Autoimmune arthritis associated with mutated interleukin (IL)-6 receptor gp130 is driven by STAT3/IL-7–dependent homeostatic proliferation of CD4+ T cells

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Mice homozygous for the F759 mutation in the gp130 interleukin (IL)-6 receptor subunit have enhanced gp130–mediated signal transducer and activator of transcription (STAT)3 activation and spontaneously developed a lymphocyte-mediated rheumatoid arthritis–like joint disease. Here, we show that the development of the disease is dependent on both major histocompatibility complex (MHC) II–restricted CD4+ T cells and IL-6 family cytokines. In spite of the necessity for CD4+ T cells, the gp130 mutation was only required in nonhematopoietic cells for the disease. The gp130 mutation resulted in enhanced production of IL-7. Conditional knockout of STAT3 in nonlymphoid cells showed that the enhancement of IL-7 production was dependent on STAT3 activation by IL-6 family cytokines. Homeostatic proliferation of CD4+ T cells was enhanced in gp130 mutant mice and acceleration of homeostatic proliferation enhanced the disease, whereas the inhibition of homeostatic proliferation suppressed the disease. Anti–IL-7 antibody treatment inhibited not only the enhanced homeostatic proliferation, but also the disease in gp130 mutant mice. Thus, our results show that autoimmune disease in gp130 mutant mice is caused by increased homeostatic proliferation of CD4+ T cells, which is due to elevated production of IL-7 by nonhematopoietic cells as a result of IL-6 family cytokine–gp130–STAT3 signaling.

The online version of this article contains supplemental material.
T cells in peripheral organs are divided into two populations according to their expression of CD44 molecules: the CD44<sup>high</sup> memory and CD44<sup>low</sup> naive phenotypes (9). It is hypothesized that the CD44<sup>low</sup> population consists of relative newcomers from thymus selection, whereas the CD44<sup>high</sup> memory cells are survivors in the peripheral environment. Especially when the peripheral T cell number is low (i.e., in the neonatal condition, or after irradiation, chemotherapy, or viral infection), both CD4<sup>+</sup> and CD8<sup>+</sup> T cells proliferate dramatically (10). This proliferation, termed “homeostatic proliferation” (HP), plays a critical role in maintaining the T cell number in the periphery (10). Homeostatic proliferating T cells show higher CD44 expression and cytokine secretion and divide faster than CD44<sup>low</sup> naive cells (9). Because the CD44<sup>high</sup> memory phenotype T cells increase with age and divide slowly, even under normal, specific pathogen-free (SPF) conditions, it is clear that HP is induced in normal healthy animals (9, 10). HP is strong in the neonatal period when thymus-derived naive T cells first migrate into the lymphopenic peripheral environment (11). Two known signals stimulate T cell HP. One is mediated by MHC–self-peptide complexes and the other is mediated by common γ cytokines, such as IL-7 and IL-15 (10, 12). In fact, CD4<sup>+</sup> T cell HP is significantly impaired in MHCII− or IL-7−deficient mice (12, 13). Furthermore, the overexpression of IL-7 in vivo induces autoimmune diseases, including dermatitis or colitis, in mice (14, 15). Recently, HP that produced IL-21 was shown to enhance autoimmune disease in NOD mice (16).

IL-6 is a pleiotropic cytokine that regulates multiple biological functions such as development of the nervous and hematopoietic systems, acute-phase responses, inflammation, and immune responses (17). In rheumatoid arthritis (RA) patients, a high concentration of IL-6 is detected in the serum and joint fluids (18). Recently, important roles for proinflammatory cytokines, such as TNF-α, IL-1, and IL-6 in the pathogenesis of RA have been reported (19, 20). The importance of IL-6 has also been shown in SKG mice, a model of spontaneously occurring RA, and in antigen-induced RA models, such as CIA and AIA (21–23). Furthermore, treatment with anti–IL-6 receptor is effective for certain patients with RA (24). There are nine IL-6 family cytokines, including IL-6, oncostatin M, LIF, CNTF, CT-1, IL-11, and IL-27 (25). All the family members share gp130 as a receptor subunit and signal transducer (17). We previously showed that gp130 transduces two major signaling pathways after stimulation with an IL-6 family cytokine. One is the JAK–STAT3 pathway, from its YxxQ motif, and the other is the SHP2–Gab–Ras–ERK–MAPK pathway, from the Y759 residue in its cytoplasmic portion (26). To investigate the in vivo function of these signaling pathways, we have established a series of knockin mice (27) and shown that one of these lines, gp130<sup>F759/F759</sup> (F759), spontaneously develops an RA-like disease in a manner dependent on mature lymphocytes ~1 yr after birth (28). The severity of the disease in the F759 is enhanced in an IL-6−dependent manner by crossing the mice with p40-Tax Tg, human T cell leukemia virus 1 transgenic mice (29).

Here, we seek to clarify the immunological mechanisms by which the gp130<sup>F759/F759</sup> mutation causes the RA-like disease. Our results from F759 showed the involvement of enhanced CD4<sup>+</sup> T cell HP caused by IL-6 family-gp130–STAT3–IL-7 cascade in nonhematopoietic cells bearing the gp130<sup>F759/F759</sup> mutation.

**RESULTS**

**IL-6, MHCII−restricted CD4<sup>+</sup> T cells and the gp130<sup>F759/F759</sup> mutation in nonhematopoietic cells are involved in the development of RA−like disease**

We first examined whether development of RA-like disease in F759 was dependent on IL-6. We prepared a double mutant, IL6KO/F759, and monitored its development of the disease. Both the severity and the incidence of the disease were significantly decreased in the IL-6−deficient F759 compared with control F759 (Fig. 1 A), indicating the involvement of IL-6 in disease development. However, the disease still developed in the absence of IL-6. Because the disease was dependent on the gp130<sup>F759/F759</sup> mutation (28), these results suggested other IL-6 family cytokines are also involved in the disease in F759.

To identify the lymphocyte populations necessary for the disease, we generated the following four double mutant mice: Igh6KO/F759, CD4KO/F759, CD8KO/F759, and C2TAKO/F759. The Igh6KO/F759 showed almost the same progression of the disease as the control F759 (Fig. 1 B), but the CD4KO/F759 had significantly less severe arthritis (Fig. 1 B). However, CD8KO/F759 had significantly increased score and incidence compared with the control mice (Fig. 1 B), showing that CD8<sup>+</sup> T cells are dispensable, or rather suppressive for disease development. We hardly detected the disease in C2TAKO/F759 (Fig. 1 B). Therefore, we concluded that MHCII−restricted CD4<sup>+</sup> T cells were necessary but that CD8<sup>+</sup> T or B cells were dispensable for the development of the arthritis in F759.

To investigate which tissues or cells needed to have the gp130 mutation for disease development, we divided all the cells in the body into two groups: hematopoietic and nonhematopoietic. We performed BM transplantation using F759 and wild-type congenic mice. Bone marrow cells derived from wild-type or F759 were transplanted into lethally irradiated wild-type or F759. Chimerism, tested using BM cells, was >95% 8 mo after transplantation (unpublished data). We observed the disease developed in the two groups of chimeric mice in which nonhematopoietic cells had the gp130<sup>F759/F759</sup> mutation, regardless of the presence of the gp130<sup>F759/F759</sup> mutation in hematopoietic cells 8 mo after BM transplantation; that is, the disease developed in the chimeric mice in which the host was F759 and the BM donor was either wild-type or F759 (F759 having wild-type BM [62.5%, n = 8] and F759 having F759 BM [57.1%, n = 7]). Conversely, chimeric mice with a wild-type nonhematopoietic environment failed to develop the disease, regardless of the presence of the gp130<sup>F759/F759</sup> mutation in the hematopoietic cells (wild-type host having wild-type BM [0%, n = 12] and wild-type host having wild-type BM [59.5%, n = 12]).
having F759 BM (0%, \( n = 10 \)). These results showed that the gp130\(^{F759/F759} \) mutation in nonhematopoietic cells was necessary for the development of the disease. Together, these results demonstrated that MHCII-restricted CD4\(^+ \) T cells were critically involved, but that the gp130 mutation in the CD4\(^+ \) T cells was not necessary for the disease. Therefore, we focused next on the roles played by CD4\(^+ \) T cells and nonhematopoietic cell populations bearing the gp130\(^{F759/F759} \) mutation in the development of the disease in F759.

**Homeostatic proliferating CD4\(^+ \) T cells are involved in the disease in F759**

Because King et al. recently demonstrated that HP is involved in autoimmunity in NOD mice (16) and because we observed that F759 had more memory/activated phenotype CD4\(^+ \) T cells than wild-type controls (Fig. 2 A) and that F759 had significant lymphoadenopathy and splenomegaly (28), we hypothesized that enhanced HP of CD4\(^+ \) T cells might be involved in the development of the disease in F759.

As expected, we found that the CD4\(^+ \) T cell HP was enhanced in F759 compared with wild-type mice after irradiation (Fig. 2 B). We next modified the speed of the HP. First, we used neonatal thymectomy to enhance HP; neonatal thymectomy suppresses T cells in the thymus from entering the peripheral environment, resulting in enhanced HP in the periphery (11). Here, we chose a day-7 thymectomy (NTx) to induce HP in vivo because Asano et al. demonstrated that NTx did not reduce CD25\(^+ \)CD4\(^+ \) T (T reg) cells numbers and did not induce any autoimmune disease, whereas a day-3 thymectomy (N3Tx) reduced the numbers of T reg cells and induced several autoimmune diseases (30). We confirmed that the CD4\(^+ \) T cells in NTxed F759 divided more rapidly than those in NTxed wild-type controls or non-NTxed F759 (Fig. 2 C).

We also noted that the NTxed F759 had prominent lymphadenopathy and splenomegaly, suggesting an enhanced HP in F759 is a critical factor for determining the size of lymph nodes and spleen. The total cell number in the lymph nodes and spleen increased dramatically in F759 compared with wild-type mice after both were NTxed (Fig. 2, D and E). The numbers of CD4\(^+ \) T cells in the lymph nodes and spleen increased significantly in the F759 compared with wild-type mice after NTx (Fig. 2 F). HP is reported to induce memory/activated phenotype CD4\(^+ \) T cells (31). Consistent with this, the majority of the CD4\(^+ \) T cells in NTxed F759 showed the memory/activated phenotype (Fig. 2 G), suggesting that the enhanced CD4\(^+ \) T HP in F759 accelerated the accumulation of memory/activated T cells.

Importantly, the NTxed F759 developed arthritis earlier than the sham-operated F759 (Fig. 3 A). We observed mononuclear cell infiltration and hyperplasia of the synovial cells in F759 after NTx (unpublished data). In contrast, we observed few signs of disease in the wild-type mice, even after NTx (Fig. 3 A). We further confirmed that the disease in the NTxed F759, like that of the untreated F759, was dependent on MHCII-restricted CD4\(^+ \) T cells (Fig. 3 B).

We next investigated whether the suppression of HP in F759 inhibits development of the disease. Because we showed a large number of CD4\(^+ \)CD25\(^- \) T cells, filler cells, suppressed
Figure 2. Increase in CD44^{high}CD4^{+} T cells, lymphadenopathy, splenomegaly, and enhanced homeostatic proliferation in the periphery of F759. (A) FACS profiles of CD4^{+} T cells isolated from sLNs of F759 and control mice (8–10 mo old). These experiments were performed three times independently; representative data are shown. The percentage of CD44^{high}CD4^{+} T cells (CD62L^{high} and CD62L^{low}) is shown with error bars indicating 1 SD. The p-value was calculated by Student’s t test (*, P = 0.0000025).

(B) CD44^{low}CD4^{+} T cells were sorted from C57BL/6/SJL mice, labeled with CFSE, and injected into irradiated wild type or C57BL/6/F759. FACS profiles of the CD45.1^{+}CD4^{+} T cells in the sLNs and spleen are shown. These experiments were performed five times independently and representative data are shown. The relative amount of CFSE^{high} (most slowly dividing [A]), CFSE^{middle} [B], and CFSE^{low} (most rapidly dividing [C]) cells, with the value for CFSE^{high} defined as 1, is shown with error bars indicating 1 SD. The p-value was calculated by Student’s t test (*, P = 0.00037 and **, P = 0.000002).

(C) CD44^{low}CD4^{+} T cells from C57BL/6/SJL were CFSE labeled and injected into nonthymectomized and N7Txed C57BL/6 or C57BL/6/F759 neonates. FACS profiles of CD45.1^{+}CD4^{+} T cells from the sLNs and spleen are shown. These experiments were performed three times independently and representative data are shown. The relative

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amount of CFSE<sub>high</sub> (A), CFSE<sub>middle</sub> (B), and CFSE<sub>low</sub> (C) cells, with the value for CFSE<sub>high</sub> defined as 1, is shown with error bars indicating 1 SD. The p-value was calculated by Student’s t-test (*, P = 0.0017; **, P = 0.0008). (D and E) The sLNs (D) and spleen (E) were from and the total cell numbers were calculated. The mean is shown with error bars indicating 1 SD. The p-value was calculated by Student’s t-test (*, P = 0.0042; **, P = 0.0038; *** P = 0.00034; and ****, P = 0.000027). (F) The sLNs and spleen were harvested from NTxed C57BL/6 and C56BL/6/F759. The numbers of CD4<sup>+</sup> T cells were calculated from FACS analyses (open circles; NTxed F759 and open triangles; NTxed wild type). The mean is shown as open (NTxed wild type) and closed (NTxed F759) bars. The p-value was calculated by Student’s t-test (*, P = 0.0065; **, P = 0.0068; *** P = 0.00034; and ****, P = 0.000021). (G) Neonatal thymectomy increased the CD44<sup>high</sup> memory/effector T cell percentage of CD62L<sup>high</sup> and CD62L<sup>low</sup> is shown with error bars indicating 1 SD. The p-value was calculated by Student’s t-test (*, P = 0.024; **, P = 0.0032; *** P = 0.00000039; and ****, P = 0.0000002).
from gp130\textsuperscript{FxxQ/FxxQ} knockin (FxxQ) mice, which are defective in gp130-mediated STAT3 activation (27) (Fig. 5 G). We performed a critical experiment using type I–collagen–Cre/STAT3\textsuperscript{fl ox/fl ox} mice (Col1a-STAT3). IL-6 plus sIL-6R stimulation did not induce IL-7 expression in vitro in lymph nodes of Col1a-STAT3 (Fig. 5 F, top right). Moreover, in vivo expression of IL-7 in lymph nodes of irradiated Col1a-STAT3 was significantly lower compared with controls (Fig. 5 F, bottom). Furthermore, we showed there is almost no IL-7 protein expression in lymph nodes of Col1a-STAT3 after irradiation (Fig. 5 H). Together with the existence of a STAT3 binding site in the IL-7 promoter region (unpublished data), these results indicated that the IL-6/IL-6 family–gp130–STAT3–IL-7 cascade existed in the nonhematopoietic cells.
Finally, we analyzed the contribution of IL-7 on the development of the disease in NTxed F759. We showed that the anti–IL-7 antibody that significantly inhibited the gp130F759/F759-mediated enhancement of CD4+ T cell HP almost completely suppressed the disease in NTxed F759, whereas control IgG2b had no effect on the disease development (Fig. 6 B and not depicted). We could not suppress the disease development after injection of anti–IL-7 antibody in the neonatal period of F759 without a thymectomy (unpublished data). This ineffectiveness of the antibody treatment might be a result of the difficulty to maintain enough amount of anti–IL-7 antibody in vivo during a long period, such as 1 yr. However, we showed that IL-7RKO/F759 did not develop the disease in age (the disease incidence was 11% in IL-7RKO/F759 and 84% in control F759 mice at 12 mo old [n = 8 and n = 12, respectively]). From all the aforementioned results, we concluded that, in the F759, the overexpression of IL-7 mediated through the IL-6/IL-6 family-gp130–STAT3 signaling in nonhematopoietic cell populations was involved in both the accelerated HP of CD4+ T cells and disease development.

**DISCUSSION**

We demonstrated previously that Rag2KO background F759 did not develop the disease (28), clearly indicating that mature lymphocytes, CD4+ T cells, CD8+ T cells, and B cells are responsible for disease development. Here, we neglected the possible involvement of CD8+ T cells and B cells for the enhancement of the disease. CD8+ T cells are suppressive, although the mechanism of this phenomenon of CD8+ T cells should be clarified by additional studies after confirming it. From these results, it is reasonable to conclude that CD4+ T cells play a role in the disease of F759. In fact, we showed significant reduction of the disease severity in CD4-deficient F759 compared with control F759, indicating that, at least in part, CD4+ T cells are involved in the disease. We should note that CD4-deficient mice contain T cells able to recognize MHCII molecules (38). This may explain why the disease developed without CD4+ cells.

We observed that CIITAKOF759 clearly prevented induction of the disease, whereas CD4-deficient F759 animals show less protection from the disease. We hypothesize three possibilities to explain this phenomenon. First, as we described in previous paragraphs, CD4-deficient mice contain many T cells able to recognize MHCII molecules (38), whereas there is a limited number of MHCII-restricted CD4+ T cells in CIITAKO mice. Second, it is known that CIITA can influence transcription of MHCII plus multiple accessory genes for MHCI antigen presentation including H2DM, Ii, Plexin A1, and cathepsin E (39–41). Because accessory molecules are not able to work for CD4+ T cell responses without CD8+ T cells and B cells for the CD4+ T cell responses without CD8+ T cells and B cells for the disease development. Here, we neglected the possible involvement of CD8+ T cells and B cells for the enhancement of the disease. CD8+ T cells are suppressive, although the mechanism of this phenomenon of CD8+ T cells should be clarified by additional studies after confirming it. From these results, it is reasonable to conclude that CD4+ T cells play a role in the disease of F759. In fact, we showed significant reduction of the disease severity in CD4-deficient F759 compared with control F759, indicating that, at least in part, CD4+ T cells are involved in the disease. We should note that CD4-deficient mice contain T cells able to recognize MHCII molecules (38). This may explain why the disease developed without CD4+ cells.

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**IL-7 expression is involved in enhanced CD4+ T cell HP and is necessary for the development of the disease in F759 neonates**

We next asked whether the enhanced CD4+ T cell HP in F759 is dependent on an enhanced production of IL-7. We transferred CFSE-labeled CD4+ T cells into F759 neonates 1 d after NTx and injected an anti–IL-7 antibody. The in vivo depletion of IL-7 by the antibody treatment strongly suppressed CD4+ T cell HP in the F759 neonates, whereas the IL-7 depletion in wild-type controls showed a minimal effect on CD4+ T cell HP, as demonstrated by Min et al. (reference 11 and Fig. 6 A), showed an enhanced IL-7 production via gp130 signaling was involved in the enhanced HP of CD4+ T cells in F759 neonates.
Figure 5. IL-6 injection induced IL-7 in vivo and gp130-STAT3 directly induced IL-7 in the fibroblasts of F759. (A) IL-6KO/F759 (nonirradiated) with i.v. injection IL-6 was prepared. Real-time PCR for IL-7 or SOCS3 was performed using RNA from the sLNs of the IL-6KO/F759. These experiments were performed two times independently and the sum of the data is shown. (B) IL-6 KO mice with irradiation 24 h before the i.v. injection of IL-6 was prepared. Real-time PCR for IL-7 was performed using RNA from the sLNs. These experiments were performed three times independently and representative data are shown. The p-value was calculated by Student’s *t* test (in A: *, *P* = 0.034; **, *P* = 0.018). In B, *, *P* = 0.016. (C) Splenic fibroblasts were prepared and stimulated with IL-6. Real-time PCR was performed for IL-7 and SOCS3. The mean is shown with error bars indicating 1 SD. Student’s *t* test was done between IL-6–treated and untreated cells. The p-value was calculated by Student’s *t* test (*, *P* < 0.05 and **, *P* < 0.01). These experiments were performed three times independently and representative data are shown. (D) Splenic fibroblasts prepared from wild-type neonates were stimulated with IL-6 plus sIL-6R. Real-time PCR was performed for IL-7 and SOCS3. The mean is shown with error bars indicating 1 SD. The p-value was calculated by Student’s *t* test (*, *P* < 0.05 and **, *P* < 0.01). These experiments were performed three times independently and representative data are shown. (F, top left) MEF were prepared from FxxQ or wild-type fetus were stimulated with IL-6 plus sIL-6R. Real-time PCR was performed for IL-7. The mean is shown with error bars indicating 1 SD. The p-value was calculated by Student’s *t* test (in A: *, *P* = 0.034; **, *P* = 0.018). In B, *, *P* = 0.016. These experiments were performed three times independently and representative data are shown. (top right) Col1a-STAT3, control (STAT3flox/flox or type I-collagen-Cre/STAT3flox/+, and F759 were irradiated, and the sLNs were harvested and cultured with IL-6 plus sIL-6R. Real-time PCR for IL-7 was performed using sLNs. The mean is shown with error bars indicating 1 SD. The p-value was calculated by Student’s *t* test (*, *P* < 0.05). These experiments were performed three times independently and representative data are shown. (bottom) Col1a-STAT3, control, and F759 were irradiated. Real-time PCR for IL-7 or SOCS3 was performed using the lymph nodes. The mean is shown with error bars indicating 1 SD. The p-value was calculated by Student’s *t* test (*, *P* < 0.05; **, *P* < 0.01). These experiments were performed three times independently and representative data are shown. (G) MEF were prepared from F759 or wild-type fetus were stimulated with IL-6 plus sIL-6R. Their protein fractions were prepared and applied on SDS-page followed by Western blotting of IL-7. (H) The sLNs irradiated Col1a-STAT3, wild-type, and F759 were harvested. Their protein fractions were prepared and applied on SDS-page followed by Western blotting of IL-7.
There are three observations that directly support this conclusion. First, thymectomy in the neonatal period enhanced both the CD4+ T cell HP and the development of the disease. Second, either the transfer of filler CD4+ T cells or, third, the depletion of IL-7 inhibited the CD4+ T cell HP and suppressed the disease.

How is the HP of CD4+ T cells enhanced in F759? The rate of HP is known to be dependent on both TCR-MHC and cytokine signaling (12, 13, 44). We identified IL-7 from nonhematopoietic cell populations as a key cytokine for enhancing the HP of CD4+ T cells in F759. Because it was an unexpected finding that gp130 signaling induced IL-7, we performed eight independent experiments to show the existence of this pathway. First, it is reported that the main source of IL-7 is nonhematopoietic cells (37), and we confirmed a connective tissue-enriched fraction, which mainly consisted of nonhematopoietic cells, expressed IL-7 in F759. Second, we showed the IL-7 mRNA and protein expression increased in F759, especially after induction of HP. Third, conversely, IL-7 mRNA and protein expression was significantly decreased in Col1a-STAT3. Fourth, we confirmed both splenic fibroblasts and MEF expressed IL-7 after stimulation of gp130 signaling. Fifth, STAT3 binding sites of gp130 were critical for IL-7 mRNA expression in MEFs. Sixth, IL-6 injection induced IL-7 expression in lymph nodes in vivo. Seventh, we showed IL-7 protein increased in MEFs from F759 compared with those from wild-type controls after stimulation of IL-6 plus sIL-6R stimulation. Eighth, IL-7 depletion suppressed the HP of CD4+ T cells in F759. Thus, we concluded that a gp130–STAT3–IL-7 cascade in nonhematopoietic cells is critical for the enhancement of CD4+ T cell HP in F759.

Because we showed that the disease development was at least in part dependent on IL-6, consistent with our previous observation that the human T cell leukemia virus 1 p40Tax-induced enhancement of the disease in F759 is dependent on IL-6 (29), we suggest that IL-6 is at least partly involved in the gp130-mediated IL-7 expression in vivo. There are nine IL-6 family members reported, which include IL-6, IL-11, IL-27, oncostatin M, LIF, and CNTF (25). Because all of their receptors share gp130 as a subunit, it is likely that other IL-6 family cytokines also play a role in the gp130–STAT3–IL-7 cascade. Therefore, we hypothesized that the reason why we had significant numbers of animals that developed the disease even in the absence of IL-6 is that other IL-6 family members function as IL-7 inducers in IL-6KO/F759. Consistent with this hypothesis, we showed that other IL-6 family members induced a significant level of IL-7 in primary fibroblasts. We also showed HP increased IL-6 expression in vivo in the F759. Interestingly, we also showed IL-7 expression was correlated with IL-6 expression in the F759 thymectomized.

How does the enhancement of CD4+ T cell HP induce the disease in F759? We have not identified a bias of the T cell receptor repertoire toward CD4+ T cells in F759 with the disease (unpublished data), although the memory/activated CD4+ T cells were increased in F759. At present,
we cannot completely exclude the possibility that a minor population of antigen-specific CD4+ T cells among the homeostatic proliferating T cells plays a pathogenic role, but our data strongly support the idea that nonspecific antigen-proliferating CD4+ T cells play a central role in causing the tissue-specific RA-like disease via HP in a manner dependent on the gp130F759/F759 mutation of nonhematopoietic cells. In this regard, it is also interesting that B cells are not required for the disease in F759, although abundant autoantibodies are produced in these mice (28). This finding suggests that the apparent adaptive autoimmunity, such as autoantibody production, observed in the F759, is merely a consequence of the inflammatory response of the disease. We hypothesize that the HP-mediated activation of CD4+ T cells in F759 is a critical inducer or source of cytokines for the induction of the disease because the HP of CD4+ T cells is a type of CD4+ T cell activation in vivo and induces the expression of many cytokines from themselves and from DCs (45, 46).

As for the role of the IL-6 family cytokines, we propose two possibilities. One is that IL-6 family cytokines act only as a stimulator of CD4+ T cell HP through IL-7 production, but not as an effector molecule for disease induction. In this case, it may be possible that some cytokines other than IL-6 family cytokines that are induced by the enhanced HP of CD4+ T cells function as effectors for the disease development. The second scenario is that IL-6 family cytokines act not only as a stimulator of the HP of CD4+ T cells but also as a direct effector molecule for disease induction. If the second scenario is fit to this F759 model, IL-6/IL-6 family–mediated gp130F759/F759 signaling in nonhematopoietic tissues, most likely in the joint, could play a major role in the development of the disease. Because IL-6 is known to be a survival factor for synovial fibroblastic cells in the presence of soluble IL-6 receptor (47) and activatable CD4+ T cells express IL-6 family cytokines (unpublished data), it is likely that a hyperactivated STAT3 status caused by gp130F759/F759 signaling is involved in the bone destruction or overproliferation of synovial fibroblastic cells in the F759. Such aberrant gp130F759/F759 signaling directly or indirectly induced in joints via the enhanced HP of CD4+ T cells in F759 could cause tissue-specific diseases.

Nevertheless, we emphasize that MHCII-restricted CD4+ T cells were required for the disease in the F759. This may indicate that we should consider the environmental factors that cooperate with genetic factors to promote autoimmune diseases in patients. We hypothesize that certain environmental factors, including infectious agents, may induce the activation of CD4+ T cells in a manner dependent on MHCII molecules. If this event causes T cell activation, resulting in the production of critical cytokines, which might be IL-6 family cytokines, at high enough levels to affect the survival and growth of the target tissue or induce chronic inflammation in the target tissue of a certain genetic background, the disease would be caused in a manner associated with MHCII as well as multiple genetic backgrounds that may affect the target tissue, as reported for many autoimmune diseases, including RA.

MATERIALS AND METHODS

Mice. F759 and FxxQ that have a human version of gp130 (S710L) were established as described previously (27). C57BL/6 were obtained from SLC, 129/B6-F759 or 129/B6-FxxQ heterozygous were crossed with C57BL/6 over more than eight generations (N8). FxxQ homozygous mice were used at 14–15 d post-conus as a result of the perinatal lethality. C57BL/6 background Igh6KO, CD4KO, CD8KO, and C2TAKO mice were purchased from The Jackson Laboratory and C57BL/6 background IL-6KO was provided by Dr. Ikuta (Kyoto University, Kyoto, Japan). IL-7RK0 was provided by Dr. Ikuta (Kyoto University, Kyoto, Japan). These mice were crossed with C57BL/6 F759 (N8) to obtain double mutant mice: Igh6KO/F759, CD4KO/F759, CD8KO/F759, C2TAKO/F759, IL-6KO/F759, and IL-7RK0/F759. C57BL/6/SJL was also purchased from The Jackson Laboratory. STAT3fl ox/fl ox mice were provided by Dr. Akira (Osaka University, Osaka, Japan). Alfa 1 collagen-Cre mice were provided by Dr. Karsenty (Baylor College of Medicine, Houston, TX). All mice were maintained under SPF conditions according to the instructions of the Osaka University Medical School. All animal experiments were performed following the guidelines of the Institutional Animal Care and Use Committees in Graduate School of Frontier Bioscience and Graduate School of Medicine, Osaka University.

Neonatal thymectomy. Neonatal thymectomy was performed as described previously (30). In brief, C57BL/6 day 3 or day 7 neonates were anesthetized on ice and surgically thymectomized. Both lobes of the thymus were completely ablated with sterile toothpicks. The sternum and skin were sewed with 7–0 surgical sutures (Akiyama-Seisasho). Some neonates, whose skin and sternum were cut and sewn, were used as sham-operated mice.

Assessment of arthritis. Mice were inspected every week for up to 16 mo and assessed for signs of arthritis basically as described previously (28, 29). We modified the method minorly. Although we had removed scores of dead mice from the assessment, which usually had high scores (28, 29), here, we put the same scores from the point of time at which they were moribund or dead until end point of the assessment of the groups. The arthritis score shown is the sum of the scores for four limbs graded from 0 to 4. Thus, the maximum arthritis score for all four limbs was 16.

Histological analysis. Joints were fixed in 4% paraformaldehyde, decalcified for 12 h in Morse’s solution (22.5% boric formate and 10% sodium acid citrate solution) followed by 12 h in 4% paraformaldehyde, and embedded in paraffin. Sections were stained with hematoxylin/eosin (28).

BM transplantation. F759 and wild-type mice were irradiated (9.5 Gray) 24 h before bone marrow injection. BM cells (2 × 106) were prepared from congenic-SJL or F759 and intravenously injected into the irradiated host mice. 8 mo after the injection, >95% of the bone marrow cells were replaced with the donor (CD45.1) cells (unpublished data).

Antibodies and reagents. Antibodies against FITC-conjugated anti–mouse CD19, NK1.1, I-A^b, PE-conjugated anti–mouse CD4, Cyochrome-conjugated anti–mouse CD44, Ge-1, and CD11b, and allophycocyanin-conjugated anti–mouse CD45.1 were purchased from eBioscience. Purified anti–mouse CD3ε, CD3β, FITC-conjugated anti–mouse CD44, CD45.2 and PE-conjugated anti–mouse CD25 were obtained from Caltag. PE-conjugated anti–mouse CD62L was obtained from BD Biosciences. FITC-conjugated anti–mouse GITR, was obtained from R&D Systems. Anti–IL–7 antibody (mouse IgG2b) was purified from M25 hybridoma culture supernatant. The M25 hybridoma was a gift from Dr. Marrack (National Jewish Medical and Research Center, Denver, CO). Control mouse IgG2b was purchased from Sigma-Aldrich.

Cell preparation and cell sorting. The LNs (inguinal, axillaries, cervical, and mesenteric) and spleen were harvested, passed through a 100-μm cell strainer (BD Falcon) with RPMI 1640, and washed. Erythrocytes were eliminated with 0.165 M NH4Cl. A T cell–enriched sample was prepared using a nylon wool column, and the CD4^+CD44high or CD4^+CD25^- T cells
were purified by a Mollo high-performance cell sorter (DakoCytomation). The purity of the CD4+ T cells was consistently >98%.

**Flow cytometry.** For cell surface staining, 10^6 T cells were incubated with fluorescence-conjugated antibodies for 30 min on ice. The cells were analyzed by flow cytometry (BD Biosciences) using CELLQuest and FlowJo software.

**In vivo IL-6 and anti-IL-7 antibody treatments.** 1 μg of human IL-6 (Toray) was intravenously injected into irradiated IL-6KO adult mice or nonirradiated IL-6KO/F759 (2 wk old). Anti-IL-7 antibody (0.1 mg) or mouse IgG2b antibody (0.1 mg) was intraperitoneally injected into 7-d-old neonatal thymectomized F759 every 2 d for 2 wk.

**Homeostatic proliferation assay.** CD4+CD44 lowCD4 T cells were labeled with CFSE (Invitrogen) and i.p. injected into thymectomized nonirradiated IL-6KO/F759 (2 wk old). Anti–IL-7 antibody (0.1 mg) or anti–IL-6 antibody plus hormone and corticosterone (Sigma-Aldrich) were intraperitoneally injected every 2 d for 2 wk. Anti–IL-7 antibody (0.1 mg) or anti–IL-6 antibody plus hormone and corticosterone (Sigma-Aldrich) were intraperitoneally injected every 2 d for 2 wk.

**Fibroblast culture and stimulation.** For splenic fibroblast, neonatal spleens (3 d after birth) of wild-type and F759 were harvested and single cells were cultured in RPMI 1640 carrying 10% FCS and 1 mg/ml collagenase (Roche) in 5% CO2 incubator for 15 min. The resulting MFs (5 × 10^4/well) were plated in six-well plates and stimulated with IL-6 plus sIL-6R (50 ng/ml each) for 6 h after 24 h of starvation in RPMI 1640 plus 0.1% FCS. Human IL-6 (Toray), human soluble IL-6 receptor (R&D Systems), mouse oncostatin M (R&D Systems), mouse CT-1 (R&D Systems), mouse CT-2 (R&D Systems), mouse IL-11 (R&D Systems), and mouse LIF (Chemicon) were used for the experiments (50 ng/ml each).

**In vitro whole lymph node culture and stimulation.** The sLNs of irradiated type I-collagen-Cre/STAT3tm1 mouse, wild type, or F759 were lyzed with NP-40 cell lysis buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl and 1% NP-40) containing protease inhibitors (Sigma-Aldrich). Protein fraction was purified by 2D Cleanup kit (BioRad Laboratories). After SDS page on a 10–20% gel, the protein fraction was applied in Western blotting analysis with anti–mouse IL-7 antibody (R&D Systems) plus horseradish peroxidase secondary antibody (Zymed Laboratories) and visualized by ECL (GE Healthcare).

**Statistical analysis.** All the data were statistically analyzed with a Student’s t test.

**Online supplemental material.** Data regarding CD25+ T cells in F759 and N7Txed F759 mice is shown in Fig. S1, A–E, which is available at http://www.jem.org/cgi/content/full/jem.20052187/DC1.

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