To our knowledge, the remaining five genes (*mptl, Laptm5, UCP2 Gnai2* and *IL16*) have not been identified previously as SS-related genes. *Mptl* was cloned as a mouse homologue of Drosophila retinal degeneration B (rdgB), and the mptl protein has been predicted to be a membrane-bound phosphatidylinositol transfer protein (PITP) [33], which transports phosphatidylinositol (PI) through the aqueous phase from one membrane compartment to another and functions as a cofactor for the synthesis of phosphatidylinositol bisphosphate (PIP2) [34]. Given that the constitutive turnover of PI is markedly augmented in some subsets of T lymphocytes in MRL/lpr mice [35], it is fair to speculate that such T cells accumulate and are related to the pathogenesis of SS. *Laptm5* is highly expressed in adult hematopoietic organs such as bone marrow, spleen, thymus, lymph nodes, and peripheral blood leukocytes [36]. The high expression of *Laptm5* in our MRL/lpr mouse model of SS could be due to an increased number of lymphocytes infiltrating the salivary glands. Further experimental studies are required to clarify the role of *Laptm5* in the pathogenesis of SS. The mitochondrial protein known as uncoupling protein 2 (*UCP2*) is highly expressed in the spleen and macrophages. A recent report suggests that *UCP2* plays a role in limiting macrophage-mediated immunity [37]. The expression of *UCP2* is induced by TNF-α [38] and the expression of TNF-α is increased in MRL/lpr mice [39]. These combined findings suggest that increased TNF-α in MRL/lpr mouse salivary glands could contribute to the up-regulation of *UCP2* and subsequent disease progression.

We then examined the expression of these genes in the salivary glands of MRL/lpr mice and NFS/sld mice that had undergone a thymectomy (Tx-NFS/sld), a new model for primary SS, by using real-time-quantitative reverse transcription-polymerase chain reaction analysis. The expression of 11 genes (*IL-16*, *Grap*, *caspase3*, *Ly-6C.2*, *Mel-14 antigen*, *cathepsin B*, *mptl*, *Laptm5*, *Gnai2, vimentin,* and *UCP2)* was higher in MRL/lpr mice than in MRL/++ mice and the expression of 9 genes (*IL-16*, *Grap*, *caspase3*, *Ly6c2, Mel-14 antigen*, *cathepsinB*, *mptl Laptm5,* and *Gnai2)* was higher in the Tx-NFS/sld mice than in the control mice that did not undergo thymectomy. In addition, the fetus microarray analysis demonstrated that the *Laptm5* gene was also highly expressed in the salivary glands of Tx-NFS/sld mice. Furthermore, immunohistochemical studies showed that mouse and human *Laptm5* protein antigens were expressed on certain infiltrated lymphocytes and on ductal cells in the salivary glands from the SS mouse model and patients with SS, but very weakly in control subjects. These results suggest that some apoptosis-related genes might be responsible for the pathogenesis of organ-specific autoimmune lesions in SS (Arthritis Rheumatism Vol. 9, S251, 2001, Arthritis and Rheumatism Vol. 50 S577 2004).

These early findings confirm the excellent specificity and reproducibility of our cDNA microarray analysis for identification of disease-related genes. A microarray system can handle thousands of genes and help in the extraction of genes that have significant relationships to the stages of a disease. In addition, an in-house cDNA microarray is advantageous in allowing the exchange of arrayed genes and assaying specific disease-related genes on one glass slide, which is useful for the diagnosis and prediction of clinical stages.

In the present study, we isolated nine SS-related genes on a cDNA microarray using the MRL/lpr SS mouse model. Further studies may allow the identification of other SS-related genes, thus allowing the performance of clustering analysis, which could provide useful information about classification of the disease, clinical course, stage of the disease, and selection of a suitable treatment. The combination of an in-house microarray and the use of an animal model is a suitable strategy for exploring a gene expression profile and should gradually evolve into a system useful for clinical investigation.

Take-home message

- The combination of cDNA microarray and use of animal models is a suitable strategy for exploring a gene expression profile and should gradually evolve into a system useful for clinical investigation.
- Fifteen highly expressed genes, *IL-16*, *Grap*, *caspase3*, *Ly-6C.2*, *Mel-14 antigen*, *cathepsin B*, *mptl*, *Laptm5*, *Gnai2, vimentin*, *UCP2*, *saposin*, *Trt, laminin receptor 1*, and *HSP 70 cognate*, in the salivary gland of MRL/lpr mouse by cDNA microarray analysis were identified.
- The expression of 9 genes, *IL-16*, *Grap*, *caspase3*, *Ly6c2, Mel-14 antigen*, *cathepsin B*, *mptl Laptm5*, and *Gnai2,* was higher in the salivary gland of

thymectomized-NFS/sld mice than in the control mice that did not undergo thymectomy.

- Five genes, *mpt1, Laptm5, UCP2 Gnai2* and *IL16*, have not been identified previously as SS-related genes.
- Human *Grap* and *Laptm5* protein antigens were expressed on certain infiltrated lymphocytes and on ductal cells in the salivary glands from patients with SS, but very weakly in control subjects.

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Endometriosis and systemic lupus erythematosus: a comparative evaluation of clinical manifestations and serological autoimmune phenomena.

Due to evidences suggesting association between endometriosis (EM) and systemic lupus erythematosus (SLE), Pasoto SG. et al. (Am J Reprod Immunol 2005;53:85–93), have performed a comparative evaluation of clinical and humoral immunologic abnormalities in both diseases. Forty-five women with histologically confirmed pelvic EM, 21 healthy-women and 15 female SLE-patients without surgically confirmed EM were prospectively evaluated. None of the EM-patients fulfilled criteria for SLE. However, EM-patients presented higher frequencies of arthralgia (62%) and generalized myalgia (18%) compared to that of normal-controls (24%, $p = 0.04$) but comparable with SLE-patients. Antinuclear antibodies (ANA) were detected in 18% of EM-patients, as compared with healthy-women ($p = 0.01$). Anti-Ro and anticardiolipin antibodies were more often in SLE (40%, 33%) than in EM-patients (2%, $p < 0.001$ and 9% $p = 0.04$). Elevated immune-complexes and low total complement were also more frequent in SLE patients. The data indicate differences of ANA antigenic specificity and complement consumption between EM and SLE. The high prevalence of generalized musculoskeletal complaints in EM justifies a multidisciplinary approach.

Two cases of antinuclear antibody negative lupus showing increased proportion of B cells lacking RP105.

B cells lacking RP105 molecule, a member of the Toll-like receptor family, were increased in the peripheral blood of 2 patients with antinuclear antibody (ANA) negative systemic lupus erythematosus (SLE). The increased proportion of RP105-lacking B cells was associated with disease activity in patients with ANA-negative SLE. Koarada S. et al (J Rheumatol 2005; 32:562-4) suggested that when there are no significant serological markers for SLE, analyses of expression of RP105 may be helpful in evaluation of activity in ANA-negative SLE. Thus they describe a new approach, using phenotyping of B cells, to evaluate activity of ANA-negative SLE.

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Development of Autoimmune Arthritis With Aging Via Bystander T Cell Activation in the Mouse Model of Sjögren's Syndrome

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Objective. **A wide spectrum of extraglandular manifestations may occur in patients with Sjögren's syndrome (SS), but the mechanisms responsible for in vivo progression are still obscure. We undertook this study to evaluate the age-related changes during the development of extraglandular autoimmune lesions, including arthritis, in the murine model of primary SS, and to evaluate the possible relationship between age-related disturbance of activation-induced cell death and the in vivo kinetics against autoantigens.**

Methods. **A total of 126 NFS/*sld* mice were investigated at ages 2, 4, 6, 10, 12, 18, 20, and 24 months. Cytokine production was tested using culture supernatants from anti-CD3 monoclonal antibody–stimulated T cells. Anti–single-stranded DNA (anti-ssDNA) antibodies, Ig isotypes (IgG1, IgG2a), rheumatoid factor (RF), and anti–type II collagen (anti-CII) antibodies were detected by enzyme-linked immunosorbent assay. Proliferative T cell responses against each of 3 recombinant α-fodrin proteins and against CII were analyzed.**

Results. **Autoimmune arthritis developed in SS model mice until age 24 months. Significant elevations in serum levels of RF, anti-ssDNA antibodies, and anti-CII antibodies were found in aging SS model mice. A high titer of serum autoantibodies against α-fodrin fragments (containing different epitopes that were originally identified in primary SS model mice) was frequently detected in young and aged SS model mice. Moreover, we found that α-fodrin autoantigen induced Th1 immune responses and accelerated disturbance of Fas-mediated T cell apoptosis in aged SS model mice.**

Conclusion. **These results indicate that age-related disturbance of activation-induced cell death via bystander T cell activation may play a crucial role in the development of autoimmune arthritis in a murine model of SS.**

The age-related decline in thymic function causes extensive remodeling of the T cell system (1,2). Rheumatoid arthritis (RA), like many other autoimmune syndromes, is a disease of adults, with the highest incidence rates reported in the elderly (3,4). The immune system undergoes profound changes with advancing age that are beginning to be understood and that need to be incorporated into the pathogenetic models of RA. Age-dependent changes in T cell homeostasis are accelerated in patients with RA (5). The repertoire of naive and memory T cells is less diverse, possibly as a result of thymic insufficiency, and it is biased toward autoreactive cells.

Activation-induced cell death (AICD) is a well-known mechanism of peripheral T cell tolerance that depends upon an interaction between Fas and Fas ligand (FasL) (6,7). Aging is associated with progressive decline in T cell functions, including a decreased response to mitogens and soluble antigens, decreased production of interleukin-2 (IL-2) and decreased expression of IL-2 receptor, a decrease in naive cells and an increase in memory cells, and a defect in the signaling pathway (8–10). AICD plays a central role, especially in killing autoreactive T cells and in preventing autoimmune responses (11–13). AICD in T cells in vivo has been proposed to limit the expansion of an immune response

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by eliminating effector cells that are no longer needed (14). It has been reported that activation of T cell clones or T cell lines induces FasL expression, and that interaction between Fas and its ligand is the major mechanism involved in AICD (15,16). A defect in AICD of effector T cells may result in the development of autoimmune disease (17), but there is no clear in vivo role of organ-specific autoantigen for AICD with aging.

Primary Sjögren's syndrome (SS) is a T cell–mediated autoimmune disease, and autoreactive T cells bearing the CD4 molecule may recognize unknown self antigen triggering autoimmunity in the salivary and lacrimal glands, leading to clinical symptoms of dryness of the mouth and eyes (sicca syndrome) (18,19). Although it is well known that a wide spectrum of extraglandular manifestations including polyarthritis may occur in SS patients (20), detailed mechanisms of in vivo progression under autoimmune conditions are still obscure. Results from many animal models of autoimmunity indicate that self tissue damage leads to the activation of autoreactive T cells specific for autoepitopes distinct from those used to initiate the disease (i.e., epitope spreading) (21–25).

The aim of this study was to analyze the age-related changes during the development of extraglandular autoimmune lesions, including arthritis, in the murine model of primary SS. We also undertook to evaluate the possible relationship between age-related disturbance of AICD and the in vivo kinetics against autoantigens.

MATERIALS AND METHODS

Mice. An animal model for primary SS was previously established in NFS/*sld* mutant mice (26). Thymectomy was performed on day 3 after birth (3d-Tx), and a total of 126 NFS/*sld* mice, consisting of 72 3d-Tx and 54 nonthymectomized (non-Tx) female mice, were investigated. They were killed by cervical dislocation at ages 2, 4, 6, 10, 12, 18, 20, or 24 months. We analyzed 5–8 mice killed at each of these ages. Moreover, representative mice were chosen from the aged group (ages 20 and 24 months) and from the young group (ages 2 and 4 months) for comparison. Female C57BL/6 (B6) mice purchased from Charles River Japan (Atsugi, Japan) were used for age-matched controls (n = 36). Their care was certified by the Animal Welfare Board to be in accordance with institutional guidelines.

Histology and immunohistology. All organs were removed from the mice and fixed with 4% phosphate buffered formaldehyde (pH 7.2), and ankles were further decalcified in 10% EDTA. Sections (4 μm) were stained with hematoxylin and eosin. Histologic grading of inflammatory arthritis was performed according to the methods of Edwards et al (27), with 1 point for each of the following features (up to a total of 5 points), as follows: hyperplasia/hypertrophy of synovial cells; fibrosis/fibroplasia; proliferation of cartilage and bone; destruction of cartilage and bone; and mononuclear cell infiltrate.

Flow cytometric analysis. Surface markers were identified by monoclonal antibodies (mAb) with an EPICS flow cytometer (Coulter, Miami, FL). Rat mAb to CD3 (Life Technologies, Grand Island, NY), B220, CD4, and CD8 (Becton Dickinson, San Jose, CA), murine Fas (Jo2; PharMingen, San Diego, CA), and murine FasL (K-10; PharMingen) were used. Double-labeled surface phenotypes, such as CD3/B220, CD4/FasL, and CD8/FasL, were analyzed. Apoptotic cells were also detected with an EPICS flow cytometer using the Annexin V-FITC Apoptosis Detection Kit (Genzyme, Cambridge, MA). For detection of T cell activation markers, spleen and inguinal lymph node cell suspensions were stained with antibodies conjugated to phycoerythrin (anti-CD3 [Invitrogen, Carlsbad, CA], anti-CD4 [Cedarlane, Hornby, Ontario, Canada], and anti-B220 [Becton Dickinson]) and fluorescein isothiocyanate (anti-CD8 [Cedarlane] and anti-Thy1.2, anti-CD44, anti-CD45RB, and anti–Mel-14 [PharMingen]) and were analyzed with an EPICS flow cytometer.

Measurement of cytokines and matrix metalloproteinase 9 (MMP-9) production. Cytokine production was tested, using culture supernatants from anti-CD3 mAb–stimulated splenic T cells, by 2-step sandwich enzyme-linked immunosorbent assay (ELISA) using a mouse IL-2, IL-4, interferon-γ (IFNγ), and IL-10 kit (Genzyme). Briefly, culture supernatants were added to microtiter plates precoated with anti–IL-2, anti–IL-4, anti-IFNγ, and anti–IL-10 capture antibodies and incubated overnight at 4°C. After addition of biotinylated detecting antibodies and incubation at room temperature for 45 minutes, avidin–peroxidase was added and incubated at room temperature for 30 minutes. Plates were washed extensively with 0.1% Tween in phosphate buffered saline (PBS) between each step. Finally, ABTS substrate containing H_2O_2 was added, and the colorimetric reaction was read at an absorbance of 450 nm using an automatic microplate reader (Flow, McLean, VA). The concentrations (in pg/ml) of IL-2, IL-4, IFNγ, and IL-10 were calculated according to the standard curves produced by various concentrations of recombinant cytokines. MMP-9 production was tested by 2-step sandwich ELISA using a human MMP-9 kit (Genzyme).

Recombinant α-fodrin fragments. Recombinant α-fodrin protein, the complementary DNA (cDNA) encoding human α-fodrin (JS-1, 1–1,784 bp; 2.7A, 2,258–4,884 bp; 3′DA, 3,963–7,083 bp) (28), was constructed by inserting cDNA into the *Eco* RI site of pGEX-4Ts. Glutathione S-transferase (GST) fusion protein was expressed and purified using a GST gene fusion system (Amersham Biosciences, Piscataway, NJ).

Measurement of anti–single-stranded DNA (anti-ssDNA) antibodies, Ig isotypes, rheumatoid factor (RF), α-fodrin fragments, and anti–type II collagen (anti-CII) antibodies. Anti-ssDNA antibodies, Ig isotypes (IgG1, IgG2a), RF, and anti-CII antibodies were detected by ELISA as described previously (29–31). For anti-ssDNA antibody detection, plates were precoated overnight with methylated bovine serum albumin (BSA), followed by calf thymic DNA that had been boiled for 15 minutes and chilled on ice. The wells were subsequently blocked with 3% BSA in PBS for 2 hours at room temperature.

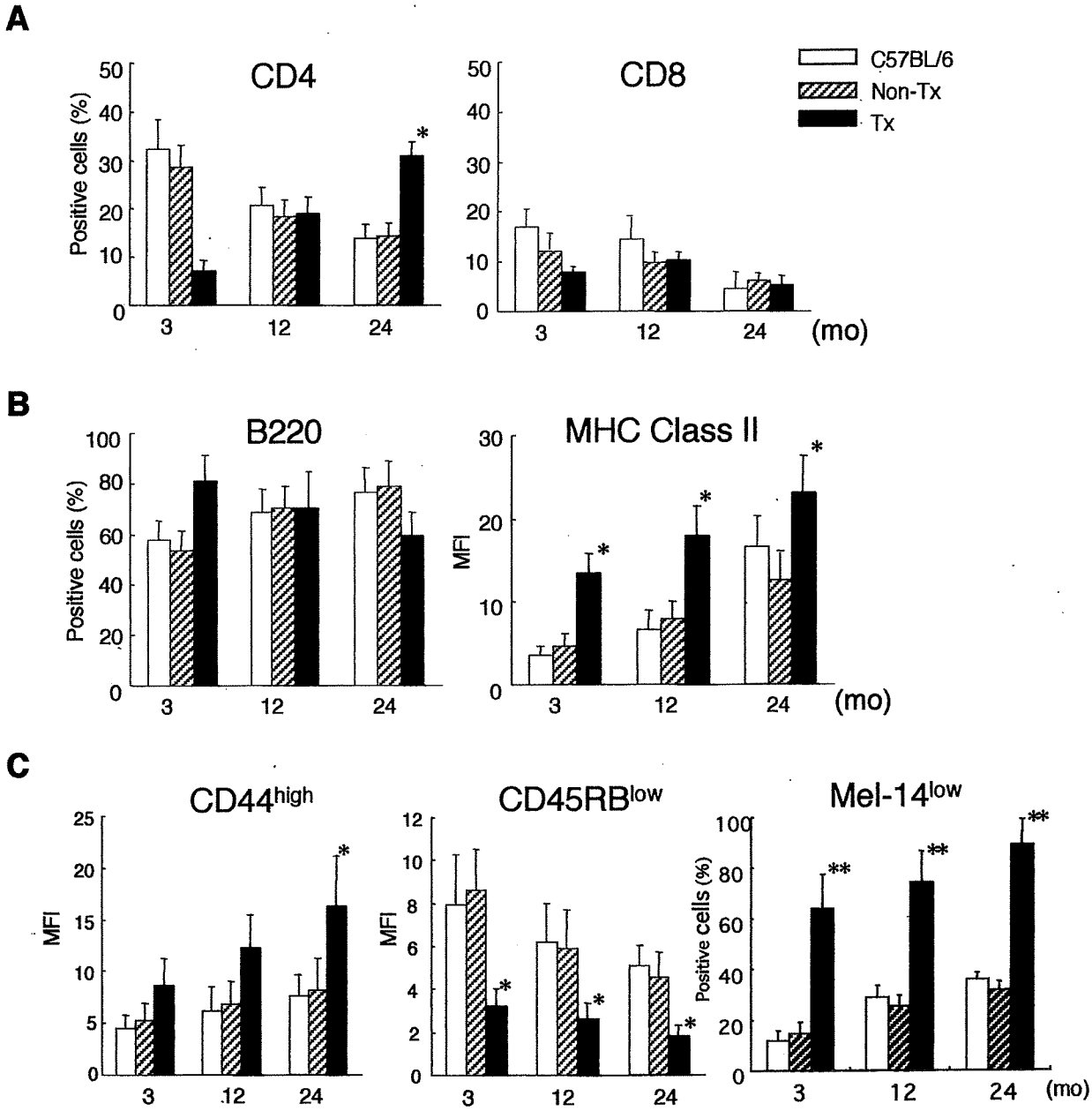

Figure 1. Age-related changes in activation markers. Shown are the results of the flow cytometric analysis of the CD4+,CD8+ T cell population, B220+ cells, class II major histocompatibility complex (MHC)–positive cells, and CD4+ T cells bearing activation markers in the spleens. Values are the mean and SD. **A,** An increase in the CD4+ T cell population, but not in the CD8+ T cell population, was detected in aged Sjögren's syndrome model mice (NFS/*sld* mice thymectomized on day 3 after birth, termed Tx mice). * = $P < 0.05$ versus nonthymectomized NFS/*sld* mice (non-Tx mice) or age-matched C57BL/6 control mice at age 24 months, by Student's *t*-test. **B,** Class II MHC–positive cells were significantly up-regulated in spleens with advancing age. * = $P < 0.05$ versus non-Tx or C57BL/6 control mice at all ages, by Student's *t*-test. No significant differences between groups were observed in the percentages of splenic B220+ cells. **C,** Significant up-regulation of CD4+ T cells bearing $CD44^{high}$, $CD45RB^{low}$, or Mel-14^{low} activation markers was observed in spleens from Tx mice with advancing age, but not in age-matched non-Tx or C57BL/6 control mice. * = $P < 0.05$; ** = $P < 0.01$, by Student's *t*-test. MFI = mean fluorescence intensity.

Serum samples were diluted (1:300) in 3% BSA in PBS, added to antigen-coated wells, incubated at 37°C for 1 hour, and washed 5 times with PBS containing 0.05% Tween 20. Subsequently, wells were incubated with peroxidase-conjugated goat anti-mouse Ig (BD PharMingen, San Diego, CA) for 1 hour and washed. We added 3,3′,5,5′-tetramethylbenzidine sub-

strate (Sigma, St. Louis, MO) and determined the absorbance spectrum with an automatic ELISA reader (Flow). Ig isotypes were assayed in the same manner using IgG1-specific and IgG2a-specific second-step conjugates and *p*-nitrophenyl phosphate substrate (Southern Biotechnology, Birmingham, AL). All assays were performed in duplicate and results were quantified against a standard curve obtained with the known positive control serum.

For the measurement of IgG-RF and IgM-RF, human IgG and IgM (Chemicon, Temecula, CA) were coated onto plates at 10 μg/ml in carbonate buffer, and the same procedures were followed as described above. Serum autoantibodies against α-fodrin fragments were detected using recombinant α-fodrin proteins. After coating with the recombinant α-fodrin protein in 96-well ELISA plates, biotinylated anti-mouse IgG (Vector, Burlingame, CA) was added as second antibody. Measurements of α-fodrin–specific autoantibodies were read with an automatic ELISA reader. For the measurement of serum antibodies to CII, native bovine CII was dissolved in 0.1*M* acetic acid at 1 mg/ml and diluted with 0.1*M* sodium bicarbonate at 10 μg/ml (pH 9.6). The microtiter plate was coated with 100 μl of CII antigen solution. After washing 3 times, 100 μl/well of serum samples that had been serially diluted in PBS/Tween 20/10% BSA and control serum were added and incubated for 1 hour at 37°C. After washing, peroxidase-conjugated goat anti-mouse IgG (at 1.4 μg/ml, 100 μl/well; Organon Teknika, Durham, NC) was added and incubated for 1 hour at 37°C. One hundred microliters of peroxidase-conjugated rabbit anti-mouse IgG (Zymed, San Francisco, CA) at a 1:1,000 dilution with PBS/Tween 20/10% BSA was added to detect anti-CII antibodies. A total of 100 μl *o*-phenylenediamine (0.5 mg/ml) dissolved in 0.1*M* citrate buffer (pH 5.0) containing 0.012% H_2O_2 was added, and the reaction was stopped using 8*N* H_2SO_4 (20 μl/well).

Proliferative T cell response. Single-cell suspensions of spleen cells were cultured in 96-well flat-bottomed microtiter plates (5×10^5/well) in RPMI 1640 containing 10% fetal calf serum, penicillin/streptomycin, and β-mercaptoethanol. Cells were cultured with 5 μg/ml of each recombinant α-fodrin protein (JS-1, 2.7A, and 3'DA), 5 μg/ml of bovine CII, and concanavalin A (EY Laboratories, San Mateo, CA) for 72 hours. During the last 8 hours of the 72-hour culture period, 1 μCi ^{3}H-thymidine was added per well, and the incorporated radioactivity was determined using an automated beta liquid scintillation counter (Aloka, Tokyo, Japan).

RESULTS

Analysis of the age-related changes in expression of activation markers. To clarify whether self-reactive T cells are spontaneously activated in aged SS model mice, we analyzed the CD4+,CD8+ T cell population, B220+ cells, class II major histocompatibility complex (MHC)–positive cells, and CD4+ T cells bearing activation markers in the spleens, by flow cytometry. The results showed that the CD4+ T cell population, CD4+ T cells bearing CD44^{high}, Mel-14^{low}, or CD45RB^{low} activation markers, and class II MHC–positive cells were significantly up-regulated in the spleens from SS model mice with advancing age, but not in the spleens from age-matched non-Tx and B6 mice (Figures 1A–C). These data suggested that the spontaneously activating CD4+ T cells and class II–expressing antigen-presenting cells appear in the spleen with advancing age in the murine model of SS.

Age-related changes in joint histopathology. We examined the in vivo age-related changes in the development of extraglandular manifestations of autoimmune lesions in NFS/*sld* SS model (3d-Tx) mice compared with those in NFS/*sld* control (non-Tx) mice and age-matched B6 mice. Inflammatory lesions in aged SS model mice were observed in the joints and in several organs, including the lung, liver, and kidney, and the most prominent histopathologic abnormalities were observed in arthritic lesions. Destructive autoimmune arthritis developed in aging SS model mice, and these lesions became more aggravated with age from 6 months until 24 months. Histologic analysis of the knee joints was performed at ages 6, 12, 18, and 24 months for all of the experiments. Analysis of the histologic results indicated that the aged group had significantly greater subsynovial inflammation, synovial hyperplasia, pannus formation and cartilage erosion, bone destruction, and overall histologic abnormality (Figure 2A). Figure 2B shows photomicrographs of representative arthritic lesions from aging SS model mice at ages 18 and 24 months as well as the absence of such lesions in 24-month-old non-Tx and B6 mice. The effects observed in aged SS model mice included synovial hyperplasia, pannus formation, bone erosion, and infiltration of mononuclear cells into the subsynovial tissues. In contrast, mononuclear cell infiltration and bone and cartilage abnormalities were absent in age-matched control mice.

Age-related changes in cytokine profile and production of various autoantibodies. As measured by ELISA, culture supernatants from anti-CD3 mAb–stimulated splenic T cells obtained from SS model mice at ages 3, 12, and 24 months contained higher levels of IL-2 and IFNγ with advancing age, while levels of IL-4 and IL-10 were not observed to differ with advancing age (Figure 3A). We detected increased serum levels of RF (Figure 3B), anti-CII antibodies (Figure 3C), and anti-ssDNA antibodies (Figure 3D) in aging SS model mice but not in control mice. Moreover, we detected an increasing IgG2a:IgG1 ratio with advancing age in sera from SS model mice compared with sera from control mice (Figure 3E).

Immune responses against recombinant α-fodrin. To determine whether an immune response could be mounted against recombinant α-fodrin protein, the cDNA encoding human α-fodrin (JS-1, 1–1,784 bp;

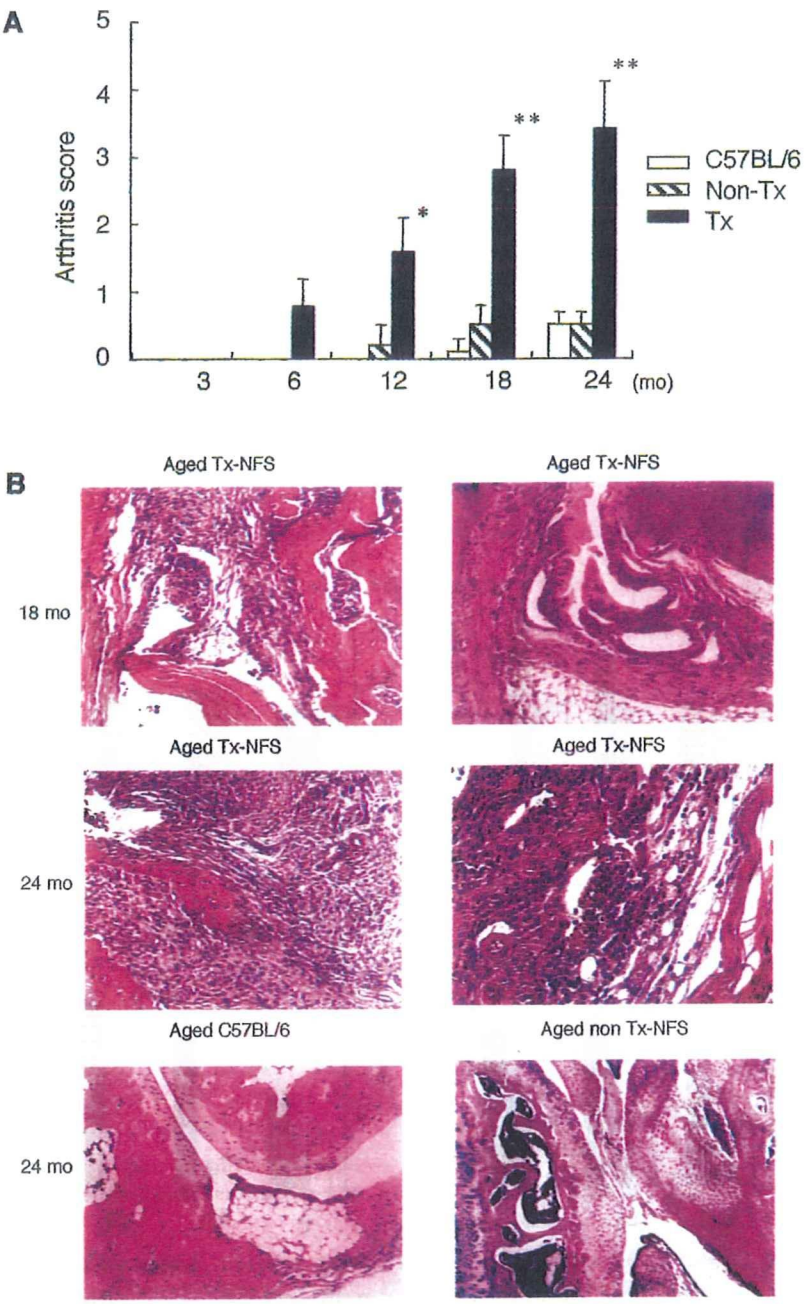

Figure 2. Age-related changes in joint histopathology. **A,** Effects of aging on joint histopathology in Sjögren's syndrome (SS) model mice. Shown are histologic scores of autoimmune arthropathy developed in aging SS model mice compared with those in age-matched non-Tx and C57BL/6 mice at ages 3, 6, 12, 18, and 24 months. Histologic evaluation of the knee joints was performed according to the methods of Edwards et al (see Materials and Methods) (27). Values are the mean and SD. * = $P < 0.05$; ** = $P < 0.01$, by Student's *t*-test. **B,** Photomicrographs of representative arthritic lesions from aging SS model mice at ages 18 and 24 months. The histopathologic effects observed in these mice included pannus formation, synovial hyperplasia, and infiltration of mononuclear cells into the subsynovial tissues. In contrast, mononuclear cell infiltration and bone and cartilage abnormalities were absent in age-matched control mice at age 24 months. See Figure 1 for other definitions. (Hematoxylin and eosin stained; original magnification × 120.)

2.7A, 2,258–4,884 bp; 3′DA, 3,963–7,083 bp) was constructed by inserting cDNA into the *Eco* RI site of pGEX-4Ts (Figure 4A). A high titer of serum autoantibodies against the N-terminal α-fodrin fragment JS-1 (originally identified in primary SS model mice) was detected in both young and aged SS model mice

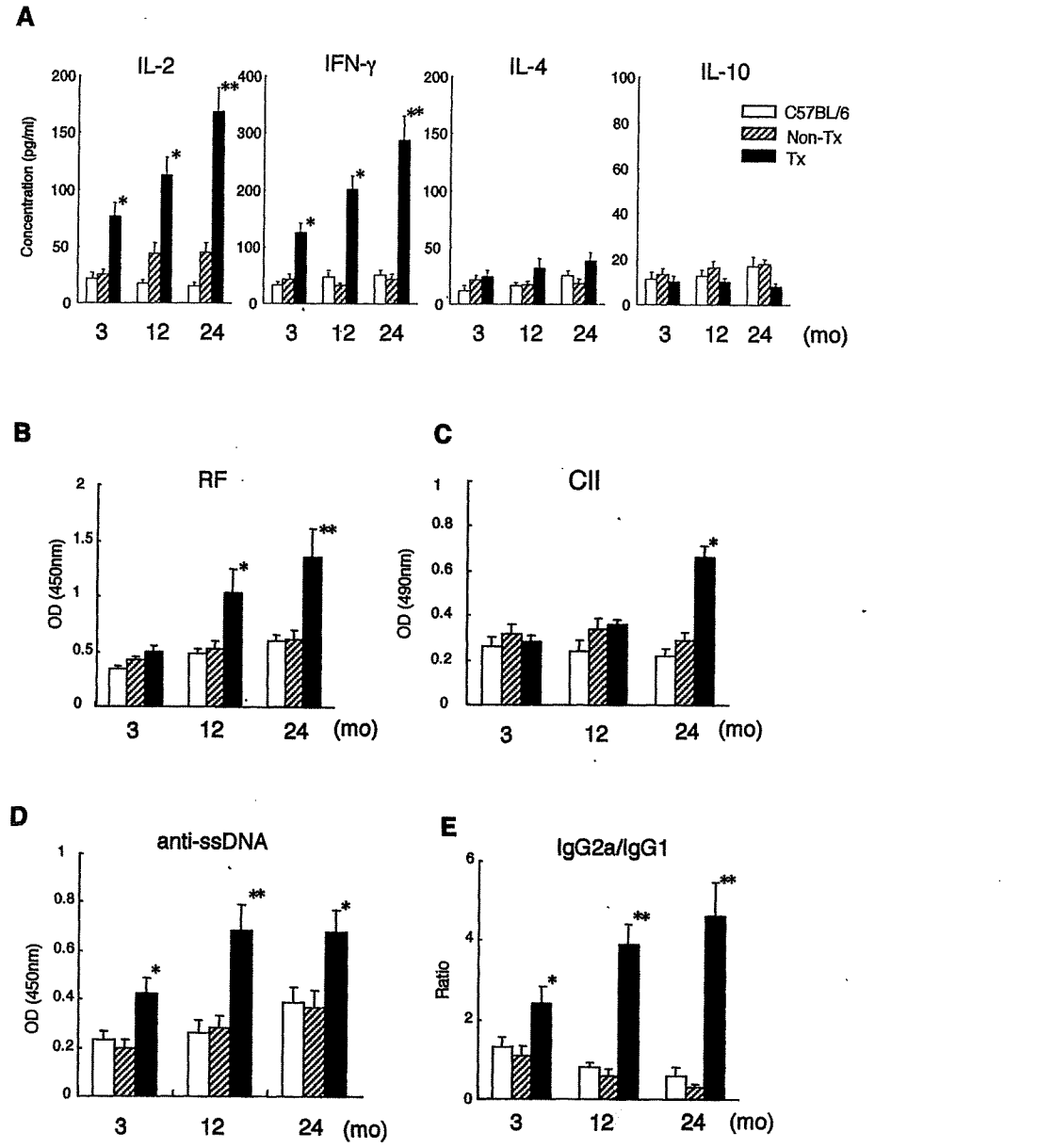

Figure 3. Age-related changes in cytokine profile and production of various autoantibodies. Values are the mean and SD. **A,** As measured by enzyme-linked immunosorbent assay, culture supernatants from anti-CD3 monoclonal antibody–stimulated splenic T cells obtained from aged Sjögren's syndrome (SS) model mice at ages 3, 12, and 24 months contained high levels of interleukin-2 (IL-2) and interferon-γ (IFNγ), while levels of IL-4 and IL-10 did not differ with advancing age. * = $P < 0.05$; ** = $P < 0.01$, by Student's *t*-test. **B,** Increased serum levels of rheumatoid factor (RF) were observed in aged SS model mice compared with those in control mice. * = $P < 0.05$; ** = $P < 0.01$, by Student's *t*-test. **C,** A significant increase in serum anti–type II collagen (anti-CII) antibodies was observed in aged SS model mice compared with control mice. * = $P < 0.05$ by Student's *t*-test. **D,** Significant increases in anti–single-stranded DNA (anti-ssDNA) antibodies were found at different ages in SS model mice compared with control mice. * = $P < 0.05$; ** = $P < 0.01$, by Student's *t*-test. **E,** An increasing IgG2a:IgG1 ratio with advancing age was detected in sera from SS model mice compared with control mice. * = $P < 0.05$; ** = $P < 0.01$, by Student's *t*-test. OD = optical density (see Figure 1 for other definitions).

by ELISA (Figure 4B). Moreover, autoantibody production against the C-terminus of the α-fodrin fragment (3′DA) was frequently detected in 12-month-old SS model mice (Figure 4B).

To address the role of autoantigen-reactive T cells, we examined the T cell proliferative responses against α-fodrin fragments (JS-1, 2.7A, and 3′DA) in the spleen cells at different ages. We detected significantly

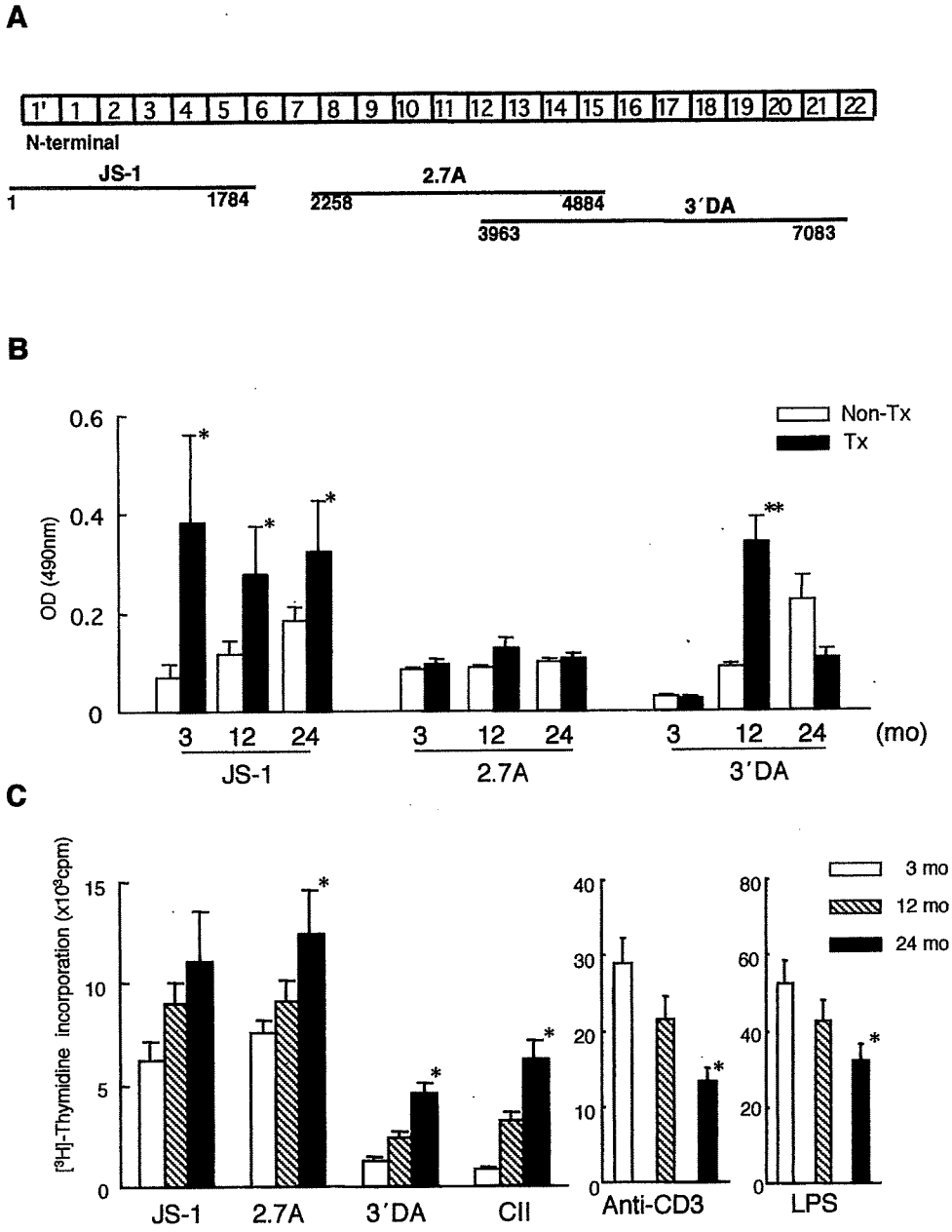

Figure 4. Immune responses against recombinant α-fodrin. **A,** Recombinant α-fodrin protein, the cDNA encoding human α-fodrin (JS-1, 1–1,784 bp; 2.7A, 2,258–4,884 bp; 3'DA, 3,963–7,083 bp), was constructed by inserting cDNA into the *Eco* RI site of pGEX-4Ts. **B,** A high titer of serum autoantibodies against the α-fodrin fragment JS-1, originally identified in primary SS model mice, was detected in both young and aged SS model mice by enzyme-linked immunosorbent assay. Moreover, autoantibody production against the C-terminus of the α-fodrin fragment (3'DA) was detected in 12-month-old SS model mice. Values are the mean and SD. * = $P < 0.05$; ** = $P < 0.01$, by Student's *t*-test. **C,** Significantly increased proliferation was detected in spleen cells from aged SS model mice stimulated with 2.7A and 3'DA protein. Moreover, a significant increase in CII-specific T cell proliferation was found in SS model mice with advancing age. In contrast, impaired proliferative responses were observed with advancing age upon stimulation with anti-CD3 and lipopolysaccharide (LPS). Values are the mean and SD of triplicate cultures. * = $P < 0.05$ by Student's *t*-test. See Figures 1 and 3 for other definitions.

increased proliferation in spleen cells from aged SS model mice stimulated with 2.7A and 3'DA protein (Figure 4C). We also examined the T cell proliferative responses against CII in the spleen cells at different ages. We found that the spleen cells in aged SS model mice showed a significant increase in CII-specific T cell

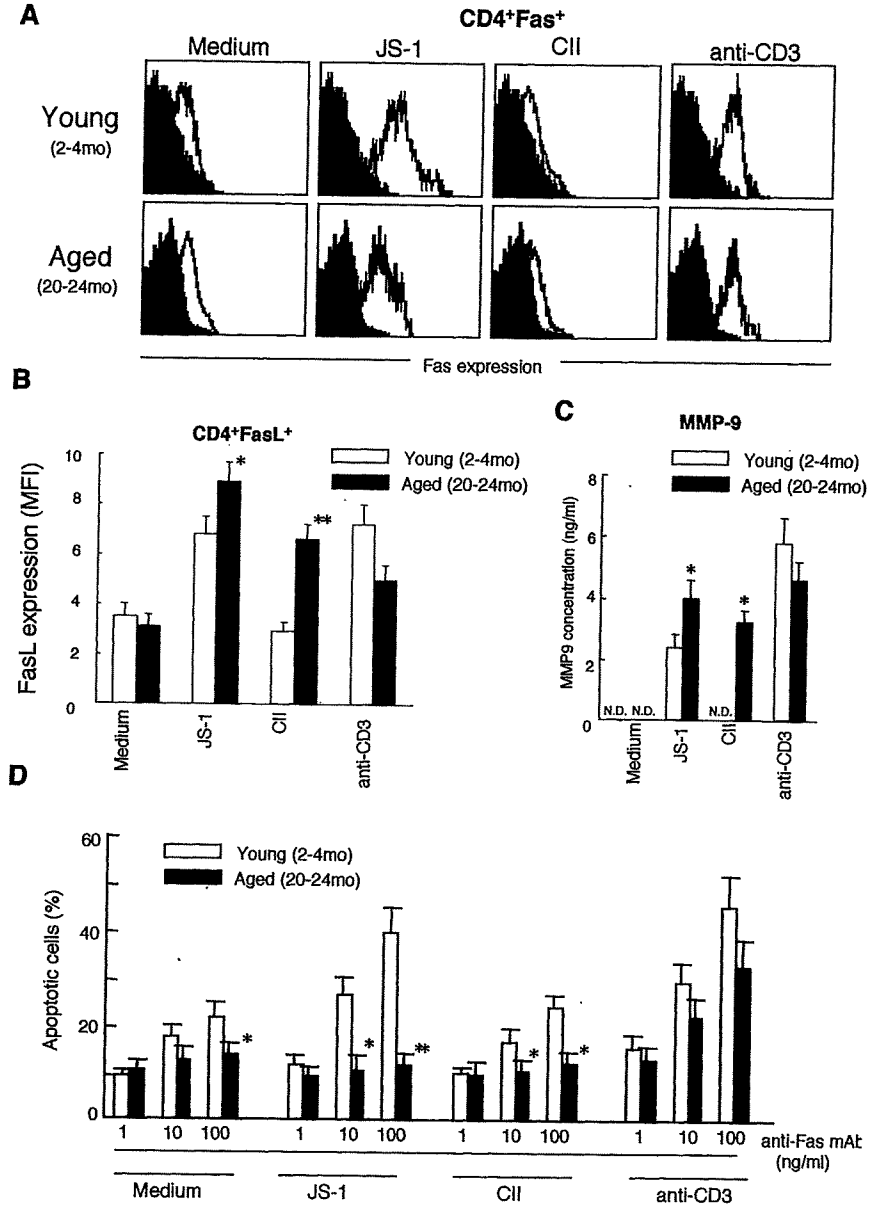

Figure 5. Expression of Fas, Fas ligand (FasL), and matrix metalloproteinase 9 (MMP-9). **A,** As shown by flow cytometry, an increased number of CD4+,Fas+ T cells was observed in JS-1–stimulated spleens from young SS model mice, but not from aged SS model mice. No significant difference was found in the numbers of CD4+,Fas+ T cells in CII-stimulated spleens from young versus aged SS model mice. **B,** A large proportion of CD4+ T cells expressing FasL was observed in spleens from aged SS model mice stimulated with either JS-1 or CII, but not in spleens stimulated with anti-CD3 monoclonal antibodies (mAb). Values are the mean and SD. * = $P < 0.05$; ** = $P < 0.01$, by Student's *t*-test. **C,** By enzyme-linked immunosorbent assay, an increased concentration of MMP-9 was detected in culture supernatant from JS-1– and CII-stimulated splenic T cells from aged SS model mice, but not in culture supernatant from anti-CD3 mAb–stimulated splenic T cells from young SS model mice. Values are the mean and SD. * = $P < 0.05$ by Student's *t*-test. **D,** Stimulation with autoantigens (JS-1 and CII) resulted in a significant, dose-dependent decrease in anti-Fas–induced CD4+ T cell apoptosis. Values are the mean and SD. * = $P < 0.05$; ** = $P < 0.01$, by Student's *t*-test. MFI = mean fluorescence intensity; ND = not detected (see Figure 3 for other definitions).

proliferation with advancing age (Figure 4C). In contrast, impaired proliferative responses were observed with advancing age upon stimulation with anti-CD3 and lipopolysaccharide. These data suggest that α-fodrin–reactive T cells may proliferate against a different antigenic epitope, which is followed by bystander T cell

activation, resulting in the development of autoimmune lesions in aged SS model mice.

Expression of Fas, FasL, and MMP-9. We next analyzed the numbers of Fas- and FasL-expressing splenic CD4+ T cells from young (ages 2–4 months) and aged (ages 20–24 months) SS model mice. An increased number of CD4+,Fas+ T cells was observed in spleens from young SS model mice, but not from aged SS model mice, stimulated with JS-1 (Figure 5A). No significant difference was found in the numbers of CD4+,Fas+ T cells in CII-stimulated spleens from young versus aged SS model mice (Figure 5A). A large proportion of CD4+ T cells expressing FasL was observed in spleens from aged SS model mice stimulated with either JS-1 or CII, but not in spleens stimulated with anti-CD3 mAb (Figure 5B). We previously detected a significantly increased concentration of MMP-9 in culture supernatant from JS-1–stimulated splenic T cells activated with anti-CD3 mAb from SS model mice (32). In the present study, we detected an increased concentration of MMP-9 in culture supernatant from JS-1– and CII-stimulated splenic T cells from aged SS model mice (Figure 5C). Moreover, it was demonstrated that autoantigen (JS-1 and CII) stimulation resulted in a significant, dose-dependent decrease in anti-Fas–induced CD4+ T cell apoptosis (Figure 5D), indicating the impairment of anti-Fas–induced T cell apoptosis in aged SS model mice. These data suggest that autoantigen stimulation may participate in immune dysregulation in the periphery in aged SS model mice.

DISCUSSION

We have used the NFS/*sld* mouse model of SS to study the age-related changes in the development of extraglandular manifestations of autoimmune lesions, and we have found that severe autoimmune arthritis developed with age in 12- and 24-month-old mice. An age-related dysregulation of immune functions in the murine model of SS resulted in a significant increase in serum levels of RF, anti-ssDNA antibodies, and anti-CII antibodies, and these changes increased with age.

Fas-mediated AICD is an important mechanism of peripheral T cell tolerance (7,33,34). Mice or humans lacking functional Fas or FasL display profound lymphoproliferative reactions associated with autoimmune disorders (35,36). We have previously demonstrated that Fas-mediated AICD is down-regulated by JS-1 autoantigen stimulation in spleen cells from SS model mice (32). In proteoglycan-induced arthritis, CD4+ T cells proliferate at a high rate in response to proteoglycan stimulation (37) and exhibit a Th1-type response (38). These observations suggest that a defect in AICD of autoreactive Th1 cells may contribute to the pathogenesis of the disease.

Our data demonstrated that splenic T cells from SS model mice contained higher levels of IL-2 and IFNγ with advancing age, and that a high titer of serum autoantibodies against α-fodrin autoantigen fragments (containing different epitopes that were originally identified in primary SS model mice) was frequently detected in young and aged SS model mice. We detected significantly increased proliferation in spleen cells from aged SS model mice stimulated with 2.7A and 3′DA protein. Our data suggest that α-fodrin autoantigen induces Th1 immune responses and accelerates disturbance of the Fas-mediated T cell apoptosis pathway in aged SS model mice.

We further observed that the spleen cells in aged SS model mice showed a significant increase in CII-specific T cell proliferation, which increased with age. CII, the main constituent of hyaline cartilage, has been proposed as one possible candidate autoantigen in rheumatoid arthritis (RA), because CII-specific antibodies are frequently found in RA patients and because an RA-like disease can be induced in certain mouse strains after immunization with CII. Our data showed a significant increase in production of serum autoantibodies against different fragments of α-fodrin autoantigen and against CII with aging, by ELISA. Moreover, significant proliferative responses against 2 α-fodrin fragments (2.7A and 3′DA) were observed in spleen cells from aged SS model mice, suggesting that bystander T cell activation may play an important role in the development of autoimmune lesions in these mice. It is possible that down-regulation of Fas-mediated AICD plays a major role in the accelerated development of autoimmune lesions with aging in the murine model of SS.

Epitope spreading has been generally proposed to contribute to the chronic pathogenesis of T cell–mediated autoimmune diseases, including experimental autoimmune encephalomyelitis (EAE) (39,40) and spontaneous diabetes in the nonobese diabetic mouse (41,42). However, it remains unclear whether T cells specific for endogenous epitopes play a significant pathologic role in tissue damage during the clinical episodes. CD4+ T cells are susceptible to AICD induced through T cell receptor (TCR)–mediated recognition of allogeneic class II MHC molecules (43,44). Our data demonstrate that autoantigen (JS-1 and CII) stimulation results in a significant, dose-dependent decrease in anti-Fas–induced CD4+ T cell apoptosis. In addition,

AICD is triggered in CD4+ T cells by the specific antigenic peptide (e.g., tetanus toxoid or myelin basic protein) presented by the appropriate class II MHC molecules (45), supporting the notion that AICD can be triggered in activated cells through the TCR-mediated recognition of antigen. Autoimmune epitope spreading has been described in patients with systemic lupus erythematosus, multiple sclerosis, and bullous pemphigus (46,47), and it is reported to be B7-1 dependent, playing a major pathologic role in EAE in mice (48). By the time a patient is diagnosed as having an autoimmune disease, significant tissue destruction has already occurred, making it difficult to identify the antigen against which the autoimmune response is directed (47).

It has been shown that membrane FasL is cleaved into a 26-kd soluble form by an MMP (49,50). We previously detected a 26-kd soluble form of FasL and MMP-9 exclusively in JS-1–stimulated splenic T cells in SS model mice (32). In the present study, we detected a significantly increased concentration of MMP-9 in culture supernatant from JS-1– and CII-stimulated splenic T cells activated with anti-CD3 mAb from aged SS model mice. It is possible that autoantigen (JS-1 and/or CII)–stimulated MMP-9 production may play an important role in down-regulation of Fas-mediated AICD, and in bystander T cell activation, resulting in accelerated development of autoimmune lesions.

In conclusion, these results suggest that age-related disturbance of AICD may play a major role in accelerated development of autoimmune lesions. The functional assays of cellular autoimmunity provide convincing evidence for impaired T cell tolerance to a set of closely related self determinants.

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Critical role of cathepsin-inhibitors for autoantigen processing and autoimmunity

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Introduction

The cysteinal lysosomal proteases, cathepsin S and cathepsin L have been shown to process invariant chain (Ii), thereby facilitating MHC class II maturation. However, their role in autoantigen processing is not established. Sjögren's syndrome (SS) is an autoimmune disorder characterized by lymphocytic infiltrates and destruction of the salivary and lacrimal glands and systemic production of autoantibodies to the ribonucleoprotein (RNP) particles SS-A/Ro and SS-B/La (Fox et al., 1986; Chan et al., 1991; Kruize et al., 1995). Previously, we reported a 120 kD α-fodrin autoantigen on the development of autoimmune exocrinopathy in SS model mice and identified autoantigen-specific T cell responses associated with Th1 cytokine production of interleukin (IL)-2 and interferon (IFN)-γ (Haneji et al., 1994, 1997). Insulin-dependent diabetes mellitus (IDDM) is a T-cell-mediated, organ-specific autoimmune disease that occurs in humans and in animal models such as the non-obese diabetic (NOD) mouse (Katz et al., 1993; Haskins and McDuffie, 1990). The autoimmune diabetes in NOD mice is characterized by lymphocytic infiltration of the islets (insulitis) followed by destruction of islet β-cells. Evidence for the role of $CD4^+$ Th1 cells in IDDM derives from recent studies of the NOD mouse in which the identical glutamic acid decarboxylase (GAD) and proinsulin are key β-cell autoantigens recognized by both T cells and B cells (Kaufman et al., 1993; Liu et al., 2002).

MHC class II molecules encounter and bind antigenic peptides as class II–peptide complexes on the cell surface of antigen-presenting cells (APCs) for recognition by $CD4^+$ T cells (Cresswell 1994; Gremain, 1994; Wolf and Ploegh, 1995). The molecular mechanisms leading to formation of class II–peptide complexes and

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presentation of antigen on the cell surface begin with synthesis of class II $\alpha\beta$ heterodimers in the endoplasmic reticulum. These class II $\alpha\beta$ heterodimers associate early during biosynthesis with a type II membrane protein, the invariant chain (Ii) (Roche and Marks, 1991; Lamb and Cresswell, 1992). Several lysosomal proteases have been implicated in the processing of Ii and antigenic peptides. Cathepsin B, the most abundant lysosomal cysteine protease, has been tied to Ii degradation using purified class II-Ii complexes (Reyes et al., 1991). Cathepsin L, a potent cysteine-class endoprotease, is specifically inhibited by a fragment of the alternatively spliced Ii form p41 (Bevec et al., 1996). Cathepsin S containing potent endoproteolytic activity is highly expressed in the spleen and professional APCs and other class II-positive cells, and is inducible with by IFN-γ (Shi et al., 1992, 1994). Katunuma and his colleagues have developed specific inhibitors of cathepsin B (CA074), cathepsin L (Clik148), and cathepsin S (Clik60), in vivo as well as in vitro (Towatari et al., 1991; Murata et al., 1991; Katunumua et al., 1999). Matsunaga et al. first reported that CA074 suppresses immune responses (Matsunaga et al., 1993), suggesting that cysteine proteases in lysosomes play an important role in the functional differentiation of MHC class II-restricted $CD4^+$ T cells. However, it is uncertain whether the inhibition of cathepsins block generation of the antigenic peptide on the development of autoimmune diseases. To address this important issue, autoantigen processing and presentation after specific inhibition of cathepsins were examined in murine model for SS and IDDM.

Materials and methods

Mice

Female NFS/N strain carrying the mutant gene *sld* (Hayashi et al., 1988), and NOD mice were reared in our specific pathogen-free mouse colony and given food and water ad libitum. Thymectomy was performed on day 3 after birth (3d-Tx) in NFS/*sld* mice. C56BL/6 mice (Charles River Japan, Inc., Atsugi, Japan), were used as controls.

Cyclophosphamide-induced diabetes

Prediabetic (7–8-week-old) female NOD mice were challenged with i.p. injection of cyclophosphamide (CY) (200 mg/kg) (Sigma-Aldrich) and analyzed 1–4 weeks after CY injection.

Assessment of diabetes

The blood glucose level was monitored weekly with a Glucometer (Kodama, Tokyo, Japan) using 50 μl blood from tail vein. Mice were monitored weekly for development of urine glucose with Keto-Diastix (Bayer-Sankyo Co., Ltd., Tokyo, Japan).

Cathepsin inhibitors

Specific inhibitors for cathepsin B (CA074), cathepsin L (Clik148), and cathepsin S (Clik60) have been developed with the help of computer-graphic modeling based on the stereo-structure as described previously (Towatari et al., 1991; Murata et al., 1991; Katunumua et al., 1999).

In vivo treatment with cathepsin inhibitors

We examined the in vivo therapeutic effects of cathepsin S-inhibitor (Clik60), cathepsin B-inhibitor (CA074), and cathepsin L-inhibitor (Clik148) in murine model for SS and IDDM in CY-treated NOD mice. Each inhibitor (Clik60, CA074, and Clik148) dissolved in PBS was administered i.p. into model mice at doses of 0.1 mg/mouse/day) and compared with non-treated model mice. All organs were removed from the mice, fixed with 4% phosphate-buffered formaldehyde (pH 7.2) and prepared for histological examination. The sections were stained with hematoxylin and eosin (H&E). Histological grading of the inflammatory lesions in the SS model was done according to the method proposed previously (White and Caserett, 1974).

Proliferation assay

Single cell suspensions of spleen cells or regional lymph node cells (LNCs) were cultured in 96-well flat bottom microtiter plates (Nunc, Roskilde, Denmark). For proliferation assay, a total of 5×10^5 cells/well were cultured for 72 h under stimulation of recombinant α-fodrin protein (JS-1, 10 μg/ml) (10), insulin (10 μg/ml), GAD (10 μg/ml), OVA (10 μg/ml), and ConA (5 μg/ml), and pulsed with 1 μCi/well of [^{3}H]thymidine (NEN Life Science Products, Boston, MA) during final 20 h of the culture. We further examined the in vitro preventive effects of cathepsin inhibitors (10^{-7}–10^{-4} M CA074, Clik148, and Clik60) for antigen-specific proliferative T cell responses. T cell purification was done using CD4mAb-bounded immunomagnetic beads (Dynal, CA).

Measurement of cytokine production

Cytokine production from spleen cells was tested by two-step sandwich ELISA using a mouse IL-2, IL-4, and IFN-γ kit (Genzyme). In brief, culture supernatants from spleen cells activated with immobilized anti-CD3 mAb (10 μg/ml)(Ceder Lane Lab.) for 3 days were added to microtiter plates precoated with anti-IL-2, IL-4, and IFN-γ capture Ab and incubated overnight at 4°C. After addition of biotinylated detecting Ab and incubation at room temperature for 45 min, avidin-peroxidase was added and incubated at room temperature for 30 min. Plates were washed extensively with 1% Tween in PBS between each step. Finally, 2,2′-azino-di-3-ethylbenzthiazoline sulfonate (ABTS) substrate containing H_2O_2 was added and the colorimetric reaction was read at an absorbance of 450 nm using an automatic microplate reader (BioRad Laboratories Inc., Hercules, CA). The concentrations of IL-2 (pg/ml), IL-4

(pg/ml), and IFN-γ pg/ml) were calculated according to the standard curves produced by various concentrations of recombinant cytokines.

Detection of serum autoantibodies against 120 kD α-fodrin

Serum autoantibody production against 120 kD α-fodrin was analyzed by immunoblotting as described previously (Haneji et al., 1997).

Measurement of fluid secretion

Detection of tear and saliva volume of the treated, and non-treated SS animal model was done according to a modified method as described (Delporte et al., 1997).

Results and discussion

Therapeutic effects of specific inhibitors of Cathepsin S (Clik60) for animal model of SS

To address the role of autoantigen-reactive T cells, we examined the proliferative T cell responses in the cervical LNCs and spleen cells from SS model mice and controls. We found that the LNCs and spleen cells in SS model mice at 8 weeks of age showed a significant increase in autoantigen (JS-1)-specific T cell proliferation (Fig. 1a), but not in C57BL/6 control mice. No significant differences were observed in the proliferative response stimulated with OVA (10 μg/ml), and ConA (5 μg/ml) among these mice. We examined the inhibitory effects of cathepsin inhibitors against autoantigen-specific T cell responses in vitro. In regional LNCs, but not in the spleens, from SS model mice, a significantly inhibitory effect of Clik60 was observed in dose-dependent manner (Fig. 1b). We next examined the in vivo therapeutic effects of Clik60, Clik148, and CA074 in murine model for SS. Treatment with i.p. injection of Clik60 (0.1 mg/mouse/day) was effective in preventing the development of autoimmune lesions in the lacrimal ($P<0.01$), parotid ($P<0.05$), and submandibular ($P<0.05$) glands of the SS model mice, but not in groups injected with CA074, and Clik148 (Fig. 2a). Moreover, the average saliva and tear volume of Clik60-treated model mice was significantly higher than that of the non-treated SS model mice (Fig. 2b). In addition, autoantigen (JS-1)-specific T cell response was significantly inhibited in LNCs from Clik60-treated mice. Culture supernatants from anti-CD3mAb-stimulated splenic T cells obtained from Clik60-treated mice contained high levels of IL-4, but low levels of IL-2 and IFN-γ by ELISA (Fig. 3a). Serum autoantibody production against 120 kD α-fodrin autoantigen was exclusively inhibited in Clik60-treated mice, but not in other groups (Fig. 3b). These results strongly suggest that Clik60 plays an important role in preventing autoantigen presentation to generate class II molecules competent for binding antigenic peptide, resulting in inhibition of autoimmunity in the salivary and lacrimal glands. A large proportion of class II ($I\text{-}A^q$)-expressing cells was observed

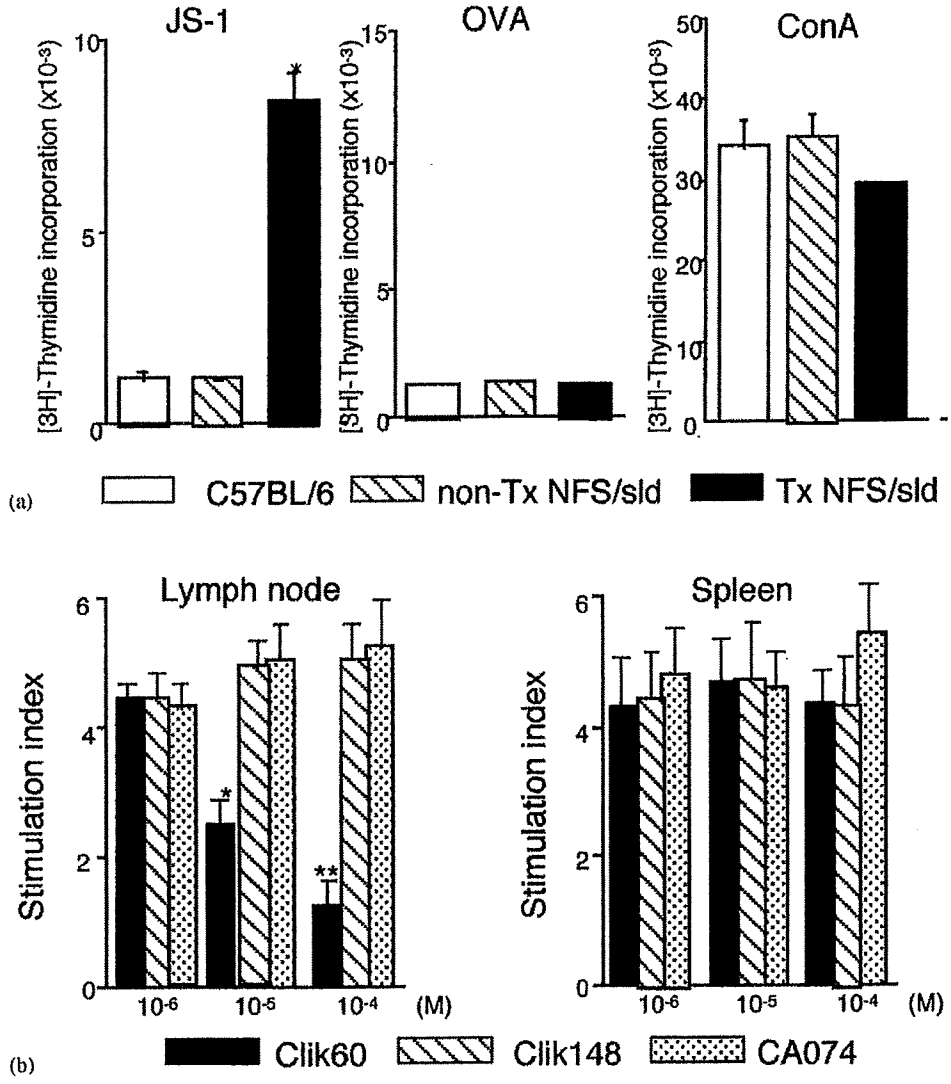

Fig. 1. (a) Detection of proliferative T cell response of LNCs from SS model mice to organ-specific autoantigen (JS-1) (*$P<0.001$, Student's t-test)(a). No differences were found in OVA (10 μg/ml), and ConA (5 μg/ml)-responsiveness. (b) In vitro preventive effect of proliferative T cell response of LNCs, but not spleen cells, to JS-1 by Clik60 in dose-dependent manner. No inhibitory effects of Clik148, and CA074 were found. Data are expressed as stimulation indices (SI)±standard error of the mean (s.e.m.). Three experiments from each group were performed at 8 weeks of age, and the mean values of index were statistically significant at $P<0.05$* and $P<0.01$** (Student's t-test). Part of this Figure included in reference by Saegusa et al. (2002).

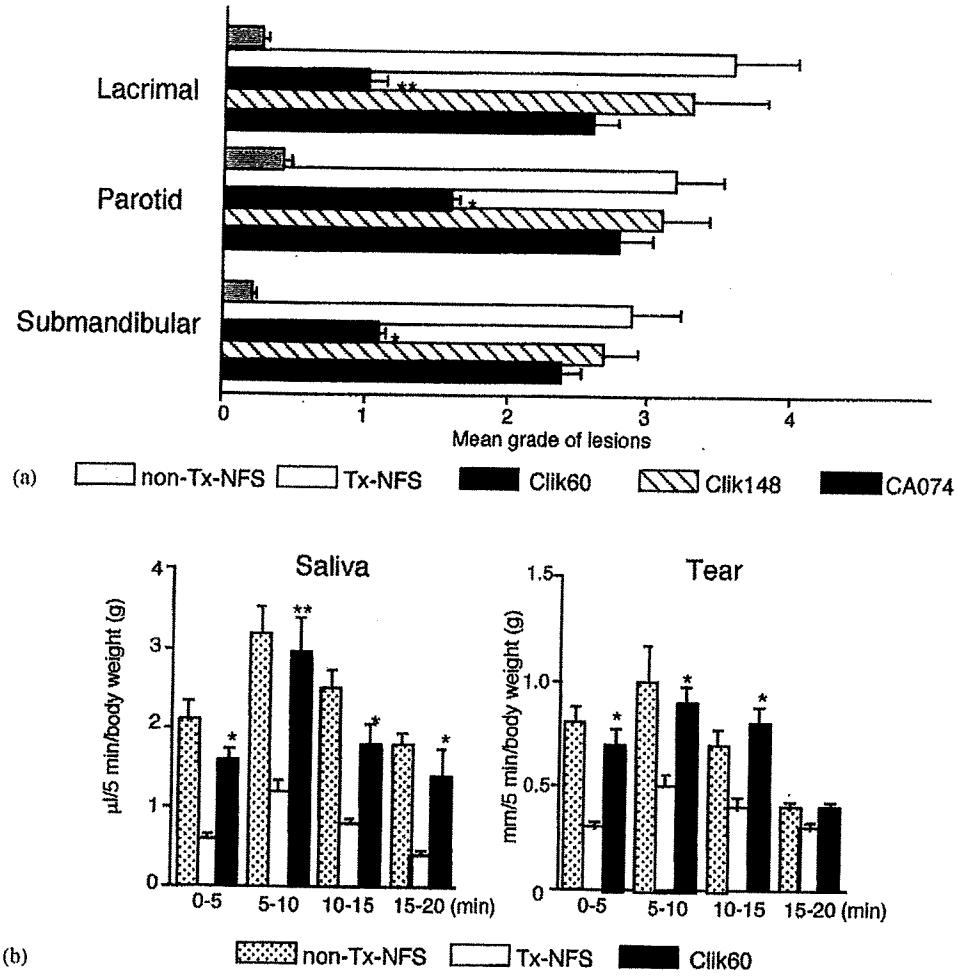

Fig. 2. (a) Effects of in vivo administration of i.p. injection of cathepsin inhibitors (Clik60, Clik148, and CA074). Each inhibitor was administered into SS model mice ($n = 10$, for each) at doses of 0.1 mg/mouse/day from 4 weeks–7 weeks, and then analyzed at 8 weeks, compared with non-treated SS model mice ($n = 7$). The treatment with i.p. injection of Clik60 was effective in preventing the development of autoimmune lesions in the lacrimal, parotid, and submandibular glands of the SS model mice, but not in groups injected with Clik148, and CA074 ($^{*}P<0.01$ and $^{**}P<0.005$, Student's t-test). (b) The average saliva and tear volume of the SS model mice treated with Clik60 was significantly higher than that of control mice. Results are expressed as mean±SEM in 5 mice examined per each group ($^{*}P<0.05$ and $^{**}P<0.005$, Student's t-test). Part of this Figure included in reference by Saegusa et al. (2002).

on mouse salivary gland (MSG) epithelial cells from SS model mice, and MHC class II molecule can be stably induced by IFN-γ-stimulation on MSG cells from syngeneic control mice (Saegusa et al., 2002). It is possible that the salivary gland