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Effective Treatment of a Mouse Model of Sjögren's Syndrome With Eyedrop Administration of Anti-CD4 Monoclonal Antibody

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Objective. **To determine whether eyedrop administration of an anti-CD4 monoclonal antibody (mAb) is effective in the treatment of Sjögren's syndrome (SS) using a mouse model of the disease.**

Methods. **The anti-CD4 mAb was administered daily into the eyes of mice with SS from ages 4 to 8 weeks or ages 10 to 12 weeks. During treatment, tear volume was monitored and after final treatment, histologic features of the lacrimal and salivary glands, the phenotypes and function of T cells, and serum titers of anti–α-fodrin antibody were examined.**

Results. **Eyedrop administration of anti-CD4 mAb before the onset of SS prevented the autoimmune pathology seen in the lacrimal glands but not that in the salivary glands. Furthermore, eyedrop administration of anti-CD4 mAb after the development of SS inhibited mononuclear cell infiltration and the destruction of parenchyma only in the lacrimal glands. Eyedrop administration of anti-CD4 mAb suppressed the local activation of CD4+ T cells rather than deleting CD4+ T cells, which reduced the expansion of pathologic CD4+ T cells against α-fodrin.**

Conclusion. **These results demonstrate the remarkable efficacy of anti-CD4 mAb eyedrops in the treatment of SS eye symptoms, which illustrates a new antibody-based therapeutic strategy for patients with eye problems caused by SS as well as other diseases.**

The immune system has acquired regulatory systems that preclude the reactivity of mature T cells against self antigens presented by major histocompatibility complex (MHC), while maintaining an ability to respond to non-self antigens presented by self MHC (1,2). The deletion of T cells that have T cell receptors with a high affinity for self antigens in the thymus is an important mechanism for self-tolerance induction and many other systems, including apoptosis, anergy of mature T cells and regulatory T cells, and control T cell tolerance (3–5). The dysregulation of T cell tolerance induction/maintenance is considered to be responsible for many types of autoimmune diseases, and a variety of mechanisms for causing autoimmune diseases have been proposed (5–9). However, the precise mechanisms of human autoimmune diseases remain unclear, and this prevents the establishment of specific therapeutic strategies for these conditions.

Sjögren's syndrome (SS) is an autoimmune disease characterized by the destruction of lacrimal and salivary glands, resulting in dry eyes and dry mouth as the major symptoms (10). Because SS patients have high titers of autoantibodies, including anti-SSA/Ro and SSB/La, abnormal T and B cell activation has been considered to cause SS (10,11). We have established and investigated an animal model of SS in NFS/*sld*-mutant mice thymectomized 3 days after birth and found that the 120-kd α-fodrin protein is a critical autoantigen in the development of SS in this mouse model (12–14). Furthermore, we and other investigators (12,15) have found that patients with SS have high titers of serum

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anti–α-fodrin antibody, suggesting that α-fodrin is a critical autoantigen for the onset or progression of human SS. Although the mechanism of autoimmune disorders in the lacrimal and salivary glands in this mouse model is still unclear, autoreactive CD4+ T cells are responsible for the destruction of the lacrimal and salivary glands in this model (16–18) as well as in human SS (19,20).

Patients with SS are treated with oral or intravenous immunosuppressive drugs, including steroids and cyclosporin A (CSA), which suppress T cell proliferation (10). Such drugs are effective in certain SS patients, but sometimes induce severe side effects (10). The characteristic symptom of SS is dry eyes (10,21), so it is better to treat this symptom with eyedrop (ED) administration of drugs rather than systemically. In this regard, topical CSA has been successfully used as a treatment for the eye symptoms of SS (22), although double-blind clinical studies of SS patients are necessary for the final determination of its efficacy.

To establish a topical therapeutic strategy for the eye symptoms of SS patients, we evaluated ED administration of anti-CD4 monoclonal antibody (mAb) in a mouse model of SS. The anti-CD4 mAb specifically affects CD4+ T cell activation, the dysregulation of which is responsible for the development of SS symptoms in mice and humans (16–18). Although an antibody is a high molecular weight glycoprotein, we found that ED administration of anti-CD4 prevented the onset as well as the progression of autoimmune responses in the lacrimal glands in mice with SS by inhibiting CD4+ T cell activation, possibly through infiltration of the antibody into the lacrimal glands. These findings suggest that it might be possible to treat the eye symptoms of humans with SS as well as other diseases with ED administration of antibodies that recognize critical molecules that cause each disease.

MATERIALS AND METHODS

Mice and experimental protocol. Female NFS/*sld* mice carrying the mutant gene *sld* (23) were bred in our specific pathogen–free mouse colony and were provided with food and water ad libitum. A thymectomy was performed on NFS/*sld* mice 3 days after birth (3d-Tx mice), as previously described (12,24). For the eyedrops, 2 μl of anti-CD4 mAb (GK1.5) dissolved in phosphate buffered saline was prepared in our laboratory (1 mg/ml) and applied with a micropipette to both eyes once a day from 4 to 8 weeks of age or from 10 to 12 weeks of age. As a control, rat IgG eyedrops (Inter-Cell Technologies, Hopewell, NJ) were used.

Histologic examination. The mice were killed at 8 or 12 weeks of age. All organs were removed, fixed in 4% phosphate buffered formaldehyde (pH 7.2), and prepared for histologic examination. Sections were stained with hematoxylin and eosin (H&E). Histologic grading of inflammatory lesions was performed according to the method proposed by White and Casarett (25), as follows: 1 = 1–5 foci composed of >20 mononuclear cells per focus, 2 = >5 such foci, but without

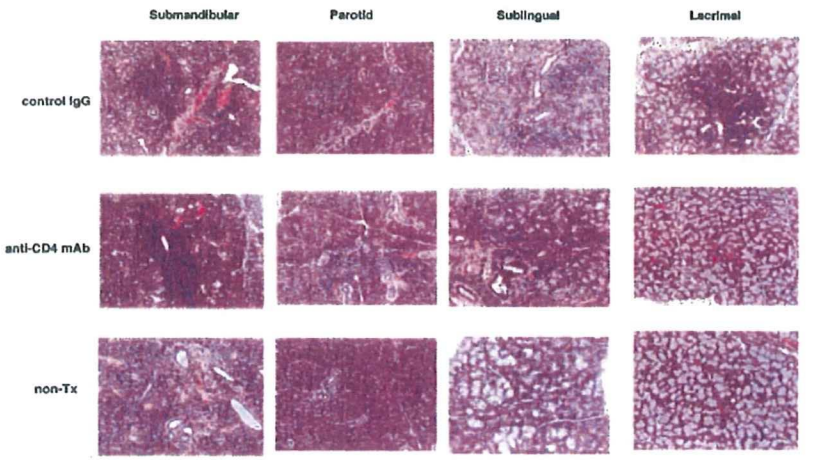

Figure 1. Suppression of autoimmune responses in lacrimal glands by treatment with eyedrop (ED) administration of CD4. Mice thymectomized 3 days after birth were treated with ED administration of control IgG or anti-CD4 monoclonal antibody (mAb) from ages 4 to 8 weeks. The lacrimal, parotid, submandibular, or sublingual glands were removed 4 weeks after initial treatment and stained with hematoxylin and eosin. As a control, nonthymectomized (non-Tx) NSF/*sld* mice were used. Results are representative of 4 independent experiments.

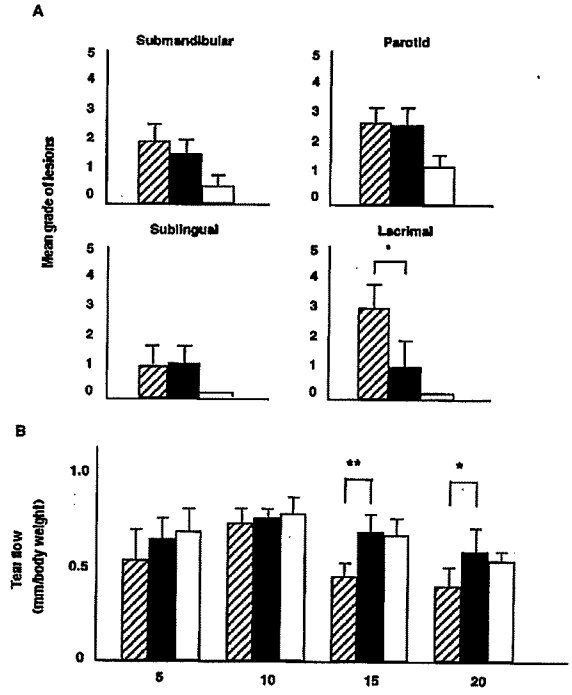

Figure 2. Prevention of the onset of autoimmune responses by ED administration of CD4 in lacrimal glands only. **A,** The 3-day-old thymectomized (3d-Tx) mice were treated with eyedrop (ED) administration of control IgG (hatched bars) or anti-CD4 mAb (solid bars) from ages 4 to 8 weeks. The lacrimal, parotid, submandibular, or sublingual glands were removed 4 weeks after initial treatment and stained with hematoxylin and eosin. As a control, nonthymectomized (non-Tx) NSF/*sld* mice were used (open bars). The histologic scores of each gland were evaluated as described in Materials and Methods. Values are the mean and SEM of 7 mice. * = $P < 0.01$. **B,** The 3d-Tx mice were treated with ED administration of control IgG (hatched bars) or anti-CD4 mAb (solid bars) from ages 4 to 8 weeks. As a control, non-Tx NSF/*sld* mice were used (open bars). The tear volume of each mouse was evaluated as described in Materials and Methods. Values are the mean and SEM of 9 mice. * = $P < 0.05$; ** = $P < 0.01$.

significant parenchymal destruction, 3 = degeneration of parenchymal tissue, 4 = extensive infiltration of the glands with mononuclear cells and extensive parenchymal destruction, and 5 = severe destructive foci with focal fibrosis, ductal dilation, and/or fatty infiltration in addition to the grade 4 lesions. Histologic evaluation of the lacrimal and salivary glands was performed in a blinded manner, and 1 tissue section from each lacrimal and salivary gland was examined.

Flow cytometric analysis. Single-cell suspensions from the lymph nodes or spleen were stained with a combination of phycoerythrin (PE)–conjugated anti-CD4 mAb and fluorescein isothiocyanate–conjugated anti-CD8 mAb or PE-conjugated anti-CD4 mAb and cytochrome-conjugated anti-CD44 mAb and analyzed with a FACSCalibur flow cytometer (Becton Dickinson, Mountain View, CA). Cells were gated according to size and scatter to eliminate dead cells and debris from analysis. All antibodies were from BD Transduction Laboratories (San Jose, CA).

Measurement of fluid secretion. Analysis of the tear and saliva volume of 15 treated and 15 untreated 3d-Tx NFS/*sld* mice and 12 non–3d-Tx NFS/*sld* mice was performed according to a previously described method (26). A total of 42 mice were examined.

Proliferation assay. Total spleen cells or cervical lymph node cells (5×10^5/well) in RPMI 1640 containing 10% fetal calf serum, penicillin/streptomycin, and 2-mercaptoethanol were stimulated with recombinant α-fodrin protein (JS-1) (12) or 2.0 μg/ml concanavalin A (Con A; Sigma, St. Louis, MO) in 96-well, flat-bottomed plates for 72 hours. Then, ^{3}H-thymidine (1 μCi/well; NEN Life Science Products, Boston, MA) was pulsed into the cell mixture during the final 20 hours of culture. Incorporation of ^{3}H-thymidine was evaluated by an automated β-liquid scintillation counter (MicroPlus; Wallace, Turku, Finland).

Enzyme-linked immunosorbent assay (ELISA). The 96-well plates were coated with JS-1 (12). After washing the protein, diluted serum from mice with SS was added. Biotinylated anti-mouse IgG (Vector, Burlingame, CA) was added as the second antibody. The JS-1–specific antibodies were measured by an automatic ELISA reader (Flow, McLean, VA).

Statistical analysis. The results of histologic, tear secretion, and flow cytometric analyses as well as proliferation assay and ELISA were evaluated by Student's *t*-test.

RESULTS

Prevention of the onset of autoimmune pathology in lacrimal glands by ED administration of CD4. The 3d-Tx NFS/*sld* mice began to develop autoimmune lesions in the lacrimal and salivary glands at 4 weeks of age or later, while no inflammatory lesions were observed in non–3d-Tx NFS/*sld* mice (12). To evaluate whether ED administration of CD4 was effective for preventing the onset of SS autoimmune pathology, anti-CD4 mAb was applied daily to both eyes of 3d-Tx mice from 4 to 8 weeks of age. Then the histologic features of the lacrimal, parotid, submandibular, and sublingual glands of anti-CD4 mAb– or control IgG-treated mice were evaluated by H&E staining (Figures 1 and 2). The lacrimal, parotid, submandibular, and sublingual glands of control IgG-treated 3d-Tx mice showed massive infiltration of mononuclear cells around ducts, as well as destruction of parenchyma, 4 weeks after initial treatment (Figure 1).

In contrast, the infiltration of mononuclear cells as well as the destruction of parenchyma was inhibited in the lacrimal glands, but not the parotid, submandibular, and sublingual glands, of 3d-Tx mice treated with ED administration of CD4 (Figure 1). The non–3d-Tx NSF/*sld* mice did not develop any autoimmune responses

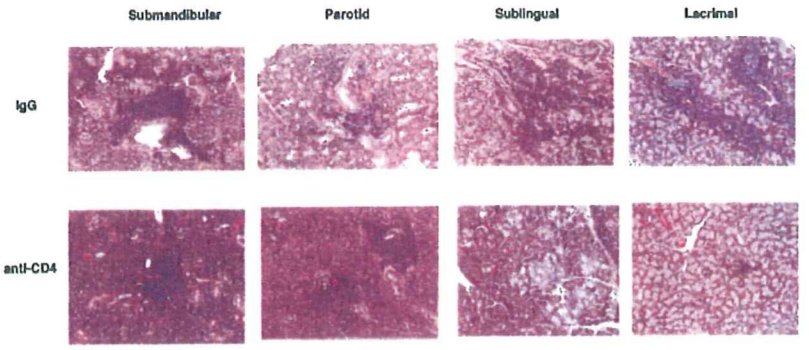

Figure 3. Suppression of established autoimmune responses in lacrimal glands by ED administration of CD4. The 3d-Tx mice were treated with ED administration of control IgG or anti-CD4 mAb from ages 10 to 12 weeks. The lacrimal, parotid, submandibular, or sublingual glands were removed 2 weeks after initial treatment and stained with hematoxylin and eosin. Results are representative of 3 independent experiments. See Figure 2 for definitions.

(Figure 1). Those histologic findings were scored 4 weeks after initial treatment, as described in Materials and Methods (Figure 2A). The 3d-Tx NFS/*sld* mice treated with ED administration of CD4 had significantly inhibited autoimmune responses only in the lacrimal glands ($P < 0.01$) (Figure 2A), although there were still very small foci of infiltrating mononuclear cells in the lacrimal glands. Furthermore, the average tear volume of mice treated with ED administration of CD4 significantly recovered to the level of control IgG-treated mice (Figure 2B). These results demonstrate that ED administration of CD4 specifically suppressed the onset of autoimmune responses in the lacrimal glands, but not the salivary glands.

Suppression of already developed autoimmune pathology by ED administration of CD4. It is necessary to consider the clinical use of a therapeutic strategy and establish one that can suppress autoimmune pathology that has already developed. Thus, in order to examine the effect of ED administration of CD4 on the autoimmune lesions of mice that had already developed SS, we initiated treatment at 10 weeks of age and continued it for 2 weeks. Then, histologic sections of the lacrimal, parotid, submandibular, and sublingual glands were examined by H&E staining 2 weeks after initial treatment. ED administration of CD4 suppressed the cell infiltration and parenchyma destruction of the lacrimal glands, but not of the parotid, submandibular, or sublingual glands (Figure 3), similar to the therapeutic effects of ED administration of CD4 before the onset of autoimmune diseases (Figures 1 and 2). The histologic scores clearly demonstrated that the therapeutic effect is limited only to the lacrimal glands (Figure 4A), indicating that ED administration of CD4 also has the potential to suppress progression of already developed autoimmune diseases. In contrast, the tear volume of mice treated with ED administration of CD4 was not significantly increased compared with that of mice treated with control IgG (Figure 4B).

CD4+ T cell number augmented and activation inhibited by ED administration of CD4. The anti-CD4 mAb used can cause the deletion of CD4+ T cells when injected into mice and can block the interaction between CD4 and class II MHC (27,28). Thus, we next examined whether the therapeutic effect of ED administration of CD4 on 3d-Tx NFS/*sld* mice was attributable to the deletion of CD4+ T cells or the inhibition of CD4+ T cell activation.

Cervical lymph node cells and spleen cells were purified from 3d-Tx NFS/*sld* mice treated with ED administration of CD4 from 4 to 8 weeks of age or 10 to 12 weeks of age. In mice treated from 4 to 8 weeks of age, the relative number of CD4+ T cells from lymph nodes was increased compared with the number of CD8+ T cells and compared with the control IgG-treated group (Figure 5A). Since the total cell number did not change with ED administration of CD4 (Figure 5A), only the total CD4+ T cells and not the CD8+ T cells increased. The relative and total numbers of both CD4+ and CD8+ T cells from lymph nodes were increased in mice treated with ED administration of CD4 from 10 to 12 weeks of age (Figure 5B). In contrast, the number of CD4+ and CD8+ T cells from the spleen did not change with ED administration of CD4 in either group (data not shown).

We next examined CD44 expression on CD4+ T

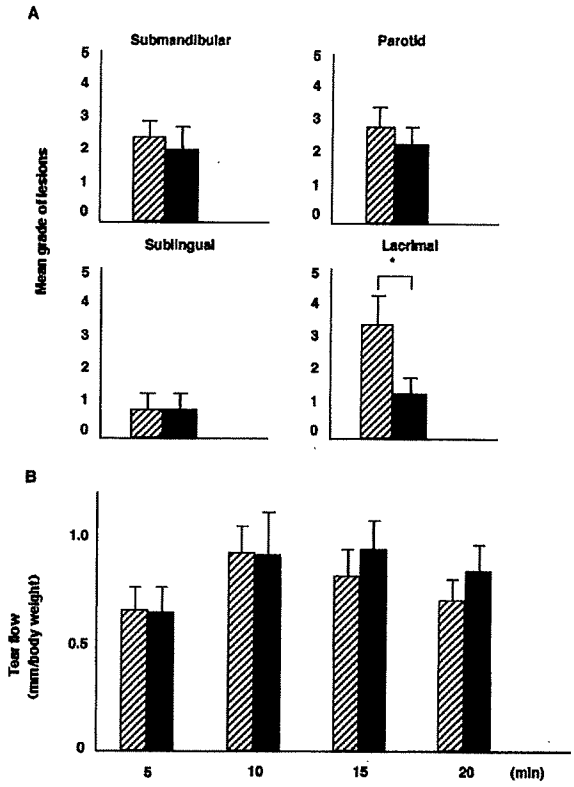

Figure 4. Prevention of the progression of autoimmune responses by ED administration of CD4 in lacrimal glands only. **A,** The 3d-Tx mice were treated with ED administration of control IgG (hatched bars) or anti-CD4 mAb (solid bars) from ages 10 to 12 weeks. The lacrimal, parotid, submandibular, or sublingual glands were removed 2 weeks after initial treatment and stained with hematoxylin and eosin. The histologic scores of each gland were evaluated as described in Materials and Methods. Values are the mean and SEM of 7 mice. * = $P < 0.05$. **B,** The 3d-Tx mice were treated with ED administration of control IgG (hatched bars) or anti-CD4 mAb (solid bars) from ages 10 to 12 weeks. The tear volume of each mouse was evaluated as described in Materials and Methods. Values are the mean and SEM of 5 mice. See Figure 2 for definitions.

cells because CD44 is known to be highly expressed on activated T cells and memory T cells (29). Lymph node CD4+ T cells from mice treated with ED administration of CD4 from ages 4 to 8 weeks and ages 10 to 12 weeks expressed lower levels of CD44 compared with those from the control IgG-treated group (Figures 5A and B). In contrast, the expression level of CD44 on splenic CD4+ T cells from 3d-Tx mice treated with ED administration of CD4 was similar to that from control IgG-treated mice (data not shown). Taken together, these results demonstrate that ED administration of CD4 inhibited the activation of CD4+ T cells infiltrating into the lacrimal glands, but did not delete the CD4+ T cells.

Reduction of JS-1–specific T cell response by ED administration of CD4. We previously reported that CD4+ T cells from 3d-Tx mice responded to the α-fodrin JS-1 peptide (13). Thus, we examined whether ED administration of CD4 affects the JS-1–specific proliferative response of lymph node cells from 3d-Tx mice. Cervical lymph node cells were purified after ED administration of CD4 from 4 to 8 weeks of age or 10 to 12 weeks of age and were stimulated with JS-1 or Con A. As previously reported, lymph node T cells from control IgG-treated mice at 8 to 12 weeks of age vigorously proliferated in response to JS-1 (Figures 6A and B). In contrast, the JS-1–specific T cell responses were lower in mice treated with ED administration of CD4 both from 4 to 8 weeks of age and 10 to 12 weeks of age than in

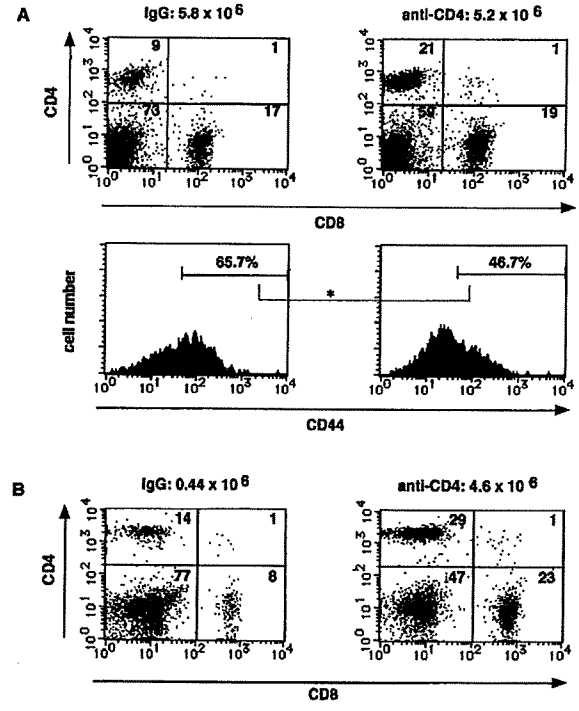

Figure 5. Flow cytometric analysis of lymph node cells. Cervical lymph node cells from 3d-Tx mice that were treated with ED administration of control IgG or anti-CD4 mAb from ages 4 to 8 weeks (**A**) or ages 10 to 12 weeks (**B**) were stained with phycoerythrin (PE)–conjugated anti-CD4 mAb and fluorescein isothiocyanate–conjugated anti-CD8 mAb or PE-conjugated anti-CD44 mAb and cytochrome-conjugated anti-CD44 mAb and analyzed by flow cytometry. The total cell number is indicated across the top. Results are representative of at least 3 independent experiments. * = $P < 0.05$ by Student's unpaired *t*-test. See Figure 2 for other definitions.

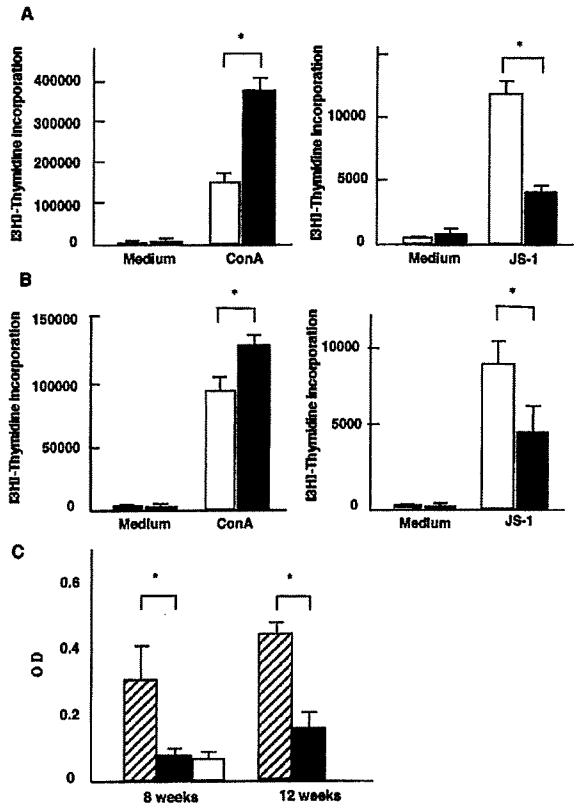

Figure 6. Proliferative response of JS-1–specific T cells and serum titer of anti–JS-1 antibody. The cervical lymph node cells from 3d-Tx mice that were treated with ED administration of control IgG (**left**) or anti-CD4 mAb (**right**) from **A**, ages 4 to 8 weeks or **B**, ages 10 to 12 weeks were stimulated with JS-1 peptide or concanavalin A (Con A) for 72 hours, and incorporation of ^{3}H-thymidine into cells during the final 12 hours was evaluated. Values are the mean and SEM of 7 mice. * = $P < 0.01$. **C**, Levels of serum autoantibody against 120-kd α-fodrin (JS-1) in 3d-Tx mice that were treated with ED administration of control IgG (hatched bars) or anti-CD4 mAb (solid bars) from ages 4 to 8 weeks or ages 10 to 12 weeks were measured by enzyme-linked immunosorbent assay. Non-Tx mice were used as controls (open bars). Values are the mean and SEM of 7 mice. OD = optical density; * = $P < 0.05$. See Figure 2 for other definitions.

control IgG-treated mice (Figures 6A and B). The inhibitory effects of anti-CD4 mAb appeared to be stronger when administered from 8 to 12 weeks of age (Figures 6A and B). The response to Con A was higher in groups treated with ED administration of CD4 (Figures 6A and B), arguing against the possibility that ED administration of CD4 suppressed total T cell responses. These results demonstrate the reduced expansion of JS-1–specific T cells in mice treated with ED administration of CD4, which is consistent with the low CD44 expression on CD4+ T cells from mice treated with ED administration of CD4.

Reduced titer of serum antibody against α-fodrin. The 3d-Tx mice have high titers of serum autoantibody against recombinant α-fodrin protein (JS-1) (12). Thus, we examined whether the local treatment with ED administration of CD4 affected serum levels of autoantibody against α-fodrin in the mice with SS. The titer of serum antibody against α-fodrin was evaluated after treatment of 3d-Tx mice with ED administration of CD4 from 4 to 8 weeks of age or 10 to 12 weeks of age. As shown in Figure 6, the titer of autoantibody against α-fodrin was lower in mice treated from ages 4 to 8 weeks and ages 10 to 12 weeks compared with control IgG-treated groups (Figure 6C). The decreased serum titer of autoantibody against α-fodrin suggests that ED administration of CD4 affected autoimmune pathology only in the lacrimal glands but was able to suppress systemic production of α-fodrin–specific autoantibody.

DISCUSSION

Patients with SS have generally been treated with systemic administration of immunosuppressive drugs (10,21). Despite the effectiveness of such drugs, it is better to establish a local therapeutic strategy for eye and mouth symptoms of SS because the systemic use of immunosuppressive drugs induces severe side effects (21). In this study, we evaluated whether ED administration of CD4 effectively inhibits autoimmune pathology in the lacrimal glands of a mouse model of SS (12). We have previously reported that anti-CD86 mAb treatment improved the autoimmune pathology in both the lacrimal and the salivary glands of mice with SS (30). We used an antibody because the specific binding ability of a mAb against the target molecule allows the establishment of a molecule-specific therapeutic strategy with fewer side effects. Although an antibody is a large molecular weight glycoprotein, we demonstrated that ED administration of CD4 effectively inhibited both the onset and the progression of autoimmune responses only in the lacrimal glands of mice with SS. Although we did not simply compare the efficacy of topical administration of anti-CD4 mAb with that of systemic administration, this successful therapeutic effect of anti-CD4 mAb would provide the possibility of establishing a new form of antibody-based therapy for patients with eye symptoms caused by SS as well as other types of diseases.

ED administration of CD4 increased the total number of CD4+ T cells in cervical lymph nodes after a

4-week treatment starting at the age of 4 weeks, and increased both CD4+ and CD8+ T cells after a 2-week treatment starting at the age of 10 weeks. After ED administration of CD4, the resultant CD4+ T cells expressed low levels of CD44 compared with those after treatment with control IgG. The results suggest that the main therapeutic effect of the anti-CD4 mAb in the pathology of SS is the inhbition of CD4+ T cell activation rather than the deletion of CD4+ T cells, although the anti-CD4 mAb both blocks the interaction between CD4 and class II MHC and deletes CD4+ T cells in vivo (27,28). The increase in T cells after ED administration of CD4 would be due to reduced CD4+ T cell activation, resulting in a decrease in the activation-induced death of CD4+ T cells, which may explain the larger relative cell numbers in the anti-CD4 mAb–treated mice than in the control IgG-treated mice. This is supported by results from a previous study (20) demonstrating increased CD4+ T cell apoptosis in humans with SS. Furthermore, in our preliminary experiments, we observed a decrease in annexin V–positive CD4+ T cells in lymph nodes from mice treated with ED administration of CD4 (data not shown).

Nevertheless, it will be important to evaluate the mechanistic basis of the effect of anti-CD4 mAb. In particular, the number of lymphocytes in the lacrimal glands significantly decreased after a 2-week treatment of ED administration of CD4 starting at 10 weeks of age, which suggests the contribution of cytolytic as well as inhibitory activity of anti-CD4 mAb to the inhibition of autoimmune pathology. In addition, the suppressed activation of CD4+ T cells might decrease the activation of antigen-presenting cells or direct the help of CD8+ T cells, which also suppresses the initial expansion of CD8+ T cells, resulting in activation-induced cell death from ages 10 to 12 weeks but not ages 4 to 8 weeks. The differential mode of activation of CD8+ T cells during the initial and progression stages of diseases suggests that CD8+ T cells are required in the progression stage of SS. We are currently performing experiments using anti-CD8 mAb eyedrops to address this issue.

One might argue whether CD25+ regulatory T cells play some role in the effect of ED administration of CD4 in this mouse model because a thymectomy was performed 3 days after birth in the mice with SS. However, we do not think that CD25+ regulatory T cells significantly contribute to the therapeutic effect of ED administration of CD4 because the relative number of CD25+ T cells in regional lymph nodes is not increased in mice treated with ED administration of CD4 (data not shown). Recently, several groups have demonstrated that regulatory T cells differentiated from naive CD25+ T cells (31,32), although there are no appropriate cell surface markers to distinguish such regulatory T cells from effector T cells. Thus, it would be interesting to evaluate the contribution of such regulatory T cells to the inhibition of autoimmune pathology by ED administration of CD4.

ED administration of CD4 inhibited CD4+ T cell activation in cervical lymph node cells but not spleen cells, indicating that anti-CD4 mAb locally affects T cell activation. Although an antibody is a high molecular weight glycoprotein, we have observed the localization of anti-CD4 mAb in the lacrimal glands but not the spleen or regional lymph nodes (Hayashi Y, et al: unpublished observations). Thus, we think that anti-CD4 mAb blocks the activation of pathologic CD4+ T cells by interfering with class II MHC interactions in the lacrimal glands. Nevertheless, it remains unclear how large glycoproteins migrate into the parenchyma of the lacrimal glands, and this must be addressed in a future study.

We showed that ED administration of CD4 reduces serum anti–JS-1 autoantibody levels. These results suggest that ED administration of CD4 inhibited local JS-1–specific CD4+ T cell activation, which impaired T cell help to activate JS-1–specific B cells. Although ED administration of CD4 inhibited autoimmune pathology only in the lacrimal glands, it almost completely eliminated serum JS-1–specific autoantibody. There are 2 possible explanations for this result. The first is that the major helper T cells to induce JS-1–specific autoantibody might be from infiltrating T cells in the lacrimal glands. However, we think this possibility is unlikely because α-fodrin protein is present both in the lacrimal and the salivary glands, and transfer of infiltrating CD4+ T cells into the salivary glands in normal mice is able to cause SS-like lesions with autoantibody production (33). The second possibility is that ED administration of CD4 can prevent T cell help to activate B cells both in the lacrimal and the salivary glands but cannot sufficiently suppress autoimmune pathology in the salivary glands. The direct contribution of CD4+ T cells in the absence of B cells to the autoimmune pathology in this mouse model should be evaluated to address this possibility.

Several groups have examined the effect of ED administration of CSA as a treatment for SS and reported on its positive effect on autoimmune pathology (22,34). However, since systemic injection of CSA induces severe side effects in renal arteries (10), it is possible that even CSA eyedrops can induce some side effects in the eye. In this regard, antibodies have the ability to bind very specifically to their target receptor,

which suggests that it is possible to establish a therapeutic strategy with fewer side effects. Indeed, the application of 2 μg of anti-CD4 mAb daily did not induce any side effects histologically (data not shown). We did not observe any changes in cell number or activation status in the spleen with ED administration of CD4, which repudiates the possibility that anti-CD4 mAb enters into the circulation.

Several aspects of this model system illustrate important issues for future trials with antibody-based therapeutics. A variety of cell surface molecules have been reported to be responsible for the progression of eye symptoms in SS as well as other diseases (17,35,36). Thus, this antibody-based topical therapy may also be applicable to other types of antibodies or diseases.

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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.

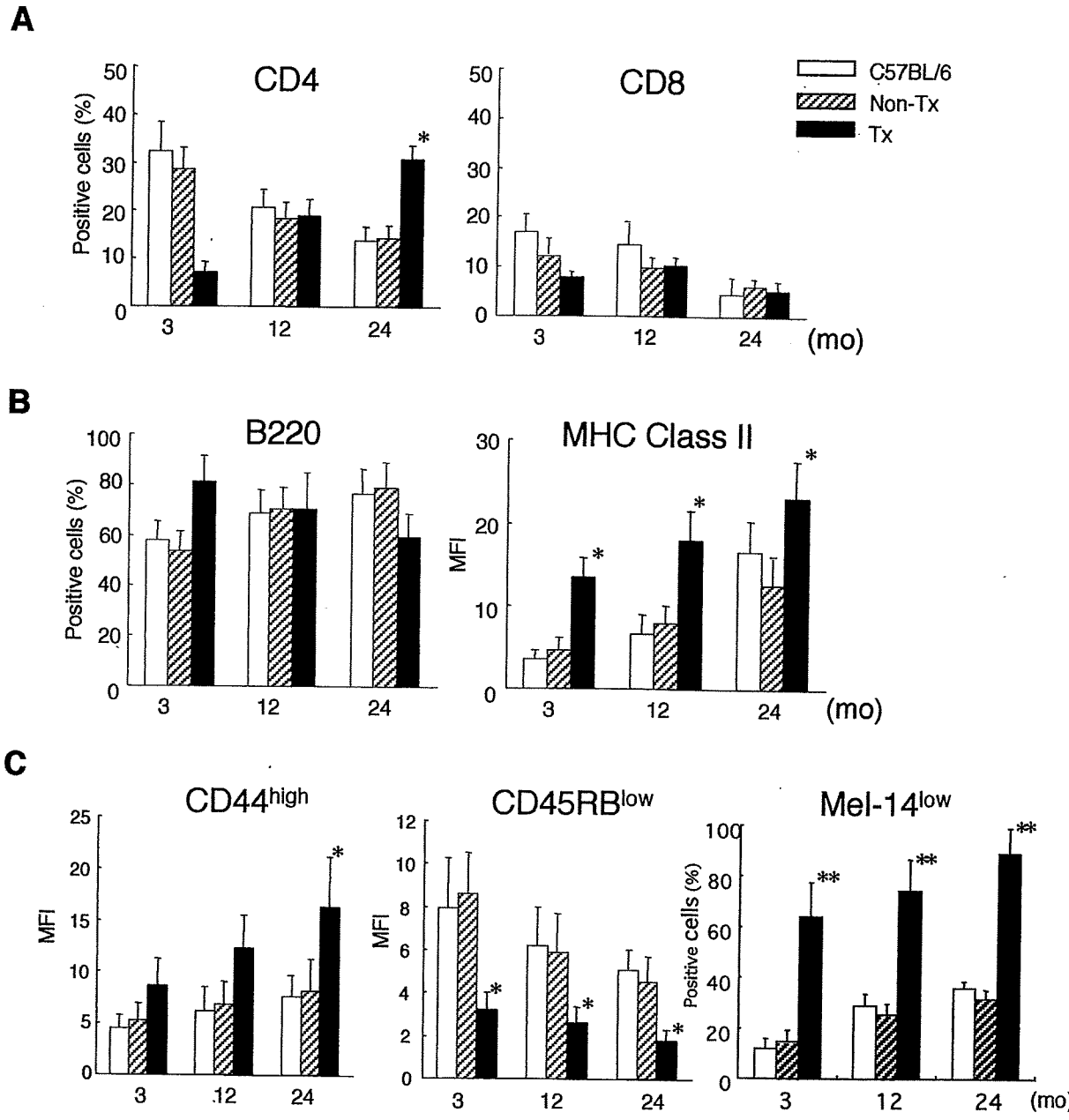

Figure 1. Age-related changes in activation markers. Shown are the results of the flow cytometric analysis of the CD4+,CD8+ T cell population, B220+ cells, class II major histocompatibility complex (MHC)–positive cells, and CD4+ T cells bearing activation markers in the spleens. Values are the mean and 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 $CD44^{high}$, $Mel\text{-}14^{low}$, 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;

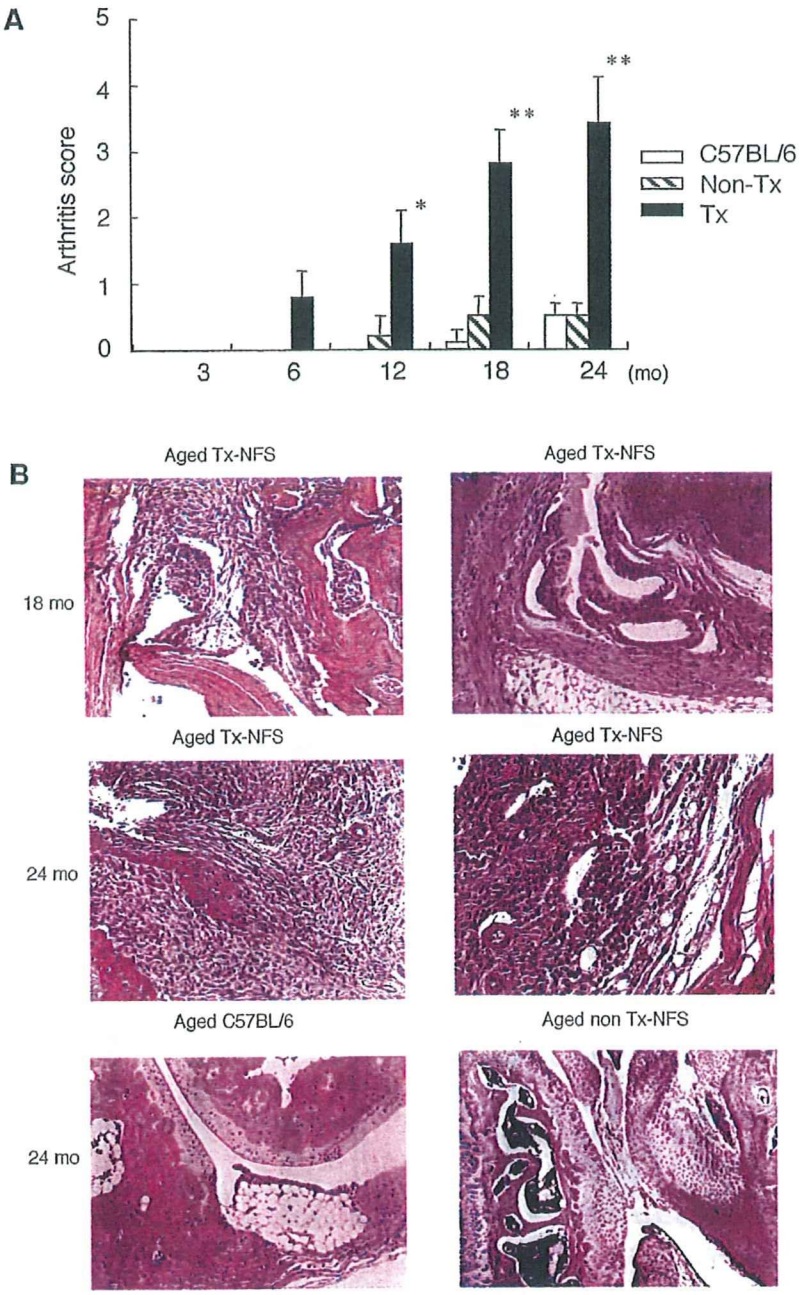

Figure 2. Age-related changes in joint histopathology. **A,** Effects of aging on joint histopathology in Sjögren's syndrome (SS) model mice. Shown are histologic scores of autoimmune arthropathy developed in aging SS model mice compared with those in age-matched non-Tx and C57BL/6 mice at ages 3, 6, 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

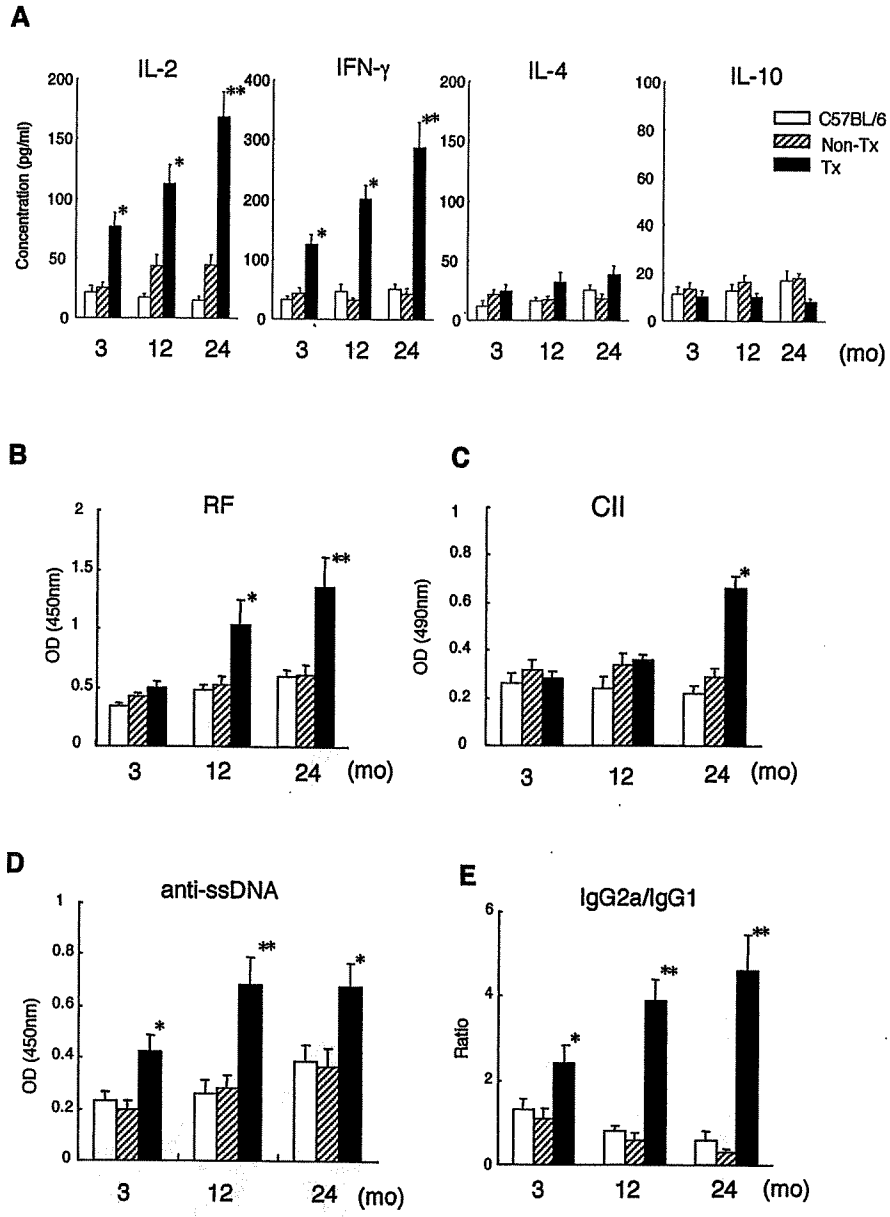

Figure 3. Age-related changes in cytokine profile and production of various autoantibodies. Values are the mean and SD. **A,** As measured by enzyme-linked immunosorbent assay, culture supernatants from anti-CD3 monoclonal antibody–stimulated splenic T cells obtained from aged Sjögren's syndrome (SS) 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

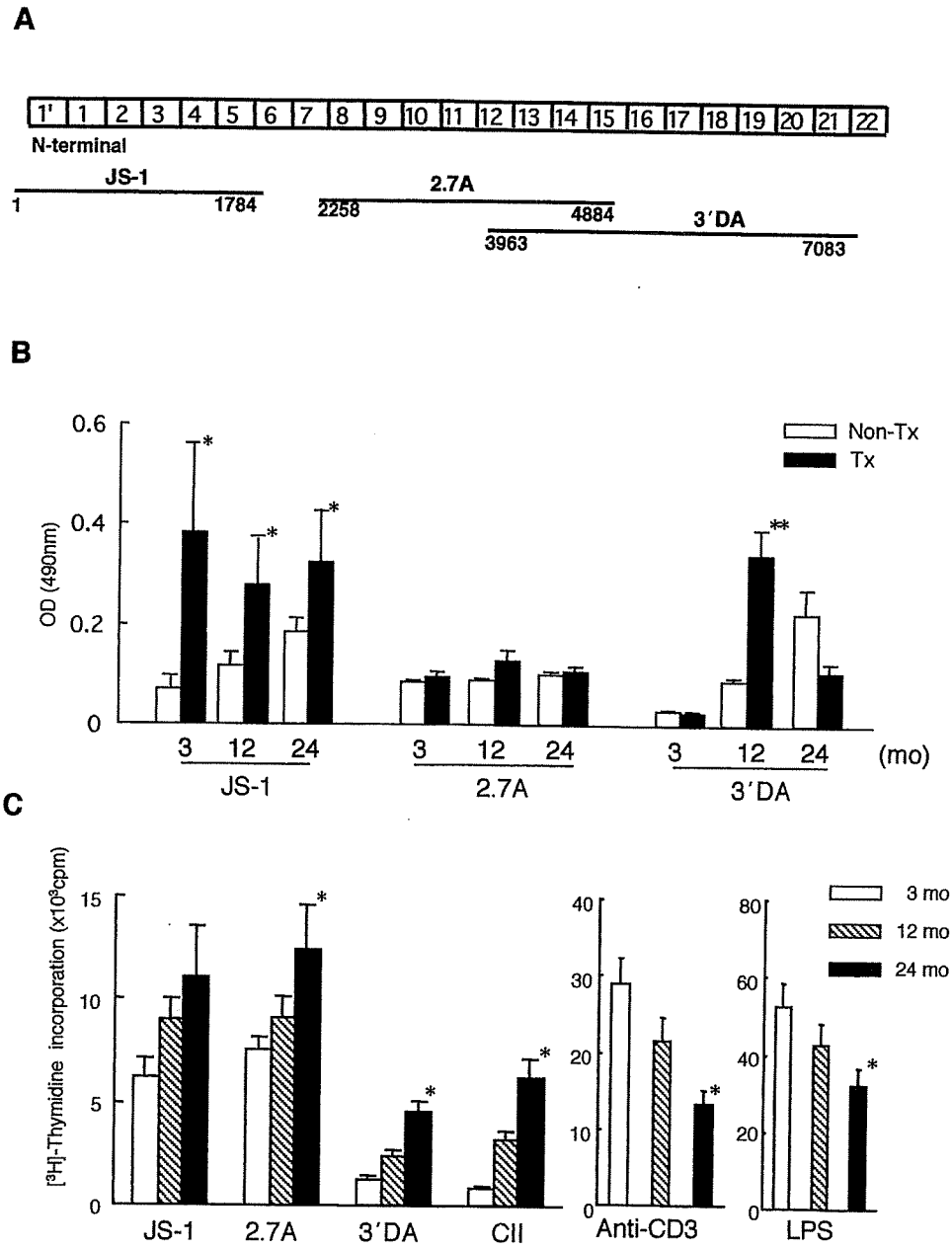

Figure 4. Immune responses against recombinant α-fodrin. **A**, Recombinant α-fodrin protein, the cDNA encoding human α-fodrin (JS-1, 1–1,784 bp; 2.7A, 2,258–4,884 bp; 3'DA, 3,963–7,083 bp), was constructed by inserting cDNA into the *Eco* RI site of pGEX-4Ts. **B**, A high titer of serum autoantibodies 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

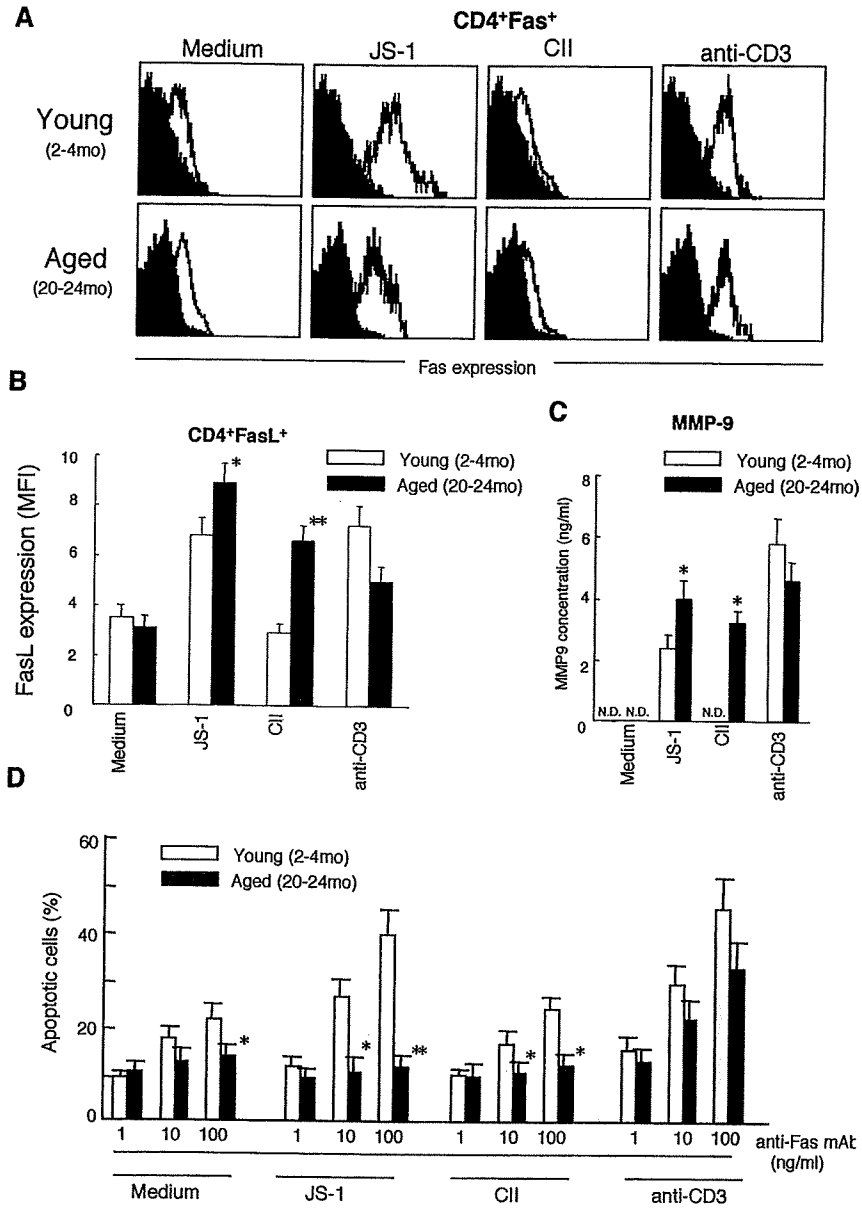

Figure 5. Expression of Fas, Fas ligand (FasL), and matrix metalloproteinase 9 (MMP-9). **A,** As shown by flow cytometry, an increased number of CD4+,Fas+ T cells was observed in JS-1–stimulated spleens from young SS model mice, but not from aged SS model mice. No significant difference was found in 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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