, we analyzed thymic sections by immunofluorescent staining. In BWF1 mice, thymic architecture is strongly affected by the disease,[4,29] but medullary localization of T_{reg} cells remained virtually unchanged even after the manifestation of severe nephritis (Figure 3, D and E). Localization of T_{reg} cells within the thymus is also confined to the medulla in BALB/c mice irrespective of their age (Supplemental Figure 1, A and B, see *http://ajp.amjpathol.org*).

Altered Distribution of T_{reg} Cells within the Secondary Lymphoid Organs of Aged BWF1 Mice

T_{reg} cells have to be located in the site of antigen presentation within the secondary lymphoid organs to make contacts with their target cells.[26,27] Because our analyses on the number, function, and site of the development of T_{reg} cells could not find any obvious defect, we examined the localization of T_{reg} cells within the secondary lymphoid organs of BWF1 mice. T_{reg} cells in aged BWF1 mice were located in the follicles and red pulp as well as periaortic lymphoid sheath in the spleen, whereas T_{reg} cells in young BWF1 mice were mostly located in the periaortic lymphoid sheath (Figure 3, F and G; Supplemental Figure 2, see *http://ajp.amjpathol.org*). Similar localization of T_{reg} cells were observed in the renal lymph node where T_{reg} cells were located in the follicles and medulla as well as paracortex in aged BWF1 mice, whereas the localization of T_{reg} cells in young BWF1 mice was relatively confined to paracortex (Figure 3, H and I; Supplemental Figure 2, see *http://ajp.amjpathol.org*). Such altered localization was not limited to T_{reg} cells, but was also seen in $Foxp3^-$ conventional T cells. In contrast, localization of T_{reg} cells in BALB/c mice was not altered with age and was similar to that of young BWF1 mice (Supplemental Figures 1, C–F, and 2, see *http://ajp.amjpathol.org*).

Changes in the Expression of Chemokine Receptors on T_{reg} Cells in Aged BWF1 Mice

Localization of T cells is tightly regulated by various chemokines and their receptors to achieve efficient induction of immune response or tolerance.[30] To elucidate the molecule(s) responsible for the altered localization of T_{reg} cells, we next analyzed the expression of chemokine receptors involved in the function of T_{reg} cells[31–34] by flow cytometry. Both $Foxp3^+$ and $Foxp3^-$ $CD4^+$ T cells in the spleen showed decreased CCR7 expression (Figure 4C) and enhanced CXCR4 expression (Figure 4E) in aged BWF1 mice. On the other hand, the expression level of CCR4, CCR5, and CXCR3 did not show marked difference between young and aged BWF1 mice (Figure 4, A, B, and D), except that CXCR3 expression was slightly enhanced on both $Foxp3^+$ and $Foxp3^-$ cells of aged BWF1 mice (Figure 4, F and H). These changes in the expression of chemokine receptors on $CD4^+$ T cells were not observed in BALB/c mice (Figure 4, G and I). Expression pattern of chemokine receptors on $CD4^+$ T cells in the target organs and lymph nodes was similar to that of splenic $CD4^+$ T cells (data not shown). Aged BWF1 mice showed a 5 to 7 fold decrease in the expression of CCL19, CCL21, and CXCL12, ligands for CCR7 and CXCR4, in the lymphoid organs (Supplemental Figure 3, see *http://ajp.amjpathol.org*). On the other hand, expression of CXCL9 and CXCL10, ligands for CXCR3, were increased 2- to 3-fold and 8- to 28-fold, respectively, in

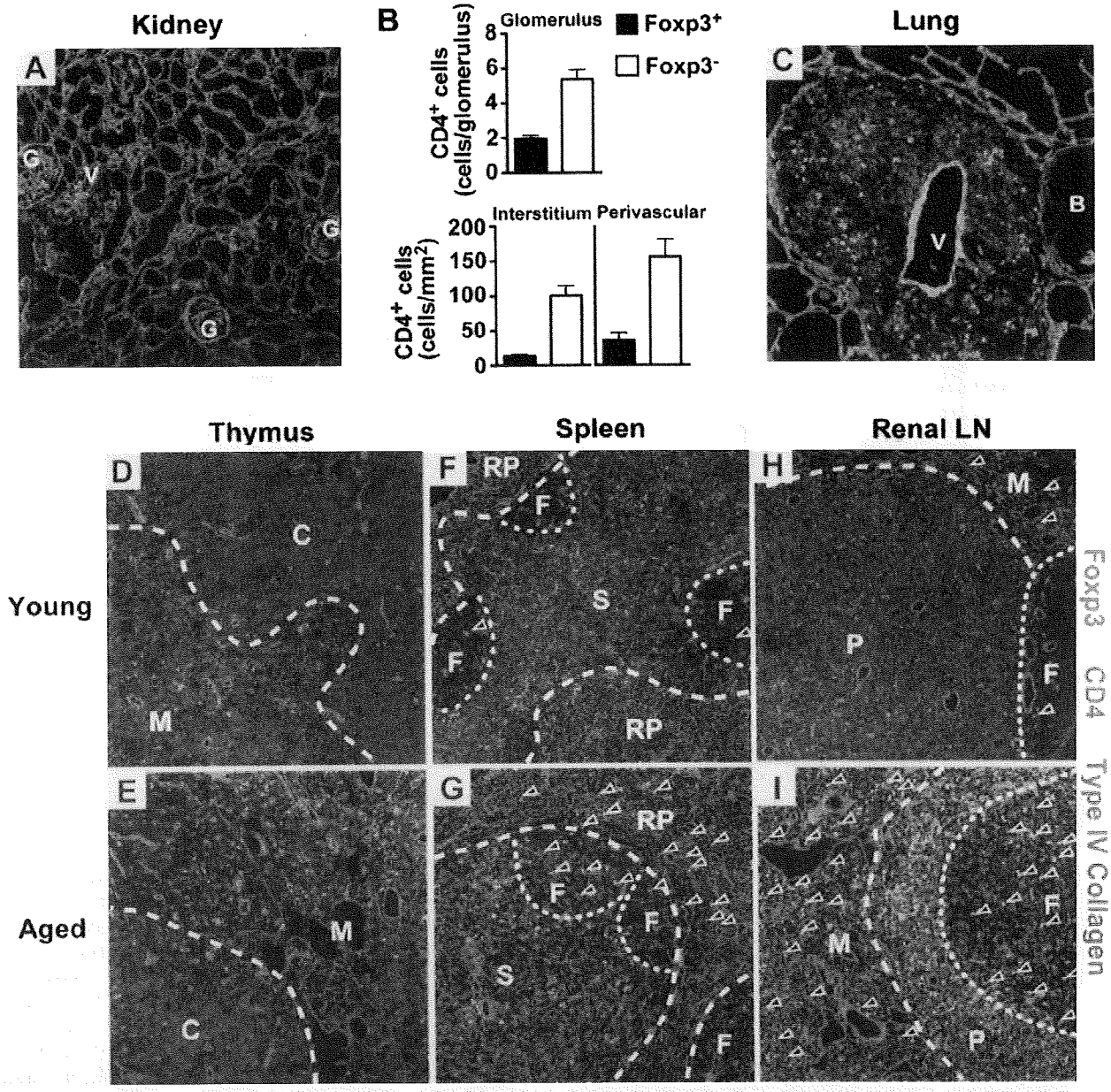

Figure 3. Altered localization of T_{reg} cells in aged BWF1 mice. **A–C:** Histological analysis of the kidney and lung of aged BWF1 mice ($n = 4$). **A** and **C:** Triple immunofluorescent staining of a 6-μm-thick cryosection of the kidney (**A**) and lung (**C**) of aged BWF1 mice with anti-Foxp3 (green), anti-CD4 (red), and anti-type IV collagen (blue). Green signal on the vascular endothelium and bronchus of the lung was also detected in isotype control (data not shown). Such nonspecific signal was not observed in $CD4^+$ cells. **B:** Summary of the number of $Foxp3^+$ (filled bar) and $Foxp3^-$ (open bar) $CD4^+$ T cells within renal compartments. Data were expressed as mean ± SEM. More than three fields were counted to calculate the mean value. **D–I:** Triple-immunofluorescent staining of 6-μm-thick cryosection of the thymus (**D, E**), spleen (**F, G**), and renal lymph node (**H, I**) of young (**D, F, H**) or aged (**E, G, I**) BWF1 mice with anti-Foxp3 (green), anti-CD4 (red), and anti-type IV collagen (blue). B, bronchus; C, cortex; F, follicle; G, glomerulus; M, medulla; RP, red pulp; P, paracortex; S, periaortic lymphoid sheath; V, blood vessel. **Arrowheads** in **D–I** indicate $Foxp3^+$ $CD4^+$ T cells located out of paracortex or periaortic lymphoid sheath. Representatives of the independent examination of four young and aged BWF1 mice are shown. Original magnifications, ×100.

the lymphoid organs and target organs, respectively (Supplemental Figure 3, see *http://ajp.amjpathol.org*).

Activated Phenotype of Both $Foxp3^+$ and $Foxp3^-$ $CD4^+$ T Cells in Aged BWF1 Mice

Altered localization of T_{reg} cells in aged BWF1 mice per se does not explain the cause of their failure to control the autoimmunity. We found that T_{reg} cells of aged BWF1 mice showed decreased expression of CD25 and CD62L (Figure 5, A, B, and I), in contrast to the enhanced or unaltered expression of activation markers CD44, CD69, and CD103 (Figure 5, C–E, and I). Various co-stimulatory molecules up-regulated on activation were reported to affect the function and/or number of T_{reg} cells,[35–37] therefore, we analyzed the expression of co-stimulatory molecules of

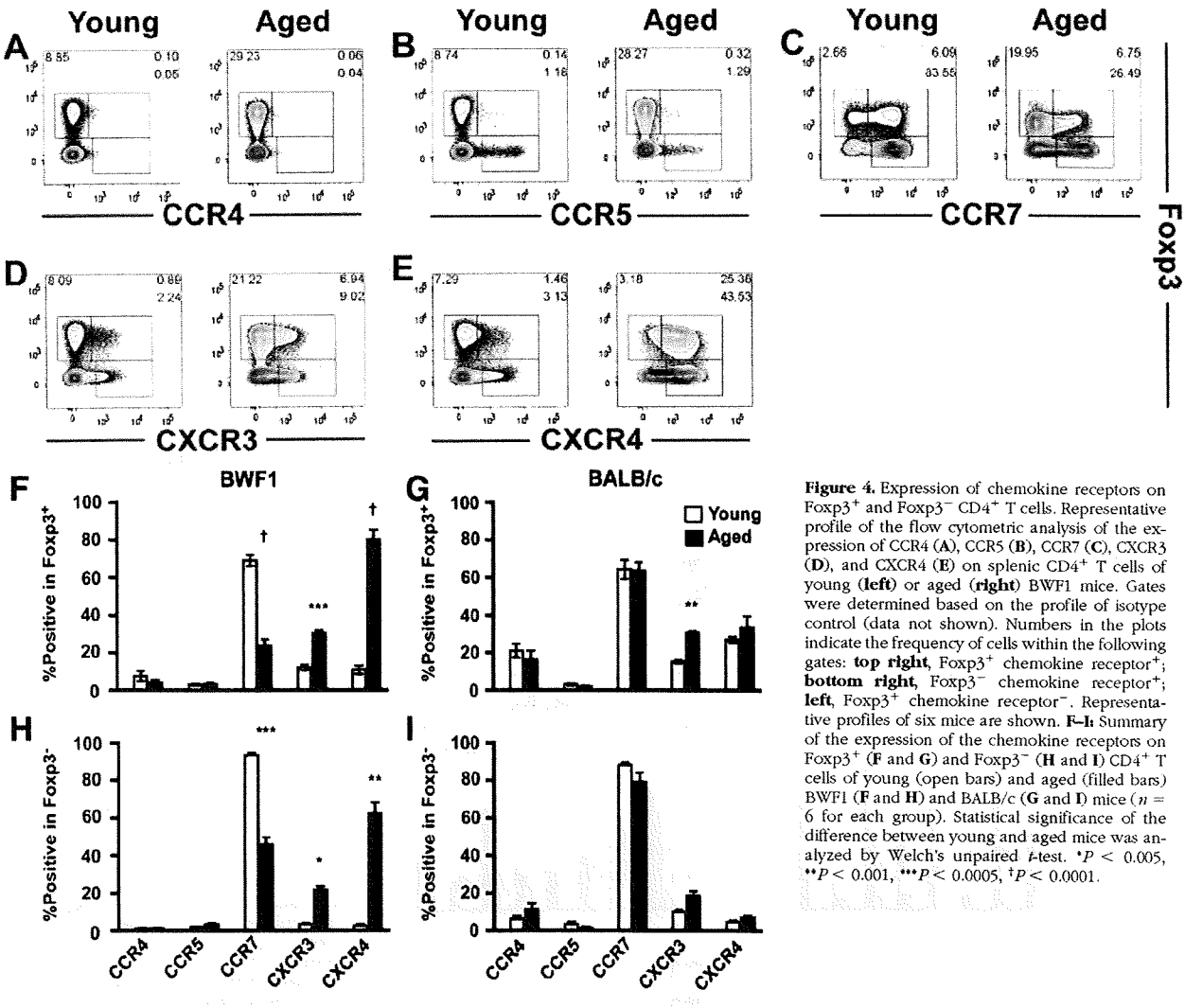

Figure 4. Expression of chemokine receptors on Foxp3$^+$ and Foxp3$^-$ CD4$^+$ T cells. Representative profile of the flow cytometric analysis of the expression of CCR4 (**A**), CCR5 (**B**), CCR7 (**C**), CXCR3 (**D**), and CXCR4 (**E**) on splenic CD4$^+$ T cells of young (**left**) or aged (**right**) BWF1 mice. Gates were determined based on the profile of isotype control (data not shown). Numbers in the plots indicate the frequency of cells within the following gates: **top right**, Foxp3$^+$ chemokine receptor$^+$; **bottom right**, Foxp3$^-$ chemokine receptor$^+$; **left**, Foxp3$^+$ chemokine receptor$^-$. Representative profiles of six mice are shown. **F–I:** Summary of the expression of the chemokine receptors on Foxp3$^+$ (**F** and **G**) and Foxp3$^-$ (**H** and **I**) CD4$^+$ T cells of young (open bars) and aged (filled bars) BWF1 (**F** and **H**) and BALB/c (**G** and **I**) mice (n = 6 for each group). Statistical significance of the difference between young and aged mice was analyzed by Welch's unpaired t-test. $^*P < 0.005$, $^{**}P < 0.001$, $^{***}P < 0.0005$, $^{\dagger}P < 0.0001$.

T_{reg} cells. Associated with their activated phenotype, costimulatory molecules OX40, 4-1BB, and ICOS were expressed on CD4$^+$ T cells in aged BWF1 mice at higher level than young BWF1 (Figure 5, F–I). Among them, expression of OX40 and ICOS was enhanced on both Foxp3$^+$ and Foxp3$^-$ T cells, whereas expression of 4-1BB was enhanced only on Foxp3$^+$ T_{reg} cells (Figure 5, I and K). Age-dependent alteration of surface phenotype in BALB/c mice was limited to slight changes in CD44 and CD62L (Figure 5, J and L).

Inability of T_{reg} Cells of BWF1 Mice to Suppress in Vitro IgG Antibody Production

Lastly, we assessed the impact of T_{reg} cells on the antibody production by B cells. Sekigawa and colleagues[24] demonstrated that CD4$^+$ T cells of aged BWF1 mice induced IgG antibody production of splenic B cells on stimulation with concanavalin A and lipopolysaccharide *in vitro*. We used this method with several modifications and found that CD25$^-$ CD4$^+$ T cells of aged, but not young, BWF1 mice induced IgG antibody production by B cells even in the absence of the stimuli (Figure 6 and data not shown). Because antibody production by B cells was totally dependent on the presence of CD4$^+$ T cells in this assay, we assumed that T_{reg} cells would suppress the antibody production by interfering with CD4 help. CD25$^+$ CD4$^+$ T cells, however, did not affect the amount of IgG antibody produced by B cells (Figure 6), demonstrating that T_{reg} cells of both young and aged BWF1 mice are unable to suppress IgG antibody production induced by CD4$^+$ T cells of aged BWF1 mice.

Discussion

Foxp3$^+$ CD4$^+$ T_{reg} cells play a pivotal role in the maintenance of dominant self tolerance, and lack of functional T_{reg} cells is associated with various autoimmune diseases. In contrast, our present study in a murine model of SLE revealed a substantially expanded pool size of T_{reg} cells with a phenotype suggesting their highly activated state, and their inability to suppress antibody production *in vitro*.

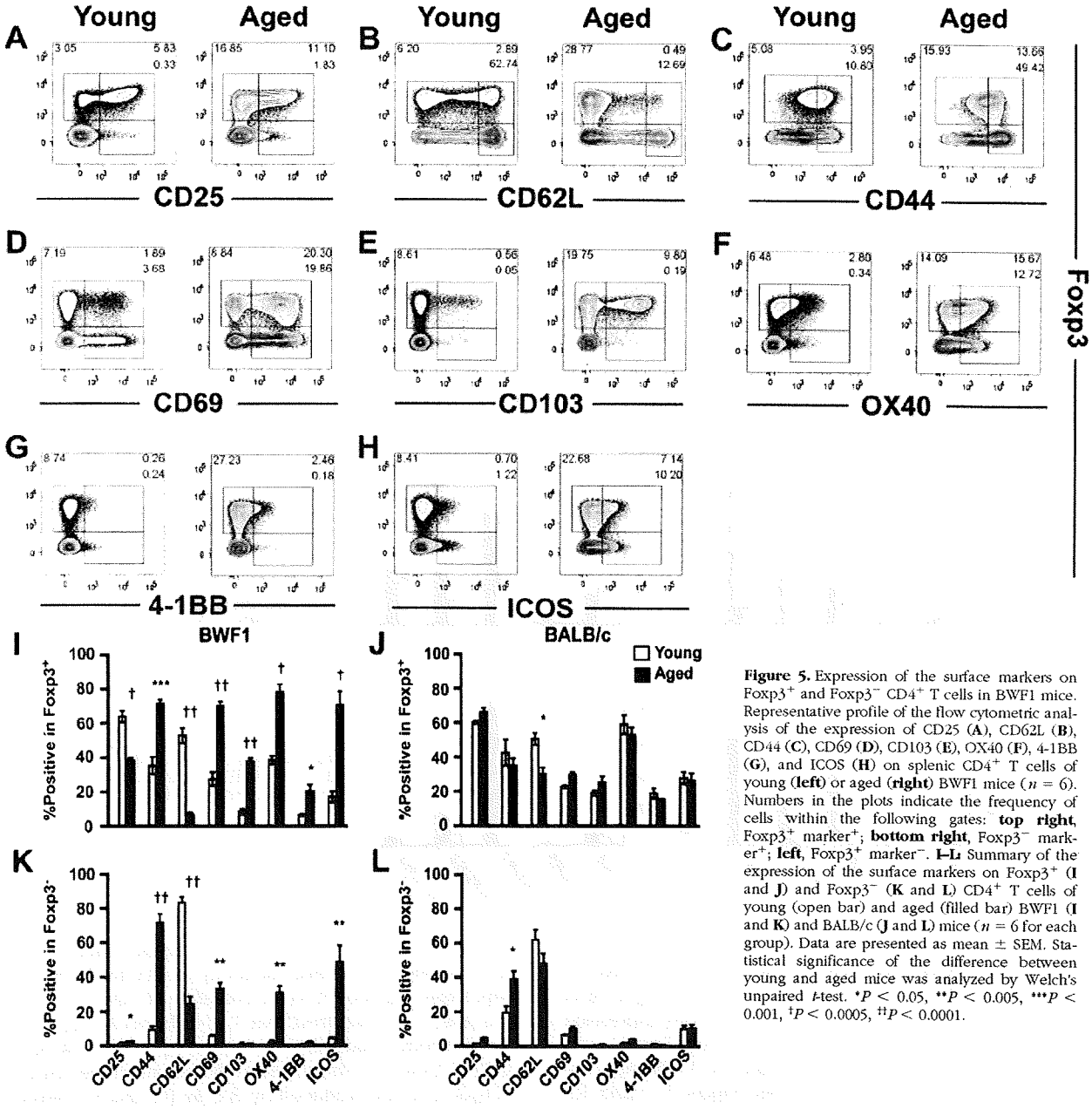

Figure 5. Expression of the surface markers on Foxp3$^+$ and Foxp3$^-$ CD4$^+$ T cells in BWF1 mice. Representative profile of the flow cytometric analysis of the expression of CD25 (**A**), CD62L (**B**), CD44 (**C**), CD69 (**D**), CD103 (**E**), OX40 (**F**), 4-1BB (**G**), and ICOS (**H**) on splenic CD4$^+$ T cells of young (**left**) or aged (**right**) BWF1 mice ($n = 6$). Numbers in the plots indicate the frequency of cells within the following gates: **top right**, Foxp3$^+$ marker$^+$; **bottom right**, Foxp3$^-$ marker$^+$; **left**, Foxp3$^+$ marker$^-$. **I–L** Summary of the expression of the surface markers on Foxp3$^+$ (**I** and **J**) and Foxp3$^-$ (**K** and **L**) CD4$^+$ T cells of young (open bar) and aged (filled bar) BWF1 (**I** and **K**) and BALB/c (**J** and **L**) mice ($n = 6$ for each group). Data are presented as mean ± SEM. Statistical significance of the difference between young and aged mice was analyzed by Welch's unpaired *t*-test. $^*P < 0.05$, $^{**}P < 0.005$, $^{***}P < 0.001$, $^{\dagger}P < 0.0005$, $^{\dagger\dagger}P < 0.0001$.

We could not detect any obvious defect in the suppressive activity of T_{reg} cells in BWF1 mice. In addition, localization of both T_{reg} cells and Foxp3$^-$ conventional CD4$^+$ T cells within the lymphoid organs was altered, but they showed concomitant migratory behavior. These data collectively suggest that T_{reg} cells in BWF1 mice had little defect in their function, and the failure of T_{reg} cells to control the disease might be predominantly caused by the extrinsic factors, such as cytokine milieu and costimulatory signals provided by antigen-presenting cells (APCs). On the other hand, it is reported that treatment of BWF1 mice with the T_{reg} cell-inducing molecules such as all-*trans*-retinoic acid or tolerogenic peptides delays or prevents the onset of murine lupus.[18–20,38] One possible explanation for the failure of T_{reg} cells to control the disease is that presence of T_{reg} cells capable of controlling the disease at an earlier stage is critical, as suggested by the previous reports in which induction of T_{reg} cells in BWF1 mice was conducted well before the onset of the disease. Another possibility is the antigen specificity of T_{reg} cells. La Cava and colleagues[19] showed that induction of T_{reg} cells specific for the peptide derived from anti-DNA antibody were associated with the therapeutic effect of this peptide in BWF1 mice. This report raises the possibility that endogenous T_{reg} cells in prediseased BWF1 mice lack population(s) with such antigen specificity, and expansion of the pool size of T_{reg} cells in aged BWF1 mice with severe nephritis does not

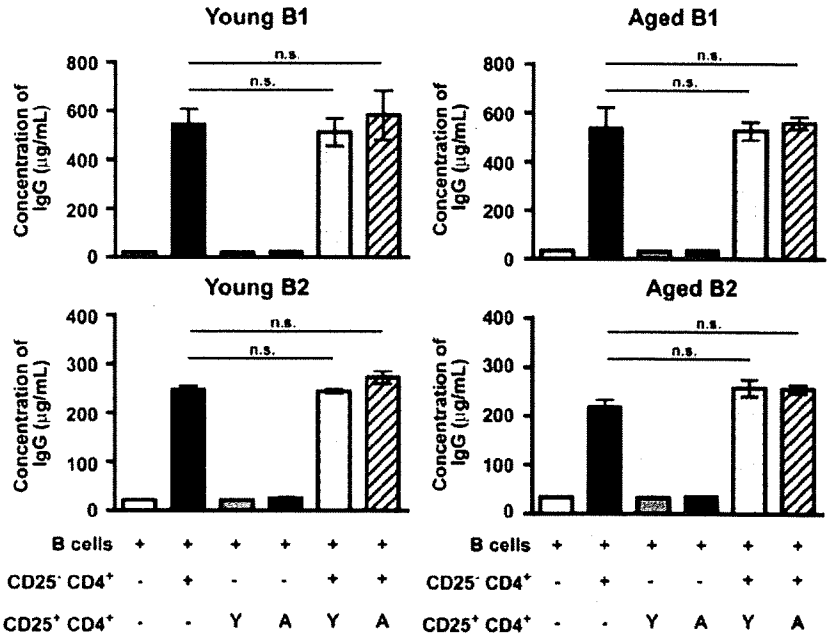

Figure 6. Inability of T_{reg} cells of BWF1 mice to suppress *in vitro* antibody production induced by $CD25^-$ $CD4^+$ T cells of aged BWF1 mice. Concentration of IgG antibody in the culture supernatant was measured by enzyme-linked immunosorbent assay after co-culture of T cells and B cells in the following combinations for 5 days. B cells alone, (white column); B cells + $CD25^-$ $CD4^+$ T cells (black column); B cells + $CD25^+$ $CD4^+$ T cells of young BWF1 (light gray column); B cells + $CD25^+$ $CD4^+$ T cells of aged BWF1 (dark gray column); B cells + $CD25^-$ $CD4^+$ T cells + $CD25^+$ $CD4^+$ T cells of young BWF1 (dotted column); B cells + $CD25^-$ $CD4^+$ T cells + $CD25^+$ $CD4^+$ T cells of aged BWF1 (striped column). $CD25^-$ $CD4^+$ T cells of aged BWF1 mice were used for all combinations. B-cell subsets used for each combination were indicated above each panel. Data are presented as mean ± SEM. n.s., not significant by one-way analysis of variance with Bonferroni's multiple comparison test. Representative of three independent experiments is shown.

compensate for that repertoire. It is therefore feasible that accumulation of T_{reg} cells is too late to control the pathogenic autoimmune response in aged BWF1 mice, or that antigen specificity of T_{reg} cells in aged BWF1 mice differ from those in young BWF1 mice. However, there are other possible mechanisms for the inability of T_{reg} cells to control the pathogenic autoimmune response in aged BWF1 mice as described below.

There are several reports suggesting a possible effect of T_{reg} cells on T-dependent B-cell responses.[19,39–41] It was, therefore, surprising that T_{reg} cells of BWF1 mice could not suppress the *in vitro* antibody production induced by $CD25^-$ $CD4^+$ T cells despite their intact suppressive activity against the proliferation of T cells *in vitro*. Possible explanations for our result are as follows: first, loss of the sensitivity of $CD25^-$ $CD4^+$ T cells of aged BWF1 mice to T_{reg} cell-mediated suppression; second, reversal of T_{reg} cell-mediated suppression by signaling through co-stimulatory molecules. OX40, 4-1BB, and ICOS have been implicated in the pathogenesis of lupus.[42–44] OX40 and 4-1BB magnify the T-cell response through induction of the proliferation of conventional T cells and inhibition of T_{reg} cell-mediated immune suppression.[37,45] The ICOS-mediated signal is essential for the induction of follicular helper T cells, thus it functions as an enhancer of B-cell response.[46] On the contrary, these molecules as well as ICOS also facilitate the expansion of T_{reg} cells.[36,45,47] B cells of aged BWF1 mice, however, did not show significant expression of ligands for these co-stimulatory molecules (data not shown). This observation implies that reversal of the suppression, if any, might take place through the other pathway(s). Also, $CD25^-$ $CD4^+$ T cells of aged, but not young, BWF1 mice contain $CXCR5^+$ $ICOS^+$ follicular helper T cells whose function may be resistant to T_{reg} cell-mediated suppression. Further studies with regard to the impact of T_{reg} cells on humoral immune response as well as the interaction between T_{reg} cells and their target cells will be required to clarify their role in antibody-mediated autoimmune diseases such as SLE.

Concomitant migratory behavior of T_{reg} cells and conventional T cells was shown to be crucial for the immunoregulatory function of T_{reg} cells.[26,31,32] Chemokines and their receptors, as well as the activation markers CD44, CD62L, CD69, and CD103, are the possible regulators of the migration of T cells. Our present data demonstrating similar localization of T_{reg} cells and conventional T cells with the comparable expression of chemokine receptors and activation markers between these cells suggest that regulation of the migratory behavior of these cells were not impaired; however, BWF1 mice still develop the fatal autoimmune response. This idea, together with our notion of intact suppressive activity, further suggests that failure of T_{reg} cells to control the disease is because of the other factor(s) residing in the microenvironment.

Collectively, we demonstrated that aged BWF1 mice developing lupus nephritis had increased $Foxp3^+$ $CD4^+$ T_{reg} cells with highly activated phenotype and altered localization, but with intact suppressive activity. Our present results may provide a clue to understanding the nature of T_{reg} cells in the lupus and also help to unveil the mechanisms of the failure of T_{reg} cells to control autoimmune responses. Further studies directed at these points would facilitate the development of novel strategies for the treatment of SLE.

Acknowledgments

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Tacrolimus, a Calcineurin Inhibitor, Overcomes Treatment Unresponsiveness Mediated by P-glycoprotein on Lymphocytes in Refractory Rheumatoid Arthritis

KATSUNORI SUZUKI, KAZUYOSHI SAITO, SHIZUYO TSUJIMURA, SHINGO NAKAYAMADA, KUNIHIRO YAMAOKA, NORIFUMI SAWAMUKAI, SHIGERU IWATA, MASAO NAWATA, KAZUHISA NAKANO, and YOSHIYA TANAKA

ABSTRACT. ***Objective.*** Tacrolimus, a calcineurin inhibitor, is used for treatment of rheumatoid arthritis (RA). It also inhibits functions of P-glycoprotein, which is involved in drug resistance. We examined the mechanisms of early response to 2-week tacrolimus treatment in patients with RA.

Methods. One hundred thirteen patients with refractory RA despite at least 3 antirheumatic agents, including methotrexate, were treated with tacrolimus (1.5–3 mg/day) and the response was assessed at 2 weeks. Expression of the multidrug resistance (*MDR-1*) gene and P-glycoprotein was assessed in peripheral blood mononuclear cells (PBMC) collected from 113 patients and 40 healthy subjects. The drug exclusion function by the P-glycoprotein was measured by the residual amount of intracellular tritium-labeled dexamethasone cell/medium ratio (C/M ratio).

Results. The disease activity of enrolled patients was 5.8 ± 1.2 (mean ± SD) by DAS28 erythrocyte sedimentation rate. A good response to tacrolimus was noted at 2 weeks in 22 of 113 patients. At baseline, PBMC of patients with RA showed upregulated expression of *MDR-1* gene and P-glycoprotein and low C/M ratio. The response to tacrolimus correlated with P-glycoprotein expression and C/M ratio. A significant improvement in C/M ratio was noted after 2 weeks of treatment. The C/M ratio correlated significantly with P-glycoprotein expression on CD4+ lymphocytes.

Conclusion. Early efficacy of tacrolimus treatment depended on its inhibitory effect on the drug exclusion function of P-glycoprotein, leading to restoration of intracellular therapeutic levels of corticosteroids and clinical improvement. Evaluation of P-glycoprotein expression on lymphocytes is potentially useful for predicting the response to RA treatment. (J Rheumatol First Release Jan 15 2010; doi:10.3899/jrheum.090048)

Key Indexing Terms:
P-GLYCOPROTEIN RHEUMATOID ARTHRITIS DRUG RESISTANCE
TACROLIMUS CALCINEURIN INHIBITOR

Rheumatoid arthritis (RA) is a systemic disease characterized pathologically by the presence of autoreactive lymphocytes and clinically by tenderness and swelling of multiple joints. Treatment of RA includes the use of antirheumatic drugs designed to correct the autoreactive lymphocytes[1]. However, some patients respond poorly to such drugs[2] or develop resistance to them after an initial response[3].

Drug resistance is partly caused by disturbance of the drug exclusion pump of P-glycoprotein on the surface of tumor cells[4-8]. P-glycoprotein is induced by transcription factor YB-1, which is activated by various external stimuli such as drugs and inflammatory cytokines and is expressed on cells following the induction of multidrug resistance gene (*MDR-1*).

We have reported the expression of P-glycoprotein in lymphocytes and that its expression pathway is similar to that of drug exclusion[9]. The transcription factor YB-1 is expressed in the cytoplasm of peripheral blood lymphocytes of healthy subjects[9]. Absence of *MDR-1* gene expression is associated with weak or no P-glycoprotein expression[9]. Thus, in peripheral blood lymphocytes of patients with RA, stimuli from inflammatory cytokines, such as interleukin 2 (IL-2), and stimuli from cells injured by longterm exposure

From the First Department of Internal Medicine, University of Occupational and Environmental Health, Japan.

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K. Suzuki, MD; K. Saito, MD, PhD; S. Tsujimura, MD, PhD; S. Nakayamada, MD, PhD; K. Yamaoka, MD, PhD; N. Sawamukai, MD, PhD; S. Iwata, MD; M. Nawata, MD; K. Nakano, MD, PhD; Y. Tanaka, MD, PhD.

Address correspondence to Dr. Y. Tanaka, First Department of Internal Medicine, University of Occupational and Environmental Health, Japan, 1-1 Iseigaoka Yahatanishi Kitakyushu 807-8555, Japan.
E-mail: tanaka@med.uoeh-u.ac.jp

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to antiinflammatory drugs and corticosteroids, cause translocation of transcription factor YB-1 from the cytoplasm into the nucleus, which in turn triggers the expression of *MDR-1*. Further, P-glycoprotein is expressed on the surface of lymphocytes, and drugs that are substrates of P-glycoprotein are exported outside the cell[9].

Similar to cyclosporine, tacrolimus is an effective drug widely used as a calcineurin inhibitor, particularly in transplant surgery[10,11]. Intracellularly, tacrolimus forms a complex with FK binding protein (FKBP-12). The complex binds to calcineurin, which results in inhibition of its activation. The complex also inhibits the activation of the transcription factor nuclear factor-ATc, thus preventing its entry into the nucleus. Consequently, the transcription of cytokines such as IL-2 and IL-4 is blocked, resulting in suppression of RA disease[12-15]. We reported previously that cyclosporine, a calcineurin inhibitor, also inhibits P-glycoprotein, and it can thus eliminate drug resistance in systemic lupus erythematosus, which is caused by drug exclusion due to expression of P-glycoprotein[9,16].

Tacrolimus also inhibits P-glycoprotein[17,18]; however, the drug resistance blocking action of tacrolimus is still unexplained, as is the consequence of this on treatment of RA. Studies indicate that the clinical effects of tacrolimus on RA appear after 1–2 months of treatment[19-25]. In our study, we report rapid improvement in clinical symptoms and laboratory tests after 2-week treatment with tacrolimus. Based on this finding, we then investigated the clinical effects of tacrolimus combined with other drugs including corticosteroids, which are substrates of P-glycoprotein. The results showed that tacrolimus suppresses drug exclusion by inhibiting P-glycoprotein, thus allowing restoration of therapeutic levels of corticosteroids within the cells.

MATERIALS AND METHODS

Patients. The subjects were 113 patients with RA who met the diagnostic criteria of the American College of Rheumatology[26] and showed resistance to antirheumatic agents. In addition, the study included peripheral blood samples from 40 healthy adults, matched by age and sex to the patients with RA. The control subjects were either staff members of our hospital or healthy subjects who visited our hospital for medical examinations. Patients with RA that was resistant to treatment were defined as patients with a Disease Activity Score (DAS28) erythrocyte sedimentation rate (ESR) score > 3.1 for RA activity despite receiving treatment with adequate doses of at least 3 antirheumatic drugs, including methotrexate (MTX), for a minimum of 3 months, and showing no or moderate response, according to the European League Against Rheumatism (EULAR) improvement criteria[27-29]. Tacrolimus was used for these patients with treatment-resistant RA. Tacrolimus was administered at a daily dose of 3 mg in patients ≤ 65 years of age and 1.5 mg for those aged ≥ 65 years. All other antirheumatic drugs used by the enrolled patients were withheld during our 4-week study, except for nonsteroidal antiinflammatory drugs and corticosteroids. One patient who underwent treatment by biological disease-modifying antirheumatic drugs was not included in our study.

We started tacrolimus at the hospital of the University of Occupational and Environmental Health, Japan. The same person evaluated the clinical efficacy of tacrolimus throughout the observation period. The expression of P-glycoprotein on lymphocytes and polymerase chain reaction (PCR) analysis of the *MDR-1* gene were conducted by an individual different from the one who evaluated the clinical effects such as the number of swollen and tender joints, and evaluation by visual analog scale. Data analysis was performed by a third individual blinded to the other 2 investigators.

The Human Ethics Review Committee of the university reviewed and approved our study, including the collection of peripheral blood samples from healthy adults and patients with RA. Each subject provided a signed participation consent form.

Measurements. The background factors investigated were sex, age, duration of RA, dosage of antirheumatic drugs including corticosteroids, history of treatment, use of concomitant drugs such as corticosteroids at the time of initiation of tacrolimus therapy, and the dosage of other drugs.

We also evaluated the severity of morning stiffness, number of swollen joints, number of tender joints, and patient-evaluated pain and overall evaluation by a visual analog scale, in addition to evaluation by the attending physician. The response to treatment was evaluated using the EULAR criteria[28,30,31]. The laboratory tests included C-reactive protein (CRP), ESR, rheumatoid factor (RF), and matrix metalloproteinase-3.

Expression of P-glycoprotein on peripheral blood mononuclear cells. Peripheral blood mononuclear cells (PBMC) were separated by specific gravity fractionation using Lymphocyte Separation Medium 50494 (Pharmacia Biotech, Uppsala, Sweden). Of the CD antigens that were expressed on PBMC, ≥ 90% were on lymphocytes (CD4, CD8–, or CD19+ cells) and ≤ 10% were on mononuclear cells. PBMC were stained and analyzed by flow cytometry using the standard procedure of FACScan (Becton Dickinson, Mountain View, CA, USA) as described[32,33]. The cells were divided to a final concentration of 2×10^5 cells/well. Polyclonal γ-globulin (10 μg/ml; Mitsubishi Welpharma Co., Osaka, Japan) was added to the culture to block Fc receptors. After culture, MRK-16 (100 μg/ml; Kyowa Medex, Tokyo, Japan), a monoclonal antibody specific for P-glycoprotein[34], was added to the culture solution. The cells were further labeled with FITC-conjugated antimouse IgG antibody (5 μg/ml; Fujisawa, Osaka, Japan). For cell staining, cells were treated with antimouse IgG that binds to nonspecific sites before using phycoerythrin-conjugated CD4 mAb or CD19 mAb (1.25 μg/ml; Becton Dickinson). Of these double-stained cells, target cells that were gated based on CD4 or CD19 expression were extracted using FACScan. Quantification of the cell surface antigens on 1 cell was performed using QIFIKIT beads (Dako, Kyoto, Japan) as reported[33,35]. The data were used to construct a calibration curve of the mean fluorescence intensity versus antibody-binding capacity. The cell specimen was analyzed on the FACScan and the antibody-binding capacity calculated by interpolation on the calibration curve. When the green-fluorescence laser detection was set at 500 nm in the FACScan, the antibody-binding capacity was equal to 202.98 × exp (0.0092 × mean fluorescence intensity) (R^2 = 0.9995). Subsequently, the specific antibody-binding capacity was obtained after correcting for the background and apparent antibody-binding capacity of the negative control antimouse IgG antibody. The specific antibody-binding capacity represented the mean number of accessible antigenic sites per cell, referred to as antigen density and expressed in sites/cell.

Cell/medium ratio (C/M ratio) of dexamethasone. To assess the function of P-glycoprotein, PBMC were collected from patients with RA and analyzed for the residual amounts of carbon-labeled butanol and tritium-labeled dexamethasone in cells. The C/M ratio represented the coefficient of intracellular and extracellular ratio of dexamethasone and was determined using the formula:

$$\text{C/M ratio} = [(^3\text{H in cell fraction}/^{14}\text{C in cell fraction})/(^3\text{H in medium fraction}/^{14}\text{C in medium fraction})]$$

Changes in the residual amount of the drug in PBMC of patients with RA were assessed by pretreatment of the cells with tacrolimus 0–50 ng/ml, sulfasalazine (SSZ) 0–50 μg/ml, or MTX 0–400 ng/ml.

Carbon-labeled n-butanol (1.61 mCi/mmol; Toho Biomedical, Tokyo, Japan) was diluted with 0.5 MBq/ml of non-carbon-labeled butanol (Sigma

Aldrich, Tokyo, Japan). Tritium-labeled dexamethasone (40.0 Ci/mmol; Perkin Elmer, Boston, MA, USA) was dissolved in dimethyl sulfoxide (DMSO; Nacalai Tesque Inc., Tokyo, Japan) and diluted with phosphate-buffered saline (PBS; adjusted to a final concentration of DMSO of 0.1%). To supply ATP, 7 mM of dextrose was added to PBS. PBMC were suspended in this solution and adjusted so that the cell density was 5×10^6 cells/ml[36]. PBMC were cultured at 37°C for 20 min in 5×10^{-5} M of carbon-labeled butanol and 3.0×10^{-8} M of tritium-labeled dexamethasone. After the culture, 100 μl aliquots of the cells were layered in 80 μl of a mixture of lauryl bromide and silicone oil (2:1 ratio; Nacalai Tesque) in an Eppendorf tube (Assist, Tokyo, Japan). After centrifugation at 10,000 rpm for 2 min, the tube was instantly frozen in liquid nitrogen. The frozen tube was divided into medium (upper layer) and mixture (cell components, bottom layer) bound parts. Soluene-350 and 10 ml Hionic-Flouor (Packard, Meriden, CT, USA) were added to the cell components. The medium part was dissolved in a mixture of toluene (Wako Pure Chemicals, Osaka, Japan), methanol (Wako), ethylene glycol monoethanol (Nacalai Tesque), and Permaflour (ratio 200:50:50:12; Packard). Both parts were irradiated using a scintillation counter.

Reverse transcription-PCR. Whole-cell RNA was obtained from PBMC of healthy adults and patients with RA and separated using the Isogen (Wako) protocol. Total RNA (500 ng) was reverse transcribed for 30 min at 42°C. Denaturing was performed for 45 s using iCycler (Bio-Rad, Richmond, CA, USA). Amplification was performed with annealing at 55°C for 45 s and extension at 72°C for 90 s using the 30-cycle specific *MDR-1* and ß-actin primers. The sequences of the primers are human ß-actin forward, 5'-TGA ACC CCA AGG CCA ACC GC-3', reverse, 5'-TTG TGC TGG GTG CCA GGG CA-3'; and human *MDR-1* forward, 5'-CCC ATC ATT GCA ATA GCA GG-3', reverse, 5'-GTT CAA ACT TCT GCT CCT GA-3'. Amplified products were electrophoresed with Marker 4 (Nippon Gene, Tokyo, Japan) on 3% agarose gels.

Statistical analysis. Continuous variables related to patients' background are presented as mean ± standard deviation. Differences between 2 groups were tested statistically by Wilcoxon test.

Correlations between background factors and response to treatment at Week 2 were analyzed by Pearson's correlation coefficient. Multivariate logistic analysis was also performed. A 2-tailed p value ≤ 0.05 was considered statistically significant. All statistical analyses were performed using JMP software, version 7.0 (SAS Institute Inc, Cary, NC, USA) on Mac OSX.

RESULTS

A total of 113 patients with RA were treated with tacrolimus. The mean age of the population was 65.5 ± 12.2 years (median 66 yrs), mean disease duration was 114 ± 120 months (median 96 mo), the ratio of men to women was 23:90, the mean dose of corticosteroid was 2.69 ± 3.69 mg/day, and the rate of concomitant treatment with corticosteroid was 56%.

The mean DAS28-ESR of the enrolled patients with RA at the time of initiation of tacrolimus treatment was 5.8 ± 1.2 (median 5.9) and the percentage of patients with a high DAS28-ESR disease activity score (≥ 5.1) was 83% (Figure 1A). However, the percentage of patients with a DAS28-ESR disease activity score < 3.2, categorized as low disease activity, increased from 0% at baseline to 23% after 2 weeks of the tacrolimus therapy. The percentages of patients with a good response, moderate response, and no response were 19%, 34%, and 47%, respectively, according to the improvement criteria for response to treatment proposed by the EULAR (Figure 1B). In general, the clinical efficacy of tacrolimus, mediated through inhibition of calcineurin, becomes evident after more than 1 month of therapy. However, in our study, about 20% of the patients showed marked response at 2 weeks after the start of treatment. The effects lasted for 12 weeks after tacrolimus treatment and DAS28 gradually decreased, and the proportion of patients who achieved more than moderate response also increased.

Next, we investigated the mechanism of the marked response to 2-week tacrolimus treatment. To examine the possibility that the early efficacy was due to the inhibitory effect of tacrolimus on P-glycoprotein, we analyzed P-glycoprotein expression on lymphocytes and drug exclusion by P-glycoprotein using the C/M ratio. P-glycoprotein was overexpressed in CD4 and CD19 lymphocytes of patients with RA, compared with the healthy controls (Figure 2A). The *MDR-1* gene was not expressed in the lymphocytes of healthy controls. In contrast, even with no stimulation, the *MDR-1* gene was clearly expressed (Figure 2B) in lymphocytes of all of 5 representative patients with RA (patients 1–3 had good response and 4 and 5 had moderate response).

We also measured intracellular concentrations of tritium-labeled dexamethasone *in vitro*. In healthy adult lymphocytes that do not express P-glycoprotein, the C/M ratio increased in a time-dependent manner. In contrast, there was no increase in C/M ratio in P-glycoprotein-expressing lymphocytes of patients with RA (Figure 3A). The addition of tacrolimus at 0–50 ng/ml, which has an inhibitory effect on P-glycoprotein, increased the C/M ratio in a dose-dependent manner (Figure 3B). However, the addition of MTX or SSZ as the control drug did not result in any significant change in the C/M ratio (Figure 3B).

Next, we analyzed the relationships among various background factors and the response to tacrolimus treatment (Table 1). The response to such treatment was not influenced by factors related to RA disease activity, including number of swollen joints, number of tender joints, visual analog scale, CRP, and ESR. Further, the response to tacrolimus treatment did not correlate with age, sex, duration of illness, mean corticosteroid dose, and tacrolimus dose. On the other hand, the rate of concomitant use of corticosteroids was significantly different among nonresponders (32/53; 60.4%), moderate responders (18/38; 47.4%), and good responders (22/22; 100%), as shown in Table 1. The percentage of patients using corticosteroids who did not achieve good response to tacrolimus (53.9%) was significantly lower than that of patients who did.

We also analyzed the correlation between response to treatment and P-glycoprotein expression and C/M ratio (Table 1). In this analysis, P-glycoprotein expression on CD4– and CD19+ lymphocytes was stratified by the response to treatment. The expression levels of P-glycoprotein in both CD4– and CD19+ lymphocytes were significantly higher in the good response group compared with the

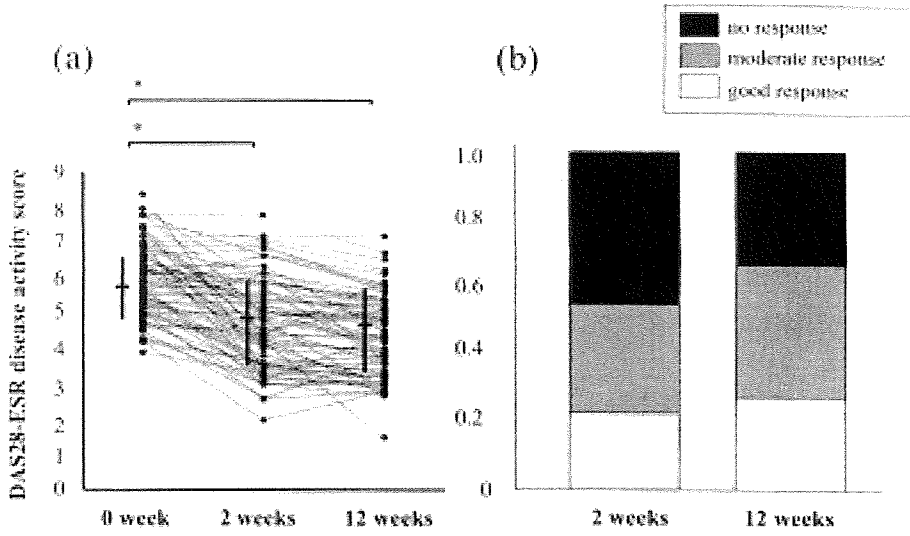

Figure 1. Changes in disease activity and treatment response at baseline (before induction of tacrolimus treatment) and at 2 and 12 weeks of treatment in patients with treatment-resistant RA. RA disease activity was assessed by DAS28-ESR. Treatment response was assessed using EULAR criteria. A. Disease activity. Data for individual patients and mean ± SD values for baseline and posttreatment for the groups. *p < 0.05, Wilcoxon test. B. Treatment response at 2 and 12 weeks after commencement of tacrolimus treatment.

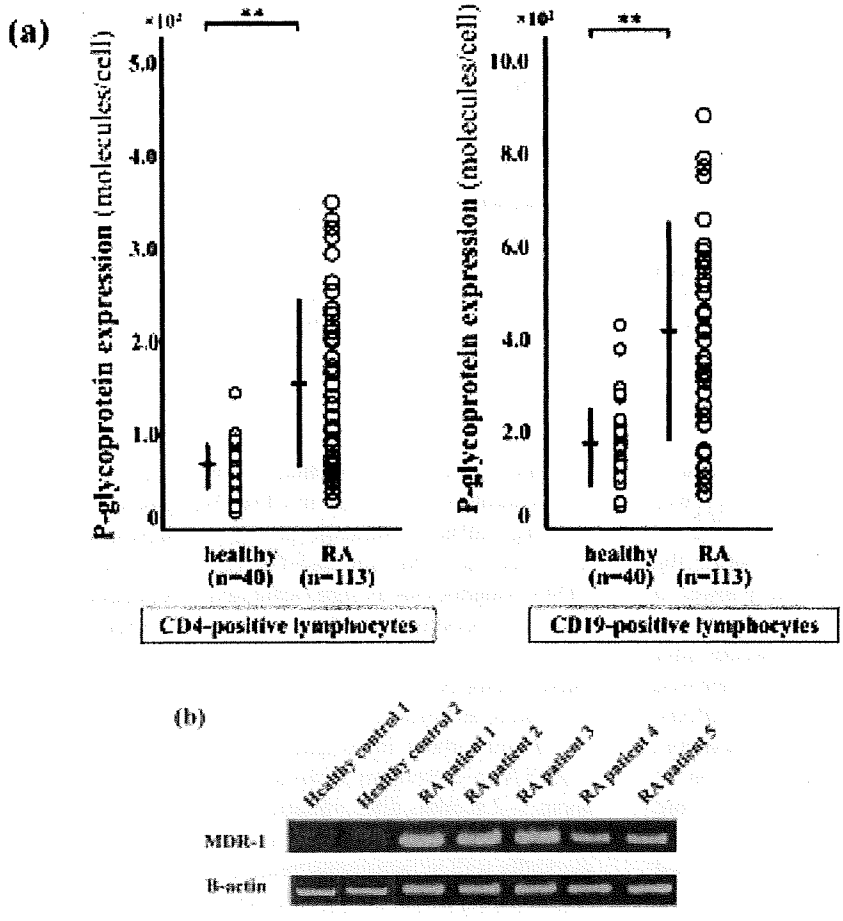

Figure 2. P-glycoprotein expression on lymphocytes of patients with RA treated with tacrolimus and healthy controls. A. Mononuclear cells were isolated from peripheral blood of patients with treatment-resistant RA and controls and stained with CD4 and CD19 antibodies and P-glycoprotein antibodies. Expression of P-glycoprotein on lymphocyte was measured. Data for individual subjects and mean ± SD values of the 2 groups are shown. **p < 0.01, Wilcoxon test. B. Multidrug resistance gene (*MDR-1*) was assessed by RT-PCR. Note the expression of *MDR-1* in patients with treatment-resistant RA but not in healthy controls. Representative results of 5 experiments with similar results. Electrophoresis was performed on the amplifier together with Marker 4 in 3% agarose gel.

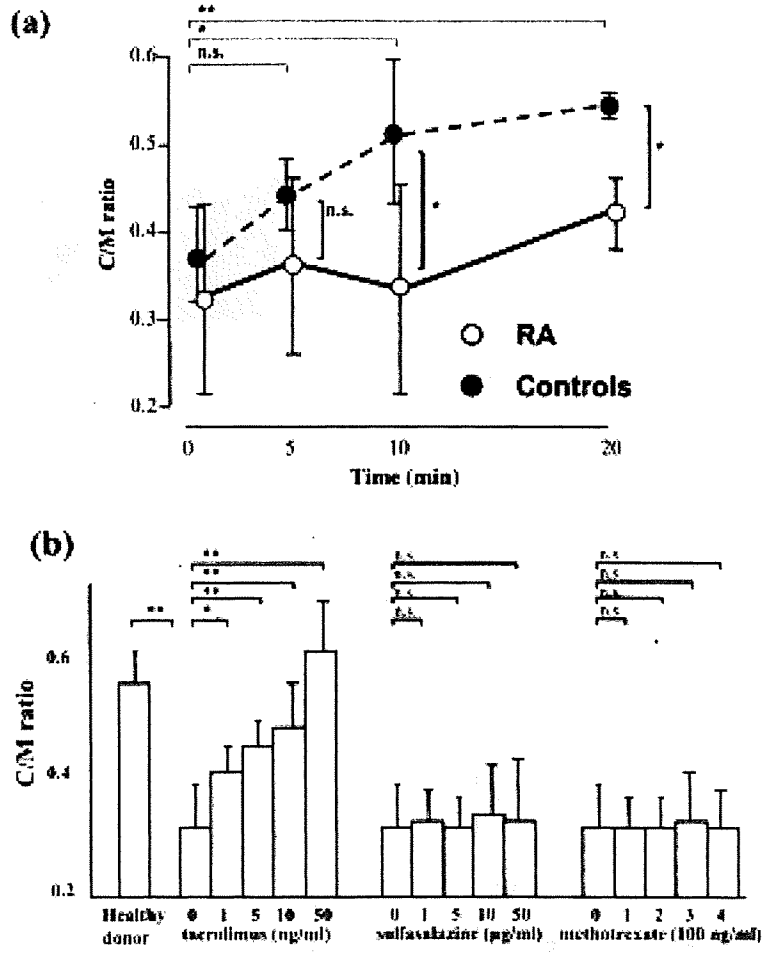

Figure 3. Serial changes in cell/medium (C/M) ratio and changes in C/M ratio after the addition of drugs. Data are mean ± SD. A. Serial changes in C/M ratio. B. Changes in C/M ratio of peripheral blood mononuclear cells from patients with treatment-resistant RA in response to tacrolimus and 2 anti-RA drugs, sulfasalazine and methotrexate. *p < 0.05, **p < 0.01, Wilcoxon test; ns: nonsignificant.

other groups (no response and moderate response; Table 1, Figure 4A). Moreover, the C/M ratio was significantly lower in the good response group compared with the moderate or no response group. Interestingly, the C/M ratio before the initiation of tacrolimus treatment was significantly lower in the good response group compared with the moderate and no response groups (Figure 4B). However, at 2 weeks after the initiation of tacrolimus treatment, the value improved significantly in the good response group compared with the moderate and no response groups (Figure 4C). In addition, P-glycoprotein expression at 2 weeks after initiation of tacrolimus therapy was similar to that at baseline (before therapy; Figure 4D).

Finally, multivariate logistic regression analysis was used to determine those factors that can explain the response to tacrolimus treatment. The analysis included the C/M ratio, P-glycoprotein expression on CD4+ lymphocytes, CRP, and morning stiffness. Multivariate logistic regression analysis identified the C/M ratio and P-glycoprotein expression on CD4+ lymphocytes as significant and independent determinants of a good response to tacrolimus treatment (Table 2).

DISCUSSION

The safety and efficacy of tacrolimus in the treatment of RA is currently under investigation. The response of patients with RA to tacrolimus treatment is normally investigated about 1–2 months after initiation of such treatment[19-25]. In our study, however, we noted that some patients responded markedly well to tacrolimus treatment after only 2 weeks. In order to explain this early response to treatment, we investi-

Table 1. Baseline clinical characteristics of patients with RA treated with tacrolimus. P value represents differences between good response and moderate and no response groups.

Characteristics	No Response (n = 53)	Moderate Response (n = 38)	Good Response (n = 22)	p
Age (yrs)	65.1 ± 12.7	66.2 ± 15.3	68.0 ± 12.2	NS
Disease duration (mo)	166 ± 143	115 ± 108	171 ± 157	NS
Morning stiffness (min)	278 ± 369	171 ± 323	195 ± 413	NS
No. of tender joints	10.2 ± 6.1	8.6 ± 4.6	9.9 ± 8.2	NS
No. of swollen joints	9.0 ± 4.3	7.3 ± 4.8	7.0 ± 3.9	NS
Visual analog scale (mm)	56.5 ± 18.6	66.5 ± 21.0	54.6 ± 24.5	NS
C-reactive protein (mg/dl)	2.8 ± 2.6	1.5 ± 1.6	2.2 ± 2.6	NS
Erythrocyte sedimentation rate (ESR, mm/h)	60 ± 29	48 ± 26	42 ± 32	NS
Rheumatoid factor (IU/l)	275 ± 341	423 ± 1103	68 ± 63	NS
Matrix metalloproteinase	296 ± 302	302 ± 371	192 ± 139	NS
Disease activity 28 ESR	5.8 ± 0.7	5.7 ± 0.8	5.3 ± 0.9	NS
Prednisone (mg)	1.3 ± 2.6	1.5 ± 3.8	2.9 ± 2.7	NS
Patients on prednisone (%)	60.4 (32/53)	47.4 (18/38)	100 (22/22)	0.05
Tacrolimus (mg)	2.1 ± 0.8	2.0 ± 0.8	1.9 ± 0.8	NS
P-glycoprotein expression on CD4 (molecule/cell)	140 ± 58	159 ± 65	248 ± 110	0.05
P-glycoprotein expression on CD19 (molecule/cell)	374 ± 171	388 ± 173	586 ± 193	0.05
C/M ratio	0.49 ± 0.12	0.49 ± 0.17	0.36 ± 0.12	0.01

C/M: cell/medium. NS: nonsignificant.

gated the inhibitory effect of tacrolimus on P-glycoprotein, both *in vitro* and *in vivo*. PBMC of patients with RA showed expression of the *MDR-1* gene, and overexpression of P-glycoprotein on the lymphocyte surface. These features were not identified in PBMC from healthy adults. Further, the C/M ratio in patients with RA was lower than that of the healthy adults, indicating enhanced drug exclusion function of P-glycoprotein. However, in *in vitro* experiments, the addition of tacrolimus to PBMC improved the C/M ratio to a level similar to that of healthy adults, and the response depended on the dose of tacrolimus added to the cell culture. We also tested the response of PBMC to 0–50 g/ml SSZ and 0–400 ng/ml MTX. These doses represent the C_{max} values used clinically. At these concentrations, SSZ and MTX, which do not inhibit P-glycoprotein, did not improve the C/M ratio. These findings indicate that the improvement in C/M ratio is not due to a secondary inhibition of P-glycoprotein through inhibition of lymphocyte activation. The results of *in vitro* experiments were confirmed *in vivo*; tacrolimus produced a significant increase in the C/M ratio of patients who showed a good response to 2-week tacrolimus therapy (based on the improvement criteria of EULAR). Multivariate logistic analysis indicated that after 2 weeks of tacrolimus treatment, overexpression of P-glycoprotein on lymphocytes in the good response group correlated significantly with a low C/M ratio before treatment. Since all patients were also treated with corticosteroids throughout the study, the above findings suggest that the early response to tacrolimus therapy was not due to tacrolimus alone but also to inhibition of extracellular exclusion of corticosteroids, mediated by the inhibitory effect of tacrolimus on P-glycoprotein expression. In addition, there were no differences in P-glycoprotein expression between initiation and 2 weeks after initiation of tacrolimus (Figure 4D). Therefore, the main effect of tacrolimus was due not to a decrease in P-glycoprotein molecules on lymphocytes, but to functional competitive inhibition of drug exclusion.

Humoral factors such as TNF-α, IL-6, and IL-1 that are locally produced in joints, or upregulation of P-glycoprotein expression through induction of transcription of *MDR-1* gene following activation of YB-1 by certain drugs that are added to the treatment for RA such as antirheumatic drugs and corticosteroids, are involved in the development of resistance to treatment in patients with RA. Often, the same drug must be used continuously over a long period to control RA disease activity. During this period, expression of the multidrug resistance genes may be induced due to increased disease activity, resulting in drug resistance. For example, increased disease activity and low response rate following longterm use have been reported during treatment with SSZ. In fact, upregulation of P-glycoprotein expression was reported in low responders to SSZ[37]. With SSZ, which is used in the treatment of RA, stimulation of cells that enhance P-glycoprotein expression causes simultaneous extracellular exclusion of the drug. Thus, the low response to SSZ is probably due to increased extracellular exclusion of the drug through P-glycoprotein. The above finding of the inhibitory effect of tacrolimus on P-glycoprotein expression suggests that tacrolimus could potentially improve the resistance to other drugs in addition to corticosteroids.

We demonstrated that tacrolimus inhibits drug exclusion mechanisms operating through P-glycoprotein in patients with good response after 2 weeks of treatment. As a drug used for management of treatment-resistant RA, tacrolimus inhibits disease activity by blocking the calcineurin pathway and improves the low response by inhibiting drug exclusion

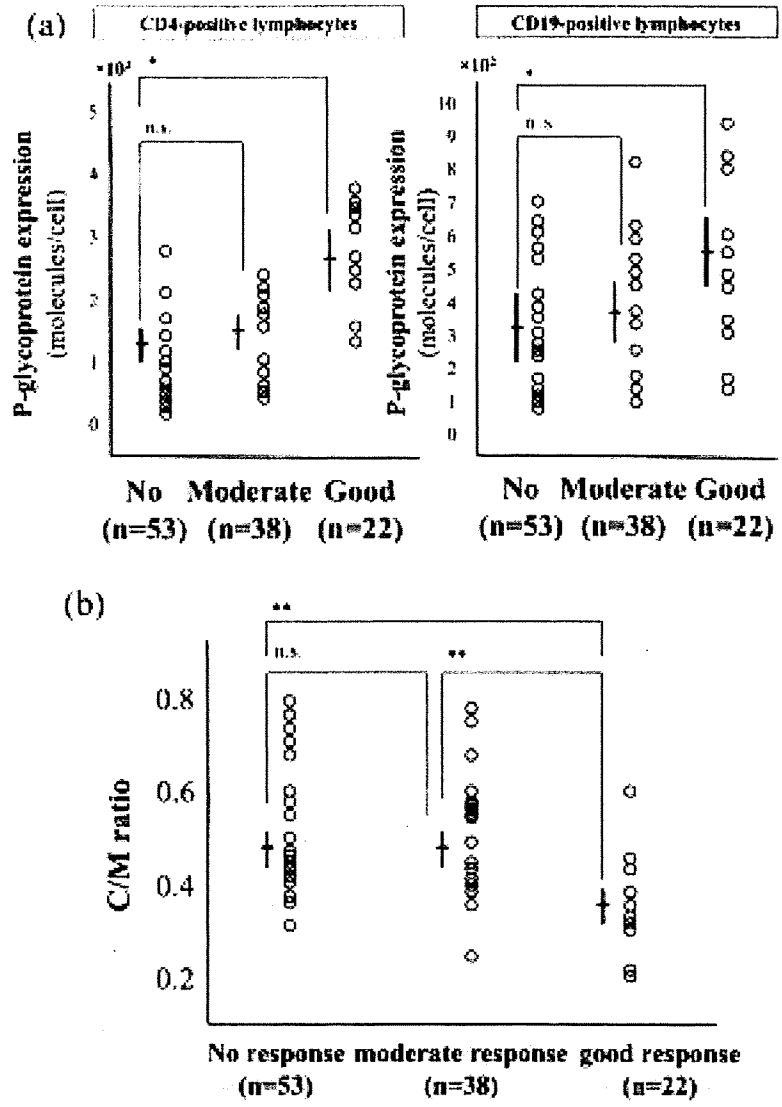

Figure 4. Responses to 2-week treatment with tacrolimus. Data represent values of individual patients and group mean ± SD values. A. Expression of P-glycoprotein on CD4 and CD19 lymphocytes of patients with treatment-resistant RA. B. C/M ratio of peripheral blood mononuclear cells (PBMC) from patients with treatment-resistant RA. C. C/M ratio measured in PBMC of patients with RA before and after 2-week tacrolimus treatment. D. Expression of P-glycoprotein on CD4 lymphocytes of patients with RA before and after 2-week tacrolimus treatment. *$p < 0.05$, **$p < 0.01$, Wilcoxon test; ns: nonsignificant.

of concomitantly used drugs through P-glycoprotein. Thus, among the existing antirheumatic drugs, tacrolimus is a potentially useful drug, although its mechanism of action is unknown. Patients on combination treatment have high expression levels of P-glycoprotein and low C/M ratio before the initiation of tacrolimus treatment because the concomitantly used drug, such as a corticosteroid, becomes a substrate for P-glycoprotein. Thus, in such patients, treatment with tacrolimus is expected to result in an increase in intracellular concentration of corticosteroids through the inhibitory effect of tacrolimus on P-glycoprotein, leading to a rapid remanifestation of the clinical efficacy of corticosteroid and thus early efficacy. In other words, it is likely that tacrolimus, through its inhibitory action on P-glycoprotein, can lower treatment resistance due to extracellular exclusion of drugs such as corticosteroids and SSZ, leading to improved response to treatment. Our results suggest that initiation of tacrolimus treatment for such patients is highly useful clinically. In addition, the determination of P-glycoprotein expression level on lymphocytes, which correlates with clinical response, is useful for predicting the response to treatment as well as helping with the choice of therapy.

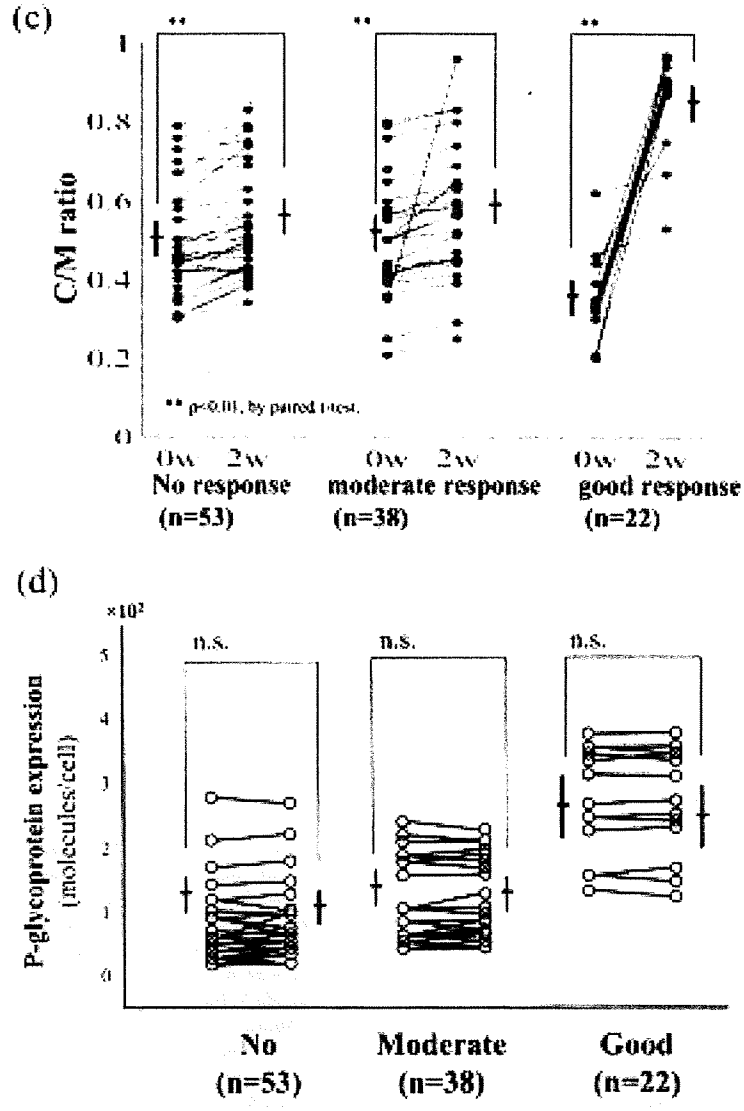

Figure 4. Continued.

Table 2. Multivariate logistic analysis of factors involved in response of patients with RA to tacrolimus treatment.

Measurement	Estimate	SE	Chi-square	Pr > z
Intercept	0.1069	2.29120	0.00	0.9628
C/M ratio	9.0576	4.44129	4.16	0.0414
P-glycoprotein expression on CD4+ lymphocyte	–0.0133	0.00609	4.79	0.0286
CRP	–0.1996	0.24054	0.69	0.4066
MS	0.00149	0.001593	0.88	0.3490

C/M: cell/medium; CRP: C-reactive protein; MS: morning stiffness.