tained at the peak of arthritis (Figures 2A and B). Surprisingly, neither IFNγ-producing CD4+ (Th1) cells nor IFNγ-producing γ/δ T cells were detected in the swollen joints at any of the time points analyzed. In contrast, Th1 cells were detected in the DLNs of swollen joints (Figure 2A). In immunized joints, IL-17–producing γ/δ T cells and Th17 cells were already observed on day 1, reached the first peak on day 10 after immunization, and then reached their highest counts at the peak of arthritis. The absolute numbers of IL-17–producing γ/δ T cells were consistently higher than the numbers of Th17 cells at most time points analyzed. In contrast to what was observed in swollen joints, Th1 cells were detected in immunized joints after immunization (Figures 2A and B). In both swollen and immunized joints, the percentages of IL-17–producing γ/δ T cells among IL-17–producing cells were higher than those in DLNs of swollen and immunized joints (Figure 2A). In nonswollen joints, both IL-17–producing T cells and IFNγ-producing T cells were rarely observed. In addition, IFNγ-producing γ/δ T cells were a minor population at the sites of CIA (Figure 2A).

Efficient stimulation of IL-17 production from γ/δ T cells by IL-1β and IL-23. A recent study showed that a subset of γ/δ T cells already differentiate to acquire an IL-17–producing function in the thymus (26). In other studies, specific expression of CCR6 on Th17 has been suggested (27–30). Therefore, the expression of CCR6 on IL-17–producing γ/δ T cells in the thymus of naive DBA1/J mice was evaluated. IL-17–producing, but not IFNγ-producing, γ/δ T cells preferentially expressed CCR6 (Figure 3A). A small number of γ/δ T cells are present in the normal joints of mice (18). To elucidate whether de novo CCR6+ IL-17–producing γ/δ T cells are present in the normal joints of naive DBA1/J mice, cells were collected from the normal joints of naive mice, and intracellular cytokine staining was performed. By analyzing cells from 2 normal paws and ankles at a time, CCR6+ IL-17–producing γ/δ T cells could be detected (Figure 3B). In addition, in mice with CIA, 92% of CCR6+ γ/δ T cells produced IL-17 (Figure 3C).

Next, the IL-17 production requirements for γ/δ T cells were analyzed. Gamma/delta T cells from naive DBA1/J mice were analyzed by stimulation with cytokines in the presence or absence of anti-γ/δ TCR–activating mAb (Figure 3D). IL-17–producing γ/δ T cells were detected with anti-γ/δ TCR mAb, IL-23, and IL-1β alone. In addition, additive stimulatory effects were observed when anti-γ/δ TCR mAb was combined with IL-23, IL-1β, or anti-CD28. Surprisingly, IL-23 plus IL-1β induced IL-17 production quite efficiently. These

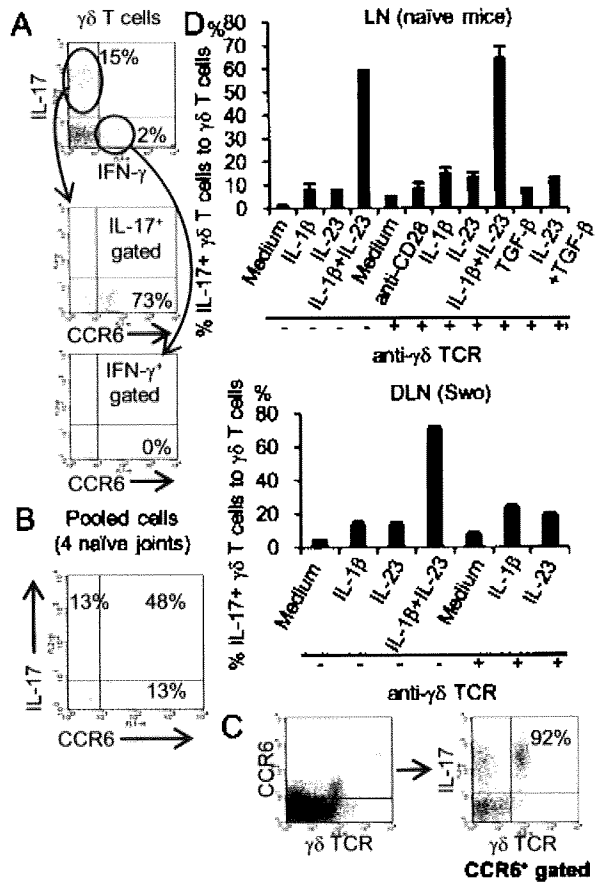

Figure 3. Efficient stimulation of IL-17 production from γ/δ T cells by IL-1β and IL-23. **A,** Thymocytes from naive mice were stimulated with phorbol myristate acetate and ionomycin for 4 hours. TCR+ cells were gated, and CCR6+ cells among IL-17–producing or interferon-γ (IFNγ)–producing γ/δ T cells were detected. **B,** Cells were collected from the paws and ankles of naive mice and stained for γ/δ TCR and CCR6. Gamma/delta TCR+ cells were gated, and CCR6+ IL-17–producing cells were detected by intracellular cytokine staining. In **A** and **B,** the percentages of cells in each quadrant are shown. **C,** Cells were collected from the DLNs of swollen joints, and IL-17–producing cells were detected by intracellular cytokine staining. CCR6+ cells were gated, and IL-17–producing cells were analyzed. The percentage of IL-17–producing cells among CCR+ γ/δ T cells is shown. **D,** Gamma/delta T cells were sorted from the peripheral lymph nodes of naive DBA1/J mice (upper panel) or from the DLNs of swollen joints of mice with CIA at the peak of arthritis (lower panel) and stimulated with cytokines, activating anti-γ/δ TCR antibodies, and anti-CD28 antibodies for 24 hours. The percentages of IL-17–producing cells among γ/δ T cells were determined by intracellular cytokine staining. Bars show the mean and SEM results from 3 different mice. TGFβ = transforming growth factor β (see Figure 1 for other definitions).

observations indicated that TCR signaling was not necessary to stimulate IL-17 production by γ/δ T cells. Furthermore, a combination of IL-23 and IL-1β was a much more potent stimulator than was TCR signaling.

Similar results were obtained with γ/δ T cells sorted from DLNs of swollen joints at the peak of CIA (Figure 3D, lower panel).

Type II collagen–independent induction and maintenance of IL-17–producing γ/δ T cells. Because IL-23 plays important roles in the maintenance of Th17 cells (31–36), we next addressed the maintaining effect of IL-23 or type II collagen on IL-17–producing γ/δ T cells. To this end, cells from the DLNs of swollen joints were cultured with IL-23, type II collagen, or medium alone (Figure 4A). Both IL-17–producing γ/δ T cells and Th17 cells were maintained in the presence of IL-23. In contrast, IL-17–producing γ/δ T cells were not type II collagen dependently maintained, whereas Th17 cells showed type II collagen dependency. To further investigate the factors that enhanced the accumulation of IL-17–producing γ/δ T cells in inflamed joints, the numbers of IL-17–producing γ/δ T cells in the differently immunized joints of mice were counted on day 10. Mice were immunized with PBS, IFA plus solution (0.05 m*M* acetic acid), IFA plus type II collagen, or CFA plus type II collagen (Figure 4B). The numbers of IL-17–producing γ/δ T cells were not significantly different between mice immunized with IFA plus solution, IFA plus type II collagen, or CFA plus type II collagen. In contrast, the numbers of IL-17–producing γ/δ T cells were significantly smaller in mice immunized with PBS compared with the 3 other treatments. The numbers of Th17 cells were significantly higher in mice immunized with IFA plus type II collagen than those in mice treated with IFA plus solution. These data indicate that IL-17–producing γ/δ T cells do not specifically respond to type II collagen and may only respond to adjuvant (IFA plus solution) or adjuvant-induced IL-23.

Next, the γ/δ TCR repertoire was analyzed (Figure 4C). The V_γ repertoire of IL-17–producing γ/δ T cells was composed of $V_{\gamma1}$, $V_{\gamma2}$, $V_{\gamma4}$, and $V_{\gamma6}$ rather than a single V_γ chain in CIA. In addition, the V_δ repertoire of IL-17–producing γ/δ T cells was composed of $V_{\delta1}$ and $V_{\delta5}$.

Exacerbation of arthritis by IL-17–producing γ/δ T cells. Next, the pathogenic roles of IL-17–producing γ/δ T cells in CIA were analyzed. When transferred to the joints of naive mice, CCR6+ γ/δ T cells did not induce arthritis. However, when transferred to the joints of mice immunized with type II collagen plus CFA, CCR6+ γ/δ T cells significantly worsened the arthritis score of joints with arthritis compared with the scores of joints treated with PBS (Figure 4D). The arthritis-exacerbating effect of CCR6+ γ/δ T cells from swollen joints was equivalent to that of CCR6+ γ/δ T cells from the DLNs of swollen joints (data not shown).

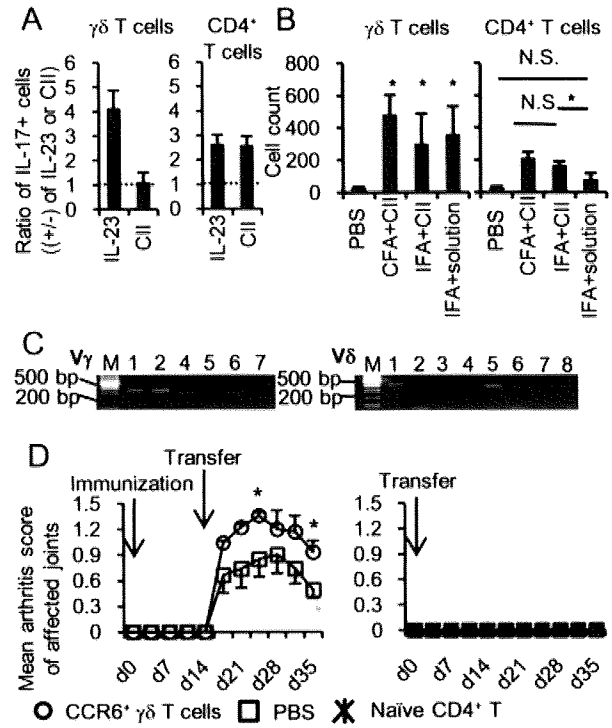

Figure 4. Exacerbation of arthritis by IL-17–producing γ/δ T cells. **A,** Cells were prepared from the DLNs of swollen joints and cultured for 7 days in the presence of IL-23, type II collagen (CII), or medium alone. IL-17–producing cells were detected by fluorescence-activated cell sorting (FACS) analysis. The ratio of the numbers of IL-17–producing cells in the presence of IL-23 or type II collagen to those in medium alone was calculated. Bars show the mean and SEM results from at least 3 different experiments. **B,** Various combinations of substances were administered into the footpads of DBA1/J mice. Ten days later, the absolute numbers of IL-17–producing cells were counted using FACS analysis. Bars show the mean and SEM results from at least 3 different mice. **C,** The use of γ/δ TCR by CCR6+ γ/δ T cells was analyzed by reverse transcription–polymerase chain reaction. **D,** CCR6+ γ/δ T cells from the DLNs of swollen joints were enriched. CCR6+ γ/δ T cells or phosphate buffered saline (PBS) alone was injected into nonimmunized wrists or ankles of mice that had been immunized with type II collagen plus Freund's complete adjuvant (CFA) 2 weeks previously. For naive mice, CCR6+ γ/δ T cells or PBS alone was injected. Values are the mean ± SEM arthritis scores in affected joints. * = $P < 0.05$ versus PBS. NS = not significant; IFA = Freund's incomplete adjuvant; M = marker (see Figure 1 for other definitions).

Absence of IL-17–producing γ/δ T cells in swollen joints of SKG mice or affected joints of patients with RA. To elucidate the pathologic differences from other arthritis models, the same analysis was performed using SKG mice (21). SKG mice carry a point mutation of the

gene encoding ZAP-70, and homozygous mice show IL-17–dependent arthritis resembling RA. Although the present study could detect only a few IL-17–producing γ/δ T cells in the DLNs of swollen joints, surprisingly, almost all of the IL-17–producing cells were Th17 cells, and the number of IL-17–producing γ/δ T cells was negligible in the swollen joints of SKG mice (Figure 5A).

SKG is a BALB/c background strain, and autoimmune arthritis in SKG mice is induced using zymosan as an adjuvant (17,21). To exclude the possibility that IL-17–producing γ/δ T cells are absent in the joints of SKG mice with arthritis because of the differences in strain and adjuvant compared with CIA, the absolute numbers of cell subsets from the joints of SKG or BALB/c mice immunized with CFA plus type II collagen were counted. Even with this protocol, IL-17–producing γ/δ T cells were not detected in SKG mice, whereas IL-17–producing γ/δ T cells were more abundant than Th17 cells in BALB/c mice (Figure 5B).

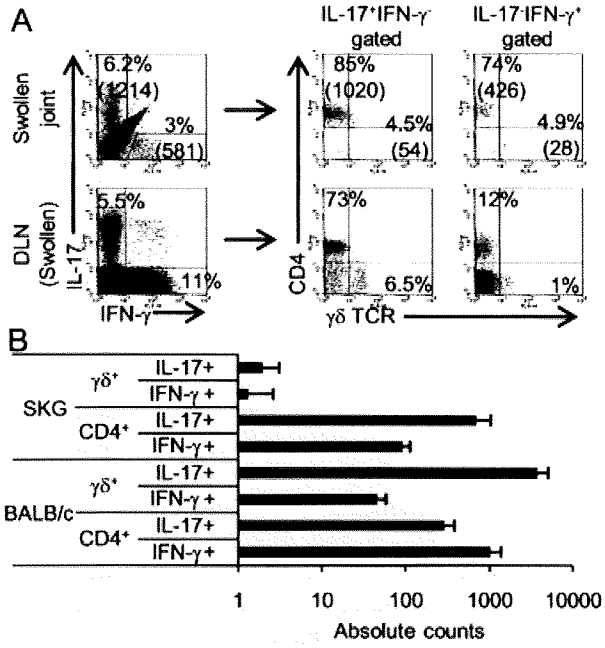

Figure 5. Absence of IL-17–producing γ/δ T cells in the swollen joints of SKG mice. **A,** Cells were collected from the ankles with maximum arthritis (and their DLNs) of SKG mice that had been treated with zymosan 7 weeks previously. Lymphocytes were gated based on their forward and side scatter. IL-17–producing cells and interferon-γ (IFNγ)–producing cells were detected by intracellular cytokine staining (left column). IL-17–producing IFNγ-negative cells (middle column) or IFNγ-producing IL-17–negative cells (right column) were gated and their expression of γ/δ TCR and CD4 was plotted. In the panels showing analysis of joints, the absolute numbers and percentages of CD4+ cells and γ/δ TCR+ cells are indicated. In the panels showing analysis of DLNs, the percentage of cells in each quadrant is noted. One experiment representative of 3 that were performed is shown. **B,** SKG or BALB/c mice were immunized with Freund's complete adjuvant plus type II collagen, and cells from the immunized joints were collected 10 days later. The absolute numbers of cells were counted using fluorescence-activated cell sorting analysis. Bars show the mean and SEM results for 3 different mice. See Figure 1 for other definitions.

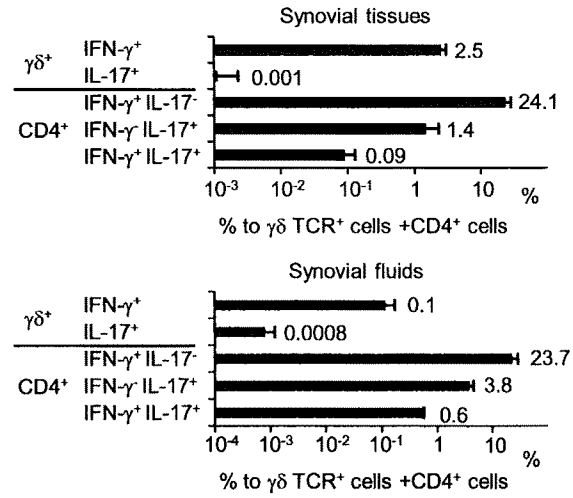

Figure 6. Absence of interleukin-17 (IL-17)–producing γ/δ T cells in the affected joints of patients with rheumatoid arthritis (RA). Cells in RA synovial tissue (n = 4) or synovial fluid (n = 7) were stained with antibodies against CD4 and γ/δ T cell receptor (TCR). IL-17–producing and interferon-γ (IFNγ)–producing cells were analyzed. The percentages of cells among total γ/δ T cells plus CD4+ T cells were determined. Bars show the mean and SEM.

Finally, cells in RA synovial tissue or fluid were analyzed to determine the presence of IL-17–producing γ/δ T cells and Th17 cells at the effector sites of arthritis. In contrast to what was observed in CIA, IL-17–producing γ/δ T cells could not be detected in the synovial tissue of affected joints, whereas IFNγ-producing γ/δ T cells were present in synovial tissue (Figure 6). Among the CD4+ T cells in synovial tissue, IL-17–producing cells were present. However, the proportions of Th1 cells among CD4+ T cells were much larger than those of Th17 cells in affected joints. Similar results were obtained in cells from synovial fluid.

DISCUSSION

The present study first focused on IL-17–producing T cells in the swollen joints of mice with CIA. It was observed that γ/δ T cells were the predominant source of IL-17 and were more abundant than Th17 cells. DX5+ NK cells did not secrete IL-17 in swollen

joints. A direct comparison of the absolute numbers of IL-17–producing γ/δ T cells with the absolute numbers of Th17 cells simultaneously in each joint of mice with CIA was performed for the first time. Although it is known that γ/δ T cells are not necessary for the induction of CIA, because γ/δ TCR–deficient mice can mount CIA (37), the present findings in the kinetics study and adoptive transfer experiments, together with previous reports (16,18,38), suggest that not only Th17 cells but also IL-17–producing γ/δ T cells contribute to the exacerbation of CIA. In contrast, α/β T cells, especially Th17 cells, are essential for the induction of CIA, because α/β TCR–deficient mice cannot mount CIA (37). In addition, IL-17–producing invariant NK T cells in CIA have been reported recently (37), but these cells were not analyzed in the current study.

The origin and functions of IL-17–producing γ/δ T cells in physiologic and pathologic conditions have been elucidated recently. It was reported that a subset of γ/δ T cells acquired an IL-17–producing function in the thymus (26) and produced cytokines immediately in response to initial stimulation. In various murine infectious disease models, these γ/δ T cells predominantly produce IL-17 and eradicate pathogens (40–43). However, the precise requirements of IL-17 production by γ/δ T cells especially in CIA are unknown, although IL-23 was known as a sufficient stimulant of IL-17 production by γ/δ T cells in naive mice (42). Here, it was demonstrated that the combination of IL-23 and IL-1β synergistically stimulated IL-17 production, but stimulation via γ/δ TCR had a limited effect. Given the enhanced expression of IL-1β and IL-23 in the inflamed joints of mice with CIA (44,45), these findings suggest that IL-17 production by γ/δ T cells in CIA might mainly be an inflammatory cytokine–driven process rather than a TCR signal–driven process.

The present study showed that IL-17–producing γ/δ T cells were CCR6 positive, and CCR6 was already expressed on IL-17–producing γ/δ T cells in the thymus of naive mice. CCL20, the only chemokine known to interact with CCR6, is physiologically expressed at epithelial surfaces (46) and fibroblast-like synoviocytes (29) and is up-regulated in inflammatory conditions (30,46). These findings suggest that CCR6 might have some roles in determining the physiologic distribution of IL-17–producing γ/δ T cells. In fact, it was found that a small number of CCR6+ IL-17–producing γ/δ T cells were present in the joints of naive mice.

Next, we focused on the differences between IL-17–producing γ/δ T cells and Th17 cells. IL-17–producing γ/δ T cells were maintained by IL-23 but not by a specific antigen (type II collagen, in this case). In contrast, Th17 cells responded to type II collagen and IL-23. Furthermore, IL-17–producing γ/δ T cells were induced equivalently in response to stimulation by IFA plus solution in the absence of type II collagen. Together with results from the previous study demonstrating that IL-17–producing γ/δ T cells are induced equally by CFA plus type II collagen and CFA (16), the present data suggest that IL-17–producing γ/δ T cells do not recognize the specific antigen (type II collagen) but rather proliferate in response to IL-23, which may be produced locally by synovial cells (44). The ligands of γ/δ T cells are largely unknown, and further analysis of possible antigens of IL-17–producing γ/δ T cells in CIA could be difficult (47). However, the present study confirmed the diverse usage of γ/δ TCR in IL-17–producing γ/δ T cells in CIA (Figure 4C), which supported the present conclusion that IL-17–producing γ/δ T cells are antigen independently induced by inflammatory cytokines.

In summary, it is speculated that the sequence of pathology of CIA is as follows. First, type II collagen–specific Th17 cells are induced by type II collagen plus CFA, which then infiltrate into the joints and cause primary inflammation. Although antigen-independent IL-17–producing γ/δ T cells could be induced simultaneously by CFA, they are not essential for the induction of arthritis. Next, primary inflammation induces local production of IL-23 from synoviocytes and increases the expression of IL-1β in joint cartilage and pannus (45). Locally produced IL-23 induces the proliferation of resident IL-17–producing γ/δ T cells. These γ/δ T cells, stimulated by IL-1β and IL-23, produce enhanced amounts of IL-17 and exacerbate the arthritis of CIA. Another, but not mutually exclusive, possibility is that primary inflammation enhances CCL20 expression in vascular endothelial cells and fibroblast-like synoviocytes (30) in inflamed joints and recruits CCR6+ IL-17–producing cells. In the ankylosing phase, the burned-out tissue does not produce inflammatory cytokines, and the activities and the number of IL-17–producing γ/δ T cells decrease to the basal level.

Finally, the cytokine profiles of T cells in the inflamed joints of SKG mice and patients with RA were compared with those in mice with CIA. In contrast to what was observed in mice with CIA, IL-17–producing γ/δ T cells were not detected in the swollen joints of SKG mice. A lack of IL-17–producing γ/δ T cells in SKG mice was not caused by the differences in strain or adjuvant. It was also observed that IL-17–producing γ/δ T cells are hardly induced in immunized joints, their DLNs, non-DLNs, and spleens of SKG mice (data not

shown) 10 days after immunization with CFA plus type II collagen. Given that TCR signals in SKG mice are attenuated because of a point mutation in ZAP-70 (21), and differentiation of γ/δ T cells needs a strong signal via the TCR (48,49), there may be some defects in γ/δ T cell differentiation in SKG mice. This speculation was supported by data showing impaired development of specific subsets of γ/δ T cells in ZAP-70–knockout mice (50). Furthermore, IL-17 production from γ/δ T cells in the synovial tissue of patients with RA has not yet been detected. In contrast to IL-17–producing γ/δ T cells, IFNγ-producing γ/δ T cells were present. In addition, among CD4+ T cells, Th1 cells were predominant; this finding was consistent with a previous report (51).

These results suggest that IFNγ-producing cells, but not IL-17–producing cells including γ/δ T cells, play predominant pathogenic roles in RA. These distinct pathogenic cell populations may result from differences between CIA and RA such as species and age-related susceptibility. Alternatively, IL-17–producing γ/δ T cells may play an important role in RA as well but are suppressed by the effects of medical treatment. It should be noted that in the present study, we could access joint materials only from patients with progressed stages of RA. Therefore, further studies with patients with recent-onset RA who have not received medical treatment are necessary to determine whether IL-17–producing γ/δ T cells are present.

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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. Usui 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. Y. Ito, Usui.

Acquisition of data. Y. Ito, Usui, Kobayashi, Iguchi-Hashimoto, H. Ito, Yoshitomi, Nakamura, Shimizu, Kawabata, Yukawa, Hashimoto, N. Sakaguchi, S. Sakaguchi, Yoshifuji, Nojima, Ohmura, Fujii, Mimori.

Analysis and interpretation of data. Y. Ito, Usui.

REFERENCES

1. Courtenay JS, Dallman MJ, Dayan AD, Martin A, Mosedale B. Immunisation against heterologous type II collagen induces arthritis in mice. Nature 1980;283:666–8.
2. Luross JA, Williams NA. The genetic and immunopathological processes underlying collagen-induced arthritis. Immunology 2001;103:407–16.
3. Wooley PH, Whalen JD, Chapdelaine JM. Collagen-induced arthritis in mice. VI. Synovial cells from collagen arthritic mice activate autologous lymphocytes in vitro. Cell Immunol 1989;124: 227–38.
4. Gregersen PK, Silver J, Winchester RJ. The shared epitope hypothesis: an approach to understanding the molecular genetics of susceptibility to rheumatoid arthritis. Arthritis Rheum 1987;30: 1205–13.
5. Tarkowski A, Klareskog L, Carlsten H, Herberts P, Koopman WJ. Secretion of antibodies to types I and II collagen by synovial tissue cells in patients with rheumatoid arthritis. Arthritis Rheum 1989; 32:1087–92.
6. Clague RB, Moore LJ. IgG and IgM antibody to native type II collagen in rheumatoid arthritis serum and synovial fluid: evidence for the presence of collagen–anticollagen immune complexes in synovial fluid. Arthritis Rheum 1984;27:1370–7.
7. Cook AD, Rowley MJ, Mackay IR, Gough A, Emery P. Antibodies to type II collagen in early rheumatoid arthritis: correlation with disease progression. Arthritis Rheum 1996;39:1720–7.
8. Weyand CM, Klimiuk PA, Goronzy JJ. Heterogeneity of rheumatoid arthritis: from phenotypes to genotypes. Springer Semin Immunopathol 1998;20:5–22.
9. Weyand CM, Goronzy JJ. T-cell responses in rheumatoid arthritis: systemic abnormalities-local disease. Curr Opin Rheumatol 1999; 11:210–7.
10. Ferretti S, Bonneau O, Dubois GR, Jones CE, Trifilieff A. IL-17, produced by lymphocytes and neutrophils, is necessary for lipopolysaccharide-induced airway neutrophilia: IL-15 as a possible trigger. J Immunol 2003;170:2106–12.
11. Weaver CT, Hatton RD, Mangan PR, Harrington LE. IL-17 family cytokines and the expanding diversity of effector T cell lineages. Annu Rev Immunol 2007;25:821–52.
12. Lubberts E, Joosten LA, Oppers B, van den Bersselaar L, Coenen-de Roo CJ, Kolls JK, et al. IL-1-independent role of IL-17 in synovial inflammation and joint destruction during collagen-induced arthritis. J Immunol 2001;167:1004–13.
13. Lubberts E, Koenders MI, Oppers-Walgreen B, van den Bersselaar L, Coenen-de Roo CJ, Joosten LA, et al. Treatment with a neutralizing anti-murine interleukin-17 antibody after the onset of collagen-induced arthritis reduces joint inflammation, cartilage destruction, and bone erosion. Arthritis Rheum 2004;50:650–9.
14. Nakae S, Nambu A, Sudo K, Iwakura Y. Suppression of immune induction of collagen-induced arthritis in IL-17-deficient mice. J Immunol 2003;171:6173–7.
15. Murphy CA, Langrish CL, Chen Y, Blumenschein W, McClanahan T, Kastelein RA, et al. Divergent pro- and antiinflammatory roles for IL-23 and IL-12 in joint autoimmune inflammation. J Exp Med 2003;198:1951–7.
16. Roark CL, French JD, Taylor MA, Bendele AM, Born WK, O'Brien RL. Exacerbation of collagen-induced arthritis by oligoclonal, IL-17-producing gamma delta T cells. J Immunol 2007;179: 5576–83.
17. Yoshitomi H, Sakaguchi N, Kobayashi K, Brown GD, Tagami T, Sakihama T, et al. A role for fungal β-glucans and their receptor Dectin-1 in the induction of autoimmune arthritis in genetically susceptible mice. J Exp Med 2005;201:949–60.
18. Arai K, Yamamura S, Hanyu T, Takahashi HE, Umezu H, Watanabe H, et al. Extrathymic differentiation of resident T cells in the joints of mice with collagen-induced arthritis. J Immunol 1996;157:5170–7.
19. Kuhnlein P, Mitnacht R, Torres-Nagel NE, Herrmann T, Elbe A, Hunig T. The canonical T cell receptor of dendritic epidermal γδ T cells is highly conserved between rats and mice. Eur J Immunol 1996;26:3092–7.
20. Nakamura R, Shibata K, Yamada H, Shimoda K, Nakayama K, Yoshikai Y. Tyk2-signaling plays an important role in host defense against Escherichia coli through IL-23-induced IL-17 production by γδ T cells. J Immunol 2008;181:2071–5.

21. Sakaguchi N, Takahashi T, Hata H, Nomura T, Tagami T, Yamazaki S, et al. Altered thymic T-cell selection due to a mutation of the ZAP-70 gene causes autoimmune arthritis in mice. Nature 2003;426:454–60.
22. Arnett FC, Edworthy SM, Bloch DA, McShane DJ, Fries JF, Cooper NS, et al. The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis. Arthritis Rheum 1988;31:315–24.
23. Prevoo ML, van 't Hof MA, Kuper HH, van Leeuwen MA, van de Putte LB, van Riel PL. Modified disease activity scores that include twenty-eight–joint counts: development and validation in a prospective longitudinal study of patients with rheumatoid arthritis. Arthritis Rheum 1995;38:44–8.
24. Steinbrocker O, Traeger CH, Batterman RC. Therapeutic criteria in rheumatoid arthritis. J Am Med Assoc 1949;140:659–62.
25. Hochberg MC, Chang RW, Dwosh I, Lindsey S, Pincus T, Wolfe F. The American College of Rheumatology 1991 revised criteria for the classification of global functional status in rheumatoid arthritis. Arthritis Rhem 1992;35:498–502.
26. Jensen KD, Su X, Shin S, Li L, Youssef S, Yamasaki S, et al. Thymic selection determines γδ T cell effector fate: antigen-naive cells make interleukin-17 and antigen-experienced cells make interferon γ. Immunity 2008;29:90–100.
27. Acosta-Rodriguez EV, Rivino L, Geginat J, Jarrossay D, Gattorno M, Lanzavecchia A, et al. Surface phenotype and antigenic specificity of human interleukin 17-producing T helper memory cells. Nat Immunol 2007;8:639–46.
28. Annunziato F, Cosmi L, Santarlasci V, Maggi L, Liotta F, Mazzinghi B, et al. Phenotypic and functional features of human Th17 cells. J Exp Med 2007;204:1849–61.
29. Singh SP, Zhang HH, Foley JF, Hedrick MN, Farber JM. Human T cells that are able to produce IL-17 express the chemokine receptor CCR6. J Immunol 2008;180:214–21.
30. Hirota K, Yoshitomi H, Hashimoto M, Maeda S, Teradaira S, Sugimoto N, et al. Preferential recruitment of CCR6-expressing Th17 cells to inflamed joints via CCL20 in rheumatoid arthritis and its animal model. J Exp Med 2007;204:2803–12.
31. Infante-Duarte C, Horton HF, Byrne MC, Kamradt T. Microbial lipopeptides induce the production of IL-17 in Th cells. J Immunol 2000;165:6107–15.
32. Harrington LE, Hatton RD, Mangan PR, Turner H, Murphy TL, Murphy KM, et al. Interleukin 17-producing CD4+ effector T cells develop via a lineage distinct from the T helper type 1 and 2 lineages. Nat Immunol 2005;6:1123–32.
33. Park H, Li Z, Yang XO, Chang SH, Nurieva R, Wang YH, et al. A distinct lineage of CD4 T cells regulates tissue inflammation by producing interleukin 17. Nat Immunol 2005;6:1133–41.
34. Veldhoen M, Hocking RJ, Atkins CJ, Locksley RM, Stockinger B. TGFβ in the context of an inflammatory cytokine milieu supports de novo differentiation of IL-17-producing T cells. Immunity 2006;24:179–89.
35. Mangan PR, Harrington LE, O'Quinn DB, Helms WS, Bullard DC, Elson CO, et al. Transforming growth factor-β induces development of the T(H)17 lineage. Nature 2006;441:231–4.
36. Bettelli E, Carrier Y, Gao W, Korn T, Strom TB, Oukka M, et al. Reciprocal developmental pathways for the generation of pathogenic effector TH17 and regulatory T cells. Nature 2006;441:235–8.
37. Corthay A, Johansson A, Vestberg M, Holmdahl R. Collagen-induced arthritis development requires αβ T cells but not γδ T cells: studies with T cell-deficient (TCR mutant) mice. Int Immunol 1999;11:1065–73.
38. Peterman GM, Spencer C, Sperling AI, Bluestone JA. Role of γδ T cells in murine collagen-induced arthritis. J Immunol 1993;151:6546–58.
39. Yoshiga Y, Goto D, Segawa S, Ohnishi Y, Matsumoto I, Ito S, et al. Invariant NKT cells produce IL-17 through IL-23-dependent and -independent pathways with potential modulation of Th17 response in collagen-induced arthritis. Int J Mol Med 2008;22:369–74.
40. Lockhart E, Green AM, Flynn JL. IL-17 production is dominated by γδ T cells rather than CD4 T cells during Mycobacterium tuberculosis infection. J Immunol 2006;177:4662–9.
41. Umemura M, Yahagi A, Hamada S, Begum MD, Watanabe H, Kawakami K, et al. IL-17-mediated regulation of innate and acquired immune response against pulmonary Mycobacterium bovis bacille Calmette-Guerin infection. J Immunol 2007;178:3786–96.
42. Shibata K, Yamada H, Hara H, Kishihara K, Yoshikai Y. Resident Vδ1+ γδ T cells control early infiltration of neutrophils after Escherichia coli infection via IL-17 production. J Immunol 2007;178:4466–72.
43. Romani L, Fallarino F, De Luca A, Montagnoli C, D'Angelo C, Zelante T, et al. Defective tryptophan catabolism underlies inflammation in mouse chronic granulomatous disease. Nature 2008;451:211–5.
44. Kim HR, Cho ML, Kim KW, Juhn JY, Hwang SY, Yoon CH, et al. Up-regulation of IL-23p19 expression in rheumatoid arthritis synovial fibroblasts by IL-17 through PI3-kinase-, NF-κB- and p38 MAPK-dependent signalling pathways. Rheumatology (Oxford) 2007;46:57–64.
45. Weiss RJ, Erlandsson Harris H, Wick MC, Wretenberg P, Stark A, Palmblad K. Morphological characterization of receptor activator of NFκB ligand (RANKL) and IL-1β expression in rodent collagen-induced arthritis. Scand J Immunol 2005;62:55–62.
46. Schutyser E, Struyf S, Van Damme J. The CC chemokine CCL20 and its receptor CCR6. Cytokine Growth Factor Rev 2003;14:409–26.
47. Konigshofer Y, Chien YH. Gammadelta T cells: innate immune lymphocytes? Curr Opin Immunol 2006;18:527–33.
48. Hayes SM, Li L, Love PE. TCR signal strength influences αβ/γδ lineage fate. Immunity 2005;22:583–93.
49. Haks MC, Lefebvre JM, Lauritsen JP, Carleton M, Rhodes M, Miyazaki T, et al. Attenuation of γδ TCR signaling efficiently diverts thymocytes to the αβ lineage. Immunity 2005;22:595–606.
50. Kadlecek TA, van Oers NS, Lefrancois L, Olson S, Finlay D, Chu DH, et al. Differential requirements for ZAP-70 in TCR signaling and T cell development. J Immunol 1998;161:4688–94.
51. Yamada H, Nakashima Y, Okazaki K, Mawatari T, Fukushi JI, Kaibara N, et al. Th1 but not Th17 cells predominate in the joints of patients with rheumatoid arthritis. Ann Rheum Dis 2008;67:1299–304.

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Letter to the Editor

Replication of association between *FAM167A(C8orf13)-BLK* region and rheumatoid arthritis in a Japanese population.

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Running title: Association of *BLK* with RA in Japanese

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Polymorphisms in the genomic region encoding B lymphoid tyrosine kinase (*BLK*) and family with sequence similarity 167, member A (*FAM167A*, also referred to as *C8orf13)* at 8p23.1 have been associated with systemic lupus erythematosus (SLE) in Caucasian[1,2] and Asian[3,4] populations. A recent genome-wide study in a north-American population demonstrated new associations with rheumatoid arthritis (RA), among which was a single nucleotide polymorphism (SNP) rs2736340 in the intergenic region of *BLK* and *FAM167A*.[5] In the HapMap Japanese samples (http://www.hapmap.org/index.html.ja), this SNP is in absolute linkage disequilibrium (r^2=1) with rs13277113, previously associated with SLE.[1-4] We demonstrated that both the population frequency of the risk genotype, rs13277113A/A, and the odds ratio (OR) for SLE were substantially higher in the Japanese compared with the Caucasian populations.[3]

Thus far, the association of *FAM167A-BLK* region with RA has not been reported in non-Caucasian populations. In this study, we examined whether the association between *BLK* and RA was replicated in Japanese.

A case-control association study was performed for 603 patients and 492 healthy controls. Because the association of *FAM167A-BLK* region with SLE is already established,[1-4] RA patients complicated with SLE were excluded. All patients fulfilled the American College of Rheumatology classification criteria for RA.[6] The patients and the healthy controls were recruited at Matsuta Clinic, University of Tsukuba, the University of Tokyo and Juntendo University. This study was reviewed and approved by the Research Ethics Committees of University of Tsukuba and other participating institutes. Written informed consent was obtained from all participants, except for some participants before 2001, prior to the enforcement of the Ethics Guidelines for Human Genome/Gene Analysis Research by the Japanese government. From such participants, oral informed consent had been obtained. In accordance with the Guidelines, the latter samples were anonymized in an unlinkable fashion, and were included in this study after review and approval by the Ethics Committee of University of Tsukuba. Genotype of rs13277113 was determined using the TaqMan SNP genotyping assay (Applied Biosystems, Foster City, CA).[3] Power calculation based on the risk allele frequency in the Japanese population (0.665) showed that this sample size provides 80% power to detect susceptibility genes with an allelic OR of 1.298. Deviation from Hardy-Weinberg equilibrium was observed neither in the patients nor in the controls.

Significant association with RA was replicated in the Japanese (Table 1). Although the OR was comparable to that in the Caucasian population (1.19 for rs2736340[5]), the risk allele frequency was considerably higher in the Japanese as compared with the Caucasians (cases 0.273 and controls 0.240 for rs2736340[5]). Population

attributable risk percent was estimated to be 22.8% in the Japanese and 9.3% in the Caucasians under the dominant model. No significant difference in rs13277113 was observed between *HLA-DRB1* shared epitope positive and negative RA (data not shown).

Our observations indicated that *FAM167A-BLK* region may be a shared genetic factor for multiple autoimmune diseases in multiple populations, but the genetic contribution may be greater in the Asian populations because of the differences in the genetic background.

Competing interests

None declared.

Funding

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References

1 Hom G, Graham RR, Modrek B, et al. Association of systemic lupus erythematosus with *C8orf13-BLK* and *ITGAM-ITGAX*. N Engl J Med 2008;358:900-9.

2 Harley JB, Alarcon-Riquelme ME, Criswell LA, et al. Genome-wide association scan in women with systemic lupus erythematosus identifies susceptibility variants in *ITGAM, PXK, KIAA1542* and other loci. Nat Genet 2008;40:204-10.

3 Ito I, Kawasaki A, Ito S, et al. Replication of the association between the *C8orf13-BLK* region and systemic lupus erythematosus in a Japanese population. Arthritis Rheum 2009;60:553-8.

4 Yang W, Ng P, Zhao M, et al. Population differences in SLE susceptibility genes: *STAT4* and *BLK*, but not *PXK*, are associated with systemic lupus erythematosus in Hong Kong Chinese. Genes Immun 2009;10:219-26.

5 Gregerson PK, Amos CI, Lee AT, et al. *REL*, encoding a member of the NF-κB family of transcription factors, is a newly defined risk locus for rheumatoid arthritis. Nat Genet 2009;41:820-3.
6 Arnett FC, Edworthy SM, Bloch DA, et al. The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis. Arthritis Rheum 1988;31:315-24.

Table 1. Association of *BLK* rs13277113 with RA in a Japanese population

		Genotype frequency			Allele frequency		Allelic association	
	n	A/A	A/G	G/G	A	G	*P*	OR (95% CI)
RA	603	308 (0.511)	242 (0.401)	53 (0.088)	858 (0.711)	348 (0.289)	0.018	1.24 (1.04-1.49)
Control	492	218 (0.443)	218 (0.443)	56 (0.114)	654 (0.665)	330 (0.335)		

OR: odds ratio, 95%CI: confidence interval.

Association was tested by χ^2- analysis using 2X2 contingency table.

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Conservative sequences in 3′UTR of TCRζ mRNA regulate TCRζ in SLE T cells

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Abstract

We have demonstrated that T-cell receptor ζ (ζ) mRNA with a 562-bp deleted alternatively spliced 3′-untranslated region (3′UTR) observed in T cells of patients with systemic lupus erythematosus (SLE) can lead to a reduction in ζ and TCR/CD3 (J. Immunol., 2003 & 2005). To determine the region in ζ mRNA 3′UTR for the regulation of ζ, ζ mRNA with 3′UTR truncations ligated into pDON-AI was used to infect murine T-cell hybridoma MA5.8 cells, which do not contain ζ. As a Western blot analysis demonstrated the importance of the regions from +871 to +950, containing conservative sequence 1 (CS1), and +1070 to +1136, containing CS2, for the production of ζ, we constructed MA5.8 mutants carrying ζ mRNA 3′UTR with deletions of these regions (ΔCS1 and ΔCS2 mutants). Western blot and FACS analyses showed significant reduction in the cell surface ζ and TCR/CD3 in both these mutants, and IL-2 production was decreased, compared with MA5.8 cells transfected with wild-type ζ mRNA. Furthermore, real-time PCR demonstrated the instability of ζ mRNA with 3′UTR deletions in these MA5.8 mutants. In conclusion, CS1 and CS2 may be responsible for the regulation of ζ and TCR/CD3 through the stability of ζ mRNA in SLE T cells.

Keywords: Systemic lupus erythematosus; TCRζ; 3′UTR; Signal transduction; Conservative sequence; T-cell receptor; IL-2; Autoimmune disease; MA5.8 cells; mRNA stability

Systemic lupus erythematosus (SLE) is well known as a prototype systemic autoimmune disease [1]. Defects in signal transduction through the TCR/CD3 complex may cause T-cell dysfunction and autoimmunity in NOD mice as well as in MRL *lpr/lpr* mice [2–4]. Several studies, including our own, have identified a functional defect in early signaling molecules on peripheral blood T cells (PBTs) as a cause of T-cell dysfunction in human SLE [5–7]. Sakaguchi et al. [8] demonstrated that a ZAP-70 mutation causes autoimmune arthritis in the SKG mouse model, supporting the notion that functional defects in early signaling molecules can cause autoimmune diseases. On the other hand, we and other groups have reported that a reduction in tyrosine phosphorylation and the diminished expression of ζ protein play crucial roles in the pathogenesis of SLE [9–13], and that an aberrant form of the ζ mRNA 3′-untranslated region (3′UTR), which is alternatively spliced and 562-bp shorter than the wild-type 3′UTR, is predominantly expressed in SLE T cells (ζmRNA/as-3′UTR) [14,15] and leads to the up-regulation of several other proteins [16]. An *in vitro* expression analysis of the ζ protein from ζmRNA/as-3′UTR using MA5.8 cell mutants and a retrovirus system showed that the predominant expression of ζmRNA/as-3′UTR leads to the down-regulation of not only ζ, but also of other TCR/CD3 components because of the instability of these ζ mRNA splice variant forms [17]. Observations in our study

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[17] and others [18] suggest that the deleted 562-bp portion of the 3′UTR in ζmRNA/as-3′UTR is critical for ζ mRNA stability. The 3′UTR region of mRNA is known to control the turnover rate of pre-synthesized mRNAs through interactions with *trans*-acting factors by altering mRNA stability and affecting the transportation and localization of mRNA [19–21]. Messenger RNA 3′UTR contains *cis*-acting elements, i.e., adenosine-uridine (AU)-rich elements (AREs), that bind to *trans*-acting proteins and participate in either the stabilization or destabilization of transcripts. Two AREs are located at positions +735 and +803 of the ζ mRNA 3′UTR, and both of these AREs are involved in the 562-bp deleted portion of the ζmRNA/as-3′UTR. Therefore, we investigated which portion, including the AREs in the ζ mRNA 3′UTR, could be responsible for the stability of ζ mRNA and the production of the TCR/CD3 complex, including ζ, using MA5.8 cell mutants and a retrovirus system. In this study, we demonstrated that two conservative sequences (CSs), rather than the AREs, are important for the production of the TCR/CD3 complex, including ζ, by influencing ζ mRNA stability.

Materials and methods

RT-PCR. RT-PCR was performed according to a previously described method [13,22]. Human ζ cDNA with 3′UTR truncations (740, 871, 950, 1070, 1136, 1330, and 1457) were amplified from the PBTs of a normal healthy control subject using primers that were designed as described in Table 1 on line. The primers for amplifying the murine CD3ε and β-actin cDNA were arranged as previously reported [22].

Cell lines and inhibition of RNA synthesis. The MA5.8 cells (lacking endogenous ζ expression) were kindly provided by Dr. Takashi Saito and the RetroPack™PT67 (BD Biosciences Clontech, Inc., Palo Alto, CA, USA) was used as the dualtropic packaging cell line. Inhibition of RNA synthesis was performed as previously reported [17].

Construction of MA5.8 mutants. MA5.8 mutants were constructed using a previously described method [17,23]. To construct the MA5.8 mutants with ζmRNA 3′UTR deletions, Fragments A, B, C, and D were amplified from the PBTs of a normal healthy control subject using primers that were designed as described in Table 1 on line. Fragment A and B or Fragment C and D were ligated into SalI-cut pDON-AI and were then transfected into RetroPack™PT67 cells.

Real-time PCR. The primers and TaqMan probes for human ζ, murine CD3 ε, and murine β-actin were designed as previously reported [23]. Amplification and detection of specific products were performed according to a previously described amplification protocol [17]. Standard curves for the quantification of mRNA were established as previously reported [23].

Western blot. Western blot was performed according to a previously described method [9]. The blots were probed with a mouse anti-human ζ mAb (TIA-2) (Coulter Immunology, Hialeah, FL, USA). TIA-2 was visualized using a peroxidase-conjugated anti-mouse IgG (GE Healthcare Bio-Science Corp., Piscataway, NJ, U.S.A.). Biotinylated proteins were detected using streptavidin-peroxidase (Southern Biotechnology Associates, Birmingham, IL, USA). The densities of the specific bands were quantified as index values using the method previously reported [17].

Flow cytometry. The flow cytometric analysis procedure has been previously described [17].

Antibody stimulation and IL-2 quantification. IL-2 quantification was determined by the method described previously [23].

Statistical analysis. Statistical significance was calculated using the Student *t*-test for unpaired data and Statview software (version 4.5; Abacus, Berkeley, CA, USA). A value of $p < 0.05$ was considered statistically significant.

Results

RT-PCR of ζ mRNA with 3′UTR truncations

To identify the region of the ζ mRNA 3′UTR responsible for the expression of ζ and the TCR/CD3 complex, human ζ cDNA with 3′UTR truncations (740 [605 bp], 871 [736 bp], 950 [815 bp], 1070 [935 bp], 1136 [1001 bp], 1330 [1195 bp], and 1457 [1322 bp]) were amplified from the PBTs of a normal healthy control (Fig. 1 on line).

Construction of MA5.8 mutants with ζ mRNA 3′UTR truncations

ζ cDNA with 3′UTR truncations (740, 871, 950, 1070, 1136, 1330, and 1457) were ligated into pDON-AI, transfected into RetroPack™PT67 cells, and used to infect MA5.8 cells to construct the MA5.8 mutants 740, 871, 950, 1070, 1136, 1330, and 1457, respectively. Also, WT and AS3′UTR mutants were constructed from full-length wild-type human ζcDNA and ζcDNA/as-3′UTR, respectively. The NEG mutant was constructed using pDON-AI without any insert DNA.

ζ protein expression in MA5.8 mutants with ζ mRNA 3′UTR truncations

In a Western blot analysis using an anti-human ζ mAb (TIA-2), the production of the ζ protein by the 740 and 871 mutants was not observed (Fig. 1). The production of ζ protein by the 1136 (15.4 index), 1330 (16.7 index), and 1457 (17.2 index) mutants was almost the same as that of the WT mutant (18.5 index). However, the ζ protein expression of the 950 (11.0 index) and 1070 (10.2 index) mutants was relatively low. Therefore, we concluded that ζ protein expression gaps might exist between the 871 and 950 mutants and between the 1070 and 1136 mutants.

Construction of MA5.8 mutants with ζ mRNA 3′UTR deletions

To determine whether the ζ mRNA 3′UTR regions from +871 to +950 and/or from +1070 to +1136 were responsible for the production of ζ protein, we constructed MA5.8 mutants containing ζ mRNA 3′UTR deletions of these two regions. Fragments A (736 bp, +136 to +871), B (680 bp, +952 to +1631), C (940 bp, +136 to +1075), and D (525 bp, +1107 to +1075) were amplified using RT-PCR (Fig. 2 on line). Then, Fragments A and B and Fragments C and D were ligated into pDON-AI, transfected into RetroPack™PT67 cells, and used to infect MA5.8 cells for the construction of the mutants. As the regions from +871 to +950 and +1070 to +1136 contain CS1 (5′-CCCUGCC UUGGGCCCUCUGGUUUGC-3′) and CS2 (5′-C

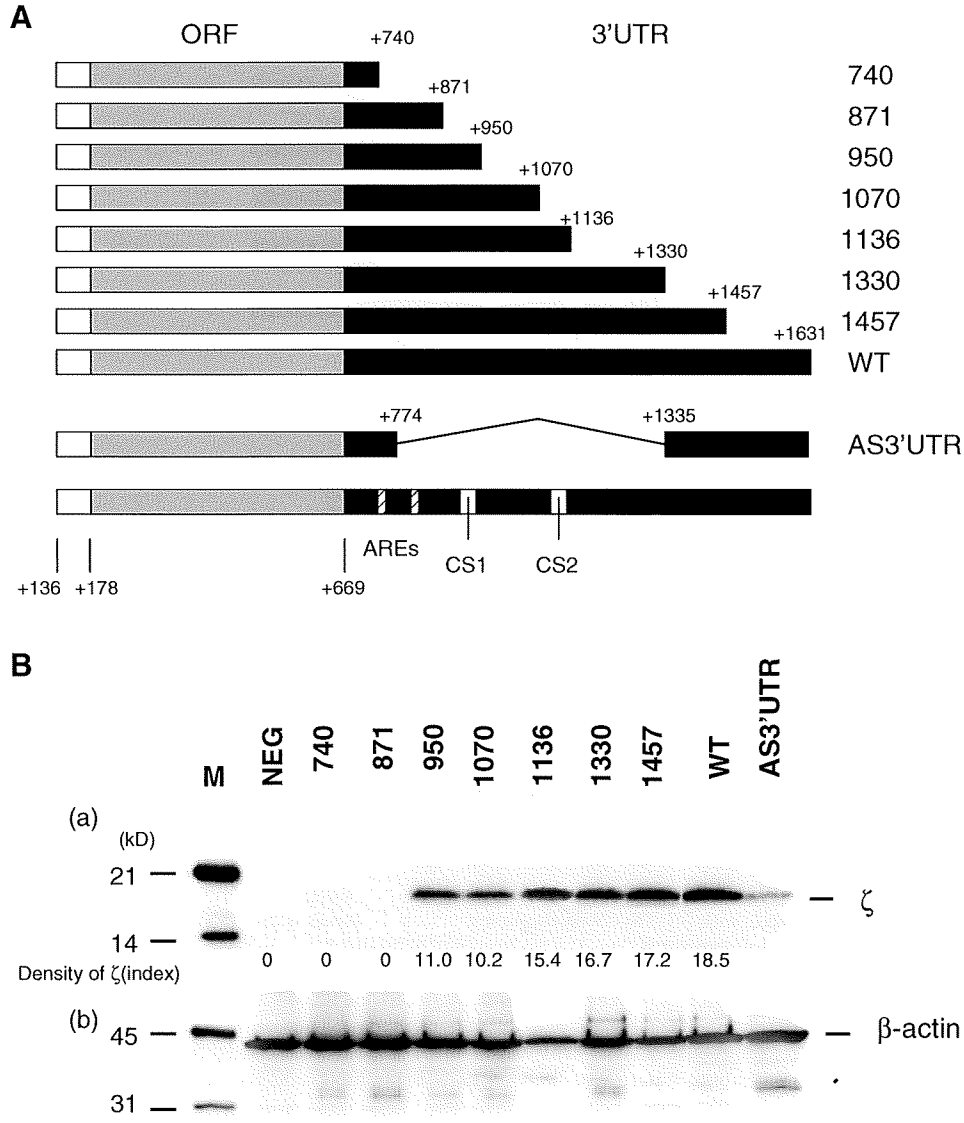

Fig. 1. (A) Scheme of human ζ cDNA with 3′UTR truncations. The shaded bars show the open reading frame (ORF), and the black bars represent the 3′UTR. The white bars show the 5′UTR. AS3′UTR refers to the ζ mRNA/as-3′UTR with a 562-bp deletion (+774 to +1335) in its 3′UTR. The wild-type ζ mRNA 3′UTR contains two A-U rich elements (AREs) (+735 and +803) and two conservative sequences (CSs: CS1, +872 to +951 and CS2, +1076 to +1106). (B) Expression of the ζ protein in MA5.8 mutants with ζ mRNA 3′UTR truncations. Cell lysates from MA5.8 and its mutants (NEG, 740, 871, 950, 1070, 1136, 1330, 1457, WT, and AS3′UTR) were electrophoresed on 15% SDS–polyacrylamide gels using a reducing method and blotted onto a PVDF membrane. The membranes were then incubated with (a) a mouse anti-human ζ mAb (TIA-2) or (b) a hamster anti-mouse β-actin mAb followed by a peroxidase-conjugated anti-mouse IgG. After treatment with chemiluminescence-enhancing reagents, the membranes were visualized on ECL X-ray films, and the densities of the 18-kDa ζ protein bands were quantified as index values.

UCCUGCUGUAAAUUUGGCUUCUGUUGUCAC-3′) (Fig. 3 on line), we defined these MA5.8 mutants as ΔCS1, and ΔCS2 mutants, respectively.

ζ protein expression in MA5.8 mutants with ζ mRNA 3′UTR CS deletions

The expression of the ζ protein in MA5.8 mutants with ζ mRNA 3′UTR CS deletions was analyzed using a Western blot and TIA-2. As shown in Fig. 2, ζ production in both the ΔCS1 and ΔCS2 mutants was slightly higher than that in the AS3′UTR but was significantly lower than that in the WT mutant. From these observations, we concluded that the ζ mRNA 3′UTR regions from +871 to +950 including CS1 and from +1070 to +1136 including CS2 were important for the production of ζ protein.

Expression of ζ and TCR/CD3 complex on the cell surfaces of MA5.8 mutants with ζ mRNA 3′UTR CS deletions

We investigated the expression of ζ and the TCR/CD3 complex on the cell surfaces of these MA5.8 mutants carrying ζ mRNA 3′UTR CS deletions using a FACS analysis. As shown in Fig. 3, the expression of ζ protein on the cell surfaces of both the ΔCS1 (mean channel fluorescence: 73.44) and the ΔCS2 (51.89) was much lower than that of

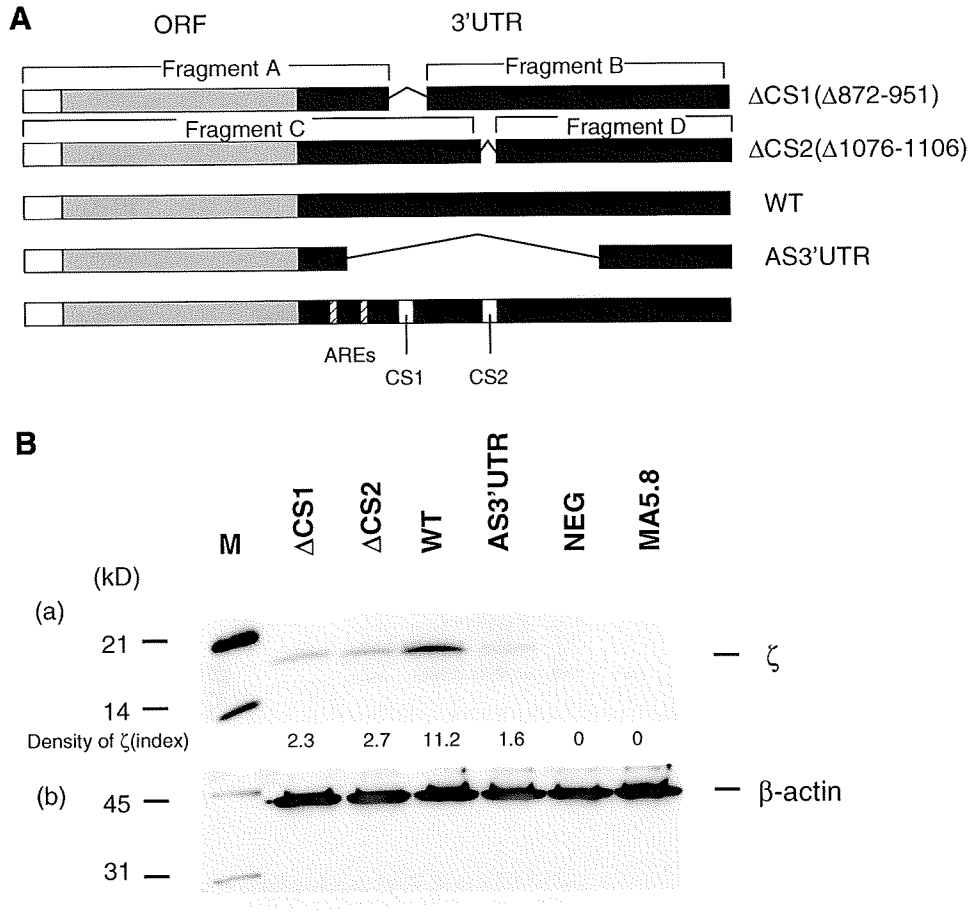

Fig. 2. (A) Scheme of the constructed ζ cDNA with 3′UTR CS deletions. Fragments A and B were ligated into SalI-cut pDON-AI to construct the ζ cDNA with the +872 to +951 deletion (ΔCS1). Fragments C and D were ligated to construct the ζ cDNA with the +1076 to +1106 deletion (ΔCS2). The shaded bars show the ORF, and the black bars represent the 3′UTR. The white bars show the 5′UTR. (B) Expression of the ζ protein in MA5.8 mutants with ζ mRNA 3′UTR CS deletions. Cell lysates from MA5.8 cells and its mutants (ΔCS1, ΔCS2, WT, AS3′UTR, NEG, MA5.8) were electrophoresed on 15% SDS–polyacrylamide gels using a reducing method and blotted onto a PVDF membrane. The membranes were then incubated with (a) a mouse anti-human ζ mAb (TIA-2) or (b) a hamster anti-mouse β-actin mAb followed by a peroxidase-conjugated anti-mouse IgG. After treatment with chemiluminescence-enhancing reagents, the membranes were visualized on ECL X-ray films, and the densities of the 18-kDa ζ protein bands were quantified as index values.

the WT mutant (138.34). Also, the production of TCR/CD3 complex on the cell surfaces of these two MA5.8 mutants with 3′UTR mRNA CS deletions, as estimated by examining CD3 ε expression, was much lower than that of the WT mutants (40.23).

Decrease in IL-2 production in MA5.8 mutants with ζ mRNA 3′UTR CS deletions

To evaluate the physiological effect of the ζ mRNA with 3′UTR CS deletions, MA5.8 mutants were stimulated with anti-mouse CD3 ε mAb (Fig. 4 on line). IL-2 production in the WT mutant on Day 1, 2, or 3 after stimulation was compared statistically with that in the ΔCS1, ΔCS2, or NEG mutant. IL-2 production in the ΔCS1 and ΔCS2 mutants on Day 1 to 3 was significantly ($p < 0.001$) lower than that in the WT mutants on Day 1 to 3, respectively. Consequently, IL-2 production in the MA5.8 mutants with ζ mRNA 3′UTR CS deletions seemed to be lower than usual.

ζ mRNA stability assay

To evaluate the relationship between the reduction in ζ protein expression and the ζ mRNA with 3′UTR CS deletions, we examined the stability of these ζ mRNA. The WT, ΔCS1, and ΔCS2 mutants were cultured and incubated with actinomycin D, and the cells were collected at 0, 6, 12, 24, and 48 h after drug exposure. ζ, CD3 ε, and β-actin cDNA in the WT, ΔCS1, or ΔCS2 mutants were quantified using real-time PCR.

As shown in Fig. 4, the relative amount of ζ mRNA in the ΔCS1 and ΔCS2 mutants rapidly decreased after treatment with actinomycin D and was significantly ($p < 0.01$) lower than that in the WT mutant over time. On the other hand, no significant decrease in the relative amount of CD3 ε mRNA, compared with ζ mRNA, was observed over time after actinomycin D treatment in both the ΔCS1 and ΔCS2 mutants. From these observations, we can conclude that the ζ mRNA with the 3′UTR CS deletions was less stable than the

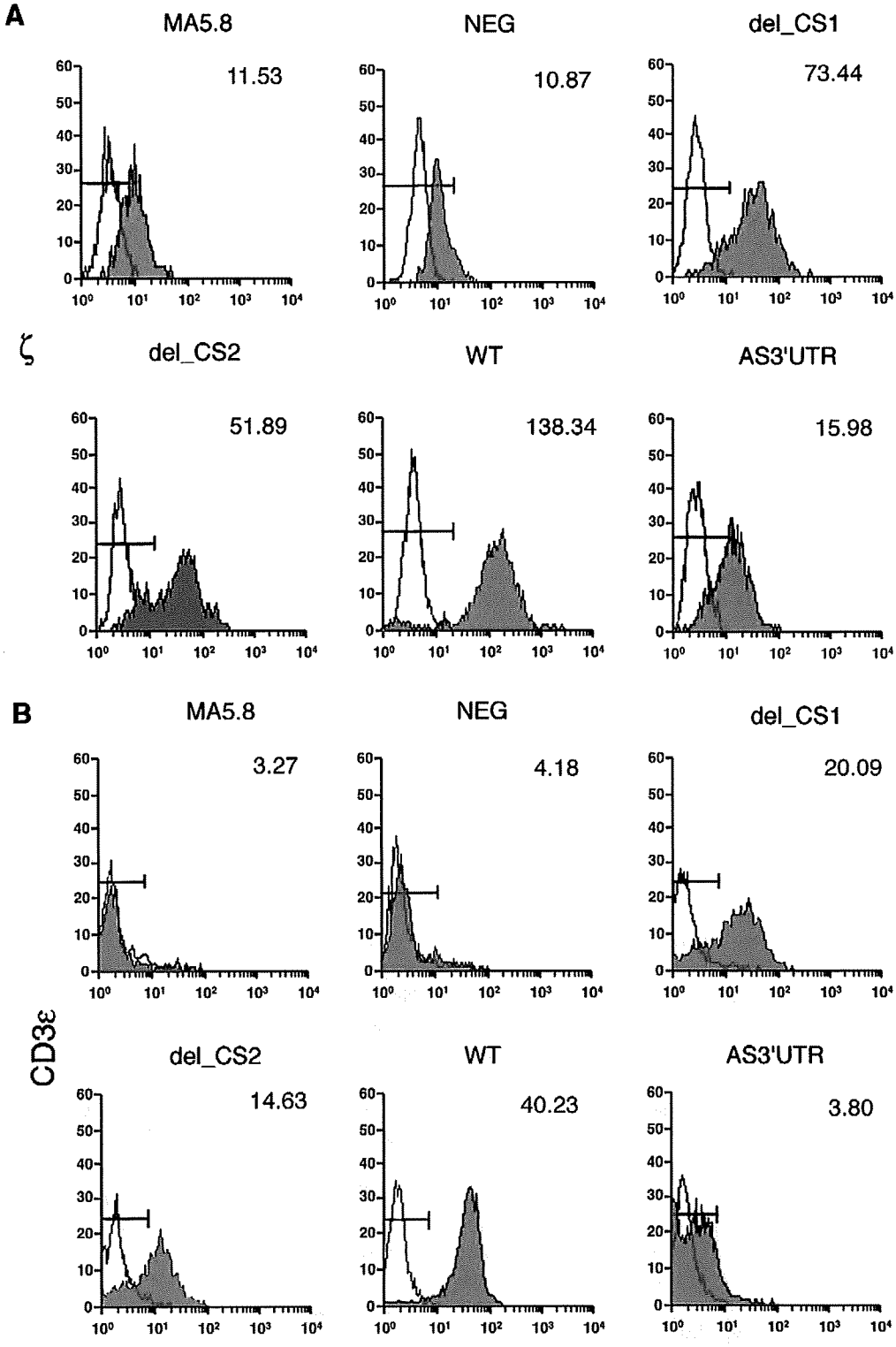

Fig. 3. Flow cytometric analysis of the MA5.8 mutants with ζ mRNA 3′UTR CS deletions. The surface expressions of (A) the ζ protein and (B) the TCR/CD3 complex on MA5.8 cells and its mutants [NEG, del_CS1(ΔCS1), del_CS2(ΔCS2), WT, AS3′UTR] were quantified using FITC-conjugated anti-human ζ mAb (TIA-2) (black profiles) and FITC-conjugated anti-mouse CD3 ε mAb (145-2C11) (black profiles), respectively. An FITC-conjugated mouse anti-human IgG (open profiles in (A)) or FITC-conjugated Armenian hamster anti-mouse IgG (open profiles in (B)) was used as the negative control. The mean channel fluorescence value is indicated within the figures at the *top right*.

wild-type ζ mRNA in the WT mutants. On the other hand, the stability of the CD3 ε mRNA was similar in the MA5.8 mutants with ζ mRNA 3′UTR CS deletions and the WT mutant.

Discussion

In this study, the down-regulation of the ζ protein in the MA5.8 mutants with ζ mRNA 3′UTR CS deletions (ΔCS1,

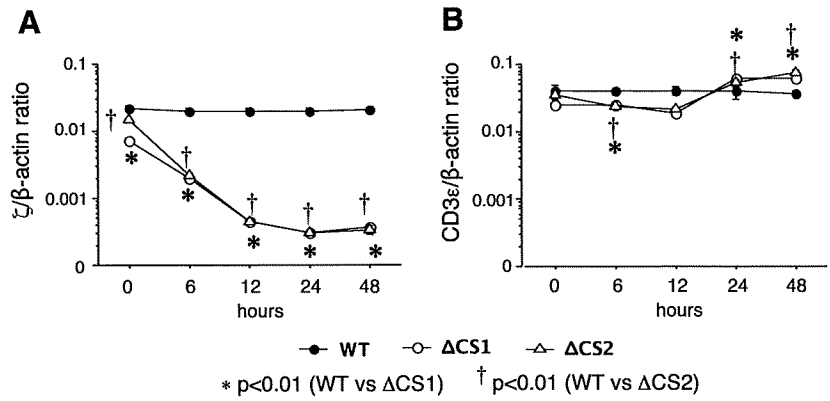

Fig. 4. Decrease in ζ mRNA stability in MA5.8 mutants with ζ mRNA 3′UTR CS deletions. MA5.8 mutants (WT, ΔCS1, ΔCS2) were cultured and incubated with 4 μg/mL of actinomycin D in the culture media. The samples were collected at various time points, and the mRNA was subsequently extracted and converted to whole cDNA. (A) ζ and (B) CD3 ε cDNA were quantified using real-time PCR and were evaluated as the ratio against β-actin cDNA. Each experiment was performed in triplicate. The bars show the mean plus or minus the SD. $^{*}p < 0.01$ for ΔCS1 (open circles) versus WT (closed circles). $^{\dagger}p < 0.01$ for ΔCS2 (open triangles) versus WT (closed circles).

ΔCS2) as confirmed using Western blot and FACS analyses, suggested that the production of the ζ protein was low in the absence of these two CSs of the ζ mRNA 3′UTR. The expression of the TCR/CD3 complex was also decreased in MA5.8 mutants without these regions, as shown by a FACS analysis.

We and other groups have previously reported that the ζ mRNA/as-3′UTR, which is predominantly expressed in SLE T cells, is less stable than the ζ mRNA/w-3′UTR and may be responsible for the reduced expression of the TCR/CD3 complex including ζ protein in SLE T cells [17,18]. Therefore, we examined the stability of ζ mRNA to investigate the reduction in ζ protein expression in the MA5.8 mutants expressing ζ mRNA 3′UTR CS deletions. From our observations, ζ mRNA with 3′UTR CS deletions appeared to be less stable and more easily degraded than ζ mRNA/w-3′UTR. Conceivably, the reduction in the stability of ζ mRNA with 3′UTR CS deletions may lead to a reduction in the expression of the intracellular ζ homodimer, leading to the absence of the expression of the TCR/CD3 complex on the cell surface. Also, the reduction in IL-2 production in these MA5.8 mutants revealed that the signal from the TCR was not transduced into the cytoplasm by anti-CD3 ε antibody stimulation in these mutants. AREs (AUUUA motifs) bind to *trans*-acting proteins and participate in either the stabilization or destabilization of the transcripts. Two AREs, which have been reported to be responsible for the stability of ζ mRNA [24], are located at positions +735 and +803 of ζ mRNA. In our study, however, both MA5.8 mutants 740 and 871 with ζ mRNA 3′UTR truncations did not express ζ protein and MA5.8 mutants ΔCS1 and ΔCS2, which contained the AREs, were easily degraded. From these observations, there might remain the possibility that the AREs at +735 and +803 are not related to the production of ζ protein or the stability of ζ mRNA. However, this possibility can be demonstrated only by using a deletion mutant lacking AREs but containing CSs. This study is now under way in our laboratory. In conclusion, the present study suggests that the regions between +872 and +951 and between +1076 and +1106 in the ζ mRNA 3′UTR are critical for ζ mRNA stability. Gramolini et al. reported that a 171-bp region in the 3′UTR of utrophin mRNA regulates utrophin mRNA stability, since the half-life of utrophin mRNA without this region is much shorter than that of the wild-type mRNA [25]. Also, Akgül et al. reported a 59-nucleotide (nt) pentobarbital-responsive element in the 3′UTR of *Drosophila* glutathione-*S*-transferase D21 (gstD21) mRNA that regulates the stability of gstD21 mRNA [26].

Interestingly, CS1 and CS2 are conserved in several mammals (Fig. 3 on line). Xie et al. reported that 106 CSs in mRNA 3′UTR are involved in post-transcriptional regulation and that half of these CSs are related to AREs, while the other half of the CSs that do not contain AREs are associated with microRNAs [27]. Since both the CS1 and CS2 in the ζ mRNA 3′UTR do not include AREs (AUUUA motifs), some miRNAs might influence the stability of the ζ mRNA by binding to the CS1 and CS2 in ζmRNA 3′UTR regions that do not contain AREs. This possibility is now being studied in our laboratory.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2007.12.145.

References

[1] J.A. Mills, Systemic lupus erythematosus, N. Engl. J. Med. 30 (1994) 1871–1879.

[2] D. Zipris, A.H. Lazarus, A.R. Crow, M. Hadzija, T.L. Delovitch, Defective thymic T cell activation by concanavalin A and anti-CD3 in autoimmune nonobese diabetic mice. Evidence for thymic T cell anergy that correlates with the onset of insulitis, J. Immunol. 146 (1991) 3763–3771.

[3] M.A. Berman, C.I. Sandborg, Z. Wang, K.L. Imfeld, F. Zaldivar Jr., V. Dadufalza, B.A. Buckingham, Decreased IL-4 production in new onset type I insulin-dependent diabetes mellitus, J. Immunol. 157 (1996) 4690–4696.

[4] S.B. Wilson, S.C. Kent, K.T. Patton, T. Orban, R.A. Jackson, M. Exley, S. Porcelli, D.A. Schatz, M.A. Atkinson, S.P. Balk, J.L. Strominger, D.A. Hafler, Extreme Th1 bias of invariant Valpha24JalphaQ T cells in type 1 diabetes, Nature 39 (1998) 177–181.

[5] A.K. Dayal, G.M. Kammer, The T cell enigma in lupus, Arthritis Rheum. 39 (1996) 23–33.

[6] S. Sierakowski, E.J. Kucharz, R.W. Lightfoot, J.S. Goodwin, Impaired T-cell activation in patients with systemic lupus erythematosus, J. Clin. Immunol. 9 (1989) 469–476.

[7] T. Takeuchi, M. Pang, K. Amano, J. Koide, T. Abe, Reduced protein tyrosine phosphatase (PTPase) activity of CD45 on peripheral blood lymphocytes in patients with systemic lupus erythematosus (SLE), Clin. Exp. Immunol. 109 (1997) 20–26.

[8] N. Sakaguchi, T. Takahashi, H. Hata, T. Nomura, T. Tagami, S. Yamazaki, T. Sakihama, T. Matsutani, I. Negishi, S. Nakatsuru, S. Sakaguchi, Altered thymic T-cell selection due to a mutation of the ZAP-70 gene causes autoimmune arthritis in mice, Nature 426 (2003) 454–460.

[9] T. Takeuchi, K. Tsuzaka, M. Pang, K. Amano, J. Koide, T. Abe, TCRζ chain lacking exon 7 in two patients with systemic lupus erythematosus, Int. Immunol. 10 (1998) 911–921.

[10] S.N. Liossis, X.Z. Ding, G.J. Dennis, G.C. Tsokos, Altered pattern of TCR/CD3-mediated protein–tyrosyl phosphorylation in T cells from patients with systemic lupus erythematosus. Deficient expression of the T cell receptor ζ chain, J. Clin. Invest. 101 (1998) 1448–1457.

[11] V. Brundula, L.J. Rivas, A.M. Blasini, M. Paris, S. Salazar, I.L. Stekman, M.A. Rodriguez, Diminished levels of T cell receptor ζ chains in peripheral blood T lymphocytes from patients with systemic lupus erythematosus, Arthritis Rheum. 42 (1999) 1908–1916.

[12] M. Pang, Y. Setoyama, K. Tsuzaka, K. Yoshimoto, K. Amano, T. Abe, T. Takeuchi, Defective expression and tyrosine phosphorylation of the T cell receptor ζ chain in peripheral blood T cells from systemic lupus erythematosus patients, Clin. Exp. Immunol. 129 (2002) 160–168.

[13] K. Tsuzaka, T. Takeuchi, N. Onoda, M. Pang, T. Abe, Mutations in T cell receptor ζ chain mRNA of peripheral T cells from systemic lupus erythematosus, J. Autoimmun. 11 (1998) 381–385.

[14] K. Tsuzaka, N. Onoda, K. Yoshimoto, Y. Setoyama, K. Suzuki, M. Pang, T. Abe, T. Takeuchi, T-cell receptor ζ mRNA with alternatively spliced 3′ untranslated region is generated predominantly in the peripheral blood T cells of systemic lupus erythematosus patients, Mod. Rheumatol. 12 (2002) 167–173.

[15] M.P. Nambiar, E.J. Enyedy, V.G. Warke, S. Krishnan, G. Dennis, M. Kammer, G.C. Tsokos, Polymorphisms/mutations of TCR ζ chain promoter and 3′ untranslated region and selective expression of TCR ζ chain with an alternatively spliced 3′ untranslated region in patients with systemic lupus erythematosus, J. Autoimmun. 16 (2001) 133–142.

[16] K. Tsuzaka, K. Nozaki, C. Kumazawa, K. Shiraishi, Y. Setoyama, K. Yoshimoto, K. Suzuki, T. Abe, T. Takeuchi, DNA microarray gene expression profile of T cells with the splice variants of TCRζ mRNA observed in SLE, J. Immunol. 176 (2006) 949–956.

[17] K. Tsuzaka, I. Fukuhara, Y. Setoyama, K. Yoshimoto, K. Suzuki, T. Abe, T. Takeuchi, TCRζ mRNA with an alternatively spliced 3′ untranslated region detected in SLE patients leads to the downregulation of TCRζ and TCR/CD3 complex, J. Immunol. 171 (2003) 2496–2503.

[18] B. Chowdhury, C.G. Tsokos, S. Krishnan, J. Robertson, C.U. Fisher, R.G. Warke, V.G. Warke, M.P. Nambiar, G.C. Tsokos, Decreased stability and translation of T cell receptor ζ mRNA with an alternatively spliced 3′-untranslated region contribute to ζ chain down-regulation in patients with systemic lupus erythematosus, J. Biol. Chem. 280 (2005) 18959–18966.

[19] G. Bassell, R.H. Singer, mRNA and cytoskeletal filaments, Curr. Opin. Cell Biol. 9 (1997) 109–115.

[20] K.C. Tsai, V.V. Cansino, D.T. Kohn, R.L. Neve, N.I. Perrone-Bizzozero, Post-transcriptional regulation of the GAP-43 gene by specific sequences in the 3′ untranslated region of the mRNA, J. Neurosci. 17 (1997) 1950–1958.

[21] J.L. Veyrune, J. Hesketh, J.M. Blanchard, 3′ Untranslated regions of c-myc and c-fos mRNAs: multifunctional elements regulating mRNA translation, degradation and subcellular localization, Prog. Mol. Subcell. Biol. 18 (1997) 35–63.

[22] J.P. Jensen, D. Hou, M. Ramsburg, A. Taylor, M. Dean, A.M. Weissman, Organization of the human T cell receptor ζ/η gene and its genetic linkage to the FcγRII-FcγRIII gene cluster, J. Immunol. 148 (1992) 2563–2571.

[23] K. Tsuzaka, Y. Setoyama, K. Yoshimoto, K. Shiraishi, K. Suzuki, T. Abe, T. Takeuchi, A splice variant of the TCR ζ mRNA lacking exon 7 leads to the down-regulation of TCR ζ, the TCR/CD3 complex, and IL-2 production in SLE T cells, J. Immunol. 174 (2005) 3518–3525.

[24] B. Chowdhury, S. Krishnan, C.G. Tsokos, J.W. Robertson, C.U. Fisher, M.P. Nambiar, G.C. Tsokos, Stability and translation of TCR ζ mRNA are regulated by the adenosine-uridine-rich elements in splice-deleted 3′ untranslated region of ζ -chain, J. Immunol. 177 (2006) 8248–8257.

[25] A.O. Gramolini, G. Belanger, B.J. Jasmin, Distinct regions in the 3' untranslated region are responsible for targeting and stabilizing utrophin transcripts in skeletal muscle cells, J. Cell Biol. 154 (2001) 1173–1183.

[26] B. Akgül, C-PD. Tu, Regulation of mRNA stability through a pentobarbital-responsive element, Arch. Biochem. Biophys. 459 (2006) 143–150.

[27] X. Xie, J. Lu, E.J. Kulbokas, T.R. Golub, V. Mootha, K. Lindblad-Toh, E.S. Lander, M. Kellis, Systematic discovery of regulatory motifs in human promoters and 3'UTRs by comparison of several mammals, Nature 434 (2005) 338–345.

Immunity
Article

Indispensable Role of the Runx1-Cbfβ Transcription Complex for In Vivo-Suppressive Function of FoxP3$^+$ Regulatory T Cells

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SUMMARY

Naturally arising regulatory T (Treg) cells express the transcription factor FoxP3, which critically controls the development and function of Treg cells. FoxP3 interacts with another transcription factor Runx1 (also known as AML1). Here, we showed that Treg cell-specific deficiency of Cbfβ, a cofactor for all Runx proteins, or that of Runx1, but not Runx3, induced lymphoproliferation, autoimmune disease, and hyperproduction of IgE. *Cbfb*-deleted Treg cells exhibited impaired suppressive function in vitro and in vivo, with altered gene expression profiles including attenuated expression of FoxP3 and high expression of interleukin-4. The Runx complex bound to more than 3000 gene loci in Treg cells, including the *Foxp3* regulatory regions and the *Il4* silencer. In addition, knockdown of *RUNX1* showed that RUNX1 is required for the optimal regulation of FoxP3 expression in human T cells. Taken together, our results indicate that the Runx1-Cbfβ heterodimer is indispensable for in vivo Treg cell function, in particular, suppressive activity and optimal expression of FoxP3.

INTRODUCTION

CD4$^+$CD25$^+$FoxP3$^+$ naturally occurring regulatory T (Treg) cells play essential roles for the maintenance of immunological self-tolerance and immune homeostasis by actively suppressing aberrant or excessive immune responses harmful to the host (Sakaguchi et al., 2006). Natural Treg cells specifically express the transcription factor FoxP3, which critically controls the development and the function of Treg cells as illustrated by *FOXP3* mutations (Ochs et al., 2005). FoxP3 deficiency or dysfunction in humans results in the development of IPEX (immune dysregulation, polyendocrinopathy, enteropathy, X-linked) syndrome, which is characterized by severe autoimmune disease, allergy, and inflammatory bowel disease (Sakaguchi et al., 2006). FoxP3 expression can confer suppressive activity to Treg cells, suppress the production of cytokines such as interleukin-2 (IL-2) and interferon-gamma (IFN-γ), and upregulate the expression of Treg cell-associated molecules including CD25 and cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) (Fontenot et al., 2003; Hori et al., 2003; Khattri et al., 2003). Recent studies have shown that the gene regulatory function of FoxP3 requires its association with other transcription factors, such as NFAT (nuclear factor of activated T cells), NF-κB (nuclear factor-κB), and Runx1 (runt-related transcription factor 1), also known as AML1 (acute myeloid leukemia 1), and with histone deacetylases and acetyltransferases (Bettelli et al., 2005; Li et al., 2007; Ono et al., 2007; Wu et al., 2006). Yet, the precise molecular mechanisms by which FoxP3 controls Treg cell function remain to be elucidated.

The Runx (AML) transcription factors consist of three members: Runx1 (AML1), Runx2 (AML3), and Runx3 (AML2) (van Wijnen et al., 2004). All Runx proteins bind to the specific DNA consensus sequences (ACCACA) via a highly conserved DNA-binding *runt* domain. Runx binding is stabilized by the association with Cbfβ (core-binding factor β), a non-DNA-binding cofactor essential for the function of all Runx proteins (Speck, 2001). The Runx-Cbfβ heterodimeric complex interacts with other DNA-binding transcription factors, coactivators, or corepressors to either activate or repress expression of the target genes in a context-dependent manner (Durst and Hiebert, 2004; Taniuchi and Littman, 2004). In addition to the essential requirement of Runx proteins for definitive hematopoiesis (de Bruijn and Speck, 2004), Runx1 and Runx3 are crucially involved in the differentiation and function of peripheral T cells (Djuretic et al., 2007; Komine et al., 2003; Naoe et al., 2007; Zhang et al., 2008) as well as thymic T cell development (Grueter et al., 2005; Sato et al., 2005; Setoguchi et al., 2008; Taniuchi et al., 2002; Woolf et al., 2003). We have previously shown that Runx1 binds to the promoter of the *Il2* and *Ifng* genes and upregulates the production of IL-2 and IFN-γ, respectively. Further, FoxP3 binds to Runx1 in Treg cells, thereby repressing *Il2* and *Ifng* and

activating the genes encoding CD25 (*Il2ra*) and CTLA-4 (*Ctla4*) (Ono et al., 2007). We have also shown that, in vitro, FoxP3 can interact with the other members of the Runx family, Runx2 and Runx3, in addition to Runx1 (Ono et al., 2007). These findings collectively suggest that the Runx-dependent transcription program operating in conventional T cells could be modulated in Treg cells through interaction with FoxP3. Yet, it remains obscure whether the Runx-mediated gene regulation is indeed required for the in vivo function of Treg cells.

In this report, we have generated mice with Treg cell-specific conditional deletion of *Cbfb* to analyze in vivo the possible contribution of Runx-dependent gene regulation to Treg cell function because all Runx proteins need to form a heterodimeric complex with Cbfβ for exerting transcriptinal activities and Cbfβ deficiency disrupts the function of the Runx complex (Speck, 2001). Here, we showed that Treg cell-specific Cbfβ-deficient mice spontaneously developed lymphoproliferation, autoimmune disease, and IgE hyperproduction and that *Cbfb*-deleted Treg cells exhibited impaired suppressive activity both in vitro and in vivo. In addition, Treg cell-specific conditional deletion of *Runx1*, but not *Runx3*, led to the development of immunological diseases similar to those observed in Treg cell-specific Cbfβ deficiency. Our findings thus indicate that the heterodimeric Runx1-Cbfβ complex is an indispensable transcription regulator for in vivo functions of Treg cells and that it is a potential therapeutic target for controlling physiological and pathological immune responses.

RESULTS

Treg Cell-Specific Deletion of *Cbfb* and the Resulting Development of Autoimmune Disease

To determine whether Runx proteins were required for in vivo function of FoxP3$^+$ Treg cells, we generated Treg cell-specific *Cbfb*-deleted mice by crossing mice harboring *LoxP*-flanked *Cbfb* allele with *Foxp3-Ires-Cre* (*FIC*) knockin mice, which faithfully express Cre recombinase in FoxP3$^+$ T cells (Naoe et al., 2007; Wing et al., 2008). FIC-mediated genomic deletion of *LoxP*-flanked region occurred in almost 100% CD4$^+$FoxP3$^+$ cells and a small population of CD8$^+$ T cells (Wing et al., 2008). With genomic DNA-PCR analyses of subpopulations of thymocytes and splenic T cells from *Cbfb$^{F/F}$: FIC* mice, inactivation of the *Cbfb* gene was initiated specifically in CD4-single positive (CD4SP) HSA^{lo}CD25^{hi} mature thymocytes and was completed in CD4$^+$CD25^{hi} splenic T cells, indicating Treg cell-specific deletion of the *Cbfb* gene (Figure 1A, left). Some of the CD4$^+$CD25$^-$ T cells also harbored the genomic *Cbfb* deletion (Figure 1A, left). This can be attributed to the presence of CD4$^+$CD25$^-$FoxP3$^+$ Treg cells in this CD4$^+$CD25$^-$ population (Figure S1 and Supplemental Data available online). As a consequence of the gene inactivation, the Cbfβ protein was undetectable in CD4$^+$CD25^{hi} splenocytes, although a substantial amount of the Cbfβ protein remained in CD4SP HSA^{lo}CD25^{hi} thymocytes (Figure 1A, right). Thus, the Cbfβ protein is gradually decreased in FoxP3$^+$ cells after *Cbfb* gene deletion in the thymus and almost completely lost in the periphery, which is consistent with a similar finding with conditional *Cbfb* deletion by *Cd4-Cre* transgene (Naoe et al., 2007).

Notably, *Cbfb$^{F/F}$: FIC* mice spontaneously developed severe lymphadenopathy and splenomegaly with significantly increased numbers of splenocytes by 14 weeks of age (Figure 1B). Various types of immune cells including CD4$^+$ T cells, CD8$^+$ T cells, B cells, macrophages, and dendritic cells increased in the enlarged spleens of *Cbfb$^{F/F}$: FIC* mice (Figure S2). Seventy percent of *Cbfb$^{F/F}$: FIC* mice developed histologically evident gastritis accompanying high titers of anti-gastric parietal cell autoantibodies in the sera, whereas control *Cbfb$^{F/+}$: FIC* littermates did not (Figures 1C and 1D). Flow cytometric analysis revealed that non-Treg T cells, i.e., CD4$^+$FoxP3$^-$ conventional T cells and CD8$^+$ T cells, in *Cbfb$^{F/F}$: FIC* mice showed an activated or memory phenotype; e.g., CD69$^+$, CD122$^+$, CD44^{hi}, and CD62L^{lo} (Figure 1E). CD4$^+$ and CD8$^+$ T cells abundantly produced cytokines such as IFN-γ, IL-2, IL-4, and IL-10, as revealed by intracellular cytokine staining after stimulation with PMA and ionomycin (Figure 1F). In addition, *Cbfb$^{F/F}$: FIC* mice showed 10-fold elevated concentrations of serum IgE and 1.5-fold increase in serum IgG (Figure 1G). Thus, Treg cell-specific Cbfβ deficiency produced autoimmune disease and led to hyperproduction of IgE.

The Effects of Treg Cell-Specific *Cbfb* Gene Deletion on Treg Cell Development and Function

To analyze the mechanism of autoimmunity caused by Treg cell-specific *Cbfb* deficiency, we attempted to determine whether thymic generation and differentiation of Treg cells, their peripheral survival, or their suppressive function was affected by the deficiency.

There was no significant difference in the number of total or FoxP3$^+$ thymocytes between *Cbfb$^{F/F}$: FIC* and *Cbfb$^{F/+}$: FIC* mice (Figure 2A and Figure S3). HSA^{lo}CD25^{hi}FoxP3$^+$CD4SP thymic Treg cells normally developed in *Cbfb$^{F/F}$: FIC* mice as in control *Cbfb$^{F/+}$: FIC* mice (Figure 2B). Whereas Runx proteins were required for the differentiation of immature thymocytes to TCRβ^{hi}HSA^{lo}CD4SP mature thymocytes (Egawa et al., 2007), the generation of FoxP3$^+$TCRβ^{hi}HSA^{lo}CD4SP mature thymocytes was not markedly impaired in *Cbfb$^{F/F}$: FIC* mice (Figure S4). Residual Cbfβ protein might be sufficient to support differentiation and maturation of FoxP3$^+$ cells in the thymus (Figure 1A).

We next examined whether Treg cell homeostasis was impaired in the periphery of *Cbfb*-deleted mice. The proportion of CD4$^+$FoxP3$^+$ Treg cells to total CD4$^+$ T cells and the absolute number of Treg cells were slightly higher in *Cbfb$^{F/F}$: FIC* mice than in *Cbfb$^{F/+}$: FIC* mice (Figures 2C and 2D). Notably, Treg cells in *Cbfb$^{F/F}$: FIC* mice showed substantially decreased expression of FoxP3, compared to those in control mice (Figure 2D). Expression of Ki-67, a cellular marker for proliferation, indicated that an equivalent or larger proportion of Treg cells were active in cell cycle in *Cbfb$^{F/F}$: FIC* mice compared with control mice (Figure 2E). In *Cbfb$^{F/F}$: FIC* mice, Ki-67$^-$ resting Treg cells showed reduced expression of FoxP3, whereas Ki-67^{hi} proliferating Treg cells expressed FoxP3 at equivalent amounts as Ki-67^{hi} Treg cells in control mice (Figure 2E). In vivo BrdU labeling also revealed that CD4$^+$CD25^{hi} splenocytes in *Cbfb$^{F/F}$: FIC* mice were more actively proliferating than those in *Cbfb$^{F/+}$: FIC* mice (Figure 2F). Treg cells in the former expressed only slightly lower amounts of CD127 (IL-7 receptor α chain) and were not apoptotic according to 7-AAD (7-amino-actinomycin D) and Annexin V staining, in accord with the previous finding that *Runx1*-deficient