Table 2 Disease severity at the time of first registry of CD and UC

	Child	Adult
IOIBD score for CD		
0–1	56 (18.1%)	473 (19.9%)
2–4	126 (40.6%)	1,203 (50.6%)
5–7	119 (38.4%)	653 (27.5%)
8–10	9 (2.9%)	48 (2.0%)
Total	310 (100.0%)	2,377 (100.0%)
P^a	<0.001	
Truelove's severity for UC		
Mild	291 (33.6%)	6,262 (45.7%)
Moderate	456 (52.7%)	6,162 (45.0%)
Severe	108 (12.5%)	1,188 (8.7%)
Fulminant	10 (1.2%)	87 (0.6%)
Total	865 (100.0%)	13,699 (100.0%)
P^a	<0.001	

Analysis of patients newly registered between 2003 and 2006

[a] Chi-square test

Table 3 Comparison of the disease location at diagnosis in childhood- and adult-onset CD and UC patients

	Child	Adult
CD		
L1 (terminal ileum)	83 (25.8%)	729 (28.7%)
L2 (colon)	62 (19.3%)	650 (25.6%)
L3 (ileocolon)	143 (44.4%)	1,004 (39.6%)
L4 (upper GI)	2 (0.6%)	0 (0.0%)
L1 + L4	12 (3.7%)	26 (1.0%)
L2 + L4	3 (0.9%)	26 (1.0%)
L3 + L4	17 (5.3%)	101 (4.0%)
Upper GI	34 (10.6%)	153 (6.0%)
Total	322 (100.0%)	2,536 (100.0%)
P^a	<0.001	
UC		
E1 (proctitis)	156 (14.0%)	3,001 (22.7%)
E2 (left sided)	360 (32.4%)	4521 (34.2%)
E3 (pancolitis)	596 (53.6%)	5,697 (43.1%)
Total	1,112 (100.0%)	13,219 (100.0%)
P^a	<0.001	

Analysis of patients newly registered between 2003 and 2006

[a] Chi-square test

Disease severity (Table 2)

As shown in Table 2, pediatric CD patients had higher IOIBD scores than adults did when they were first registered ($P < 0.001$, chi-square test). Percentage of patients with IOIBD score ≥ 5 was 41.3% in children and 29.5% in adults. Similarly, in children, moderate, severe and fulminant UCs were more frequent than in adult patients at the time of first registry (66.4 vs. 54.3%), while mild disease was less frequent (46.4 vs. 56.9%, respectively. $P < 0.001$, chi-square test).

Disease extent (Table 3)

Table 3 shows the affected site at the time of first registry in CD. Ileum and upper gastrointestinal (GI) involvement was observed more commonly in pediatric patients ($P < 0.001$, chi-square test). Extensive colitis (E3 in the Montreal classification) was more common in childhood-onset than in adult-onset UC (53.6 vs. 43.1%), whereas left-sided colitis (E2) and proctitis (E1) were more common in adult-onset than in childhood-onset patients (46.4 vs. 56.9%. $P < 0.001$, chi-square test).

Discussion

IBD is one of the most suffered chronic diseases to affect children and adolescents. Its etiology is unknown, and it is not easy to treat. Analysis of epidemiological data in the pediatric population should enable us to develop better understanding of the etiology of IBD and provide better clinical care for children with the disease [2, 6]. Using data from the nationwide registry of the Japanese government, which includes both adult and childhood data, we characterized pediatric IBD by comparing its clinical features with those in adults.

The database used in this study does not yet cover all IBD patients in Japan, and it is estimated that only 50–70% of patients are included. One major reason that not all patients have been filed is that some data are still in the process of being put into the computer at local MHLW offices. The delay in data handling results mainly from the shortage of office manpower, which is clearly independent of the age of the patients. Registration rates varied by prefecture from 0 to over 90%; 14 of 47 prefectures showed registration rates above 90%, whereas 13 prefectures did not register any patients in 2003. Therefore, there should not have been much bias in the conclusion obtained in this study that analyzed age-related changes. Another concern is that patients with mild symptoms tend not to register for this survey because of their low medical costs. However, our result regarding severity did not differ from previous studies without financial support [26]; furthermore, this sampling bias should be same in both children and adults so that it would not affect the results regarding differences in severity between children and adults.

As for IBD, a population-based study with a large enough number of patients to determine the proportion of

pediatric patients is lacking. The only data available to compare directly the incidence of IBD between adults and children are from a prospective study on the incidence of IBD in a restricted area in Denmark [1]. This study found that 6% with CD and 7% with UC had IBD onset before the age of 16 years.

In the present survey, the proportion of pediatric patients with newly registered CD and UC was 10.6 and 5.9%, respectively. In most cases, registration was done soon after diagnosis, and we assume that these numbers were close to the real incidence of the disease. The figures were, in fact, close to those of the Danish study [1]. The proportion of people who are ≤16 years old in the whole Japanese population is 13.8% [24]; therefore, the age-adjusted incidence of CD is 1.3 times greater in adults than that in children, and the incidence of UC in adults is estimated to be 2.3 times greater.

The proportion of patients below 16 years of age in 2005 was 2.6% for CD and 2.0% for UC. Therefore, the estimated prevalence of CD and UC in children adjusted by age was 5.3 and 6.9 times lower than in adults, respectively.

Incidence and prevalence of IBD vary considerably with country and race. It has been suggested that in Asian countries, incidence and prevalence of CD and UC are less than those in Western countries, especially in Northern Europe and North America. This geographic difference explains why the number of UC patients was twice that of CD in the present study. It has been reported that UC is more common than CD in Asian countries [15], which is distinct from Western countries where CD is more common than UC. The trend that UC is more common than CD in Japanese adults is also true in children. However, the ratio of UC to CD in children was 2.7, which is significantly lower than that in adults (5.1).

The age distribution of CD and UC in childhood in the present study was similar to that reported in Western countries [1, 8]. The number of patients increased abruptly after 8–10 years of age in those studies and in ours. The only exception is a recent analysis in the US in which an abrupt increase in the incidence after 8–10 years of age was obvious in CD, but not in UC. The reason for this discrepancy is not clear, and more data will be needed to reach a conclusion.

For CD in Western countries, it has been shown that women are more commonly affected than men, with a male to female ratio of 0.6–0.9 [25]. In children, boys are more likely to have CD than girls, with the male to female ratio being 1.3–1.6 [7, 8, 18]. In a recent study from Scotland, the male to female ratio was 0.6 in adult-onset patients and 1.5 in childhood-onset patients, which confirmed previous reports [28].

It has been recognized previously that, in Asian countries, CD is more common in males than in females, even in adults, although the reason for this is not known [15]. The gender distribution for CD in the present study was consistent with that previously reported in Asia, with a male-to-female ratio in adults of 2.4. Interestingly, the male to female ratio in childhood CD was significantly less (1.3) than in adults, and was close to that in Western countries. These facts suggest that some environmental, hormonal or lifestyle factors in Japanese adult male or female patients have some influence on the onset of CD.

In most studies from Western countries, male to female ratios for UC are close to 1 in adults [15, 25–28] and children [1, 12, 28, 29]. In the present study, we confirmed that there was no major difference in this ratio between children and adults, both being close to 1. One exception is a recent population-based study in Minnesota that showed a male predominance in incidence of UC, which was most obvious in childhood (male:female 3.4:1.3 for 0–19 years old) [19].

The present study showed that, in adults, 1.7% of CD and 1.8% of UC patients had a family member with the same disease. In children, the rates were higher: 3.0% for CD ($P < 0.001$) and 4.1% for UC ($P < 0.001$). However, these frequencies were still lower when compared to those in Western countries [12]. In a study by Kugathasan et al. [8], 11% of newly diagnosed pediatric IBD cases had first- or second-degree relatives with a history of IBD, and Feeney et al. [30] have reported that 30% of pediatric CD patients did so.

It has been shown that, in Japan, the first-degree relatives of UC patients have a 25-fold greater frequency of UC than in the general population, which is comparable to Western countries [27]. Therefore, as Yang et al. [15] have suggested, the lower rate of family history of UC is likely to reflect the relatively low prevalence of UC in Japan. Similarly, the lower familial occurrence of CD than UC can be explained by the lower prevalence of CD compared to UC in Japan.

In CD, age-related differences in anatomic distribution of the disease are controversial. Polito et al. [3] have reported that pediatric CD patients have the disease in the small intestines more often than older patients do. Others have reported that there are no definite differences in anatomic localization of the disease between children and adults [5, 7]. CD in very young children (≤5 years old) tends to have only colonic involvement [31]. The present study did not show any definite difference between pediatric-onset and adult-onset patients in the anatomical distribution of the disease.

Most adult studies with a large number of subjects have shown that 14–37% of patients with UC have total colitis, whereas 44–49% have proctosigmoiditis [25, 32]. In a recent large series of Finnish children, 61.2% of those with UC had total colitis [14]. Studies in the UK [7] and US [8]

have revealed that, in approximately 90% of pediatric UC patients, the entire colon or the portion beyond the splenic flexure was involved. These results suggest that children have more widespread colonic involvement in UC than adults do. In the study by Langholz et al. [1], in which pediatric and adult data were compared, although total colitis in children was less frequent (29%) than that described above, it was still significantly more frequent than that in adults (16%). Our data showed a similar trend that extensive colitis (E3) was more common in children than in adults, whereas left-sided colitis (E2) and proctitis (E1) were less common.

CD varies in its severity in adults and in children, although no studies have compared severity between age groups. In the present study, we found that the mean IOIBD score at initial diagnosis was slightly higher in children than in adults. It must also be noted that some problems specific to children, such as growth failure, are not included in the IOIBD score. Further prospective investigation that involves both adults and children is warranted.

In a small series of pediatric UC patients reported by Gryboski et al. [33], 11% had severe, 37% moderate and 53% mild disease. In 171 patients reported by Hyams et al. [34], 57% had moderate or severe, and 43% had moderate disease. These data were not compared with those of adult patients.

In the present study, moderate, severe and fulminant UC were more frequent among children, while mild disease was less frequent in children than in adults. These results were consistent with the fact that the disease severity in UC correlates with disease extent [25], and children with UC tend to have more extensive disease than adults.

Our data should be carefully interpreted regarding disease severity, which changes with time, either because of natural history or treatment. In this study, severity was determined based on the data at first registration. As mentioned above, most patients were registered soon after a diagnosis was made. However, diagnosis of IBD in children can be delayed because of the much lower incidence in that population, which might cause some increase in disease severity at the first registration.

In summary, incidence and prevalence of CD and UC are lower in children than in adults. The effect of gender on the incidence or prevalence of CD, but not of UC, differs with age. A family history for the disease in question is more common in children than in adults. Disease severity is greater in CD and UC in children than in adults. UC in childhood-onset patients results in more extensive disease than is the case for adult-onset patients. In conclusion, this nationwide surveillance in Japan showed that CD and UC in children have clinical features that are distinct from those in adults.

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Conflict of interest statement None.

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IL-2 is positively involved in the development of colitogenic CD4$^+$ IL-7Rα^{high} memory T cells in chronic colitis

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IL-2 and IL-7 share a common γ-chain receptor and are critical for T-cell homeostasis. We aimed to clarify the reciprocal roles of IL-2 and IL-7 in the development and persistence of chronic colitis. We performed a series of adoptive transfers of IL-2$^{-/-}$ CD4$^+$CD45RB^{high} T cells into RAG-2$^{-/-}$ mice and assessed the role of IL-2 in the induction of IL-7Rα on colitogenic CD4$^+$ T cells and the development of chronic colitis. RAG-2$^{-/-}$ mice transferred with WT but not with IL-2$^{-/-}$ CD4$^+$CD45RB^{high} T cells developed Th1/Th17-mediated colitis. Consistently, re-expression of IL-7Rα was severely impaired on IL-2$^{-/-}$ but not on WT CD4$^+$ T cells from the transferred mice. To exclude a contribution of the preclinical autoimmunity of IL-2$^{-/-}$mice, WT Ly5.1$^+$ or IL-2$^{-/-}$ Ly5.2$^+$ CD4$^+$CD45RB^{high} T cells from GFP mice previously transplanted with the same number of WT and IL-2$^{-/-}$ BM cells were transferred into RAG-2$^{-/-}$ mice. RAG-2$^{-/-}$ mice transferred with IL-2$^{-/-}$-derived CD4$^+$ CD45RB^{high} T cells did not develop colitis, but their splenic CD4$^+$ T cells changed from effector-memory to central-memory type. These results show that IL-2 is critically involved in the establishment and maintenance of IL-7-dependent colitogenic memory CD4$^+$IL-7Rα^{high} T cells.

Key words: Animal models · CD4$^+$ T cell · Cytokines · Memory cell · Mucosal immunity

Introduction

The inflammatory bowel diseases (IBD), Crohn's disease and ulcerative colitis, are caused by chronic inflammatory responses in the gut wall [1–3]. Although the aetiology of IBD is uncertain, there is much evidence suggesting that the pathogenesis of IBD involves dysregulated recognition of intestinal bacterial antigens, resulting in the generation of colitogenic CD4$^+$ effector and memory T cells [4–10]. However, how colitogenic CD4$^+$ T cells are generated and maintained in patients with IBD remains unknown.

During T-cell priming and maintenance of colitogenic CD4$^+$ effector and memory T cells, cytokines may provide critical signals. IL-2 is produced by activated T cells early after antigenic stimulation and is essential for proliferation of T cells in the effector phase, at least *in vitro* [11, 12]. More recently, it has been shown that exposure to IL-2 in the effector phase is required for successful long-term survival of CD4$^+$ T cells and their

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differentiation into memory T cells [13]. Interestingly, IL-2 does not seem to be needed for T-cell proliferation *in vivo* because IL-$2^{-/-}$ and IL-$2R\alpha^{-/-}$ mice develop autoimmune diseases, including chronic colitis, and extensive proliferation of T cells [14, 15]. Thus, the development of such diseases *in vivo* is explained entirely by the lack of $CD4^+CD25^+Foxp3^+$ Treg cells, which are dependent on IL-2 for their development and maintenance [16–18].

In contrast to IL-2, IL-7 is produced not by lymphocytes but by stromal cells in the BM and thymus and by epithelial cells [19–21]. This cytokine is important for supporting the survival of naïve and memory $CD4^+$ T cells, but not that of effector $CD4^+$ T cells [21–23]. Our previous studies on the pathogenesis of IBD demonstrated that: (i) IL-7 is constitutively produced by intestinal goblet epithelial cells [20], (ii) IL-7 transgenic mice developed chronic colitis [24], (iii) mucosal $CD4^+$IL-$7R\alpha^{high}$ T cells in $CD4^+CD45RB^{high}$ T-cell-transferred colitic mice are colitogenic [25, 26], and (iv) IL-7 is essential for the persistence of colitis because IL-$7^{-/-}$ × RAG-$1^{-/-}$ mice transferred with colitogenic $CD4^+$ T cells did not develop colitis [27].

IL-2 and IL-7 share a common γ-chain receptor and are critical cytokines for T-cell homeostasis [21, 22]. To clarify the reciprocal roles of IL-2 and IL-7 in the development and persistence of chronic colitis, we performed a series of adoptive transfers of WT or IL-$2^{-/-}$ $CD4^+CD45RB^{high}$ T cells into RAG-$2^{-/-}$ mice.

Results

IL-$2^{-/-}$ mice sustain substantial numbers of naïve $CD4^+$ T cells that have reduced levels of IL-7Rα

IL-$2^{-/-}$ mice develop spontaneous autoimmune syndrome from 4–6 wk of age and develop IBD at 10–12 wk of age [14]. We first analysed the phenotypic characteristics of $CD4^+$ T cells of young (3.5-wk-old) IL-$2^{-/-}$ and WT mice. The proportions of $CD4^+$ $CD44^{high}$ or $CD45RB^{low}$ memory T cells and $CD4^+CD44^{low}$ or $CD45RB^{high}$ naïve T cells in the spleen (SP) of IL-$2^{-/-}$ mice were reciprocally higher or lower, respectively, than those of the paired WT mice (Fig. 1A and B). However, it is noteworthy that naïve T cells were detected in the SP of young IL-$2^{-/-}$ mice at this stage, indicating that continuous generation of naïve T cells occurs in these mice. As IL-2 is essential for the development and maintenance of $CD4^+CD25^+Foxp3^+$ Treg [17], expression of CD25 and Foxp3 on/in SP $CD4^+$ T cells in IL-$2^{-/-}$ mice was markedly impaired compared with that in WT mice (Fig. 1A and B). In contrast, SP $CD4^+CD45RB^{high}$ populations from IL-$2^{-/-}$ and WT mice did not contain Treg (Fig. 1B). Interestingly, the positive frequency of IL-7Rα expression on SP $CD4^+$ T cells in IL-$2^{-/-}$ mice significantly reduced, which is in sharp contrast to that in WT mice (Fig. 1A and C). This suggests that SP $CD4^+$ T cells in IL-$2^{-/-}$ mice include a substantial number of effector T cells at a young age despite the absence of clinical manifestations or impaired naïve or memory T cells. Surprisingly, we found that the positive frequency of IL-7Rα expression on the SP $CD4^+$ $CD45RB^{high}$ naïve T cells of IL-$2^{-/-}$ mice was also significantly reduced compared with that of WT mice (Fig. 1B and D), suggesting that IL-2 is involved in the development and maintenance of $CD4^+$ naïve T cells (Fig. 1B). This was also the case with the SP $CD4^+CD45RB^{low}$ T cells of IL-$2^{-/-}$ mice, which may be explained by an increased number of effector T cells and/or impaired development of memory T cells.

RAG-$2^{-/-}$ mice transferred with IL-$2^{-/-}$ $CD4^+$ CD45 Rb^{high} T cells do not develop colitis

Since the positive frequency of IL-7Rα expression on IL-$2^{-/-}$ $CD4^+CD45RB^{high}$ T donor cells reduced (Fig. 1), the possibility remained that the impaired naïve $CD4^+$ T cells themselves were critically involved in the development of spontaneous colitis in IL-$2^{-/-}$ mice. To first assess the effect of IL-2 deficiency on the initial developmental process of colitis, $CD4^+CD45RB^{high}$ T cells from young WT or non-colitic IL-$2^{-/-}$ mice were injected intraperitoneally into RAG-$2^{-/-}$mice (Fig. 2A). As a negative control, RAG-$2^{-/-}$ mice were transferred with WT $CD4^+$ $CD45RB^{high}$ T cells and WT $CD4^+CD25^+$ Treg (Fig. 2A). Consistent with previous report [27], the RAG-$2^{-/-}$ mice transferred with WT $CD4^+CD45RB^{high}$ T cells manifested weight loss from 5 wk after transfer (Fig. 2B). Clinical symptoms of colitis as shown by clinical scores were apparent 7 wk after transfer (Fig. 2D). In contrast, RAG-$2^{-/-}$ mice transferred with WT $CD4^+CD45RB^{high}$ T cells and Treg showed no weight loss or clinical symptoms of colitis (Fig. 2B and D). However, RAG-$2^{-/-}$ mice transferred with IL-$2^{-/-}$ $CD4^+CD45RB^{high}$ T cells did not show wasting (Fig. 2B) or clinical symptoms of colitis throughout the observation period of 7 wk after transfer (Fig. 2D). Overall, the assessment of colitis according to clinical score showed a clear difference between RAG-$2^{-/-}$ mice transferred with IL-$2^{-/-}$ and those transferred with WT $CD4^+CD45RB^{high}$ T cells (Fig. 2D). At 7 wk after transfer, the colon of RAG-$2^{-/-}$ mice transferred with WT $CD4^+CD45RB^{high}$ T cells, but not with IL-$2^{-/-}$ $CD4^+$ $CD45RB^{high}$ T cells or a combination of WT $CD4^+CD45RB^{high}$ and Treg, was enlarged and had a greatly thickened wall (Fig. 2C). Enlargement of the SP was also present in RAG-$2^{-/-}$ mice transferred with WT $CD4^+CD45RB^{high}$ T cells but not in other groups (Fig. 2C).

Histological examination showed a massive infiltration of mononuclear cells in the lamina propria (LP) of the colon of RAG-$2^{-/-}$ mice transferred with WT $CD4^+CD45RB^{high}$ T cells (Fig. 2E). In contrast, the inflammation was mostly abrogated and only a few mononuclear cells were observed in the LP of the colon of RAG-$2^{-/-}$ mice transferred with IL-$2^{-/-}$ $CD4^+$ $CD45RB^{high}$ T cells as well as in RAG-$2^{-/-}$ mice transferred with WT $CD4^+CD45RB^{high}$ T cells and Treg (Fig. 2E). This difference was confirmed by histological scoring of colon sections (Fig. 2F). Further quantitative evaluation of LP $CD4^+$ T-cell infiltration was done using flow cytometry (Fig. 2G). The number of LP $CD4^+$ cells recovered from the colon of RAG-$2^{-/-}$ mice transferred with WT $CD4^+CD45RB^{high}$ T cells far exceeded the number originally

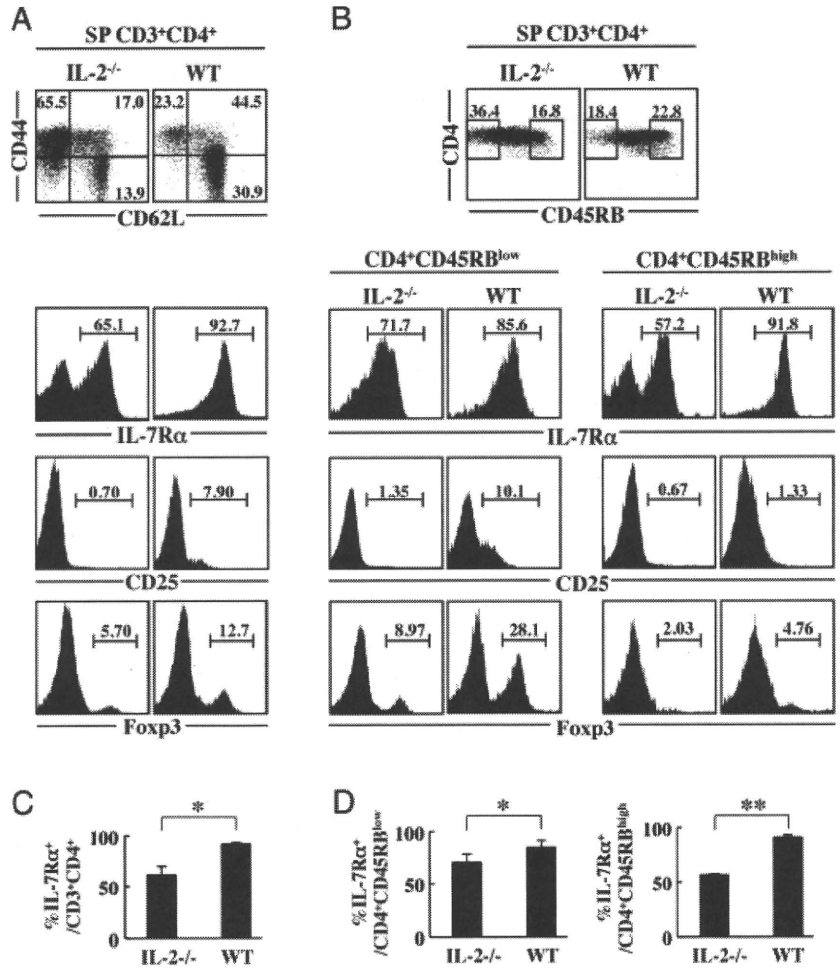

Figure 1. Phenotypic characteristics of IL-2$^{-/-}$ CD4$^+$CD45RB^{high} T cells. (A) Identification and characterization of SP CD4$^+$ T cells in young IL-2$^{-/-}$ and WT mice. FACS analysis shows the expression of CD44 and CD62L, IL-7Rα, CD25, or Foxp3 on/in SP CD4$^+$ T cells. (B) Characterization of SP CD4$^+$CD45RB^{high} and CD45RB^{low} T cells in young IL-2$^{-/-}$ and WT mice. (C) The percentage of IL-7Rα$^+$ cells in SP CD3$^+$CD4$^+$ T cells was determined using a FACSCalibur. (D) The percentage of IL-7Rα$^+$ cells in the CD4$^+$CD45RB^{low} or CD45RB^{high} T-cell populations was determined using a FACSCalibur. Data are representative of six mice *per* group. (A and B) or show mean+SEM (C and D; $n = 6$ *per* group). $^*p = 0.011$ (Mann-Whitney *U*) and $^{**}p = 0.011$ (Student's *t*).

injected, indicating extensive T-cell proliferation and survival in the inflamed colon, which was mostly abrogated in RAG-2$^{-/-}$ mice transferred with WT CD4$^+$CD45RB^{high} T cells and Treg or RAG-2$^{-/-}$ mice transferred with IL-2$^{-/-}$ CD4$^+$CD45RB^{high} T cells (Fig. 2G).

As shown in Fig. 1B, however, there were two populations of IL-2$^{-/-}$CD4$^+$CD45RB^{high} T cells, IL-7Rα^{high} and IL-7Rα^{low}. Thus, it is possible that IL-7Rα^{low} cells suppress IL-7Rα^{high} cells when they are transferred into RAG-2$^{-/-}$ mice as IL-2$^{-/-}$CD4$^+$ CD45RB^{high} T cells. To rule out this possibility, we performed another *in vivo* experiment. CD4$^+$CD45RB^{high} T cells from the SP of 4- to 5-wk-old IL-2$^{-/-}$ mice were divided into two populations, IL-7Rα^{high} and IL-7Rα^{low} by cell sorting, and each population was separately transferred into RAG-2$^{-/-}$ hosts. As a positive control, a same number of WT CD4$^+$CD45RB^{high} T cells were again transferred into RAG-2$^{-/-}$ mice (Fig. 3A). Mice transferred with IL-7Rα^{high} or IL-7Rα^{low} did not develop clinical or histological aspects of colitis, whereas mice transferred with WT CD4$^+$ CD45RB^{high} T cells did develop severe colitis (Figs. 3B–D). However, a proportion of IL-7Rα^{high} IL-2$^{-/-}$CD4$^+$CD45RB^{high} T cells converted to IL-7Rα^{low} and *vice versa* (data not shown) after they were transferred to RAG-2$^{-/-}$ mice, which shows that the expression of IL-7Rα on IL-2$^{-/-}$CD4$^+$CD45RB^{high} T cells is flexible, rather than fixed on naïve cells.

To determine the effect of these transfers on Th1/Th17 development, we next measured IFN-γ and IL-17 production by anti-CD3/CD28-stimulated CD4$^+$ LP T cells. As shown in Fig. 4A, the production of IFN-γ and IL-17 by anti-CD3/CD28-stimulated CD4$^+$ LP T cells from RAG-2$^{-/-}$ mice transferred with IL-2$^{-/-}$ CD4$^+$CD45RB^{high} T cells or from RAG-2$^{-/-}$ mice transferred with WT CD4$^+$CD45RB^{high} T cells and Treg was significantly lower than that from RAG-2$^{-/-}$ mice transferred with WT CD4$^+$ CD45RB^{high} T cells. To assess the cell surface markers on isolated SP and LP cells in each group, we then performed flow cytometric analysis. As shown in Fig. 4B, the transferred IL-2$^{-/-}$ SP and LP CD4$^+$ T cells may differentiate into CD44^{high} memory T cells in

the absence of colitis, as was the case with the paired $CD4^+$ T cells in colitic RAG-$2^{-/-}$ mice transferred with WT $CD4^+$ $CD45RB^{high}$ T cells and non-colitic RAG-$2^{-/-}$ mice transferred with WT $CD4^+CD45RB^{high}$ T cells and WT Treg. It is noteworthy that the percentages of the central memory type of $CD44^{high}CD62L^+$ T cells (T_{CM}) in the SP (Fig. 4B and C, left) and

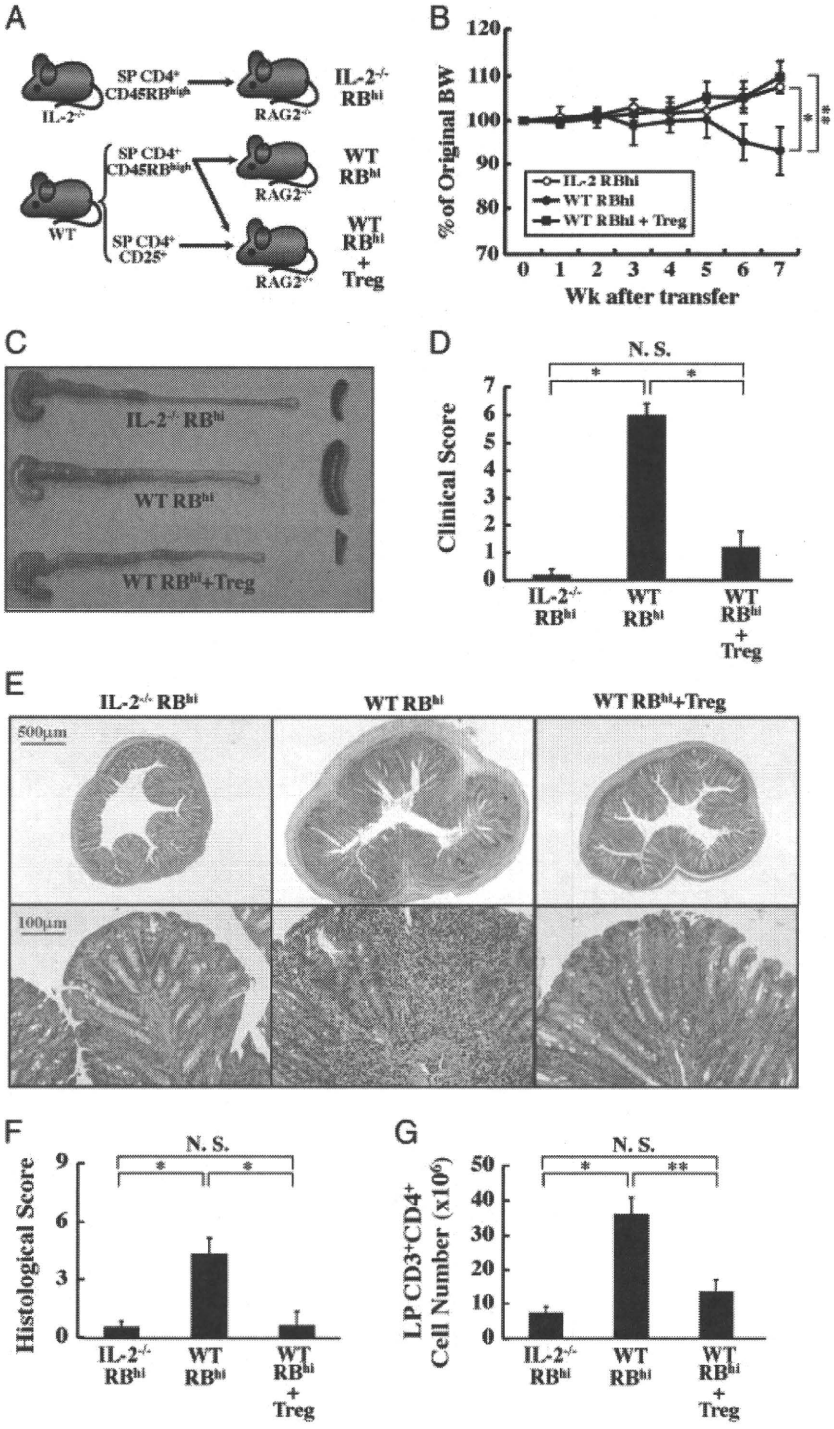

Figure 2. RAG-$2^{-/-}$ mice transferred with IL-$2^{-/-}$ $CD4^+CD45RB^{high}$ T cells do not develop colitis. We performed two independent experiments. (A) Experimental design. RAG-$2^{-/-}$ mice were transferred with SP WT or IL-$2^{-/-}$ $CD4^+CD45RB^{high}$ T cells (3×10^5 cells *per* mouse) or a mixture of WT $CD4^+$ $CD45RB^{high}$ T cells (3×10^5) and WT $CD4^+CD25^+$ Treg (1×10^5). (B) The change in body weight over time is expressed as a percentage of the original weight. $^*p = 0.049$ (Welch's *t*), and $^{**}p = 0.049$ (Student's *t*). (C) Gross appearance of the colon and spleen. (D) Clinical scores. Data are expressed as the mean ± SEM of six mice *per* group. $^*p = 0.014$ (Mann-Whitney *U*.) (E) Histological results for the colons of each group. Original magnification, × 40 (upper) and × 200 (lower). (F) Histological scoring. $^*p = 0.014$ (Mann-Whitney *U*). (G) The number of $CD4^+CD3^+$ cells in colonic LP for each group. Cell number was determined using FACS. $^*p = 0.0049$ (Welch's *t*), and $^{**}p = 0.014$ (Mann-Whitney *U*). Data are expressed as the mean ± SEM of eight mice *per* group.

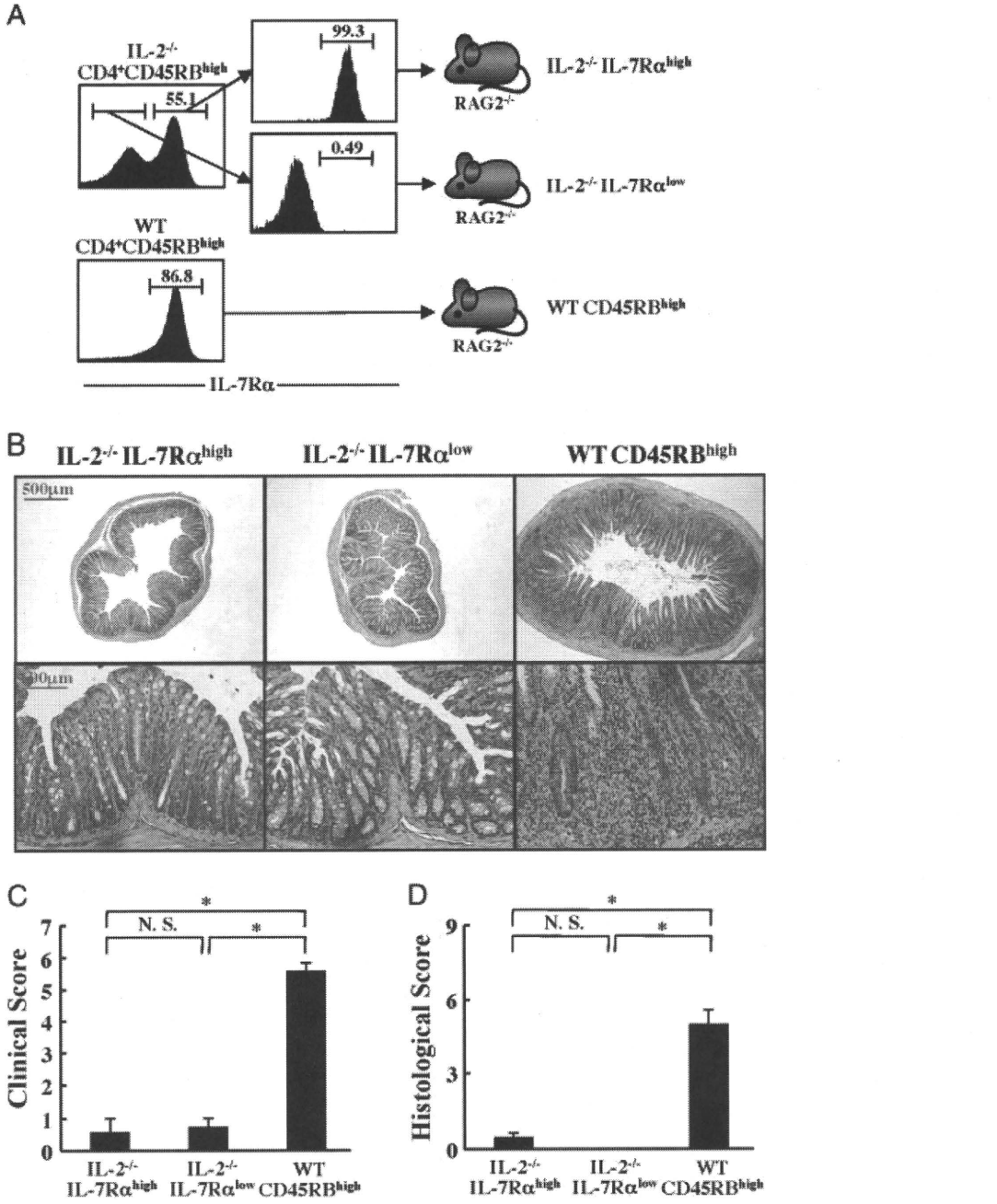

Figure 3. Either RAG-$2^{-/-}$ mice transferred with IL-$2^{-/-}$ $CD4^+CD45RB^{high}IL\text{-}7R\alpha^{high}$ T cells or those transferred with IL-$2^{-/-}$ $CD4^+CD45RB^{high}$ $IL\text{-}7R\alpha^{kow}$ T cells do not develop colitis. We performed one independent experiment. (A) Experimental design. RAG-$2^{-/-}$ mice were transferred with $IL\text{-}7R\alpha^{high}CD4^+CD45RB^{high}$ T cells (3×10^5) or $IL\text{-}7R\alpha^{low}CD4^+CD45RB^{high}$ T cells (3×10^5) from the spleen of IL-$2^{-/-}$ mice. As a positive control, we transferred the same number of WT $CD4^+CD45RB^{high}$ T cells into RAG-$2^{-/-}$ mice. (B) Histological results for the colons of each group. Original magnification, × 40 (upper) and × 200 (lower). (C) Clinical scores. (D) Histological scoring. Data are expressed as the mean ± SEM of six (C) and five (D) mice *per* group. N.S., not significant. *$p = 0.014$ (Mann-Whitney *U*). N.S., not significant.

MLN (data not shown) IL-$2^{-/-}$ $CD4^+$ T cells were significantly higher than those in the SP and MLN of colitic RAG-$2^{-/-}$ mice transferred with WT $CD4^+CD45RB^{high}$ T cells or non-colitic RAG-$2^{-/-}$ mice transferred with WT $CD4^+CD45RB^{high}$ T cells and WT Treg. In contrast, LP $CD4^+$ T cells in all groups exhibited characteristics of effector-memory type of $CD44^{high}CD62L^-$ (T_{EM}) cells, which is consistent with the non-lymphoid nature of the LP (Fig. 4B and C, right) [28, 29]. Furthermore, the positive frequency of IL-7Rα expression on SP and LP IL-$2^{-/-}$ $CD4^+$ T cells also significantly reduced, as compared with the paired cells from colitic RAG-$2^{-/-}$ mice transferred with WT $CD4^+$ $CD45RB^{high}$ T cells or non-colitic RAG-$2^{-/-}$ mice transferred with

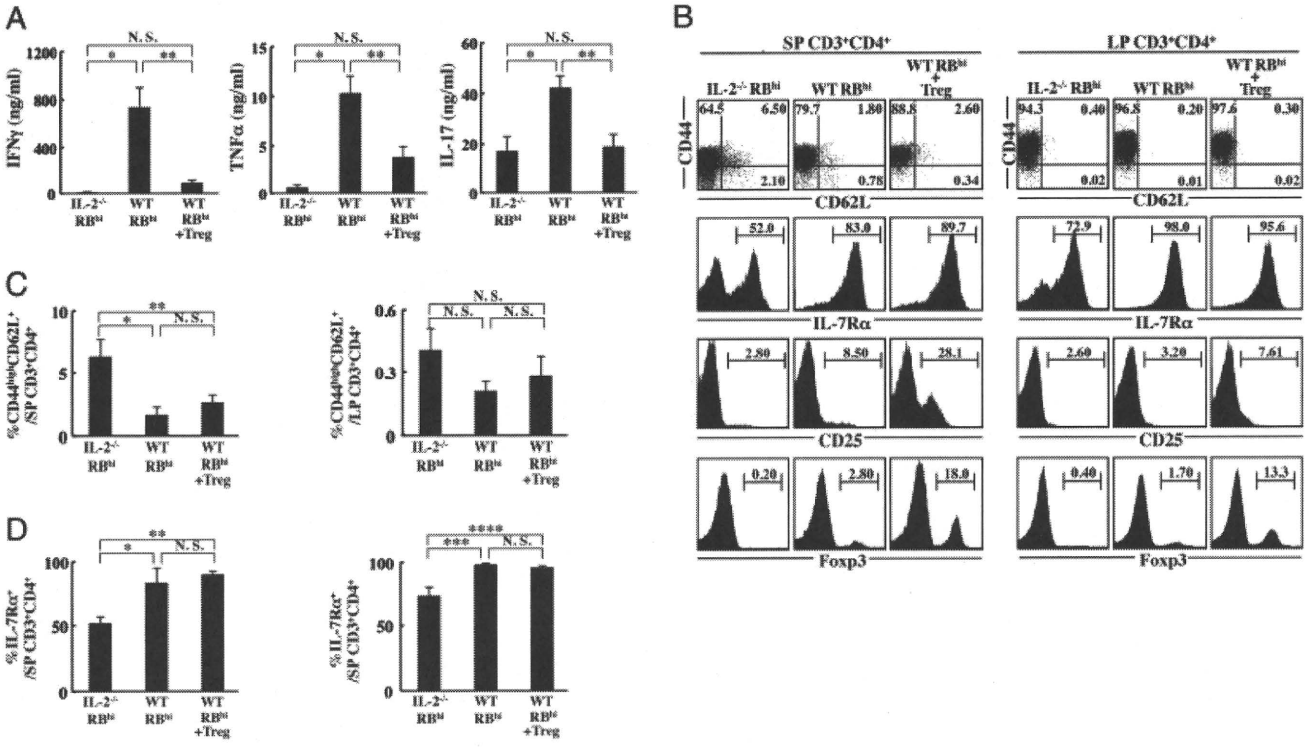

Figure 4. IL-7Rα expression of memory CD4$^+$ T cells in IL-2$^{-/-}$ CD4$^+$CD45RB^{high} T-cell-transferred RAG-2$^{-/-}$ mice was impaired. We performed two independent experiments. (A) Cytokine production by LP CD4$^+$ T cells. LP CD4$^+$ T cells were stimulated with plate-coated anti-CD3 mAb and soluble anti-CD28 mAb for 48 h. Cytokine concentration in the supernatants were measured using ELISA. Data are expressed as the mean ± SEM of eight mice *per* group. *$p = 0.021$ (Mann-Whitney *U*), and **$p = 0.014$ (Mann-Whitney *U*). (B) Expression of various cell surface markers on SP and LP CD3$^+$CD4$^+$ T cells was determined by FACS. Representative results from eight mice *per* group for CD44, CD62L, IL-7Rα, CD25, and Foxp3 are shown. (C) The percentage of T_{CM} cells was determined using FACS. Data are expressed as the mean ± SEM of eight mice. *$p = 0.0001$ (Student's *t*), **$p = 0.047$ (Student's *t*), and N.S., not significant. (D) The percentage of CD3$^+$CD4$^+$ T cells positive for IL-7Rα$^+$ is shown. Data are expressed as the mean ± SEM of eight mice *per* group. *$p = 0.0001$ (Student's *t*). **$p = 0.0009$ (Mann-Whitney *U*), ***$p = 0.001$ (Mann-Whitney *U*), ****$p = 0.017$ (Mann-Whitney *U*), and N.S., not significant.

WT CD4$^+$CD45RB^{high} T cells and WT Treg (Fig. 4B and D). In addition, CD4$^+$CD25$^+$Foxp3$^+$ Treg did not emerge in the SP or LP of RAG-2$^{-/-}$ mice transferred with IL-2$^{-/-}$ CD4$^+$CD45RB^{high} T cells, which is in contrast to RAG-2$^{-/-}$ mice transferred with WT CD4$^+$CD45RB^{high} T cells, which contained a substantial number of inducible Treg (Fig. 4B), albeit fewer than RAG-2$^{-/-}$ mice transferred with WT CD4$^+$CD45RB^{high} T cells and WT Treg, which contained a mixture of naturally occurring and inducible Treg (Fig. 4B).

Paracrine IL-2 from WT CD4$^+$ T cells enables IL-2$^{-/-}$ LP CD4$^+$ T cells to induce mild colitis in an IL-7-dependent manner

To further assess the role of IL-2 signalling in the expansion of CD4$^+$ donor cells, we performed *in vivo* competition experiments. First, the same number of CD4$^+$CD45RB^{high} donor cells from Ly5.1$^+$ WT and Ly5.2$^+$ IL-2$^{-/-}$ mice were co-injected intraperitoneally into RAG-2$^{-/-}$ mice (Fig. 5A). As expected, recipient mice developed severe colitis 6 wk after co-transfer (data not shown), and a significantly lower proportion of Ly5.2$^+$ IL-2$^{-/-}$ CD4$^+$ T cells were observed in the inflamed LP and SP compared with the paired Ly5.1$^+$ WT CD4$^+$ T cells (Fig. 5B). Furthermore, the positive frequency of IL-7Rα expression on IL-2$^{-/-}$ LP CD4$^+$ cells was markedly reduced, as compared with that on WT LP CD4$^+$ cells (Fig. 5C).

We next addressed the question of whether LP IL-2$^{-/-}$ CD4$^+$ T cells sustained in colitic RAG-2$^{-/-}$ mice transferred with a mixture of WT and IL-2$^{-/-}$ CD4$^+$CD45RB^{high} T cells (Fig. 5A) have the potential to induce colitis when transferred to new RAG-1$^{-/-}$ mice, because it was considered possible that a small but substantial number of IL-2$^{-/-}$ LP CD4$^+$ T cells in those mice (Fig. 5) would gain colitogenicity by using paracrine IL-2 from surrounding WT LP CD4$^+$ T cells. If they were colitogenic, it would also be necessary to examine whether IL-7 is also needed for the development of colitis by those cells to assess whether they are effector or memory T cells (Fig. 5A), as is the case with colitic WT LP CD4$^+$ T cells as previously demonstrated by our group [27]. To this end, we isolated LP WT (Ly5.1$^+$) and IL-2$^{-/-}$ (Ly5.2$^+$) CD4$^+$ T cells from colitic RAG-2$^{-/-}$ mice previously transferred with the same number of WT and IL-2$^{-/-}$ CD4$^+$ CD45RB^{high} T cells and then separately retransferred them into new IL-7$^{+/+}$ × RAG-1$^{-/-}$ or IL-7$^{-/-}$ × RAG-1$^{-/-}$mice (Fig. 5A). As expected, IL-7$^{+/+}$ × RAG-1$^{-/-}$ recipients transferred with WT CD4$^+$ T cells (WT → IL-7$^{+/+}$) developed severe colitis 4–6 wk

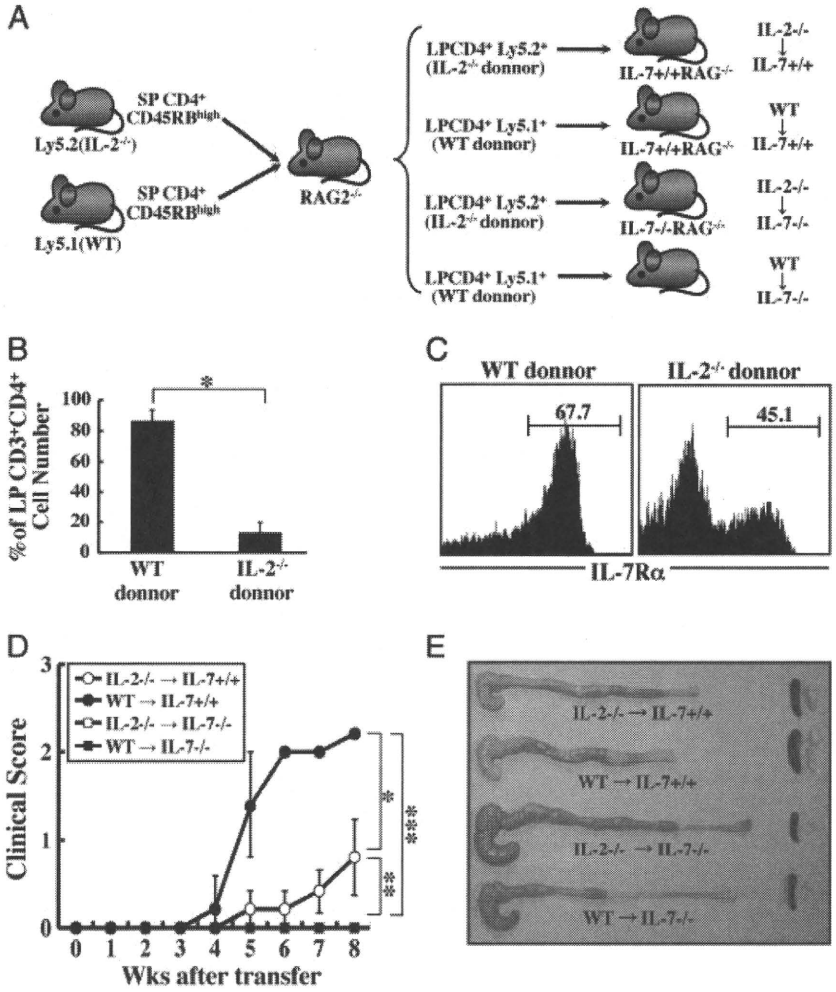

Figure 5. IL-7 is essential for the development of colitis in mice transferred with IL-2$^{-/-}$ LP CD4$^+$ T cells obtained from colitic mice previously transferred with a mixture of WT and IL-2$^{-/-}$ CD4$^+$CD45RB^{high} T cells. We performed one independent experiment. (A) Experimental design. RAG-2$^{-/-}$ mice were co-injected i.p. with a mixture of WT (Ly5.1$^+$) and IL-2$^{-/-}$ (Ly5.2$^+$) CD4$^+$CD45RB^{high} T cells. Eight weeks after transfer, WT and IL-2$^{-/-}$ LP CD4$^+$ T cells were isolated and separately retransferred into IL-7$^{+/+}$ × RAG-1$^{-/-}$ or IL-7$^{-/-}$ × RAG-1$^{-/-}$ mice. (B) Numbers of WT (Ly5.1$^+$) and IL-2$^{-/-}$ (Ly5.2$^+$) LP CD3$^+$CD4$^+$ T cells recovered from colitic RAG-2$^{-/-}$ donor mice. Data are expressed as the mean ± SEM of five mice *per* group. *$p = 0.049$ (Mann-Whitney *U*). (C) Expression of IL-7Rα on SP WT and IL-2$^{-/-}$ CD4$^+$ T cells of colitic RAG-2$^{-/-}$ donor mice was determined using FACS. (D) Clinical score after retransfer into new IL-7$^{+/+}$ × RAG-1$^{-/-}$ or IL-7$^{-/-}$ × RAG-1$^{-/-}$ mice. Data are expressed as the mean ± SEM of five mice *per* group. *$p = 0.025$, **$p = 0.034$, ***$p = 0.034$ (Mann-Whitney *U*). (E) Gross appearance of the colon, SP, and MLN 8 wk after retransfer.

after transfer, which was characterized by significant weight loss, diarrhoea, higher total clinical scores (Fig. 5D), and thickening of the colonic wall with inflammation (Fig. 5E). In contrast, IL-7$^{-/-}$ × RAG-1$^{-/-}$ recipients transferred with WT CD4$^+$ T cells (WT → IL-7$^{-/-}$) appeared healthy, exhibited no signs of colitis until 8 wk after transfer (Fig. 5D), and exhibited no apparent thickening of the colonic wall (Fig. 5E). As expected, IL-7$^{-/-}$ × RAG-1$^{-/-}$ recipients transferred with IL-2$^{-/-}$ CD4$^+$ T cells (IL-2$^{-/-}$ → IL-7$^{-/-}$) did not develop colitis. However, IL-7$^{+/+}$ × RAG-1$^{-/-}$ recipients transferred with IL-2$^{-/-}$ CD4$^+$ T cells (IL-2$^{-/-}$ → IL-7$^{+/+}$) exhibited clinical signs of colitis and a thickened colonic wall (Fig. 5E) 8 wk after transfer, albeit less severely than WT → IL-7$^{+/+}$ mice (Fig. 5E).

Histological examinations revealed no evident pathological changes in the colons of WT → IL-7$^{-/-}$ or IL-2$^{-/-}$ → IL-7$^{-/-}$ mice in contrast to colitic WT → IL-7$^{+/+}$ or IL-2$^{-/-}$ → IL-7$^{+/+}$ mice, which showed prominent epithelial hyperplasia with massive infiltration of mononuclear cells (Fig. 6A). This was confirmed by assessing each histological score (Fig. 6B). Furthermore, the score of IL-2$^{-/-}$ → IL-7$^{+/+}$ mice was significantly less than that of WT → IL-7$^{+/+}$ mice (Fig. 6B). Consistent with this, the average number of LP CD4$^+$ T cells recovered from colitic IL-2$^{-/-}$ → IL-7$^{+/+}$ mice was significantly less than that of WT → IL-7$^{+/+}$ mice (Fig. 6C). The number of LP CD4$^+$ cells in IL-2$^{-/-}$ → IL-7$^{-/-}$ or WT → IL-7$^{-/-}$ mice was almost zero (Fig. 6C). Furthermore, the positive frequency of IL-7Rα expression on SP CD4$^+$ T cells obtained from IL-2$^{-/-}$ CD4$^+$ T-cell-transferred mice was markedly reduced in both IL-7$^{+/+}$ and IL-7$^{-/-}$ recipients, whereas that from WT CD4$^+$ T-cell-transferred mice was not impaired in IL-7$^{+/+}$ or IL-7$^{-/-}$ recipients (Fig. 6D).

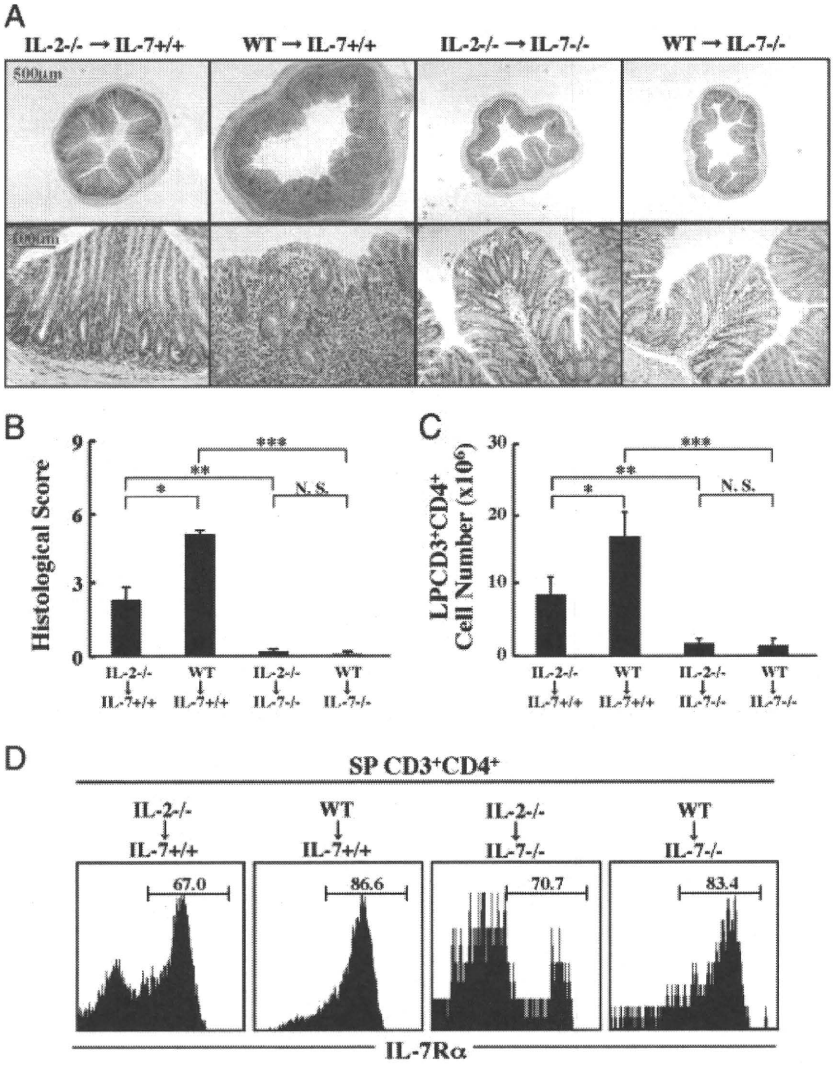

Figure 6. IL-7 is absolutely essential for the development of colitis in mice transferred with IL-$2^{-/-}$ LP $CD4^+$ T cells despite down-modulated IL-7Rα expression. We performed one independent experiment. (A) Histopathology of the distal colon 8 wk after retransfer. Original magnification, × 40 (upper) and × 200 (lower). (B) Histological score 8 wk after retransfer. Data are expressed as the mean ± SEM of five mice *per* group. $^*p = 0.009$, $^{**}p = 0.016$, $^{***}p = 0.009$ (Mann-Whitney *U*). (C) Number of LP $CD3^+CD4^+$ T cells recovered 8 wk after retransfer. Data are expressed as the mean ± SEM of five mice *per* group. $^*p = 0.049$ (Student's *t*), $^{**}p = 0.033$ (Welch's *t*), and $^{***}p = 0.014$ (Mann-Whitney *U*). (D) Expression of IL-7Rα on SP $CD3^+CD4^+$ T cells was determined using FACS. Representative FACS data of five mice are shown.

IL-$2^{-/-}$ $CD4^+CD45\ RB^{high}$ T cells do not induce colitis, even if they were developed in IL – $2^{-/-}$ and WT BM chimaeric mice

To precisely prepare similar naïve WT and IL-$2^{-/-}$ $CD4^+$ $CD45RB^{high}$ T cells in the same *in vivo* setting, we generated mixed BM chimeras. To this end, irradiated GFP mice were first transplanted with a mixture of the same number of BM cells obtained from young WT and IL-$2^{-/-}$ mice (Fig. 7A). Six weeks after the BM transplantation, various cell markers were compared between the transplanted WT and IL-$2^{-/-}$ BM-derived GFP^- $CD4^+$ cells. As shown in Fig. 6B, almost all the cell markers, including IL-7Rα, were identical in the transplanted WT and IL-$2^{-/-}$ BM-derived $CD4^+$ cells. Thus, GFP^- $Ly5.1^+$ WT and $Ly5.2^+$ IL-$2^{-/-}$ $CD4^+CD45RB^{high}$ T cells were isolated, and then the separated cells or a mixture of the same number of the two cell types was transferred into RAG-$2^{-/-}$ mice. Again, we found that RAG-$2^{-/-}$ mice transferred with GFP^- $Ly5.1^+$ WT $CD4^+$ $CD45RB^{high}$ T cells or a mixture of GFP^- $Ly5.1^+$ WT and $Ly5.2^+$ IL-$2^{-/-}$ $CD4^+CD45RB^{high}$ T cells showed similar signs of wasting disease (Fig. 7B), clinical symptoms (Fig. 7D), and clinical and histological scores (Fig. 7E and F) 8 wk after transfer, which differs from the findings for non-colitic RAG-$2^{-/-}$ mice transferred with GFP^- $Ly5.2^+$ IL-$2^{-/-}$ $CD4^+CD45RB^{high}$ T cells (Fig. 7C–F). The number of LP $CD4^+$ T cells recovered from RAG-$2^{-/-}$ mice transferred with GFP^- $Ly5.1^+$ WT $CD4^+CD45RB^{high}$ T cells or a mixture of GFP^- $Ly5.1^+$ WT and $Ly5.2^+$ IL-$2^{-/-}$ $CD4^+CD45RB^{high}$ T cells was significantly higher than that recovered from mice transferred with $Ly5.2^+$ IL-$2^{-/-}$ $CD4^+CD45RB^{high}$ T cells (Fig. 8A). The production of IFN-γ,

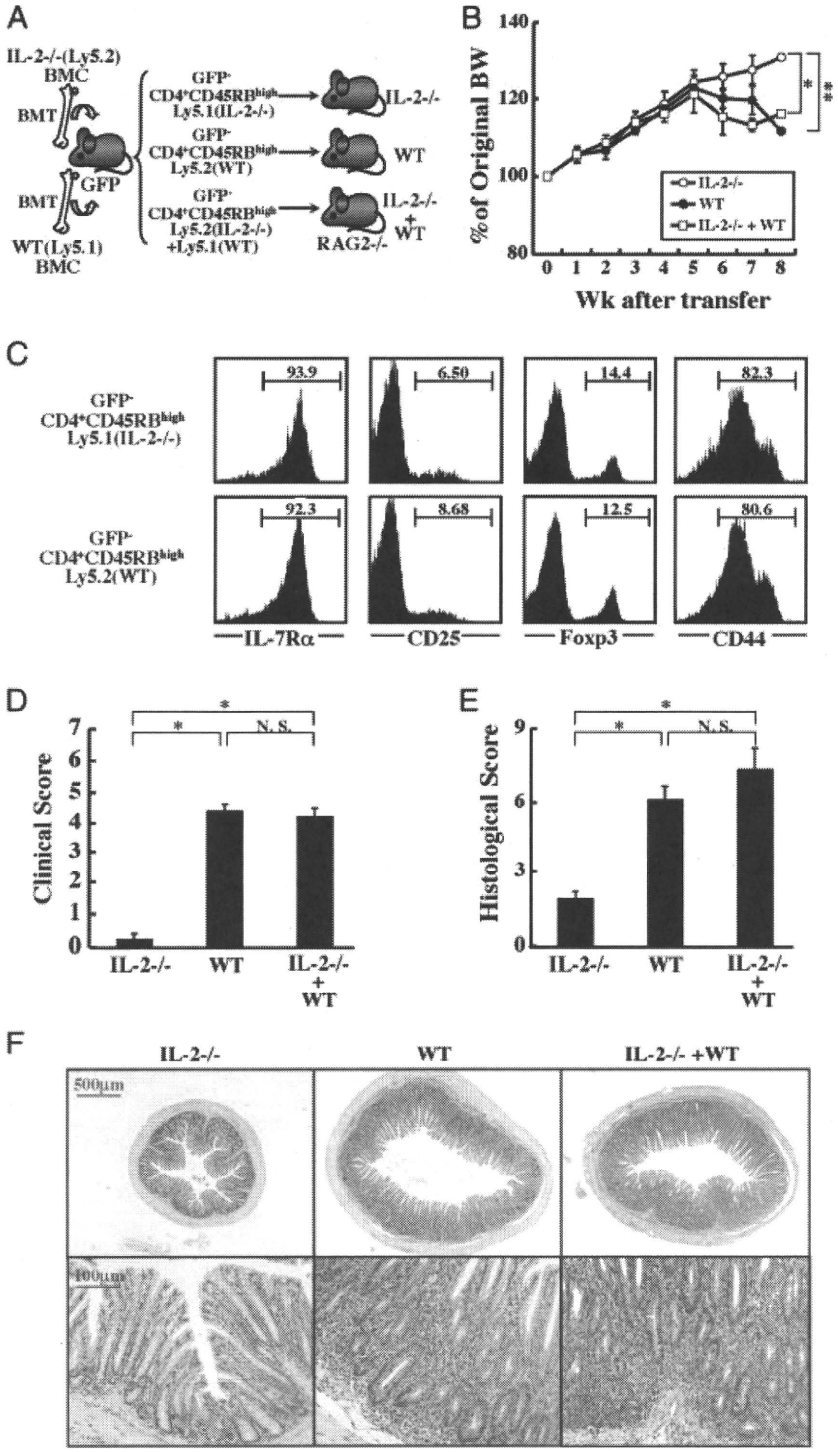

Figure 7. RAG-$2^{-/-}$ mice transferred with SP IL-$2^{-/-}$ $CD4^+CD45RB^{high}$ T cells from GFP mice previously transplanted with WT and IL-$2^{-/-}$ BM cells do not develop colitis. We performed one independent experiment. (A) Experimental design. Irradiated (7.5 Gy) GFP mice were transplanted with a mixture of the same number (5×10^6 cells) of CD3-depleted BM cells from WT ($Ly5.1^+$) and IL-$2^{-/-}$ ($Ly5.2^+$) mice. Eight weeks after the BM transplantation, WT ($Ly5.1^+$) and IL-$2^{-/-}$ ($Ly5.2^+$) GFP$^-$ $CD4^+CD45RB^{high}$ T cells were isolated using FACSAria. The cells (3×10^5 cells *per* mouse) or cell mixtures (3×10^5 cells of each) were transferred into new RAG-$2^{-/-}$ mice ($n = 5$). Mice were sacrificed 8 wk after transfer. (B) The change in body weight over time is expressed as a percentage of original weight. Data are expressed as the mean ± SEM of five mice *per* group. $^*p = 0.008$, and $^{**}p = 0.041$ (Mann-Whitney *U*). (C) Phenotypic characterization of SP GFP$^-$ WT and IL-$2^{-/-}$ $CD4^+CD45RB^{high}$ donor T cells after BM transplantation. (D) Clinical score 8 wk after transfer. Data are expressed as the mean ± SEM of five mice *per* group. $^*p = 0.0143$ (Mann-Whitney *U*). (E) Histological score 8 wk after transfer. Data are expressed as the mean ± SEM of five mice *per* group. $^*p = 0.0143$ (Mann-Whitney *U*). (F) Histopathology of the distal colon 8 wk after transfer. Original magnification, × 40 (upper) and × 200 (lower).

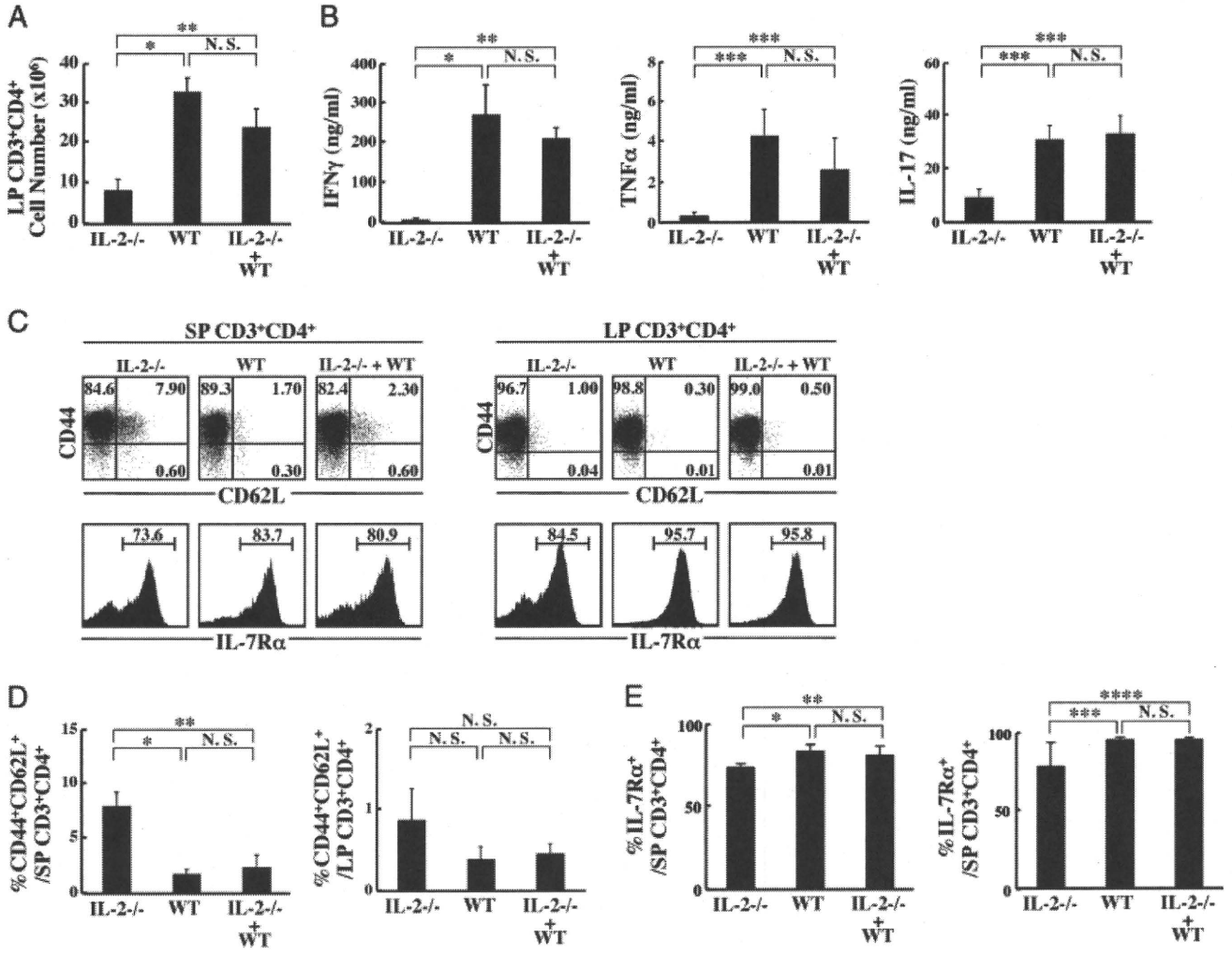

Figure 8. Marked down-modulation of IL-7Rα expression in non-colitic RAG-2$^{-/-}$ mice transferred with SP IL-2$^{-/-}$ CD4$^+$CD45RB^{high} T cells. (A) Numbers of LP CD3$^+$CD4$^+$ T cells recovered from each mouse 8 wk after transfer. Data are expressed as the mean ± SD of five mice *per* group. $^*p = 0.037$ (Student's *t*), $^{**}p = 0.05$ (Mann-Whitney *U*), and N.S., not significant (B) Cytokine production (IFN-γ, TNF-α, and IL-17) by LP CD4$^+$ T cells. Data are expressed as the mean ± SEM of five mice *per* group. $^*p = 0.003$, $^{**}p = 0.007$, $^{***}p = 0.0143$, and N.S., not significant. (C) Expression of various cell surface markers on SP (left) and LP (right) CD3$^+$CD4$^+$ T cells of each group was assessed using FACS. (D) The percentage of SP (left) and LP (right) T_{CM} cells was determined using FACS. Data are expressed as the mean ± SEM of five mice. $^*p = 0.0015$, $^{**}p = 0.0017$ (Mann-Whitney *U*), and N.S., not significant. (E) Percentage of SP (left) and LP (right) CD3$^+$CD4$^+$ T cells positive for IL-7Rα$^+$. Data are expressed as the mean ± SEM of five mice *per* group. $^*p = 0.0002$ (Welch's *t*), $^{**}p = 0.0065$ (Mann-Whitney *U*), $^{***}p = 0.0027$ (Mann-Whitney *U*), $^{****}p = 0.011$ (Mann-Whitney *U*), and N.S., not significant.

TNF-α, and IL-17 by anti-CD3/CD28 mAb-stimulated LP CD4$^+$ T cells obtained from RAG-2$^{-/-}$ mice transferred with WT CD4$^+$ CD45RB^{high} T cells or a mixture of WT and IL-2$^{-/-}$ CD4$^+$ CD45RB^{high} T cells was significantly higher than that produced in RAG-2$^{-/-}$ mice transferred with IL-2$^{-/-}$ CD4$^+$CD45RB^{high} T cells (Fig. 8B).

We next examined cell surface markers in terms of T_{CM}/T_{EM} and IL-7Rα after transfer. Again, the proportion of T_{CM} in the SP of RAG-2$^{-/-}$ mice transferred with IL-2$^{-/-}$ CD4$^+$CD45RB^{high} T cells was significantly higher than that in RAG-2$^{-/-}$ mice transferred with WT CD4$^+$CD45RB^{high} T cells or a mixture of WT and IL-2$^{-/-}$ CD4$^+$CD45RB^{high} T cells (Fig. 8C and D). In contrast, LP CD4$^+$ T cells were exclusively T_{EM} cells irrespective of the cells transferred (Fig. 8C and D). It is noteworthy that the positive frequency of IL-7Rα expression on the SP and LP of RAG-2$^{-/-}$ mice transferred with IL-2$^{-/-}$ CD4$^+$CD45RB^{high} T cells significantly reduced, as compared with that in the paired cells of other groups, irrespective of the presence or absence of colitis (Fig. 8C and E).

Discussion

In the present work, we demonstrated that IL-2 is essential for the development and perpetuation of chronic colitis. First, IL-2 is needed for the normal expression of IL-7Rα on naïve CD4$^+$ T cells during the development of mature naïve CD4$^+$ T cells. Second, IL-2 is required for retaining IL-7Rα expression on colitogenic

$CD4^+$ T cells during conversion from effector to memory $CD4^+$ T cells, resulting in the acquisition of IL-7 dependency for the perpetuation of chronic colitis.

IL-$2^{-/-}$ mice, as well as IL-2Rα $(CD25)^{-/-}$, IL-2Rβ $(CD122)^{-/-}$, and IL-2Rγ $(CD132)^{-/-}$ mice [12], all of which are severely defective in the IL-2/IL-2R signalling pathway, are known to spontaneously develop chronic colitis. Thus, it was considered possible that the abnormal naïve $CD4^+$ T cells in IL-$2^{-/-}$ mice themselves might be critically involved in the development of colitis in such colitis models. It was also considered possible that IL-2 itself may not be needed for the development of pathogenic effector and memory $CD4^+$ T cells, but is solely essential for the development and maintenance of Treg, as was recently emphasized [16–18]. To solve these issues, we used an adoptive transfer model of colitis using WT or IL-$2^{-/-}$ $CD4^+$ $CD45RB^{high}$ T cells, which are almost all naïve T cells, excluding Treg. Surprisingly, we found that RAG-$2^{-/-}$ mice transferred with IL-$2^{-/-}$ $CD4^+CD45RB^{high}$ T cells did not develop colitis until 8 wk after transfer. Furthermore, IL-$2^{-/-}$ $CD4^+CD45RB^{high}$ T-cell-derived cells differentiated into $CD44^{high}$ memory T cells in the LP and SP, similar to WT T-cell-derived cells (Figs. 2 and 3). It should be noted that inducible $CD4^+CD25^+Foxp3^+$ Treg were present in RAG-$2^{-/-}$ mice transferred with WT, but not IL-$2^{-/-}$, $CD4^+CD45RB^{high}$ T cells. These results clearly demonstrate that IL-2 is positively involved in the development of colitis without the effect of naturally occurring Treg, although IL-2 supports the development of inducible Treg, which are generally thought to suppress the development of colitis. However, an unresolved discrepancy remains between the present finding that IL-2 is positively important for the development and perpetuation of colitis and the previous finding that IL-$2^{-/-}$ mice spontaneously develop colitis together with various autoimmune diseases. Indeed, we showed that adoptive retransfer of IL-$2^{-/-}$ LP $CD4^+$ T cells obtained from colitic RAG-$2^{-/-}$ mice previously transferred with a mixture of WT and IL-$2^{-/-}$ $CD4^+CD45RB^{high}$ T cells induced colitis, albeit less severely than adoptive retransfer of WT LP $CD4^+$ T cells, suggesting that IL-2 is not absolutely essential for the development of colitis, but is a fine tuner for the full development of colitis. Indeed, we confirmed that RAG-$2^{-/-}$ mice transferred with IL-$2^{-/-}$ $CD4^+CD45RB^{high}$ T cells develop mild colitis 20 wk after transfer (data not shown). It is also possible that other cells, such as B cells and NK T cells may affect the development of colitis in IL-$2^{-/-}$ mice. Further investigation of this issue is needed.

The positive frequency of IL-7Rα expression on LP IL-$2^{-/-}$ $CD4^+CD44^{high}$ T cells was significantly reduced, as compared with the paired WT cells, indicating that IL-2 was needed for the expression of IL-7Rα on some subpopulation of $CD4^+$ T cells during the differentiation of memory $CD4^+CD44^{high}$ T cells in all the adoptive transfer experiments. Importantly, since we demonstrated that mice transferred with isolated IL-$7R\alpha^{high}$ or IL-$7R\alpha^{low}$ $CD4^+CD45RB^{high}$ T cells obtained from young IL-$2^{-/-}$ mice did not develop colitis (Fig. 3), it is not likely that IL-$7R\alpha^{low}$ cells suppress IL-$7R\alpha^{high}$ cells when they are co-transferred into RAG-$2^{-/-}$ mice. Rather, IL-2 may be critically involved in the retainment of IL-7Rα expression on colitogenic $CD4^+$ T cells. In other words, the lack of IL-2 may induce the exhaustion of colitogenic $CD4^+$ T cells, resulting in the apoptosis of those cells. Furthermore, it is a matter of controversy whether IL-2 positively or negatively controls IL-7Rα expression [13, 30]. One group demonstrated that IL-2 is a negative regulator of IL-7Rα expression during *in vitro* stimulation with anti-CD3/anti-CD28 mAb [30], whereas another group argued that IL-2 promotes the generation of $CD4^+$IL-$7R\alpha^+$ memory T cells based on findings from a physiologically relevant *in vitro* and *in vivo* priming system with antigen and antigen-presenting cells [15]. Our result from adoptive transfer in the *in vivo* colitis model supports the latter result. In our previous study with the same model of colitis using IL-2-sufficient $CD4^+CD45RB^{high}$ T cells [27], we observed that the expression of IL-7Rα on $CD4^+$ T cells was down-modulated approximately 1 wk after transfer and was then up-regulated until 4 wk after transfer. Thus, our current result suggests that IL-2 is needed for re-expression of IL-7Rα during the differentiation of functionally colitogenic memory $CD4^+$ T cells. In accordance with this, we showed that IL-$7^{-/-}$ × RAG-$2^{-/-}$ mice retransferred with IL-$2^{-/-}$ LP $CD4^+$ T cells obtained from colitic RAG-$2^{-/-}$ mice previously transferred with a mixture of WT and IL-$2^{-/-}$ $CD4^+CD45RB^{high}$ T cells did not develop colitis, whereas IL-$7^{+/+}$ × RAG-$2^{-/-}$ mice retransferred with those cells did develop colitis, albeit to a lesser extent than IL-$7^{+/+}$ × RAG-$2^{-/-}$ mice retransferred with WT $CD4^+CD45RB^{high}$ T-cell-derived colitic LP $CD4^+$ T cells. IL-2 may play a crucial role in the re-expression of IL-7Rα on colitogenic memory $CD4^+$ T cells, which may be critical for the acquisition of their IL-7 dependency in chronic colitis. Furthermore, since we showed that mice transferred with IL-$2^{-/-}$ $CD4^+CD45RB^{high}$ T cells retained IL-$7R\alpha^{high}$ $CD4^+$ T-cell population partly as well as IL-$7R\alpha^{low}$ population, but did not develop colitis, it is possible that non-colitogenic $CD4^+$ memory T cells could re-express IL-7Rα highly in IL-2-independent manner. Further investigation of this issue is needed.

Nevertheless, our model is open to criticism concerning the role of IL-2 in the development of functional memory $CD4^+$ T cells when impaired IL-$2^{-/-}$ $CD4^+CD45RB^{high}$ T cells are used because their IL-7Rα expression is down-modulated before transfer. To this end, we next used donor WT and IL-$2^{-/-}$ $CD4^+$ $CD45RB^{high}$ T cells after preparing a mixed BM chimera that received a mixture of WT and IL-$2^{-/-}$ BM cells. As mentioned previously, the phenotypic characteristics of the IL-$2^{-/-}$ $CD4^+$ T cells in the BM chimera mice, including IL-7Rα expression, were similar to those of WT $CD4^+$ T cells, indicating that paracrine IL-2 secretion from WT $CD4^+$ T cells supports the normal development of naïve IL-$2^{-/-}$ $CD4^+CD45RB^{high}$ T cells in the BM and/or thymus and prevents preclinical autoimmunity *via* Treg that develop from WT precursor cells. However, we found that RAG-$2^{-/-}$ mice transferred with the IL-$2^{-/-}$ $CD4^+CD45RB^{high}$ T cells sufficiently expressing IL-7Rα- did not develop colitis along with the down-modulated expression of IL-7Rα on both LP and SP $CD4^+$ T cells after the transfer, which is in contrast to mice transferred with the WT $CD4^+CD45RB^{high}$ T cells, which

developed severe Th1/Th17-mediated colitis. These results reinforce our evidence that IL-2 is needed for the development of colitogenic memory $CD4^+$ T cells.

Finally, it should be noted that our study might not reflect the pathogenesis of human IBD directly because our colitis model is in the extreme "lymphopenic" environment for the induction of colitis, in which IL-7 and IL-2 might be important for rapid proliferation of T cells. Further studies will be needed to address this point using other colitis models in non-lymphopenic conditions.

Collectively, our findings indicate that, at least in the absence of Treg, IL-2 is critically involved in the development and perpetuation of colitis in three ways: (i) in the normal development of naïve $CD4^+$ T cells, (ii) in the re-expression of IL-7Rα on colitogenic memory $CD4^+$ T cells, and (iii) in the conversion from T_{CM} to T_{EM}/effector T cells. Although it will be necessary to consider the double-edged role of IL-2 in colitogenic $CD4^+$ T cells and Treg in other models of colitis, which may affect the balance between these two cell types, the current study appears to shed light on the positive involvement of IL-2 in chronic colitis. Thus, it will be necessary to assess when strategies for blocking and promoting IL-2 for IBD treatment should begin.

Materials and methods

Mice

C57BL/6-Ly5.1 and C57BL/6-Ly5.2-RAG-$2^{-/-}$ mice were obtained from Taconic Laboratory (Hudson, NY) and the Central Experimental Animal Institute (Kawasaki, Japan). IL-$2^{-/-}$ mice were obtained from the Jackson Laboratory (Bar Harbor, ME). C57BL/6-Ly5.2- IL-$7^{-/-}$ × RAG-$1^{-/-}$ mice were kindly provided by Dr. Rosa Zamoyska (National Institute for Medical Research, London, UK) [31]. GFP transgenic mice were originally generated by Dr. Masaru Okabe (Osaka University, Japan) [32]. All mice were maintained under specific pathogen-free conditions at the Animal Care Facility of Tokyo Medical and Dental University. All experiments were approved by the regional animal study committees.

Antibodies

Biotin-conjugated anti-IL-7Rα (A7R34) was obtained from Immuno-Biological Laboratories (Takasaki, Japan). The following mAb were obtained from BD PharMingen (San Diego, CA): 145–2C11, FITC- or PerCP- anti-CD3; RM4–5, FITC-, PE-, PerCP- or APC- anti-CD4; 16A, FITC- or APC- anti-CD45RB; IM7, PE-anti-CD44; PC61, PE-anti-CD25; MEL-14, FITC-anti-CD62L; H1.2F3, FITC-anti-CD69; A20, FITC- or PE-anti-Ly5.1 (CD45.1); and 104, FITC- or PE-anti-Ly5.2 (CD45.2). Biotinylated antibodies were detected with PE–streptavidin (BD Phar Mingen).

In vivo adoptive transfer experiments

Adoptive transfers of WT or IL-$2^{-/-}$ $CD4^+CD45RB^{high}$ T cells into RAG-$2^{-/-}$ mice were performed to avoid the effect of $CD4^+CD25^+$ Treg. Exp. 1: Ly5.1-derived WT (IL-$2^{+/+}$) or Ly5.2-derived IL-$2^{-/-}$ ($n = 8$) $CD4^+CD45RB^{high}$ T cells (3×10^5 cells) were injected intraperitoneally into RAG-$2^{-/-}$ mice. As a negative control, RAG-$2^{-/-}$ mice were transferred with $CD4^+CD45RB^{high}$ T cells (3×10^5 cells) and Treg (1×10^5 cells). Exp. 2: $CD4^+CD45RB^{high}$ T cells from SP of 4- to 5-wk-old IL-$2^{-/-}$ mice were divided into two populations, IL-$7R\alpha^{high}$ or IL-$7R\alpha^{low}$, by cell sorting and then each population was transferred into RAG-$2^{-/-}$ hosts. As a positive control, we transferred the same number of WT $CD4^+CD45RB^{high}$ T cells into RAG-$2^{-/-}$ mice. ($n = 5$). Exp. 3: First, SP $Ly5.1^+$ and $Ly5.2^+$ $CD4^+$ T cells were separately isolated from mice transferred with $Ly5.1^+$ WT and $Ly5.2^+$ IL-$2^{-/-}$ $CD4^+CD45RB^{high}$ T cells using FACSAria. Second, the isolated $Ly5.1^+$ or $Ly5.2^+$ T cells were separately transferred into new IL-$7^{+/+}$ × RAG-$2^{-/-}$ or IL-$7^{-/-}$ × RAG-$2^{-/-}$ mice. Exp. 4: First, preparations from the femurs and tibias of WT or IL-$2^{-/-}$ mice were incubated with biotin-conjugated anti-CD3 mAb and anti-biotin magnetic beads, followed by bead depletion using a MACS separation system (Miltenyi Biotec, Auburn, CA). Then 5×10^6 T-cell-depleted BM cells from each mouse were injected intravenously into lethally irradiated (7.5 Gy) GFP transgenic mice. Second, mice were sacrificed to isolate SP naïve GFP^- $Ly5.1^+$ WT and GFP^- $Ly5.2^+$ IL-$2^{-/-}$ $CD4^+$ $CD45RB^{high}$ T cells 8–12 wk post-transplantation. Third, the isolated $Ly5.1^+$ WT or $Ly5.2^+$ IL-$2^{-/-}$ $CD4^+CD45RB^{high}$ T cells were transferred into RAG-$2^{-/-}$ mice.

Disease monitoring and histological examination

The recipient mice were weighed immediately after T-cell transfer and three times *per* week thereafter. They were observed for clinical signs of illness as previously described [9]. Histological examination was performed as described before [9].

Flow cytometry

To detect the surface expression of a variety of molecules, isolated splenocytes or LP mononuclear cells were pre-incubated with an FcγR-blocking mAb (CD16/32; 2.4G2, BD PharMingen) for 20 min, followed by incubation with specific antibodies for 30 min on ice. Biotinylated antibodies were detected with PE–streptavidin. Intracellular Foxp3 staining was performed using a PE-anti-mouse Foxp3 staining set (eBioscience) according to the manufacturer's instructions. Background fluorescence was assessed by staining with control-irrelevant isotype-matched mAb.

Cytokine ELISA

To measure cytokine production, 1×10^5 LP $CD4^+$ T cells were cultured in 200 μL of culture medium at 37°C in a humidified

atmosphere containing 5% CO_2 in 96-well plates (Costar, Cambridge, MA) precoated with 5 μg/mL of hamster anti-mouse CD3ε mAb (145–2C11, BD PharMingen) and 2 μg/mL of hamster anti-mouse CD28 mAb (37. 51, BD PharMingen) in PBS overnight at 4°C. Culture supernatants were removed after 48 h and assayed for cytokine production. Cytokine concentrations were determined by specific ELISA according to the manufacturer's recommendations (R&D Systems, Minneapolis, MN).

Statistical analysis

We examined the normality of each group. If either group was not normally distributed, we assessed the difference between the two groups using the Mann–Whitney *U*-test. If both groups were normally distributed, we assessed the variance of the population to which each group belonged using the *f*-test. When homoscedasticity of both populations occurred, we assessed the difference between two groups using Student's *t*-test. In the absence of homoscedasticity, we assessed the difference using Welch's *t*-test. We used Statcell software for all statistical analyses. Results are expressed as the mean+SEM. Differences were considered statistically significant when $p<0.05$.

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Abbreviations: **IBD:** inflammatory bowel disease · **LP:** lamina propria · **SP:** spleen · T_{CM}**:** central memory T cell · T_{EM}**:** effector memory T cell

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Delta-like 1 expression promotes goblet cell differentiation in Notch-inactivated human colonic epithelial cells

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ABSTRACT

Notch signaling has previously been implicated in the regulation of the cell fate of intestinal epithelial cells. However, the expression and function of Notch ligands in the human intestine remain largely unknown. In the present study, we showed that Notch ligands Delta-like 1 (Dll1) and Delta-like 4 (Dll4) are expressed in a goblet cell-specific manner in human colonic tissue. Additionally, we found that Dll1 and Dll4 expression was regulated in-parallel with Atoh1 and MUC2, which are both under the control of the Notch-Hes1 signaling pathway. Because knockdown of Dll1 expression completely abrogated the acquisition of the goblet cell phenotype in Notch-inactivated colonic epithelial cells, we postulate that Dll1 might function as a cis-acting regulatory element that induces undifferentiated cells to become goblet cells. Our results suggest a link between Dll1 expression and human goblet cell differentiation that might be mediated by a function that is distinct from its role as a Notch receptor ligand.

Introduction

The mammalian intestinal epithelium consists of cells of four different lineages, each of which plays a distinct but indispensable role in supporting the function of intestinal tissues [1]. Goblet cells are one of three secretory-type cells that are present in the intestinal epithelium. They are characterized by the expression of MUC2 and play a crucial role in mucosal defense. Loss of goblet cell function leads to development of spontaneous colitis [2] and can subsequently lead to development of colon cancer [3]. Thus, maintenance of a proper number of functional goblet cells is required for homeostasis of the intestinal mucosal environment.

Recent studies in mice have revealed several key molecules that are required for the differentiation and maturation of intestinal goblet cells [4]. In one of these studies, Kim et al. found that Atoh1, which is expressed in goblet cells of the human colon, to be able to directly activate transcription of the human MUC2 gene [5]. In addition, our recent study showed that Atoh1 is a key regulator of goblet cell differentiation in the human intestine [6,7]. However, little is known about the upstream signals that regulate the Atoh1-MUC2 axis in goblet cells. We have previously shown that Wnt signaling plays a crucial role in the post-transcriptional regulation of Atoh1 [6,7]. Others have suggested that Notch-Hes1 signaling is involved in regulating the differentiation of intestinal cells into absorptive and goblet cells through a mechanism called lateral inhibition. [8]. In this model, absorptive cells arise from cells in which a high level of Notch activation is maintained. These cells express high levels of the Notch target gene Hes1. Conversely, secretory-type cells, including goblet cells, arise from cells expressing Notch ligands in which Notch signaling is inactivated. These cells instead express high levels of Atoh1, and inhibit neighboring cells from achieving the same cell fate.

Although this lateral inhibition model has been well-accepted as the mechanism that determines the cell fate of intestinal epithelial cells, little has been shown about where this process takes place in vivo. Our previous study, in which we used human tissues, showed that Notch is activated in crypt cells but is completely absent in goblet cells [9]. As described in the lateral inhibition model, the distribution of Notch-activated cells and goblet cells appeared side-by-side, suggesting that goblet cells might express Notch ligands and thereby activate the Notch receptors of neighboring cells. However, the expression and function of Notch ligands in the human intestine has never been described.

In the mammalian Notch system, there are five ligands that can activate the Notch receptor: Delta-like (Dll) 1, 3, and 4 and Jagged-1, and 2 [10]. These five ligands not only act in trans as ligands for the Notch receptor but might also act in cis to modulate

Abbreviations: NICD, Notch intracellular domain; Dll, Delta-like.

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intracellular Notch activity or gene expression [10]. Previous reports have shown that Notch ligands are expressed in the murine intestine [11]. In the mouse intestine, Dll1-expressing cells have been postulated to be progenitor cells of secretory-type epithelial cells, including goblet cells [12]. However, the functional role that Dll1 expression has on secretory and goblet cell differentiation remains to be elucidated.

In the present study, we sought to examine the expression and function of Notch ligands in human colonic tissue. We found that Dll1 and Dll4 were expressed in a goblet cell-specific manner and that Dll1 expression was necessary for acquisition of the goblet cell phenotype in Notch-inactivated colonic epithelial cells. Our results not only describe the important role that Dll1 expression plays in goblet cell differentiation, but they also suggest the possible existence of a cis-acting function of the Dll1 protein.

Materials and methods

Cell culture. Cell cultures and plasmid DNA transfections were performed as described previously [13]. The inhibition of endogenous Notch signaling was performed by addition of LY-411,575 (1 μM), which was synthesized according to the method of Wu et al. [14]. The cell lines expressing the Notch1 intracellular domain (TET-On NICD1 cells) and the cell line expressing FLAG-tagged Hes1 (TET-On FLAG-Hes1 cells) under the control of tetracycline or doxycycline (DOX, 100 ng/ml, Clontech) were generated from LS174T cells as described elsewhere [9]. Generated cells were supplemented with Blastcidin (7.5 μg/ml, Invitrogen) and Zeocin (750 μg/ml, Invitrogen).

Reverse transcription (RT)-PCR assays. Total RNA preparation and RT-PCR was performed as described previously [9]. We used primer sequences for human β-actin, G3PDH, MUC2, Hes1, and Atoh1 that have been previously described [6,9]. Primer sequences for the other genes and the number of PCR cycles that were used for semi-quantitative analysis are summarized in Supplemental Table 1. Results are presented as the means of the data collected from two rounds of assays. Each assay was performed in triplicate. We used paired two-sample Student's *t*-tests to analyse these data.

Plasmids. The expression plasmids used for this study were generated as described previously [9].

Immunoblot analysis. Immunoblot assays were performed as described previously [9]. We used anti-cleaved Notch1 (1:1000, Cell Signaling Technology), anti-Hes1 (1:4000, a kind gift from Dr. T. Sudo, Toray Industry), anti-Delta (recognizing both Dll1 and Dll4, 1:200, C-20, Santa Cruz Biotechnology), anti-Jagged-1 (1:200, H-114, Santa Cruz Biotechnology), and anti-β-actin (1:5000, SIGMA) primary antibodies.

Human intestinal tissue specimens. Human tissue specimens were obtained from patients who underwent endoscopic examination or surgery at Yokohama Municipal General Hospital or Tokyo Medical and Dental University Hospital. Written informed consent was obtained from each patient, and the study was approved by the ethics committee of both Yokohama Municipal General Hospital and Tokyo Medical and Dental University.

Immunohistochemistry. Immunohistochemistry using intestinal tissues was performed as previously described [15]. We used anti-Delta (recognizing both Dll1 and Dll4, 1:200, C-20, Santa Cruz Biotechnology) and anti-Jagged-1 (1:500, H-114, Santa Cruz Biotechnology) primary antibodies.

si-RNA mediated gene-knockdown. si-RNA-mediated gene knockdown was performed as previously described [13]. Briefly, a siRNA targeted to Dll1 mRNA (100 nM, Dharmacon) or a non-targeting siRNA (100 nM, Dharmacon) was transfected into cells using Lipofectamine RNAiMAX (Invitrogen) following the manufacturer's protocol.

Results

Notch ligands are expressed in human colonic epithelial cells

Although several reports have shown that Notch ligands are expressed in the intestinal epithelium of rats and mice [11,16], they have not been shown to be expressed in human intestinal epithelial cells. Our previous report clearly showed that Notch1 activation is present in the crypt cells of the human intestinal epithelium [9]. These results suggested that Notch ligands might also be present in the human intestinal epithelium. Therefore, we first examined whether the RNA transcripts of Notch ligands could be detected within human intestinal biopsy specimens by semi-quantitative RT-PCR (Fig. 1A). Our results showed that other than Dll3, all Notch ligands are clearly expressed in the human colonic tissue. Analysis of the human colonic epithelial cell lines, LS174T and HT29, showed the same expression profile. These results suggested that of the five mammalian Notch ligands, four (Dll1, Dll4, Jagged-1, and Jagged-2) are expressed in human colonic tissue, possibly by epithelial cells.

To determine the precise distribution of Notch ligand-expressing cells, we performed immunohistochemical analysis using human colonic tissues. Immunostaining for Jagged-1 revealed that it was ubiquitously expressed in colonic crypt cells (Fig. 1B, right). In sharp contrast, immunostaining using an antibody which detects both Dll1 and Dll4 (anti-Delta) revealed selective staining positivity for goblet cells (Fig. 1B, left). Immunostaining for Jagged-2 was not successful. These results revealed that Jagged-1 and Dlls (Dll1 and Dll4) are both expressed by human colonic epithelial cells. However, Dll ligands are expressed by goblet cells and Jagged-1 is expressed by crypt cells. Therefore, these results suggest that the expression of Dll1 and Dll4 might have some functional significance in relation to the development of goblet cells within the human colonic epithelium.

Both the goblet cell phenotype and Dll ligand expression are downregulated upon activation of the Notch-Hes1 pathway in human colonic epithelial cells

Because our results suggested that Dll1 and Dll4 might play some role in the development of human goblet cells, we next examined the relationship between goblet cell differentiation and Dll ligands expression using a cell-based model.

In our previous report, we showed that forced expression of Notch1 intracellular domain (NICD1) suppressed the goblet cell phenotype of LS174T cells [9]. Using a sub-line of LS174T cells in

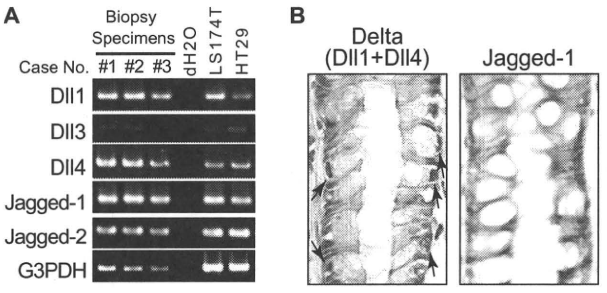

Fig. 1. Notch ligands are expressed in human colonic epithelial cells. (A) Expression of Notch ligands in human colonic biopsy specimens and in the human colonic epithelial cell lines LS174T and HT29 was analysed by semi-quantitative RT-PCR. (B) Expression of the Delta-like ligands Dll1, Dll4, and Jagged-1 in human colonic tissue was examined by immunohistochemistry. For the staining of Delta-like ligands, an anti-Delta antibody, which recognizes the c-terminal region of both Dll1 and Dll4, was used (Original magnification: 800×). The black arrows indicate goblet-shaped epithelial cells that stained positive for Dlls.