CD4+CD25^{high}CD127$^{low/-}$ Treg Cell Frequency from Peripheral Blood Correlates with Disease Activity in Patients with Rheumatoid Arthritis

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ABSTRACT. ***Objective.*** To investigate whether the frequency of peripheral blood (PB) regulatory T cells (Treg) correlates with the clinical disease activity of rheumatoid arthritis (RA).

Methods. PB Treg cells, defined as the CD4+CD25^{high}CD127$^{low/-}$ population, were examined by flow cytometry in 48 patients with RA, including 13 who had never received disease-modifying antirheumatic drugs (DMARD), 19 with active disease who were receiving (n = 14) or had received (n = 5) DMARD, and 16 receiving DMARD whose disease was in remission. The clinical disease activity of the patients was defined by the 28-joint Disease Activity Score (DAS28). The association of DAS28, C-reactive protein (CRP), or erythrocyte sedimentation rate (ESR) with the frequency of PB Treg cells was examined.

Results. The frequency of PB Treg cells in patients with RA was significantly low compared with that of healthy controls (n = 14). Among the 3 populations of patients with RA, Treg cell frequency was lowest in patients with active RA. In contrast, the Treg cell frequency of patients with RA in remission was similar to that of healthy controls. Accordingly, the frequency of CD4+CD25^{high}CD127$^{low/-}$ Treg cells negatively correlated with DAS28, CRP, and ESR in patients with RA.

Conclusion. The data suggest that Treg cells, defined as the CD4+CD25^{high}CD127$^{low/-}$ population, may contribute to the pathogenesis of RA and be an indicator of disease activity. (First Release Sept 15 2011; J Rheumatol 2011;38:2517–21; doi:10.3899/jrheum.110283)

Key Indexing Terms:
REGULATORY T CELL
RHEUMATOID ARTHRITIS
CD127 (INTERLEUKIN 7Rα)
DISEASE ACTIVITY

CD4+ regulatory T cell (Treg)-cell deficiency or absence is known to correlate with the development or exacerbation of autoimmune diseases, implying a crucial role for Treg cells in maintaining immunological self-tolerance[1,2]. In recent years, Treg cell counts and function have also been examined in patients with rheumatoid arthritis (RA)[3,4,5,6,7,8]. Treg cell function in patients with active RA is assumed to be impaired, a trend that seems to be reversed by tumor necrosis factor (TNF) antagonist therapy[6,7]; however, Treg cell counts in peripheral blood (PB) have varied across studies[3,4]. These discrepancies can probably be ascribed to differences in the labeling and definition of CD4+CD25+ T cells[9]. Among CD4+CD25+ T cells, those exerting suppressive effects[9,10] are only those expressing large amounts of CD25, e.g., CD4+CD25^{high} T cells, which highly express forkhead box P3 (FOXP-3). The intracellular staining process for FOXP-3 is somewhat time-consuming as compared with cell surface staining in clinical practice; thus, a more convenient marker on the cell surface closely correlating with FOXP-3 expression is awaited. In this regard, Saleem, *et al* recently reported that the frequency of CD62L^{+} Treg cells in PB from RA is associated with sustained remission during TNF antagonist therapy[11].

Another candidate cell surface molecule for the identification of Treg cells is CD127. Two recent studies have demonstrated that downregulation of the interleukin (IL)-7 receptor α chain, CD127, distinguishes Treg cells from activated T cells, demonstrating a significant correlation between the FOXP-3 and CD127$^{low/-}$ phenotype at the same time that it functionally suppresses the CD127$^{low/-}$ population[12,13].

We examined whether the frequency of Treg cells correlates with the clinical disease activity of RA by staining cells

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with CD4, CD25, and CD127. The frequency of CD4+CD25^{high}CD127$^{low/-}$ Treg cells negatively correlated with the 28-joint Disease Activity Score (DAS28), C-reactive protein (CRP), and the erythrocyte sedimentation rate (ESR). This is a more convenient method of detecting Treg cells in clinical practice and may follow the data of Saleem, *et al*[11], suggesting that Treg cells contribute to the pathogenesis of RA.

MATERIALS AND METHODS

Study population. Patients with RA (n = 48) and healthy controls (n = 14) were included in our study. All of the patients fulfilled the 1987 criteria of the American College of Rheumatology for RA[14]. All patients were seropositive for rheumatoid factor and/or anticitrullinated protein antibodies. Informed consent was obtained from all patients and controls. The Institutional Review Board of Nagasaki University approved the study. Clinical response to the therapy was evaluated by DAS28 (high disease activity > 5.1, moderate disease activity < 5.1 and > 3.2, low disease activity < 3.2, remission < 2.6). Patients were divided into 3 groups: (1) those naive to disease-modifying antirheumatic drugs (DMARD; n = 13); (2) those with active RA (n = 19) whose disease activity was moderate (DAS28 > 3.2), including both those who were receiving (n = 14) or had received (n = 5) DMARD; and (3) those whose disease was in clinical remission (DAS28 < 2.6) with concomitant use of DMARD (n = 16). All patients in the group with active RA and the remission group were on stable therapy. Patient characteristics are shown in Table 1. For the controls, the median (range) of age was 34.5 (27-50) years and the sex ratio was 4:10 (men:women). They were statistically younger than patients with RA. We examined a correlation of age and each population of T cell frequency among 14 controls by Spearman's rank correlation and did not find any association (data not shown).

Cell isolation and analysis by flow cytometry. Peripheral blood samples were collected in heparin. Peripheral blood mononuclear cells (PBMC) were isolated by standard Ficoll-Hypaque density centrifugation and used for flow cytometry. Freshly isolated PBMC were stained with 3 colors: FITC-labeled CD4; PE-Cy5-labeled CD25 and PE-labeled CD127; or PE-Cy5-labeled CD4, PE-labeled CD25, and FITC-labeled FOXP-3 (clone: PCH101, intracellular staining) by the standard protocol. All antibodies were products of eBioscience (San Diego, CA, USA). For flow cytometric analysis, lymphocytes were gated morphologically.

Statistical analyses. Within-group comparisons were made using the Mann-Whitney U test. Changes from the baseline were compared using Wilcoxon's signed-rank test. Correlations were assessed with Spearman's correlation coefficient test. The overall significance level for statistical analysis was 5% (2-sided). P values < 0.05 were considered statistically significant.

RESULTS

Patient characteristics are shown in Table 1. CRP, ESR, and DAS28 in the remission group were significantly lower than in the other 2 groups. CRP and ESR in the patients with active RA who were receiving or had received DMARD were significantly higher than those of DMARD-naive patients with RA, but DAS28 was not significantly different between the 2 groups.

Although most CD4+CD25+CD127$^{low/-}$ T cells were positive for FOXP-3, a portion of this population was negative for FOXP-3 (Figure 1A). On the other hand, almost all CD4+CD25^{high}CD127$^{low/-}$ T cells were positive for FOXP-3 (Figure 1A).

Phenotypes of peripheral blood CD4+ T cells of patients with RA and controls were compared (Table 2). There were no significant differences in the frequencies of CD4+CD25– T cells and CD4+CD25+ T cells between DMARD-naive patients with RA and healthy volunteers. The frequency of CD4+CD25+CD127$^{low/-}$ T cells (Figure 1A) was significantly lower in the patients with active RA who were receiving or had received DMARD than in controls ($p < 0.05$).

We counted the frequency of CD4+CD25^{high}CD127$^{low/-}$ T cells, using the cutoff value of < 5% CD127 expression among CD4+ T cells (Figure 1B). We have adopted this method to identify the CD4+CD25^{high}CD127$^{low/-}$ T cells accurately in each individual. The frequency of this population was lower in the DMARD-naive RA patient group than in the healthy controls ($p < 0.01$), and was lower in the active RA group taking DMARD than in the DMARD-naive RA group ($p < 0.01$). Further, the frequency of this population was higher in the remission group than in the active RA group with DMARD ($p < 0.0001$).

We investigated the correlation between the phenotype of

Table 1. Patient characteristics. Within-group comparisons were made using the Mann-Whitney U test and the chi-squared test (Fisher's exact probability test when appropriate).

Characteristics	Healthy Controls	DMARD-naive RA Group	Active RA Group	Remission Group
Patient, no.	14	13	19	16
Age, yrs, median (range)	34.5 (27–50)	59 (39–81)	57 (19–79)	58 (26–76)
Sex (men/women)	4/10	3/10	1/18	4/12
Disease duration, yrs, median (range)	—	0.25 (0.15–1.5)	3.5* (0.5–28)	2.5* (0.33–22)
CRP, mg/dl, median (range)	—	0.54** (0.04–5.35)	2.59**# (0.16–10.08)	0.07 (0.01–0.12)
ESR, mm/h, median (range)	—	48** (12–120)	57.4**# (1–127)	13.5 (5–26)
DAS28, median (range)	—	4.68** (3.54–7.75)	5.70** (3.21–8.16)	1.83 (1.13–2.54)
Therapy	—	—	MTX: 13, SASP: 1	MTX: 11, SASP: 2, BU: 1, ETN: 1, IFX: 3
Concomitant glucocorticoid, n (dose < 7.5 mg daily)	—	2	10***#	0

* $p < 0.0001$ vs DMARD-naive RA group. ** $p < 0.0001$ vs remission group. *** $p < 0.001$ vs remission group. # $p < 0.05$ vs DMARD-naive RA group. DMARD: disease-modifying antirheumatic drugs; RA: rheumatoid arthritis; CRP: C-reactive protein; ESR: erythrocyte sedimentation rate; DAS28: 28-joint Disease Activity Score; BU: bucillamine; ETN: etanercept; IFX: infliximab; MTX methotrexate; SASP: salazosulfapyridine.

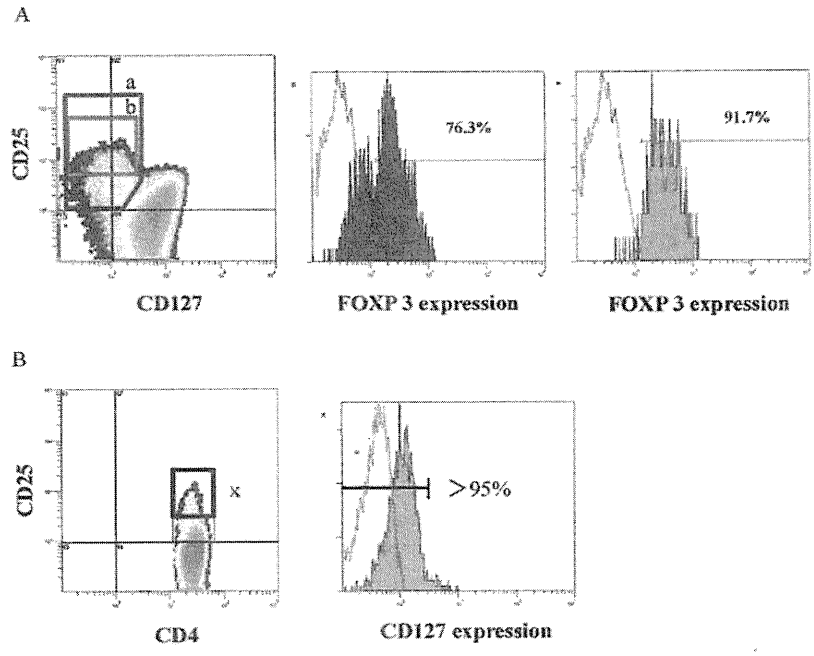

Figure 1. The CD4+CD25^{high}CD127$^{low/-}$ population as the phenotype of Treg cells. The graphs show representative data of several healthy samples. A. Plots are gated for CD4+ T cells. CD25+CD127$^{low/-}$ cells and CD25^{high}CD127$^{low/-}$ cells are found in box a and box b. Expressions of FOXP-3 in boxes a and b are shown in the second and third panels. B. Mononuclear cells were stained for CD4, CD25, and CD127. Plots are gated for CD4+ T cells. The CD4+CD25^{high} population, with the cutoff of CD127 expression at < 5% among CD4+ T cells (right side), is boxed as CD4+CD25^{high}CD127$^{low/-}$ T cells (left side, box x). Box x is individually adjusted.

Table 2. Phenotype of peripheral blood CD4+ T cells in patients with rheumatoid arthritis (RA) and in healthy controls. Data are percentage of CD4+ T cells; median (range). Within-group comparisons were made using the Mann-Whitney U test.

Study Participants	CD4+CD25–	CD4+CD25+	CD4+CD25+CD127$^{low/-}$	CD4+CD25^{high}CD127$^{low/-}$
Healthy controls, n = 14	84.7 (72.4–87.2)	15.3 (12.8–27.6)	3.63 (2.34–7.54)	3.76 (2.11–9.80)
DMARD-naive RA group, n = 13	78.3 (63.9–88.3)	21.7 (11.7–36.1)	3.12 (1.72–5.73)	2.23** (0.57–5.18)
Active RA group, n = 19	79.9 (63.9–87.2)	20.1 (12.8–36.1)	2.76* (1.25–5.31)	1.35***$^{\#}$ (0.41–2.21)
Remission group, n = 16	80.6 (66.6–90.1)	19.4 (9.9–33.4)	3.35 (2.13–7.21)	2.98$^{\$}$ (1.34–4.89)

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.0001$ vs HC, $^{\#}$ $p < 0.01$ vs DMARD-naive RA group, $^{\$}$ $p < 0.0001$ vs active RA group. DMARD: disease-modifying antirheumatic drugs.

peripheral blood Treg cells and the markers of disease activity such as CRP, ESR, and DAS28 in the 48 patients with RA (Table 3). The frequencies of CD4+CD25+ T cells and CD4+CD25+CD127$^{low/-}$ T cells were not correlated with disease activity. However, the frequency of CD4+CD25^{high}-CD127$^{low/-}$ T cells was negatively correlated with CRP, ESR, and DAS28, respectively ($p < 0.0001$).

As mentioned, CD4+ T cells were almost all positive for CD127 (Figure 2A); however, a large CD127$^{low/-}$ population was detected among CD4+CD25+ T cells in healthy individuals (Figure 2B). In patients with RA, the expression of this population was lower than in healthy individuals (Figure 2C), but it recovered after the disease went into clinical remission (Figure 2D).

DISCUSSION

Recent data obtained from patients with RA during TNF-antagonist therapy have suggested that TNF downmodulates the function of human CD4+CD25+ Treg cells[6,7,8]. Therefore, Treg cells may dynamically fluctuate, depending on the disease status of RA, and reflect the disease activity of RA. We have focused on a convenient cell surface staining method to identify Treg cells and tried to investigate the association of Treg cell frequency with the disease activity of RA.

CD25 and CD127 were used to identify the Treg cell population in our study. Since FOXP-3 is strongly expressed in CD4+CD25^{high}CD127$^{low/-}$ population, CD4+CD25^{high}- CD127$^{low/-}$ cells can be estimated as Treg

Table 3. The correlations between regulatory T cells (Treg) and rheumatoid arthritis (RA) disease activity in 48 patients with RA. The correlations were assessed using Spearman's correlation coefficient test.

	CRP		ESR		DAS28	
	r	p	r	p	r	p
CD4+CD25+	0.03	NS	0.12	NS	0.04	NS
CD4+CD25+CD127$^{low/-}$	–0.21	NS	–0.20	NS	–0.17	NS
CD4+CD25^{high}CD127$^{low/-}$	–0.65	< 0.0001	–0.58	< 0.0001	–0.61	< 0.0001

CRP: C-reactive protein; ESR: erythrocyte sedimentation rate; DAS28: 28-joint Disease Activity Score; NS: not significant.

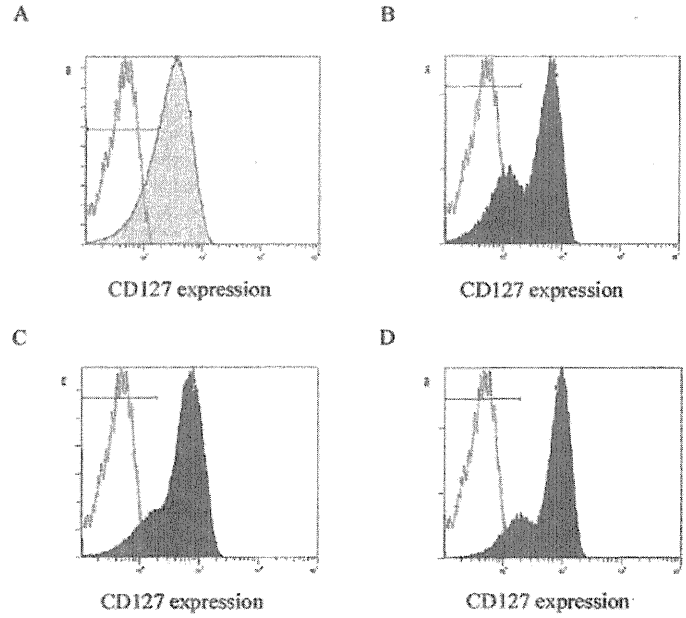

Figure 2. Changes in the proportion of the CD127$^{low/-}$ population among CD4+CD25+ T cells. Expression of CD127 among CD4+ T cells (A) and CD4+CD25+ T cells (B-D) are shown. Panels A and B describe peripheral blood mononuclear cell (PBMC) samples collected from healthy individuals. Although CD4+ T cells are likely almost all positive for CD127 (A), the expression of CD127 among CD4+CD25+ T cells is differential, with both CD127$^{low/-}$ cells and CD127^{+} cells in healthy controls (B). Panels C and D describe PBMC samples collected from a patient with early RA (male, disease duration 3 months). The proportion of CD127$^{low/-}$ cells among CD4+CD25+ T cells decreased before this patient was treated (28-joint Disease Activity Score 4.27, panel C). After this patient's disease went into clinical remission by treatment with bucillamine, the expression of CD127 among CD4+CD25+ T cells recovered to nearly the same level as that in healthy controls (D). The frequencies of CD4+CD25^{high}CD127$^{low/-}$ cells were 5.73% before therapy and 7.21% after clinical remission.

cells[12,13]. Additionally, the clinical differences between patients with RA and controls as well as the clinical measures among patients with RA were most predominantly found in the CD4+CD25^{high}CD127$^{low/-}$ population. We have set the cutoff of CD127 expression at < 5% in the individual case; thus, our definition may correctly identify the frequency of naturally arising Treg cells (Figure 3). The controls were younger than the patients with RA in our study. Although there was no correlation between Treg cell frequency and age of the control, a previous study[4] demonstrated a weak negative correlation between age and Treg cell frequency. The use of glucocorticoids was more frequent in the active RA group as compared with the DMARD-naive RA group, as well as the remission group. The influence of glucocorticoids regarding the function or number of Treg cells might be controversial[15,16]. Therefore, age-matched studies involving glucocorticoid-naive patients are necessary to confirm our results.

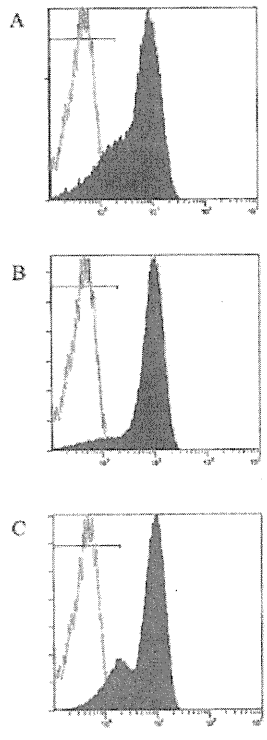

Figure 3. The proportion of the CD127$^{low/-}$ population among CD4+CD25+ T cells in the 3 RA groups. A. The group naive to disease-modifying antirheumatic drugs. B. The group with active RA. C. The remission group. Expressions of CD127 among CD4+CD25+ T cells are shown. The frequencies of CD4+CD25+CD127$^{low/-}$ cells were 3.28%, 2.24%, and 5.77% in panels A, B, and C, respectively.

DMARD may alter the function of T cells in patients with RA. However, we have found that Treg cell frequency may depend not on the use of DMARD but on the RA disease activity. The Treg cell frequency was lowest in patients with active RA, while that of patients with RA in remission was similar to that of controls, despite the administration of DMARD in both groups. In addition, the Treg cell frequency was not statistically different between DMARD-naive patients with RA and patients receiving DMARD whose RA was in remission, and an inverse correlation was found between the disease activity of RA and the Treg cell frequency. In fact, we found fluctuation of Treg cell frequency depending on the disease activity. The difference of our study as compared with previous reports is to estimate Treg cells as FOXP-3^{bright} cells. As shown in previous reports[3,4], the difference of CD4+CD25+ T cell frequency between the patients with RA and controls was not significant. In addition to CD127, a similar result is obtained when estimating Treg cells as CD62 ligand^{+} FOXP-3^{bright} cells[11]. Since we have not performed followup analysis of Treg cell frequency in each case, a prospective followup study should be performed to establish that the CD4+CD25^{high}CD127$^{low/-}$ Treg cell population does in fact reflect changes in the disease activity of RA. Further examinations, including studies with a larger number and with followup observation, are needed to confirm our findings.

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Th2 and Regulatory Immune Reactions Contribute to IgG4 Production and the Initiation of Mikulicz Disease

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Objective. **Mikulicz disease has been considered to be a subtype of Sjögren's syndrome (SS). However, recent studies have suggested that Mikulicz disease is an IgG4-related disease and is distinguishable from SS. In addition, it has been reported that both interleukin-4 (IL-4) and IL-10 induce IgG4 production and inhibit IgE. This study was undertaken to examine the expression of these cytokines in patients with Mikulicz disease and patients with SS.**

Methods. **Labial salivary gland (LSG) sections from 15 patients with Mikulicz disease and 18 patients with SS were examined for subsets of the infiltrating lymphocytes, expression patterns of messenger RNA (mRNA) for cytokines/chemokines, and relationships between the IgG4:IgG ratio and the expression of mRNA for IL-4 or IL-10.**

Results. **Immunohistochemical analysis showed lymphocyte infiltration of various subsets in the LSGs of SS patients, and the selective infiltration of IgG4-positive plasma cells and Treg cells in the LSGs of Mikulicz disease patients. The levels of mRNA for both Th1 and Th2 cytokines and chemokines in LSGs from patients with SS were significantly higher than in controls, while the expression of both Th2 and Treg cells was significantly higher in the patients with Mikulicz disease than in controls. Furthermore, the expression of IL-4 or IL-10 in the LSGs was correlated with the IgG4:IgG ratio.**

Conclusion. **These results suggest that the pathogenesis of Mikulicz disease is different from that of SS. Mikulicz disease is a unique inflammatory disorder characterized by Th2 and regulatory immune reactions that might play key roles in IgG4 production.**

Mikulicz disease has been considered to be a subtype of Sjögren's syndrome (SS), based on the histopathologic similarities between the two diseases (1). However, Mikulicz disease shows several differences in comparison with typical SS. In Mikulicz disease, enlargement of the lacrimal and salivary glands is persistent, salivary secretion is either normal or moderately dysfunctional, patients have a good response to corticosteroid treatment, and hypergammaglobulinemia and low frequencies of anti-SSA and anti-SSB antibodies are found on serologic analyses. Since Yamamoto et al (1–3) reported that serum IgG4 levels are elevated and IgG4-positive plasma cells infiltrate into the gland tissue in Mikulicz disease, these symptoms have also been recognized in autoimmune pancreatitis (4), primary sclerosing cholangitis (5), tubulointerstitial nephritis (6), interstitial pneumonia (7), Ridel's thyroiditis (8), and Küttner's tumor (9). These diseases are now called "IgG4-related diseases" (2,10). IgG4 is a Th2-dependent Ig and has low affinity for target antigen. Interleukin-4 (IL-4) directs naive human B cells to switch to IgG4 and IgE production (11).

CD4+ T helper cells including at least 5 subsets have been identified. Th0, Th1, Th2, Th17, and Treg cells are generally considered to maintain the balance

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and homeostasis of the immune system and possibly to induce various diseases by their impaired regulation. The difference in the functions of Th1 and Th2 cells has been characterized by the patterns of cytokines secreted by these Th cells. Th1 cells induced by IL-12 are mainly responsible for cell-mediated immunity, while Th2 cells induced by IL-4 are responsible for humoral immunity. These Th subsets are then mutually controlled by the cytokine that each produces. Several studies have indicated that many autoimmune diseases or allergic diseases are caused by the collapse of the Th1/Th2 balance. Th0 cells are produced by both Th1 and Th2 cytokines and are considered to be precursors of Th1 and Th2 cells. Treg cells are essential for the maintenance of immunologic self-tolerance and immune homeostasis. Recently, a subset of IL-17–producing T cells (Th17 cells) distinct from Th1 and Th2 cells was described and was shown to play a crucial role in the induction of autoimmunity and allergic inflammation (12). Furthermore, it has been demonstrated that chemokines are intimately involved in the Th1/Th2 balance and immune responses in various diseases, such as rheumatoid arthritis (13), systemic lupus erythematosus (13,14), SS (13,15), systemic sclerosis (13,16), idiopathic inflammatory myopathy (13), and atopic dermatitis (17).

The relationship of Th1/Th2 imbalance to the pathogenesis of SS has been investigated widely, and a polarized Th1 balance has been associated with the immunopathology of the disease (18–20). Numerous interferon-γ (IFNγ)–positive CD4+ T cells are detected in the salivary glands of SS patients, and an intracellular cytokine assay demonstrated subsequent promotion of Th1 cells in SS (21). We have previously shown that SS was initiated and/or maintained by Th1 cytokines and subsequently progressed in association with Th2 cytokines (22). Ogawa et al (15) reported that Th1 chemokines, such as IFNγ-inducible 10-kd protein (IP-10) and monokine induced by IFNγ, are involved in the accumulation of T cell infiltrates in the salivary glands of patients with SS. These findings suggest that Th1 cells play a central role in the pathogenesis of SS.

In contrast, patients with Mikulicz disease frequently have a history of bronchial asthma and allergic rhinitis and show severe eosinophilia and elevated serum IgE levels. We previously reported that peripheral CD4+ T cells from patients with Mikulicz disease revealed deviation of the Th1/Th2 balance to Th2 and elevated the expression of Th2-type cytokines (23,24). Moreover, recent studies have indicated that peripheral blood CD4+ T cells in patients with IgG4-related lacrimal gland enlargement showed a Th2 bias and elevated serum IgE levels (24). Therefore, it is suggested that Mikulicz disease has a Th2-predominant phenotype. The findings of a previous study showing that autoimmune pancreatocholangitis, which is an IgG4-related disease, could also be characterized by the overproduction of Th2 and regulatory cytokines (25) deserve our attention.

To date, pathogenetic differences between immune responses in SS and Mikulicz disease are not well understood. In this study, we identified the expression patterns of cytokines, chemokines, and chemokine receptors in the salivary glands of these diseases to clarify the involvement of characteristic immune responses in the development of Mikulicz disease.

PATIENTS AND METHODS

Patients. Fifteen patients with Mikulicz disease (12 women and 3 men with a mean ± SD age of 56.3 ± 13.0 years) and 18 patients with SS (16 women and 2 men with a mean ± SD age of 54.6 ± 12.8 years) who were referred to the Department of Oral and Maxillofacial Surgery at Kyushu University Hospital were included in the study. Mikulicz disease was diagnosed according to the following criteria (3): persistent symmetrical swelling (lasting longer than 3 months) of >2 lacrimal and major salivary glands, elevated serum levels of IgG4 (>135 mg/dl), and infiltration of IgG4-positive plasma cells in the tissue (ratio of IgG4-positive cells:IgG-positive cells >40%) on immunostaining. SS was diagnosed according to the criteria of both the Research Committee on SS of the Ministry of Health and Welfare of the Japanese Government (1999) (26) and the American–European Consensus Group criteria for SS (27).

All patients exhibited objective evidence of salivary gland involvement based on the presence of subjective xerostomia and a decreased salivary flow rate, abnormal findings on parotid sialography, and focal lymphocytic infiltrates in the labial salivary glands (LSGs) and submandibular glands. All patients with SS had primary SS with strong lymphocytic infiltration in the LSGs, had no other autoimmune diseases, and had never been treated with corticosteroids or any other immunosuppressants. LSG biopsies were performed as described by Greenspan et al (28). As controls, LSGs biopsy specimens were obtained from 18 patients with mucoceles who had no clinical or laboratory evidence of systemic autoimmune disease. These control LSGs were all histologically normal. Written informed consent was obtained from all patients and healthy controls.

Histologic analysis of LSGs. Formalin-fixed and paraffin-embedded sections (4 μm) of LSG specimens were prepared and stained with hematoxylin and eosin for conventional histologic examinations. The degree of lymphocytic infiltration in the specimens was judged by focus scoring (28,29). One standardized score is the number of focal inflammatory cell aggregates containing 50 or more mononuclear cells in each 4-mm^2 area of salivary gland tissue (30). All of the patients with Mikulicz disease and patients with SS

in this study had strong lymphocytic infiltration (focus scores of 10–12).

Immunohistochemical analysis of LSGs. For the immunohistochemical analysis of lymphocyte subsets, 4-μm formalin-fixed and paraffin-embedded sections were prepared and stained by a conventional avidin–biotin complex technique as previously described (31). The mouse monoclonal antibodies used to analyze lymphocyte subsets were anti-CD4 (clone B12; MBL), anti-CD20 (clones L26 and M0755; Dako), and anti-FoxP3 (clone mAbcam 22510; Abcam). The mouse monoclonal antibody and rabbit polyclonal antibody used to analyze IgG4-positive and IgG-positive plasma cells were anti-IgG (A0423; Dako) and anti-IgG4 (The Binding Site). HDP-1 (antidinitrophenyl [anti-DNP] IgG1) was used as a control mouse monoclonal antibody. The polyclonal antibodies used to analyze the cytokines were anti–IL-4 (clone ab9622), anti–IL-10 (clone ab34843), anti–IFNγ (clone ab9657) (all from Abcam), and anti–IL-17 (clone sc-7927; Santa Cruz Biotechnology). SS1 (anti–sheep erythrocyte IgG2a), NS8.1 (anti–sheep erythrocyte IgG2b), and NS4.1 (anti–sheep erythrocyte IgM), were used as control rabbit polyclonal antibodies. The mouse monoclonal antibodies used to analyze the chemokines and chemokine receptors were anti–IP-10 (clone ab73837; Abcam), anti-CXCR3 (clone ab64714; Abcam), anti–thymus and activation–regulated chemokine (anti-TARC) (54015; R&D Systems), anti–macrophage-derived chemokine (anti-MDC) (57203; R&D Systems), and anti-CCR4 (MAB1567; R&D Systems). HDP-1 (anti-DNP IgG1) was used as a control mouse monoclonal antibody. The sections were sequentially incubated with primary antibodies, biotinylated anti-mouse IgG secondary antibodies (Vector Laboratories), avidin–biotin–horseradish peroxidase complex (Vector Laboratories), and 3,3′-diaminobenzidine (Vector Laboratories). Mayer's hematoxylin was used for counterstaining. Photomicrographs were obtained using a light microscope equipped with a digital camera (CoolSNAP; Photometrics). Stained IgG4-positive cells and IgG-positive cells were counted in 1-mm^2 sections from 5 different areas, and the ratio of IgG4-positive cells to IgG-positive cells was calculated.

RNA extraction and complementary DNA (cDNA) synthesis. Total RNA was prepared from the LSG specimens by the acid guanidinium–phenol–chloroform method as previously described (32–34). Three micrograms of the total RNA preparation was then used for the synthesis of cDNA. Briefly, RNA was incubated for 1 hour at 42°C with 20 units of RNasin ribonuclease inhibitor (Promega), 0.5 μg of oligo(dT)$_{12\text{-}18}$ (Pharmacia), 0.5 m*M* of each dNTP (Pharmacia), 10 m*M* of dithiothreitol, and 100 units of RNase H reverse transcriptase (Life Technologies).

Quantitative estimation of messenger RNA (mRNA) by real-time polymerase chain reaction (PCR). Quantitative cDNA amplification was performed according to the recommendations of the manufacturer and as previously described (32–34). The cDNAs for the cytokines, chemokines, and chemokine receptors were analyzed by real-time PCR using LightCycler FastStart DNA Master SYBR Green 1 (Roche Diagnostics) in a LightCycler real-time PCR instrument (version 3.5; Roche Diagnostics). The cytokines, chemokines, and chemokine receptors examined were IL-2, IFNγ, IL-12, IP-10, CXCR3, IL-4, IL-5, TARC, MDC, CCR4, IL-10, transforming growth factor β (TGFβ), FoxP3, IL-17, and IL-6. The markers of lymphocytes examined were IgG and IgG4.

The primer sequences used were as follows: for β-actin (260 bp), forward 5′-GCAAAGACCTG-TACGCCAAC-3′, reverse 5′-CTAGAAGCATTTGCGGTGGA-3′; for CD3δ (184 bp), forward 5′-GATGTCATTGCCACTCTGC-3′, reverse 5′-ACTTGTTCCGAGCCCAGTT-3′; for IL-2 (416 bp), forward 5′-ACTCACCAGGATGCTCACAT-3′, reverse 5′-AGGTAATCCATCTG-TTCAGA-3′; for IFNγ (355 bp), forward 5′-AGTTATATCTTGGCTTTTCA-3′, reverse 5′-ACCGAATAATTAGTCAGCTT-3′; for IL-4 (203 bp), forward 5′-CTGCCTCCAAGAACACAACT-3′, reverse 5′-CACAGGACAGGAATTCAAGC-3′; for IL-5 (104 bp), forward 5′-ATGAGGATGCTTCTGCATTTG-3′, reverse 5′-TCAACTTTCTATTATCCACTCG-3′; for IL-6 (115 bp),

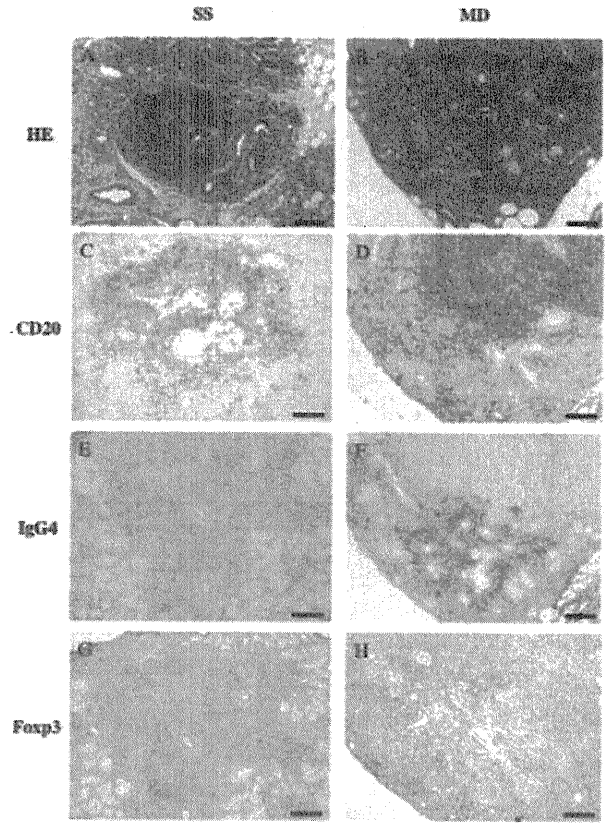

Figure 1. Selective infiltration of IgG4-positive plasma cells and FoxP3-positive Treg cells in the labial salivary glands (LSGs) of patients with Mikulicz disease (MD). Sections from the LSGs of a representative patient with Sjögren's syndrome (SS) and a representative patient with Mikulicz disease were immunostained with hematoxylin and eosin (H&E) (**A** and **B**) and anti-CD20 (**C** and **D**), anti-IgG4 (**E** and **F**), and anti-FoxP3 (**G** and **H**) monoclonal antibodies. Counterstaining with Mayer's hematoxylin was subsequently performed. Bars = 100 mm; original magnification × 100.

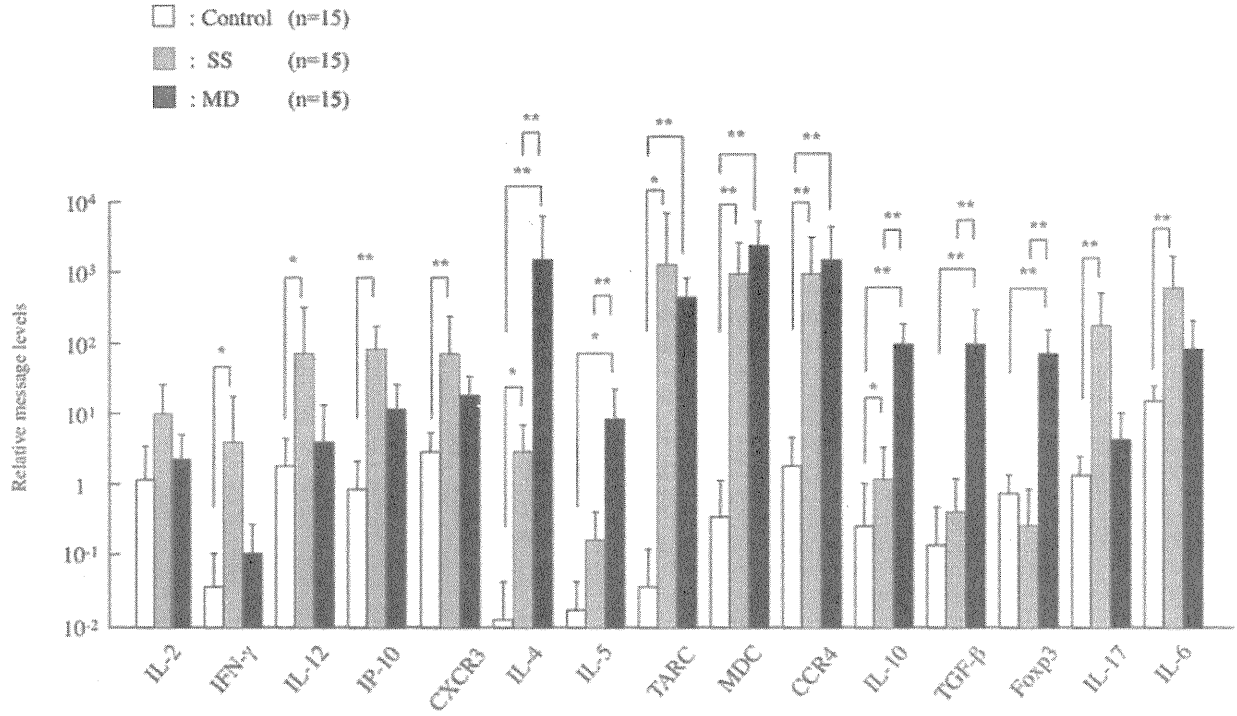

Figure 2. Expression patterns of mRNA for cytokines, chemokines, and chemokine receptors in LSGs from controls, patients with SS, and patients with Mikulicz disease. Levels of interferon-γ (IFNγ), interleukin-2 (IL-2), IL-12, IFNγ-inducible 10-kd protein (IP-10), and CXCR3 (Th1 type); IL-4, IL-5, thymus and activation–regulated chemokine (TARC), macrophage-derived chemokine (MDC), and CCR4 (Th2 type); IL-10, transforming growth factor β (TGFβ), and FoxP3 (Treg cell type); and IL-6 and IL-17 (Th17 type) were quantitatively estimated as described in Patients and Methods. Levels of mRNA in LSGs from SS patients and patients with Mikulicz disease were compared with those in control LSGs. Bars show the mean ± SD. * = $P < 0.05$; ** = $P < 0.01$ by Mann-Whitney U test. See Figure 1 for other definitions.

forward 5′-GGCACTGGCAGAAAACAA-3′, reverse 5′-CTCCAAAAGACCAGTGATGA-3′; for IL-10 (351 bp), forward 5′-ATGCCCCAAGCTGAGAACCAA-3′, reverse 5′-TCTCAAGGGGCTGGGTCAGCTA-3′; for IL-12 (187 bp), forward 5′-CCTGACCCACCCAAGAACTT-3′, reverse 5′-GTGGCTGAGGTCTTGTCCGT-3′; for IL-17 (186 bp), forward 5′-GCAGGAATCACAATCCCAC-3′, reverse 5′-TCTCTCAGGGTCCTCATTGC-3′; for FoxP3 (207 bp), forward 5′-CCCCTTGCCCCACTTACA-3′, reverse 5′-GCCACGTTGATCCCAGGT-3′; for TGFβ (142 bp), forward 5′-GCCCCTACATTTGGAGCCTG-3′, reverse 5′-TTGCGGCCCACGTAGTACAC-3′; for IgG (129 bp), forward 5′-CAAGTGCAAGGTCTCCAACA-3′, reverse 5′-TGGTTCTTGGTCAGCTCATC-3′; for IgG4 (132 bp), forward 5′-ACTCTACTCCCTCAGCAGCG-3′, reverse 5′-GGGGGACCATATTTGGAC-3′; for IP-10 (288 bp), forward 5′-CCTTAAAACCAGAGGGGAGC-3′, reverse 5′-AGCAGGGTCAGAACATCCAC-3′; for CXCR3 (184 bp), forward 5′-CTGGTGGTGCTGGTGGACAT-3′, reverse 5′-AGAGCAGCATCCACATCCG-3′; for MDC (253 bp), forward 5′-CGCGTGGTGAAACACTTCTA-3′, reverse 5′-GAATGCAGAGAGTTGGCACA-3′; for TARC (140 bp), forward 5′-TAGAAAGCTGAAGACGTGGT-3′, reverse 5′-GGCTTTGCAGGTATTTAACT-3′; for CCR4 (214 bp), forward 5′-GTGCTCTGCCAATACTGTGG-3′, reverse 5′-CTTCCTCCTGACACTGGCTC-3′; and for CD3α (184 bp), forward 5′-GATGTCATTGCCACTCTGC-3′, reverse 5′-ACTTGTTCCGAGCCCAGTT-3′.

In order to provide a meaningful comparison between different individuals or samples, we calculated the relative amounts of the PCR products of these molecules to the amounts of the PCR products of CD3δ (for the standardization of T cell mRNA) or the PCR products of β-actin (for the standardization of total cellular mRNA) in each sample, as previously described (22,23,35,36). The CD3δ PCR product levels were used for T cell–specific molecules, such as IL-2, IL-5, IL-12, and IFNγ, while the β-actin PCR product levels were used for T cell–nonspecific molecules, such as IL-4, IL-6, IL-10, IL-17, and chemokines, which were produced by a variety of cell types.

Statistical analysis. The statistical significance of the differences between the groups was determined by the Mann-Whitney U test and Spearman's rank correlation. All statistical analyses in this study were performed using JMP software, version 8 (SAS Institute). P values less than 0.05 were considered significant.

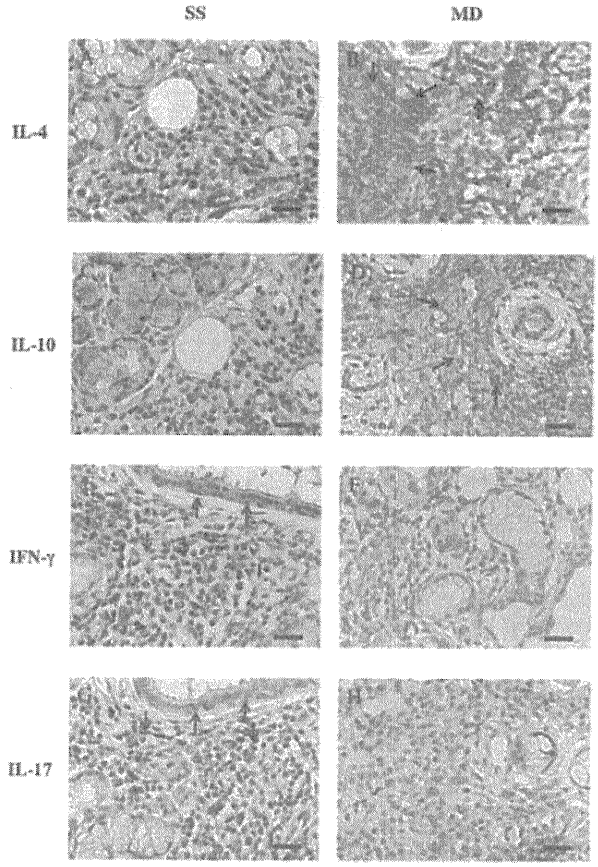

Figure 3. Expression of interleukin-10 (IL-10), IL-4, interferon-γ (IFNγ), and IL-17 in LSG specimens from patients with SS and patients with Mikulicz disease. IL-10 and IL-4 were prominently expressed around the germinal centers in LSG specimens from patients with Mikulicz disease. Sections from LSGs of a representative patient with SS and a representative patient with Mikulicz disease were immunostained with anti–IL-10 (**A** and **B**), anti–IL-4 (**C** and **D**), anti-IFNγ (**E** and **F**), and anti–IL-17 (**G** and **H**) polyclonal antibodies. Counterstaining with Mayer's hematoxylin was subsequently performed. **Arrows** indicate key features of infiltrating cells. Bars = 50 mm; original magnification × 400. See Figure 1 for other definitions.

RESULTS

Results of histologic analysis of lymphocyte subsets in the LSGs. Representative sections showing histologic findings and lymphocyte subsets in LSG specimens from patients with Mikulicz disease and patients with SS are shown in Figure 1. Specimens from patients with SS showed lymphocytic infiltration of various subsets with atrophy or severe destruction of the acini, while specimens from patients with Mikulicz disease showed selective infiltration of IgG4-positive plasma cells and FoxP3-positive Treg cells around the acinar and ductal cells with a lot of lymphoid follicles and mild destruction of the acini.

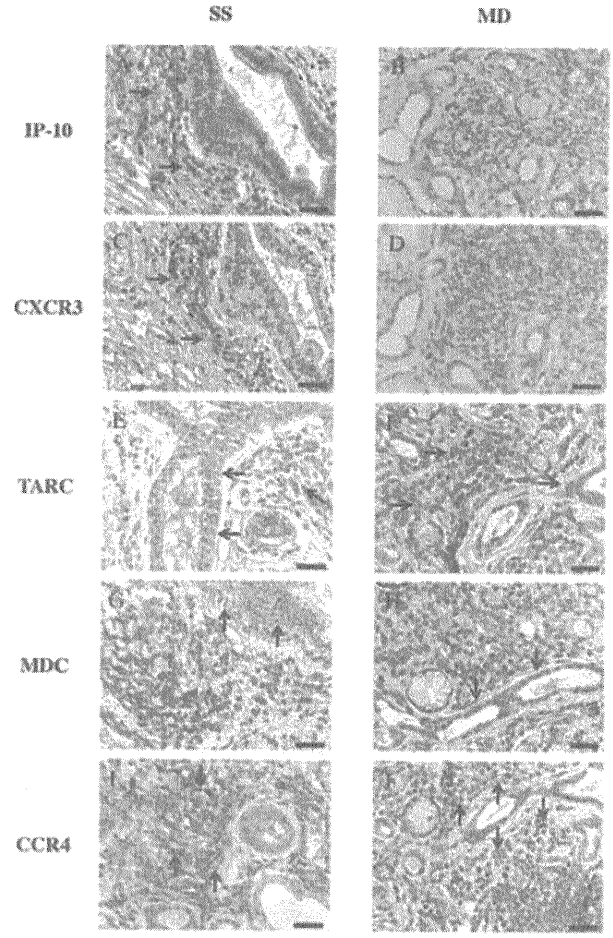

Figure 4. Expression of interferon-γ–inducible 10-kd protein (IP-10), CXCR3, thymus and activation–regulated chemokine (TARC), macrophage-derived chemokine (MDC), and CCR4 in LSG specimens from patients with SS and patients with Mikulicz disease. TARC and MDC were strongly expressed around the germinal centers in LSG specimens from patients with Mikulicz disease. Sections from LSGs of a representative patient with SS and a representative patient with Mikulicz disease were immunostained with anti–IP-10 (**A** and **B**), anti-CXCR3 (**C** and **D**), anti-TARC (**E** and **F**), anti-MDC (**G** and **H**), and anti-CCR4 (**I** and **J**) monoclonal antibodies. Counterstaining with Mayer's hematoxylin was subsequently performed. **Arrows** indicate key features of infiltrating cells. Bars = 50 mm; original magnification × 400. See Figure 1 for other definitions.

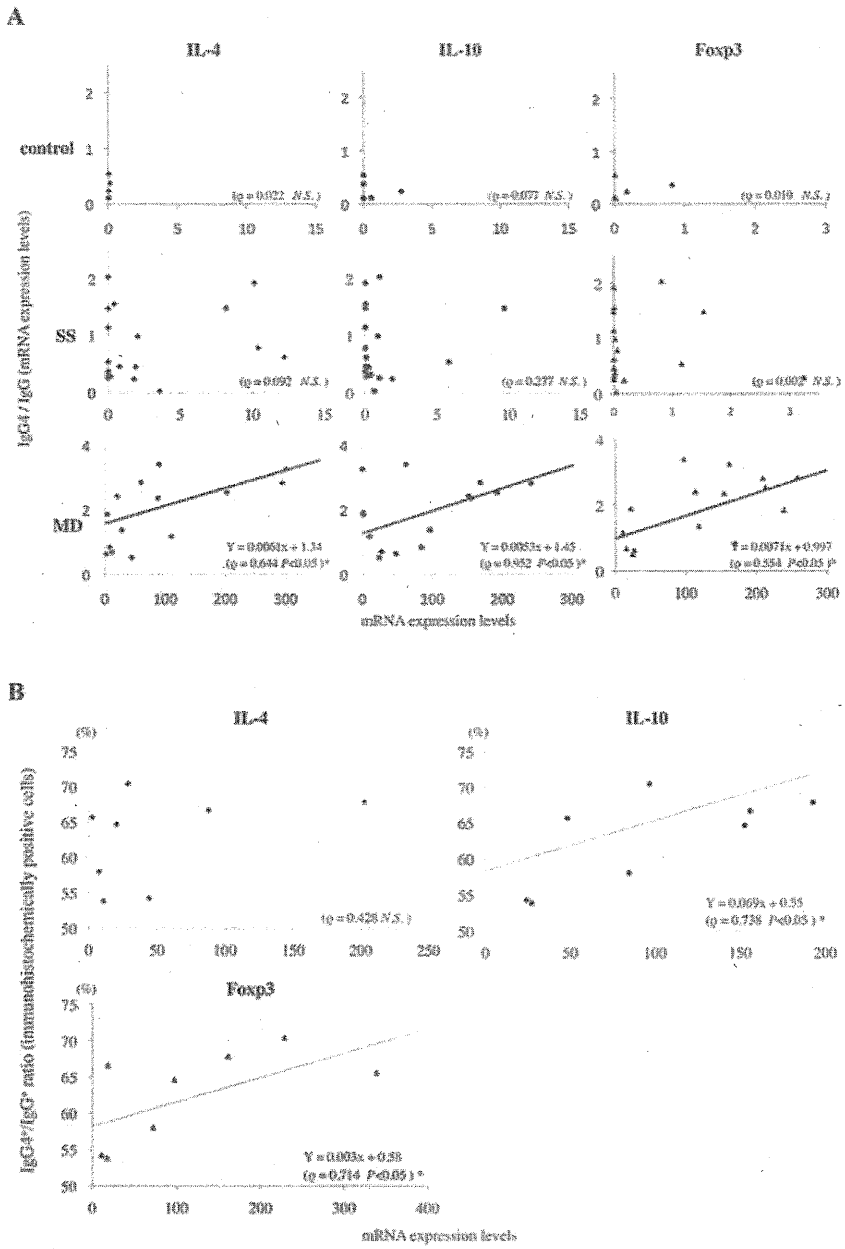

Figure 5. Correlation of the production of IgG4 with the production of interleukin-4 (IL-4), IL-10, and FoxP3 in LSGs from patients with Mikulicz disease. **A,** Correlations between the ratio of IgG4 mRNA to IgG mRNA and the expression of mRNA for IL-4, IL-10, and FoxP3 in LSGs from patients with SS, patients with Mikulicz disease, and healthy controls. Real-time polymerase chain reaction products for IL-4, IL-10, FoxP3, IgG, and IgG4 were quantitatively estimated as described in Patients and Methods. The ratio was calculated as IgG4-positive cells (%) = IgG4-positive cells/IgG-positive cells × 100. The counts were obtained in 1-mm^2 sections from 5 different areas. **B,** Correlations between the frequencies of IgG4-positive cells and the expression of mRNA for IL-4, IL-10, and FoxP3 in LSGs from patients with Mikulicz disease. * = $P < 0.05$ by Spearman's rank correlation. NS = not significant (see Figure 1 for other definitions).

Expression of mRNA for cytokines, chemokines, and chemokine receptors in the LSGs. In order to compare the expression of mRNA for cytokines, chemokines, and chemokine receptors in LSGs from patients with Mikulicz disease and LSGs from patients with SS, the relative expression compared to CD3δ was estimated and compared for cytokines and chemokine receptors primarily expressed by T cells, and the relative expression compared to β-actin was estimated for cytokines and chemokines produced by a variety of cell types.

The expression of mRNA for IFNγ, IL-12, IP-10, CXCR3, IL-4, TARC, MDC, CCR4, IL-10, IL-17, and IL-6 in LSGs from SS patients were higher than those in control LSGs (Figure 2). The expression of mRNA for IL-4, IL-5, TARC, MDC, CCR4, IL-10, TGFβ, and FoxP3 in LSGs from patients with Mikulicz disease were higher than those in control LSGs (Figure 2). In addition, the levels of expression of mRNA for IL-4, IL-5, IL-10, TGFβ, and FoxP3 in LSGs from patients with Mikulicz disease were higher than in LSGs from patients with SS (Figure 2).

Protein levels of cytokines, chemokines, and chemokine receptors in the LSGs. The specimens were immunohistochemically examined to evaluate the distributions of these proteins in LSGs from patients with SS and patients with Mikulicz disease. The Th1-type cytokine IFNγ and Th17-type cytokine IL-17 were strongly expressed and detected in and around the ductal epithelial cells in LSGs from SS patients only (Figures 3E and G). Although IL-10 and IL-4 were detected in LSGs from both patients with Mikulicz disease and patients with SS, they were prominently expressed around germinal centers in LSGs from Mikulicz disease patients but not in LSGs from SS patients (Figures 3B and D). In LSGs from patients with SS, IP-10 and CXCR3 were detected in a higher number of infiltrating lymphocytes than in LSGs from patients with Mikulicz disease (Figures 4A and C). In LSGs from patients with Mikulicz disease, TARC and MDC were strongly expressed, especially around germinal centers (Figures 4F and H). In LSGs from both patients with SS and patients with Mikulicz disease, CCR4 was detected in high numbers of infiltrating lymphocytes (Figures 4I and J).

Relationship between IgG4 production and cytokine expression in the LSGs. The relationships between IgG4 production and the expression of mRNA for IL-4, IL-10, and FoxP3 in the LSGs were examined. These molecules were all positively correlated with the ratio of IgG4 mRNA to IgG mRNA in LSGs from patients with Mikulicz disease, but no relationships were confirmed in those from SS patients (Figure 5A). Furthermore, IL-10 mRNA and FoxP3 mRNA in LSGs from patients with Mikulicz disease were correlated with the ratio of IgG4 to IgG in immunohistochemically positive cells (Figure 5B).

DISCUSSION

Mikulicz disease presents with bilateral and persistent swelling of the lacrimal and salivary glands, and it has been considered to be part of primary SS or a subtype of primary SS since the findings by Morgan and Castleman were published in 1953 (37). However, Yamamoto et al (38,39) reported differences in the clinical and histopathologic findings between Mikulicz disease and SS. Serologically, Mikulicz disease patients show hypergammaglobulinemia, hypocomplementemia, and high levels of serum IgG4, but are negative for anti-SSA and anti-SSB antibodies. Immunohistologic analysis of samples from patients with Mikulicz disease revealed the selective infiltration of IgG4-positive plasma cells, which was not observed near acinar and ductal cells. In contrast, similar specimens from SS patients showed no IgG4-positive plasma cells (38,39). In this study, samples from patients with Mikulicz disease showed selective infiltration of IgG4-positive plasma cells and FoxP3-positive cells around acinar and ductal cells with mild destruction of the acini, while samples from patients with SS showed no infiltration of IgG4-positive plasma cells and FoxP3-positive cells, and had atrophy or severe destruction of acini (Figure 1).

In order to examine the differences in infiltrating lymphocytes between LSGs from patients with SS and LSGs from patients with Mikulicz disease, we analyzed the levels of cytokines, chemokines, and chemokine receptors. The levels of Th1-, Th2-, and Th17-type molecules in LSGs from SS patients were significantly higher than those in LSGs from controls. The levels of Th2 and Treg-type molecules in LSGs from patients with Mikulicz disease were significantly higher than those in LSGs from controls. Furthermore, immunohistochemical staining indicated that IFNγ and IL-17 were strongly detected in and around ductal epithelial cells in LSGs from SS patients only, while IL-4 and IL-10 were detected in LSGs from both patients with SS and patients with Mikulicz disease. In particular, these cytokines were prominently expressed around germinal centers in specimens from patients with Mikulicz disease but not in specimens from patients with SS.

It is generally accepted that CD4+ Th cells play a crucial role in the pathogenesis of SS. Several studies of autoimmune diseases have demonstrated pathoge-

netic roles for Th1 cells and the possible protective role for Th2 cells (40,41). Our previous studies of SS suggested that the mutual stimulation of Th1 cells and their target organs via the production of various cytokines plays a key role in the induction and maintenance of SS and results in the eventual destruction of the target organ (22,42,43). Subsequently, additional Th2 cells then stimulate B cells to differentiate, proliferate, and produce immunoglobulins and, thus, play a role in the lymphoaggressiveness of SS. Regarding the possible roles of Th2 cells in the induction of B cell abnormalities, these cells might have an important association with the progression of SS. In contract, Zen et al (25) reported that autoimmune pancreatocholangitis, an IgG4-related disease, is characterized by immune reactions that are predominantly mediated by Th2 cells and Treg cells.

The results of the present study concerning the levels of cytokines, chemokines, and chemokine receptors in the LSGs are consistent with the model of SS and Mikulicz disease as distinct diseases. Immunohistochemical staining indicated that MDC and TARC were detectable in and around the ductal epithelial cells and germinal centers, while CCR4 was expressed on the infiltrating lymphocytes in the LSGs in both SS patients and patients with Mikulicz disease. The interactions of CCR4 with MDC and TARC are suggested to play a critical role in the accumulation of Th2 cells and, consequently, the progression of SS and Mikulicz disease. TARC and MDC are natural ligands for CCR4 on Th2 cells (44,45). In contrast, IP-10 was detected in and around the ductal epithelial cells, while CXCR3 was expressed on the infiltrating lymphocytes in the LSGs in SS patients only. IP-10 is a natural ligand for CXCR3 on Th1 cells (15).

It is well known that allergic immune responses are the development of allergen-specific Th2-type cytokines IL-4 and IL-13, which are responsible for IgG4 and IgE induced by B cells (46). In our previous studies, we demonstrated that Th2 immune reactions contributed to Mikulicz disease and IgG4-related tubulointerstitial nephritis (23,24,35). The expression profile of cytokines demonstrated in this study suggested that Mikulicz disease was characterized by an intense expression of Th2 and regulatory cytokines (Figure 2). In addition, recent studies have shown that class switching of IgG4 is caused by costimulation with IL-4 and IL-10, and that IL-10 decreased IL-4–induced IgE switching but elevated IL-4-induced IgG4 production (47).

Treg cells exert their effects through the modulation of both T and B cell responses, and two subsets of

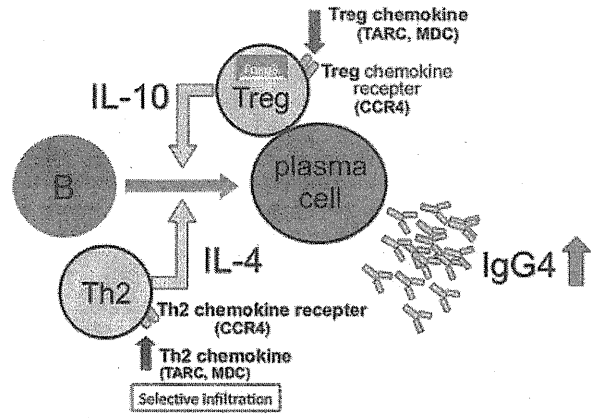

Figure 6. Schematic model of the mechanisms underlying IgG4 production. TARC = thymus and activation–regulated chemokine; MDC = macrophage-derived chemokine; IL-10 = interleukin-10.

Treg cells, CD4+CD25+FoxP3+ Treg cells (48) and IL-10–producing Tr1 cells (49), are crucial in regulating effector T cell function. CD4+CD25+FoxP3+ Treg cells are known to affect the pathogenesis of cases of autoimmune hepatitis and primary biliary cirrhosis (50). Miyoshi et al (51) showed a positive correlation between the number of mature Treg cells ($CD4+CD25^{high}$ Treg cells) and IgG4. These results indicated that increased numbers of $CD4+CD25^{high}$ Treg cells may influence IgG4 production in autoimmune pancreatocholangitis, whereas decreased numbers of naive Treg cells (CD4+CD25+CD45RA+) may be involved in the pathogenesis of the disease (51). Therefore, we examined the relationships between IgG4 and IL-4, IL-10, and FoxP3.

We found that IL-4, IL-10, and FoxP3 were positively correlated with the ratio of IgG4 mRNA to IgG mRNA in samples from patients with Mikulicz disease analyzed by real-time PCR and comparison with the IgG4 to IgG ratio of immunohistochemically positive cells. In particular, IL-10 and FoxP3 levels were strongly correlated with IgG4 production. These results suggested that Th2 and Treg cells might be involved in the pathogenesis of Mikulicz disease. The findings of the present study provided additional support for the model of Mikulicz disease as distinct from SS (Figure 6). However, accumulation of case reports and further examinations are required to elucidate the pathogenesis of the disease.

In this study, we clarified the pathogenesis of Mikulicz disease and found that it is a unique IgG4-related disease, characterized by Th2 and regulatory

immune reactions, which apparently differs from SS. A more thorough understanding of the complex mechanisms of the disease might lead to pharmacologic strategies to interrupt the interactions between chemokines and chemokine receptors or to disrupt the cytokine network as a further means of inhibiting the initiation and/or progression of Mikulicz disease.

AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Nakamura had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study conception and design. Tanaka, Moriyama, Nakashima, Miyake, Nakamura.

Acquisition of data. Tanaka, Moriyama, Hayashida, Maehara, Shinozaki, Kubo.

Analysis and interpretation of data. Tanaka, Moriyama, Nakashima.

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Clusterin Promotes Corneal Epithelial Cell Growth through Upregulation of Hepatocyte Growth Factor by Mesenchymal Cells In Vitro

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Purpose. Although the cornea expresses high levels of clusterin (CLU), the role of CLU in the cornea is poorly understood. This study was performed to investigate the possible role of CLU in corneal epithelial homeostasis.

Methods. CLU was overexpressed in 3T3 cells by transfection of a vector encoding full-length CLU (*Clu*-3T3). Colony-forming efficacy (CFE) was compared in mouse corneal cell line (TKE2) and human primary corneal/limbal epithelial cells that were cocultured with *Clu*-3T3 and mock-3T3. To determine whether feeder cells have a contact effect, cocultures without feeder-epithelium contact were also performed. Neutralizing antibody against CLU was used to assess the effects of secretory CLU in TKE2 cells cocultured with *Clu*-3T3 cells. The expression of growth factors associated with limbal stem/progenitor cell maintenance and growth were analyzed by RT-PCR and Western blot analysis.

Results. TKE2 cells cocultured with *Clu*-3T3 feeders showed higher CFE and were larger in colony size than TKE2 cells cocultured with mock-3T3 feeders. Increased CFE of TKE2 was observed without direct contact with *Clu*-3T3 cells, which was significantly blocked by treatment with CLU neutralizing antibody. *Clu*-3T3 cells expressed higher levels of HGF than mock-3T3 cells, which were significantly suppressed with anti-HGF neutralizing antibodies. Collectively, the promotion of colony-forming and cell proliferation by *Clu*-3T3 cells was partially mediated by the induction of HGF.

Conclusions. Clusterin indirectly enhances the CFE of corneal/limbal epithelial cells by inducing the production of HGF by feeder cells, suggesting a role in epithelial-mesenchymal interaction. (*Invest Ophthalmol Vis Sci.* 2011;52:2905–2910) DOI:10.1167/iovs.10-6348

Clusterin (CLU) is a glycoprotein first isolated from ram rete testis fluid; it is also known as apolipoprotein J, sulfated glycoprotein-2, glycoprotein III, and testosterone-repressed message-2. It is composed of two 35- to 40-kDa subunits (α and β) encoded by a single gene, and it forms a heterodimer stabilized by disulfide bonds.[1–4] It has been implicated in diverse physiological functions, including cell-cell interactions,[5] complement inhibition, lipid transportation, cell survival, apoptosis,[6] aging,[7] and cell protection from cytotoxic stress.[8–10] CLU is ubiquitously expressed in many tissues and body fluids; however, it was also found to be the most abundant gene transcript in the human corneal epithelium.[11,12]

Despite these initial reports, the function of CLU in the homeostasis and survival of human corneal/limbal epithelial cells is still not clearly understood. Recent studies suggested that CLU is involved in cell development and proliferation.[13,14] CLU expression is also upregulated by a multitude of factors, including cellular injury and stress and cell growth, differentiation, and aging.[6,7,15] Given that the corneal epithelium is a nonkeratinized, stratified squamous epithelium constantly undergoing proliferation and migration, we hypothesized that CLU plays a role in the maintenance of corneal epithelial homeostasis. When the corneal epithelium is injured, limbal epithelial stem cells govern the renewal of the corneal epithelium by generating transient-amplifying cells (TACs) that migrate centripetally from the limbus to the corneal basal layer.[16,17] TACs further proliferate and differentiate into terminally differentiated cells to maintain ocular surface homeostasis.[18,19] In this study, we investigated the effect of CLU on cultured murine corneal epithelial cells and primary human limbal epithelial cells using a coculture system, with NIH-3T3 feeder cells overexpressing CLU.

Materials and Methods

Murine Corneal/Limbal Epithelial Cell Line (TKE2) Culture

TKE2 is a murine limbal/corneal epithelium-derived progenitor cell line.[20] TKE2 cells were maintained in defined keratinocyte serum-free medium (KSFM; Gibco-Invitrogen Corp., Carlsbad, CA) supplemented with 10 ng/mL human recombinant epithelial growth factor, 1% penicillin/streptomycin, and growth supplement supplied by the manufacturer until use. Cell cultures were incubated at 37°C, under 95% humidity and 5% CO_2, and culture medium was changed every 3 to 4 days.

Primary Human Corneal/Limbal Epithelial Cell Culture

Human primary corneal/limbal epithelial cells (HLECs) were isolated from the limbus of eye bank corneas after the central corneal buttons were used for transplantation. Iris, endothelium, and conjunctiva were surgically removed from corneal limbus, and the limbus was treated with 2.5 U/mL neutral protease (Dispase II; Roche, Basel, Switzerland) in F12/Dulbecco's modified Eagle's medium (DMEM) at 4°C overnight.

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The epithelium was separated from the stroma with a cell scraper and was dispersed in 0.05% trypsin EDTA at 37°C for 30 minutes. HLECs were suspended in defined KSFM. Unless indicated otherwise, 5×10^4 HLECs were seeded in 25-cm^2 flasks. The cultures were incubated at 37°C, under 95% humidity and 5% CO_2, and the medium was changed every 3 days.

Establishment of a 3T3 and STO Cell Line Stably Expressing Clusterin

pCAGIPuro plasmid was a kind gift from Hitoshi Niwa (Laboratory for Pluripotent Cell Studies, Riken Center for Developmental Biology, Kobe, Japan). The plasmid, with full-length CLU cDNA containing a COOH-terminal HA, was transfected into NIH-3T3 cells and STO cells using reagent (LipofectAMINE 2000; Life Technologies, Inc., Carlsbad, CA) (*Clu*-3T3, mock-3T3, *Clu*-STO, mock-STO). The selection of clones was carried out for 2 weeks with 10 μg/mL puromycin (Life Technologies, Inc.) and were maintained in DMEM (Gibco-Invitrogen Corp., Carlsbad, CA) supplemented with 10% FCS and 1% penicillin/streptomycin. No predictable morphologic changes owing to CLU transfection were observed in either cell line.

Colony-Forming Efficiency

To evaluate the proliferative potential of cell colonies, *Clu* or mock-3T3 and STO cells were used in a colony-forming efficiency (CFE) assay, as previously described.[21,22] *Clu* or mock-transfected cells in DMEM containing 10% FCS were plated at a density of 3×10^5 cells in six-well culture plates (Iwaki, Naperville, IL). The next day, transfected cells were treated with mitomycin C (MMC; Nakarai Tesque, Kyoto, Japan) (4 μg/mL) for 2.5 hours at 37°C, and then single cells were seeded at 300 cells/well in a mixture of equal parts defined KSFM and supplemental hormonal epithelial medium[23] containing 5% FCS. For separate cultures, corneal/limbal epithelial cells were incubated with cell culture inserts (Transwell; Corning Inc., Corning, NY) to inhibit contact between epithelial and feeder cells. CFE was calculated by the percentage of colonies at day 14 generated by the number of epithelial cells plated in the well. Colony size (mm^2) and number of colonies ($n = 3$) were quantified using ImageJ software (developed by Wayne Rasband, National Institutes of Health, Bethesda, MD; available at http://rsb.info.nih.gov/ij/index.html). Growth capacity was evaluated on day 14 when cultured cells were stained with rhodamine B (Wako, Osaka, Japan) for 30 minutes.

Western Blot Analysis

Western blot analysis was performed using standard Western blotting methods. *Clu*-3T3, mock-3T3 cells, and TKE2 cells were dissociated with lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Nonidet P-40 [Calbiochem, Darmstadt, Germany]) and were homogenized. Culture supernatants of *Clu*-3T3 or mock-3T3 cells were concentrated five times with spin column (Microcon; Millipore, Bedford, MA). Protein concentration of the supernatant was measured using a BCA protein assay kit (Pierce, Rockford, IL). All samples were then diluted in 2× sample buffer (100 mM Tris-HCl [pH 6.8], 4% SDS [Invitrogen], 20% glycerol [Wako], and 12% 2-mercaptoethanol [Wako]) and were boiled. Ten micrograms of each sample were loaded on a 10% Tris-HCl gels (Ready Gels J; Bio-Rad Laboratories Inc., Hercules, CA) and were transferred onto polyvinylidene difluoride membranes (Millipore, Billerica, MA). Membranes were reacted with antibodies against CLU (M-18; Santa Cruz Biotechnology, Santa Cruz, CA), hepatocyte growth factor (HGF) (D-19; Santa Cruz Biotechnology), and β-actin (mabcam8226; Abcam, Cambridge, MA) for 60 minutes at room temperature. After three washes in TBST, donkey biotinylated anti-rabbit IgG (Zymed, San Francisco, CA) was added for 30 minutes at room temperature. The protein bands were visualized by enhanced chemiluminescence (GE Healthcare, Piscataway, NJ).

RT-PCR

Total cellular RNA was isolated from *Clu*-3T3 or mock-3T3 cells with purification mini kits (RNeasy; Qiagen, Hilden, Germany), according to the manufacturer's specifications. One microgram of total RNA was converted to cDNA (iScript cDNA Synthesis kit; BioRad) and was subsequently used for reverse transcription-polymerase chain reaction (RT-PCR) analysis. PCR was performed in a thermal cycler as follows: 94°C for 5 minutes, followed by 30 amplification cycles (94°C, 30 seconds; 60°C, 30 seconds; 72°C, 1 minute). Primer sets for mouse glyceraldehyde-3-phosphate dehydrogenase (*gapdh*) (sense, 5′-TGACGTGCCGCCTGGAGAAA-3′; antisense, 5′-AGTGTAGCCCAAGATGCCCTTCAG-3′), mouse *hgf* (sense, 5′-CACCTCCTCCTGCTTCATGT-3′; antisense, 5′-CACCTGTTGGCACACTCATC-3′), mouse *kgf* (sense, 5′-TTGACAAACGAGGCAAAGTG-3′; antisense, 5′-TTGCAATCCTCATTGCATTC-3′), mouse *tgf1* (sense, 5′-TGGATGCTCTTCAGTTCGTG-3′; antisense, 5′-GGGAGGCTCCTCCTACATTC-3′), mouse *fgf2* (sense, 5′-AGCGGCTCTACTGCAAGAAC-3′; antisense, 5′-CCGTTTTGGATCCGAGTTTA-3′), and mouse *egf* (sense, 5′-TCCCAGCGAGAAAGACTGAT-3′; antisense, 5′-TTGGCCATTTCAATCACAGA-3′) were synthesized at Operon Biotechnologies (Tokyo, Japan). Equal amounts of PCR-amplified products were visualized by ethidium bromide. The mRNA expression levels for each gene were normalized to the *gapdh* gene.

Quantitative Real-Time PCR

Total RNA was extracted (RNeasy; Qiagen) and digested (DNase I; Qiagen) according to the manufacturer's instructions. Single-strand cDNA was synthesized with reverse transcriptase (SuperScript II; Invitrogen). Semiquantitative real-time PCR was performed with a double-stranded DNA-binding dye (SYBR Green I; Applied Biosystems, Foster City, CA) using a sequence detection system (ABI Prism 7700; Applied Biosystems). Expression levels of mRNA were normalized by the median expression of a housekeeping gene (*gapdh*). Copy number was expressed as the number of transcripts per nanogram of total RNA. Primer sequences for mouse *gapdh* and *hgf* are described here.

ELISA for Mouse Clusterin

Cells were incubated for 48 hours in 96-well plates. Supernatants of *Clu*-3T3 and mock-3T3 cells were stored at −70°C until further ELISA assay. Mouse CLU concentrations were measured by an enzyme-linked immunosorbent assay kit (Life Diagnostic, West Chester, PA). The detection limit of the kit was approximately 3.9 ng/mL.

Immunohistochemistry for Mouse Clusterin

Frozen sections of normal mouse cornea were fixed for 10 minutes in cold acetone. Four-well chamber slides of TKE2 were fixed for 10 minutes in 100% methanol. These were permeabilized cell membranes with 1% nonionic surfactant (Triton X-100; Sigma, St. Louis, MO) for 10 minutes at room temperature. Frozen sections and slides were blocked by incubation with 10% normal goat serum (Chemicon International Inc., Temecula, CA) for 30 minutes at room temperature. Antibodies to CLU (1:40) (H-330; Santa Cruz Biotechnology) were applied and incubated for 60 minutes at room temperature, followed by incubation with Cy3-conjugated secondary antibody. Isotype antibodies were used as negative controls. After three washes with TBST (0.825 mM Tris, 136.9 mM NaCl, 1.34 mM KCl, 0.1% Tween 20), the sections were incubated with 1 μg/mL 4′,6-diamidino-2-phenylindole (DAPI; Dojindo Laboratories, Tokyo, Japan) at room temperature for 5 minutes. Finally, sections were washed three times in TBST and coverslipped using an aqueous mounting medium (PermaFluor; Beckman Coulter, Marseille, France).

Statistical Analysis

Differences between two paired groups were analyzed by the paired Student's *t*-test and considered significant at $P < 0.05$. Values are expressed as the mean ± SD.

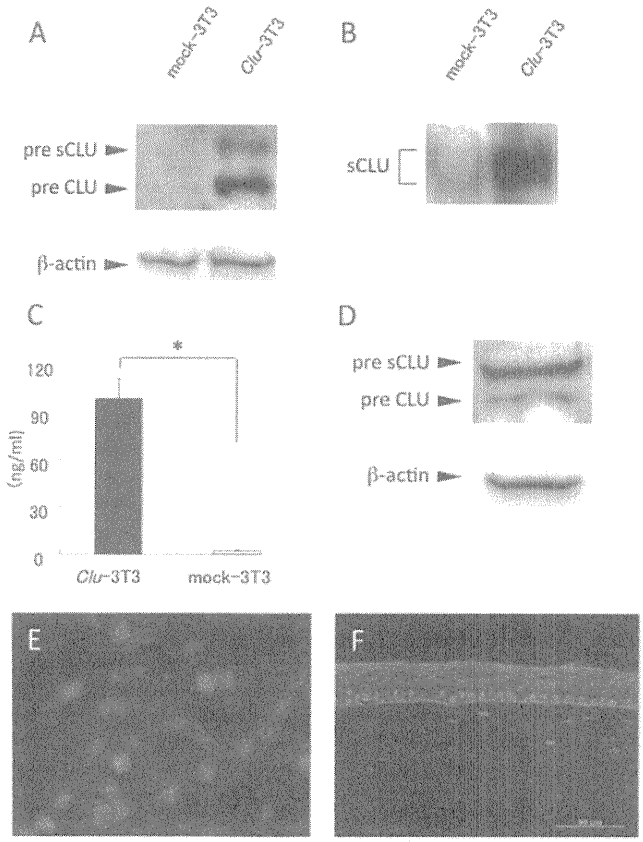

FIGURE 1. Clusterin expression in *Clu*-3T3 and TKE2 cells. (A) Western blot analysis of cell lysate shows the expression of sCLU precursor form (pre sCLU) and CLU precursor form (pre CLU) in *Clu*-3T3 cells but not in mock-3T3 cells (control). (B) Western blot analysis of culture supernatant shows that sCLU was detected more in *Clu*-3T3 than in mock-3T3. (C) ELISA of secreted CLU protein concentration in culture supernatant of *Clu*-3T3 and mock-3T3 cells ($n = 3$; mean ± SD; $^*P < 0.01$). (D) Western blot and (E) immunocytochemistry show the expression of CLU by TKE2 corneal epithelial cells. (F) CLU expression (*red*) in the mouse corneal epithelium shown by immunohistochemistry.

RESULTS

Clusterin Expression in *Clu*-3T3 Cells

Western blot analysis showed higher levels of intracellular CLU (mature form; pre-sCLU and precursor holoprotein form; pre-CLU) expression levels in the *Clu*-3T3 cells than mock-3T3 cells (Fig. 1A). In the culture supernatant, expression of the secretory form, sCLU, was higher as well in *Clu*-3T3 cells (Fig. 1B). This trend was also found in *Clu*-STO and mock-STO cells (data not shown). Furthermore, the concentration of sCLU in the condensed supernatant of *Clu*-3T3 cells was detected by ELISA (approximately 100 ng/mL) but was under detection levels in mock-3T3 cells (Fig. 1C). Western blot analysis (Fig. 1D) and immunocytochemistry (Fig. 1E) confirmed that TKE2 corneal cells expressed CLU. Immunohistochemistry of the mouse cornea also confirmed the expression of CLU in vivo (Fig. 1F).

CFE of TKE2 and Corneal/Limbal Cells Using *Clu*-3T3 Feeder Cells

To observe the effects of CLU on the clonal growth of corneal epithelial cells, colony-forming assays were performed in mouse corneal epithelial cells (TKE2 cells) cocultured with mitomycin C–treated *Clu*-3T3 and mock-3T3 feeder cells for 14 days. TKE2 cells cultured with CLU-3T3 cells showed a higher colony-forming ability than did those in mock-3T3 cells, with a 50% increase in CFE (Figs. 2A, 2B). Larger colonies were obtained with *Clu*-3T3 cells than with mock-3T3 cells. To test the effect of colony formation in response to culture supernatants produced by *Clu*-3T3 cells, we performed the colony-forming assay using culture inserts to separate epithelial cells from feeder cells (Fig. 2C). CFE of TKE2 cultured separately from *Clu*-3T3 cells was slightly lower than cocultured cells with feeder contact; however, significantly higher colony-forming capacity was observed compared with mock-3T3 feeder cells (Fig. 2D).

Human corneal/limbal epithelial cells (HLECs) formed colonies by coculturing with 3T3 cells in the same fashion, although the CFE was relatively low level compared with TKE2 (Fig. 3A). Once again, *Clu*-3T3 cells showed a higher CFE in HLECs compared with mock-3T3, showing that the phenomenon was observed irrespective of animal species (Fig. 3B). We examined the reproducibility of results using a different line of feeder cells. As shown in Figures 2C and 2D, STO cells produced colonies that were slightly smaller than 3T3 cells under the same conditions, but significant differences in CFE were observed with *Clu*-STO cells as with *Clu*-3T3 cells (Figs. 2C, 2D). Because of the stability and reproducibility of TKE2 combined with 3T3 cells, all subsequent experiments were performed with this combination of cells.

Effect of CLU Neutralizing Antibody on CFE of Corneal/Limbal Epithelial Cells

The data presented thus far suggest that stable overexpression of CLU was sufficient to cause the increased CFE of mouse and human corneal/limbal epithelial cells for both 3T3 and STO cells. We further investigated whether enhanced CFE was mediated through overproduction of sCLU. TKE2 cells were cocultured with *Clu*-3T3 or mock-3T3 cells in the presence of

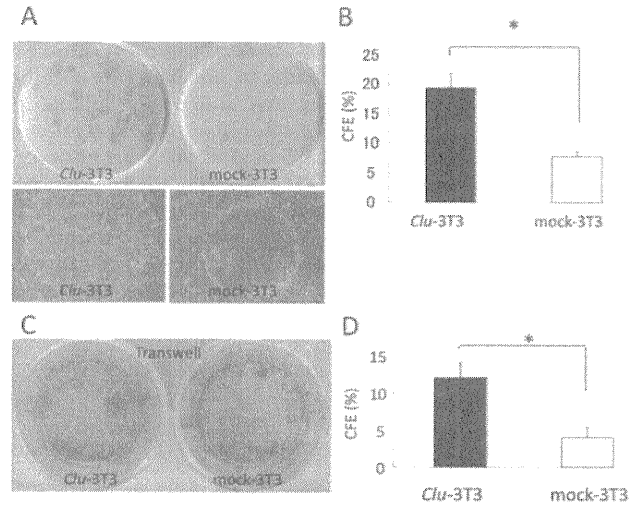

FIGURE 2. Effect of *Clu*-3T3 cells on TKE2 colony formation. (A) Murine corneal epithelial cells, clone TKE2, were cocultured with mitomycin C–treated *Clu*-3T3 and mock-3T3 cells at a density of 300 cells/well of six-well plates. After 14 days, colonies were stained with rhodamine B to show larger colony sizes with *Clu*-3T3 compared with mock-3T3 cells. (B) CFE on *Clu*-3T3 was significantly higher than mock-3T3 feeder cells ($n = 3$; mean ± SD; $^*P < 0.01$). (C) TKE2 cells cultured with mitomycin C–treated *Clu*-3T3 and mock-3T3 cells using transwell cultures to separate epithelial cells from feeder cells. (D) CFE was also significantly higher with *Clu*-3T3 cells than with mock-3T3 cells ($n = 3$; mean ± SD; $^*P < 0.05$).

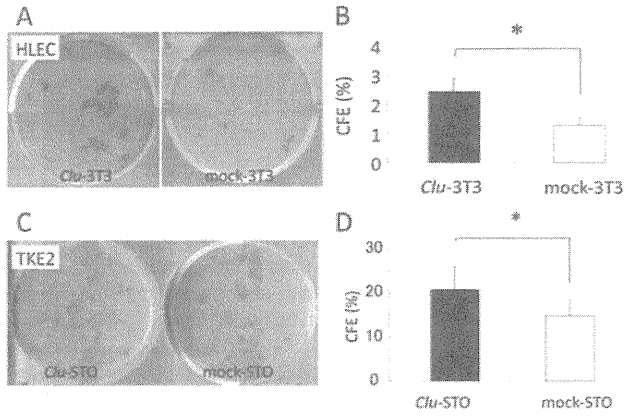

FIGURE 3. CFE of different combinations of feeder and epithelial cells. (A) Primary human corneal/limbal epithelial cells were cocultured with mitomycin C–treated *Clu*-3T3 and mock-3T3 cells. After 14 days, colonies were stained with rhodamine B. (B) CFE of HLE cells was significantly higher in *Clu*-3T3 feeder cells than in mock control (n = 3; mean ± SD; $^*P < 0.01$). (C, D) Increase in CFE of TKE2 cells was observed using *Clu*-STO compared with mock-STO cells (n = 3; mean ± SD; $^*P < 0.05$).

CLU neutralizing antibody or control antibody for 14 days to observe CFE. As shown in Figure 4, the CFE of TKE2 on *Clu*-3T3 cells was significantly reduced from 35.0% ± 2.6% to 22.2% ± 3.4% with CLU neutralizing antibody treatment. Colony size with CLU neutralizing antibody treatment was smaller than isotype control. CFE was not affected by isotype control antibody treatment.

Expression of Growth Factors by *Clu*-3T3 Cells

Given that neutralizing antibodies against CLU suppressed the CFE of corneal/limbal epithelial cells, we sought to investigate whether sCLU directly promotes CFE and cell proliferation in corneal/limbal epithelial cells. Interestingly, we found that several different concentrations (0.1–3 μg/mL) of mouse recombinant CLU had no effect on the CFE and cell proliferation of TKE2 cells (data not shown). Therefore, we hypothesized that sCLU acts on 3T3 feeder cells in an autocrine fashion, which, in turn, produces factors that indirectly regulate epithelial proliferation and CFE. We examined the expression of growth factors associated with limbal stem/progenitor cell maintenance and growth (EGF, FGF2, HGF, IGF1, KGF) and compared the expression of these growth factors in CLU-3T3 to those in mock-3T3 by RT-PCR. As a result, we found that higher levels of *hgf* were expressed in *Clu*-3T3 cells than in mock-3T3 cells, whereas *kgf* and *igf-1* expression was reduced (Fig. 5A). The increased expression of *hgf* was confirmed by quantitative real-time PCR. Higher levels of HGF protein were also detected by Western blot analysis of cell lysates (Fig. 5C).

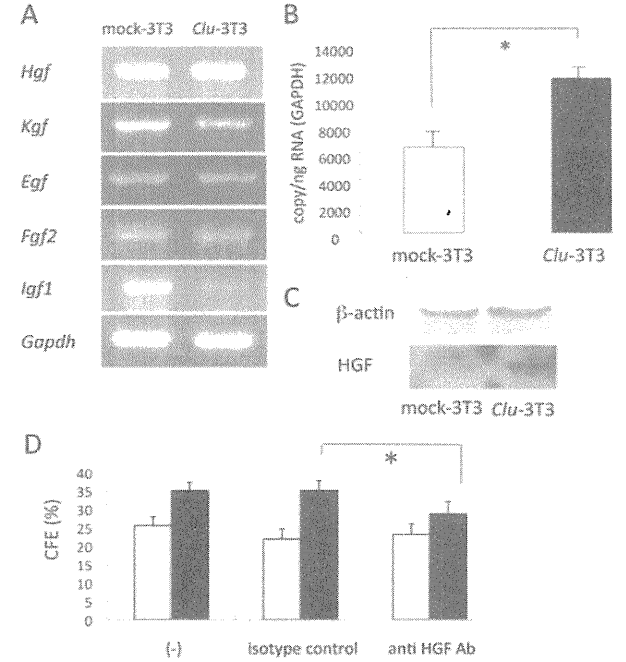

FIGURE 5. Growth factor expression by *Clu*-3T3 cells and CFE of HLE cells. (A) Total RNA isolated from *Clu*-3T3 and mock-3T3 cells were subjected to RT-PCR analysis of mRNAs for *hgf, kgf, egf, fgf2, tgf1*, and *gapdh* (internal standard). Most growth factors were downregulated in *Clu*-3T3 cells, excluding *hgf*. (B) Higher expression of *hgf* mRNA by *Clu*-3T3 cells was confirmed by quantitative real-time PCR. The data were corrected by *gapdh* expression (n = 3; mean ± SD $^*P < 0.05$). (C) Western blot of cell lysates also shows the secretion of HGF protein by *Clu*-3T3 cells. (D) Anti-HGF neutralizing antibodies suppressed the CFE of HLE cells cocultured with *Clu*-3T3 cells (n = 3; mean ± SD; $^*P < 0.05$).

These results suggested that the promotion of CFE and cell proliferation by CLU-transfected cells is partially mediated by the secretion of HGF by feeder cells.

Discussion

Homeostasis and wound healing of the corneal epithelium are orchestrated by multiple factors, including growth factors, that modulate cell migration and proliferation, cell death, and stem cell maintenance.[24,25] Among several growth factors identified, HGF is predominantly expressed by fibroblasts and stimulates corneal epithelial cell proliferation through the activation of their cognate receptors.[26,27] HGF has mitogenic and morphogenic activities in various cell types,[28,29] and its receptor, c-Met, is highly expressed on corneal epithelial cells.[30,31] In this study, we demonstrated that CLU-transfected 3T3 cells can

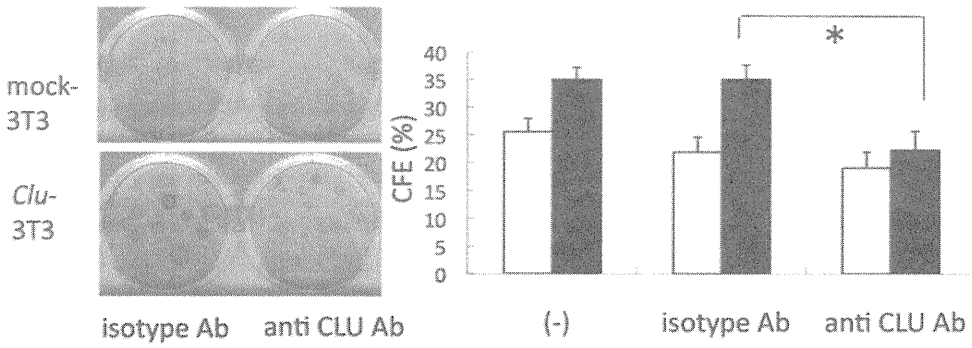

FIGURE 4. Effect of anti-mouse CLU neutralizing antibody on TKE2 colony formation. TKE2 cells were cocultured with mitomycin C–treated *Clu*-3T3 and mock-3T3 cells and treated with CLU antibody (0.5 μg/mL), which binds to sCLU. After 14 days, colonies were stained with rhodamine B (*left*), and CFE was evaluated (*right*). CFE on *Clu*-3T3 (*solid bars*) and mock-3T3 (*empty bars*) was inhibited by the antibody, with statistical significance in the *Clu*-3T3 group (n = 3; mean ± SD; $^*P < 0.05$).

upregulate HGF, which then enhances the proliferation of corneal/limbal epithelial cells. Because CLU is expressed predominantly by corneal epithelial cells in vivo,[11,12] our data suggest that CLU and HGF may be paracrine components of epithelial-mesenchymal interaction during epithelial wound healing.

We also considered the possibility of enhanced cellular motility using a scratch migration assay, but we found no difference in results using 3T3 and CLU-3T3 supernatants (data not shown). Other factors, such as inhibition of apoptosis by CLU,[32] or HGF[33] may also be involved in the increased CFE observed in our experiments. Several reports have demonstrated conflicting roles of CLU in cell proliferation. CLU inhibited the epidermal growth factor-stimulated proliferation of prostate cancer cells,[34] and transient overexpression of CLU also decreased proliferative activity in SV40-immortalized prostate epithelial cells.[35] On the other hand, the overexpression of secretory CLU resulted in significant enhancement of cell proliferation in both MIN6 insulinoma cells and primary pancreatic duct cells.[13,36] In this study, we found that several different concentrations (0.1–3.0 μg/mL) of mouse recombinant CLU had no direct effects on the CFE or cell proliferation of TKE2 cells (data not shown). CLU promoted cell proliferation indirectly through HGF produced by *Clu*-3T3. Therefore, CLU seems to have different functions depending on cell type, especially when paracrine interaction with other cells is involved. The indirect effect shown in this study may explain the different responses of cells against CLU.

sCLU has been reported to be a ligand of the LRP-2/megalin receptors.[37] However, we did not detect LRP-2 receptor transcripts in the TKE2 and 3T3 cells (data not shown), despite the suppressive effect of CFE using CLU neutralizing antibody in the coculture experiment (Fig. 4). CLU has been shown to bind with a wide range of soluble ligands,[4,38] acting as a chaperone for stressed proteins.[39] Therefore, the upregulation of HGF by sCLU may be triggered by some other mechanism that does not involve the LRP-2/megalin receptors. Further studies are required to elucidate this point.

Our results do not explain all the effects of CLU on epithelial cell proliferation because treatment with anti-HGF neutralizing antibody did not completely inhibit the CFE of TKE2 cells. This raises the possibility that CLU may modulate the expression of the other cell adhesion molecules and soluble growth factors in addition to HGF. CLU may also play a role in cellular protection against environmental stress, which may explain the high levels of CLU expressed in the cornea, one of the tissues most heavily exposed to oxygen and ultraviolet radiation. Additional studies are needed to reveal the full scope of CLU function in corneal homeostasis.

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