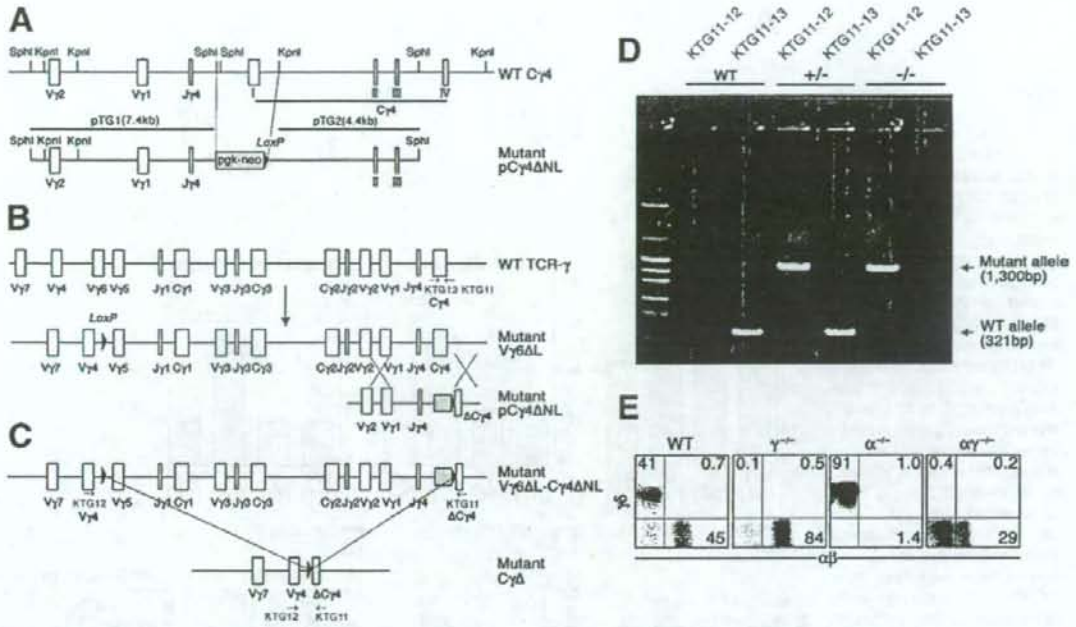

Figure 2. Generation of TCR-γ-deficient mice and subsequent production of TCRαγ double mutant mice. (*A*) Schematic representation of WT and mutant (pCγ4ΔNL) genomic Cγ4 loci together with the 3 DNA fragments used to construct the mutant pCγ4ΔNL vector. The resulting targeting vector (pCγ4ΔNL) carrying a *loxP*-flanked *pgk-neo* gene cassette in place of exon 1 of Cγ4 gene used a neomycin resistance gene driven by the *pgk* promoter as positive selection marker is shown. Restriction enzyme sites, *SphI* and *KpnI* (*solid bars*), exon structures, Vγ and Jγ (*open boxes*), and *loxP* site (*solid triangle*) are indicated. (*B*) Schematic representation of the ES clone carrying WT TCRγ gene and mutant Vγ6ΔL ES clone carrying the allele in which the Vγ6 region was replaced by a single *loxP* site and mutant pCγ4ΔNL targeting vector. Exon structures, Vγ and Jγ (*open boxes*) and *loxP* site (*solid triangle*) are indicated. (*C*) Schematic representation of generation of the mutant mice that carry the TCRγ-deficient (CγΔ) allele by Cre-mediated recombination during embryonic development. Exon structures, Vγ and Jγ (*open boxes*), and *loxP* site (*solid triangle*) are indicated. (*D*) The mutant mice that carry the TCRγ-deficient (CγΔ) allele were intercrossed to produce TCRγ$^{+/+}$ (WT), TCRγ$^{+/-}$ (γ$^{+/-}$), and TCRγ$^{-/-}$ (γ$^{-/-}$) mice, and the corresponding WT and mutant alleles were typed by PCR analysis of tail DNA with each set of primers indicated. (*E*) γδ T cells are absent from the IEL compartment of γ$^{-/-}$ and αγ$^{-/-}$ mice. γ$^{-/-}$ Mice were crossed with α$^{-/-}$ mice to obtain WT (α$^{+/-}$ × γ$^{+/-}$), γ$^{-/-}$ (α$^{+/-}$ × γ$^{-/-}$), α$^{-/-}$ (α$^{-/-}$ × γ$^{+/-}$), and αγ$^{-/-}$ (α$^{-/-}$ × γ$^{-/-}$) littermate mice.

mation characterized by elongation of crypts was much milder in αγ$^{-/-}$ mice as compared with α$^{-/-}$ mice (Figure 3*A*). Although the body weight was comparable between αγ$^{-/-}$ and α$^{-/-}$ mice, it was evident that colonic weight was significantly decreased in αγ$^{-/-}$ mice as compared with α$^{-/-}$ mice (Figure 3*B*). The disease score characterized by the thickening of colonic mucosa with crypt elongation and inflammatory cell infiltration was also significantly lower in αγ$^{-/-}$ mice than that rated in α$^{-/-}$ mice (Figure 3*C*). Although approximately 20% of 20- to 60-week-old α$^{-/-}$ mice displayed anorectal prolapse (ARP), it was not discerned in any of age-matched αγ$^{-/-}$ mice (Figure 3*D*). Notably, no difference was observed in the age of onset of colitis and in the incidence of colitis (~80%) among 20- to 32-week-old αγ$^{-/-}$ and α$^{-/-}$ mice. In addition, in comparison with administration of PBS (as control), adoptive transfer of γδ T cells that were purified from α$^{-/-}$ mice did not increase the incidence of colitis in the recipient αγ$^{-/-}$ mice. However, the transfer of γδ T cells exacerbated the severity of colitis in the recipient αγ$^{-/-}$ mice. As shown in Figure 3*E*, more severe inflammatory cell infiltration was observed in the inflamed colon of the recipient αγ$^{-/-}$ mice with γδ T-cell transfer as compared with control αγ$^{-/-}$ mice. Therefore, it is possible that γδ T cells may be involved in the exacerbation, but not induction, of UC-like colitis.

Decrease in the Colonic Neutrophils and Monocytes in the Absence of γδ T Cells

The above results indicate that the spontaneous colitis in α$^{-/-}$ mice is ameliorated by the absence of γδ T cells in αγ$^{-/-}$ mice. With these findings in mind, flow cytometric analysis of colonic LP cells isolated from WT, γ$^{-/-}$, α$^{-/-}$, and αγ$^{-/-}$ littermate mice at approximately 28 weeks of age was performed, and the representative results of 5 independent experiments are presented in Figure 4*A*. In this experiment, WT, γ$^{-/-}$, α$^{-/-}$, and αγ$^{-/-}$ littermate mice yielded 5.1 × 10^5, 6.1

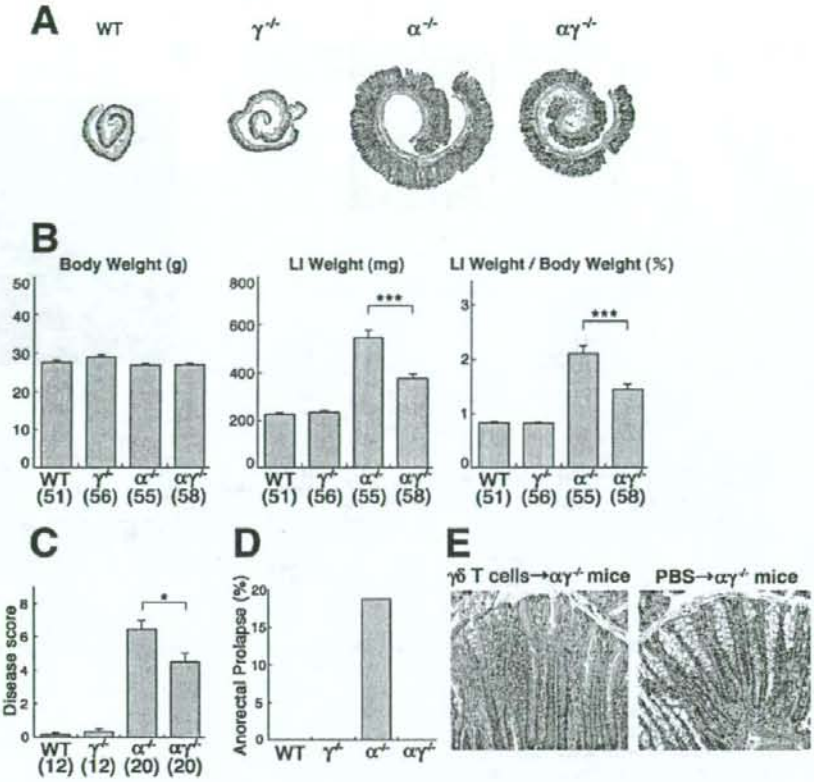

Figure 3. Colonic mucosal inflammation is milder in $\alpha\gamma^{-/-}$ mice lacking γδ T cells than in $\alpha^{-/-}$ mice possessing γδ T cells. (*A*) Representative histologic pictures of colon from WT, $\gamma^{-/-}$, $\alpha^{-/-}$, and $\alpha\gamma^{-/-}$ littermate mice. These mice were maintained in the same cages under specific pathogen-free conditions and examined at 28 weeks of age. (*B*) Body weight, wet weight of large intestine (LI weight), and ratio (%) of LI weight to body weight in WT, $\gamma^{-/-}$, $\alpha^{-/-}$, and $\alpha\gamma^{-/-}$ littermate mice at 20 to 32 weeks of age are shown. Values in parentheses are numbers of mice examined. ***$P < .001$. (*C*) Disease scores (0–10) of colitis in 24- to 32-week-old WT, $\gamma^{-/-}$, $\alpha^{-/-}$, and $\alpha\gamma^{-/-}$ mice were assessed. Values in parentheses are numbers of mice examined. *$P < .05$. (*D*) Incidence of ARP in 24- to 60-week-old WT, $\gamma^{-/-}$, $\alpha^{-/-}$, and $\alpha\gamma^{-/-}$ mice. (*E*) The histologic findings (original magnification, ×20) of colon of recipient $\alpha\gamma^{-/-}$ mouse groups that received transfer of γδ T cells (*left panel*) and that received PBS (*right panel*).

× 10^5, 32.3 × 10^5, and 12.0 × 10^5 colonic LP cells, respectively. Based on the absolute numbers of infiltrated cells and the flow cytometry results shown in Figure 4*A*, it was evident that fewer Mac-1$^+$Ly-6G$^-$ cells and Mac-1$^+$Ly-6G$^+$ cells were present in the colonic LP cell population of $\alpha\gamma^{-/-}$ mice as compared with those of $\alpha^{-/-}$ mice. Monocytes express Mac-1 but not Ly-6G, whereas neutrophils express both Mac-1 and Ly-6G.[21] Therefore, our results suggest that, in addition to monocyte infiltration (Figure 4*A*), there is a marked infiltration of neutrophils in the inflamed colonic LP of $\alpha^{-/-}$ mice. We also confirmed our previous finding[15] that a remarkable increase in γδ T cells was observed in the inflamed colonic LP of $\alpha^{-/-}$ mice (Figure 4*A*).

Immunohistochemical examination of inflamed colons from $\alpha^{-/-}$ and $\alpha\gamma^{-/-}$ mice at approximately 28 weeks of age was performed to further confirm flow cytometric results. Consistent with the flow cytometric observations, significantly smaller numbers of Mac-1$^+$ cells and Ly-6G$^+$ cells were observed in the colonic LP of $\alpha\gamma^{-/-}$ mice as compared with those in the colonic LP of $\alpha^{-/-}$ mice (Figure 4*B*).

To investigate whether γδ T cells contribute to the generation of colonic environment for enhancing the migration of neutrophils and monocytes into the inflamed colon, we examined chemotactic activity of colonic extracts from $\alpha^{-/-}$ and $\alpha\gamma^{-/-}$ mice to neutrophils and monocytes (Figure 5*A*). As a result, chemotactic activity to neutrophils of colonic extracts from $\alpha\gamma^{-/-}$ mice was significantly weaker than that from $\alpha^{-/-}$ mice, whereas the chemotactic activities to monocytes of both extracts were almost comparable (Figure 5*B*). The marked infiltration of neutrophils into the inflamed colonic LP of $\alpha^{-/-}$ mice is most likely mediated by some factors, such as MIP-2 (Figure 5*A*), that are enhanced in the presence of γδ T cells.

Taking all of these results together, and in conjunction with our previous findings,[22] colonic γδ T cells of $\alpha^{-/-}$ mice exert aggravating effect on the UC-like colitis by increasing primarily the influx of neutrophils into the inflamed mucosa.

Attenuation of Colonic Proinflammatory Cascades by the Absence of γδ T Cells

In view of the severe colitis, increased infiltration of Mac-1$^+$Ly-6G$^+$ and Mac-1$^+$Ly-6G$^-$ cells, and marked production of neutrophil chemotactic factor(s) in the inflamed colonic LP of $\alpha^{-/-}$ mice, quantitative real-time

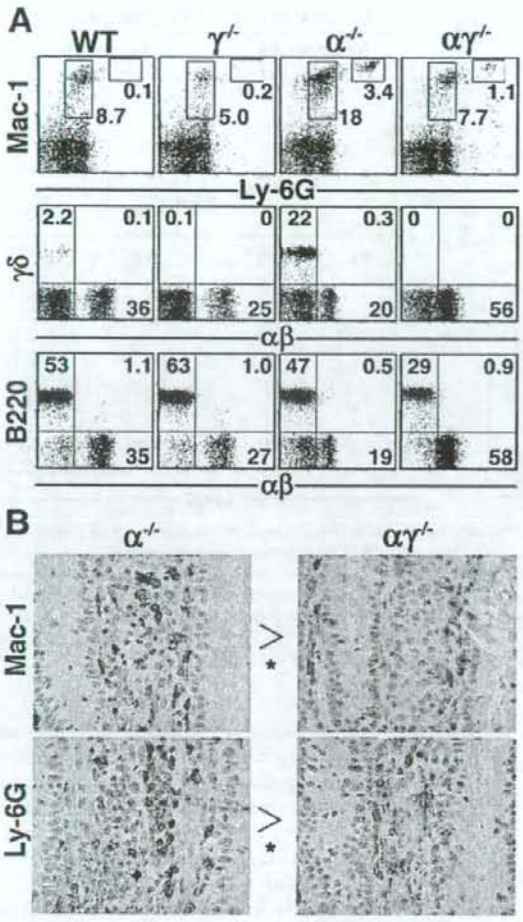

Figure 4. Decrease in colonic Mac-1$^+$Ly-6G$^-$ and Mac-1$^+$Ly-6G$^+$ cells in the absence of γδ T cells. WT (n = 3), $\gamma^{-/-}$ (n = 3), $\alpha^{-/-}$ (n = 4), and $\alpha\gamma^{-/-}$ (n = 4) mice from 28 weeks of age were examined. (*A*) Flow cytometric profiles of colonic LP cells. Absolute numbers of LP cells isolated from these WT, $\gamma^{-/-}$, $\alpha^{-/-}$, and $\alpha\gamma^{-/-}$ individuals were 5.1 × 10^5, 6.1 × 10^5, 32.3 × 10^5 and 12.0 × 10^5, respectively. (*B*) Representative immunohistochemical verification of the prominent infiltrations of Mac-1$^+$ and Ly-6G$^+$ cells into the inflamed colonic LP of $\alpha^{-/-}$ mice. Five tissue sections prepared from ascending colon to rectum per mouse, namely, 20 sections obtained from inflamed large intestines of $\alpha^{-/-}$ mice and those obtained from inflamed large intestines of $\alpha\gamma^{-/-}$ mice, were examined in a blinded fashion by 5 independent investigators, and the statistical difference in absolute numbers of Mac-1$^+$ and Ly-6G$^+$ cells between large intestinal mucosa from $\alpha^{-/-}$ and $\alpha\gamma^{-/-}$ mice were determined by 2-sided Mann–Whitney *U* test. $^*P < .05$.

RT-PCR analysis and measurement of the amounts of representative proinflammatory cytokines as well as chemokines were performed to dissect further the role of γδ T cells in the UC-like colitis in $\alpha^{-/-}$ mice.

To this end, messenger RNA (mRNA) and proteins prepared from the large intestines of WT, $\gamma^{-/-}$, $\alpha^{-/-}$, and $\alpha\gamma^{-/-}$ mice were examined. Inflamed colonic tissues from $\alpha^{-/-}$ and $\alpha\gamma^{-/-}$ mice contained at least 10 times higher levels of cytokine (Table 1)- and chemokine (Table 2)-specific mRNA than those of WT and $\gamma^{-/-}$ mice except for IL-7 and IL-10 mRNA. In contrast to the mRNA from colonic tissues of $\alpha^{-/-}$ mice, those of $\alpha\gamma^{-/-}$ mice contained significantly smaller amounts of cytokine (TNF-α, IL-1β, IL-6, and TGF-β)- and chemokine (KC and MIP-2)-specific mRNA. With these observations in mind, we measured the amounts of representative cytokines as well as chemokines that had exhibited the differences in mRNA levels between the colonic tissues of $\alpha^{-/-}$ and $\alpha\gamma^{-/-}$ mice. First, in situ production of TNF-α, IL-1β, and IL-6 but not TGF-β proteins was significantly downregulated in the inflamed colonic mucosa of $\alpha\gamma^{-/-}$ mice as compared with that of $\alpha^{-/-}$ mice (Table 1). Second, KC and MIP-2 chemokines that are involved in the chemoattract of neutrophils and/or monocytes[23] were significantly decreased in large intestines of $\alpha\gamma^{-/-}$ mice compared with those in large intestines of $\alpha^{-/-}$ mice (Table 2).

To investigate the cell types responsible for the increases in these proinflammatory cytokines and chemokines, real-time RT-PCR analysis of mRNA present in the purified cell subsets from the inflamed colonic LP of $\alpha^{-/-}$ and $\alpha\gamma^{-/-}$ mice was performed (see Supplementary Figure 1 online at www.gastrojournal.org). The IL-1β and MIP-2 mRNA were expressed preferentially by Gr-1$^+$ cells, F4/80$^+$ cells, and CD11c$^+$ cells in the colon, whereas IL-6 mRNA was mainly expressed by Gr-1$^-$F4/80$^-$CD11c$^-$ cell populations. Expression levels of TNF-α- and KC-specific mRNA were comparable between all cell populations (Gr-1$^+$ cells, F4/80$^+$ cells, CD11c$^+$ cells, and Gr-1$^-$F4/80$^-$CD11c$^-$ cells) examined. Finally, the expression levels of these cytokine- and chemokine-specific mRNA in every cell subset were lower in cells from $\alpha\gamma^{-/-}$ mice than those in cells from $\alpha^{-/-}$ mice (see Supplementary Figure 1 online at www.gastrojournal.org).

Discussion

The $\alpha^{-/-}$ mice spontaneously develop colitis that shares many features with human UC.[16,24] Commensal enteric flora is required for the development of this colitis as indicated by the absence of colitis in $\alpha^{-/-}$ mice that are maintained under germ-free conditions.[22,25] The number of colonic γδ T cells drastically decreases in the $\alpha^{-/-}$ mice under germ-free conditions.[22] However, the study to identify the role of γδ T cells in the UC-like chronic colitis in $\alpha^{-/-}$ mice has been hampered by the difficulty in generating TCRαδ double mutant ($\alpha\delta^{-/-}$) mice because of the genomic organization of these TCR genes.[20] In the present study, we overcame this problem by newly generating $\gamma^{-/-}$ mice and subsequently crossing these mice with $\alpha^{-/-}$ mice to generate TCRα double mutant mice that lacked γδ T cells. By using these $\alpha\gamma^{-/-}$ mice, we herein provide a novel insight into the role of γδ T cells

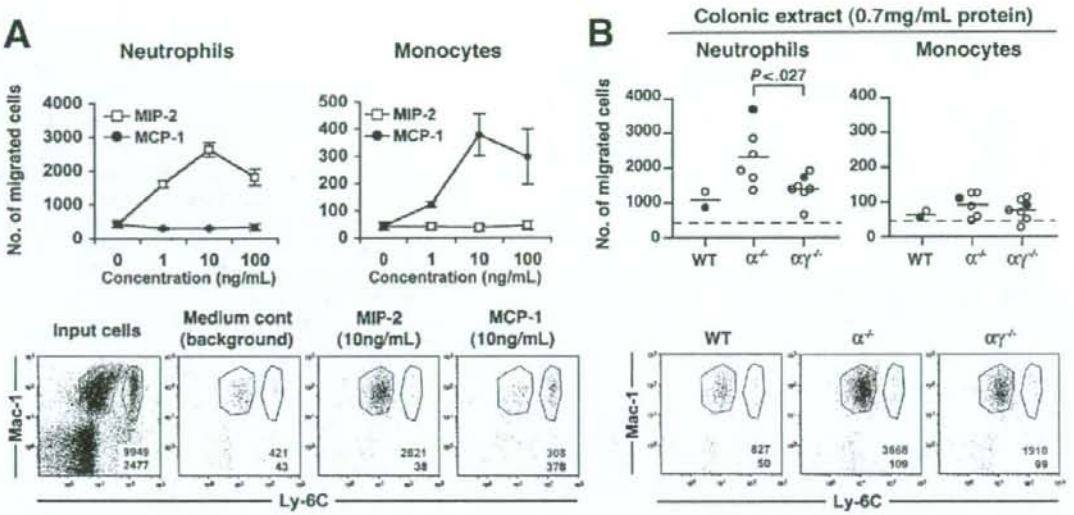

Figure 5. Chemotactic activity of colonic extracts from WT, $\alpha^{-/-}$, and $\alpha\gamma^{-/-}$ mice to neutrophils and monocytes. (*A*) The number of neutrophils and monocytes migrated in response to the increasing concentration of MIP-2 (*open square*) and MCP-1 (*solid circle*). The representative flow cytometric profiles are shown in the *lower panels*, and *red* and *blue gates* indicate the neutrophils and monocytes, respectively. *Colored numbers* represent means of the number of cells in each gate. (*B*) Chemotactic responses of neutophils and monocytes to colonic extracts (0.7 mg/mL protein) from WT, $\alpha^{-/-}$, and $\alpha\gamma^{-/-}$ mice, and each *circle* represents an individual mouse. *Horizontal bars* show mean values, and *dotted lines* indicate the number of migrated cells in medium alone. The representative flow cytomeric profiles of 3 individual animals indicated by the *solid red* and *blue circles* (*upper panels*) are shown in the *lower panels*, and *red* and *blue gates* indicate the neutrophils and monocytes, respectively.

that contributes to the exacerbation of UC-like colitis in $\alpha^{-/-}$ mice.

There is growing evidence supporting the fact that γδ T cells are part of the innate immune system and play an active multifaceted immunoregulatory role in the coordinated innate and acquired immune responses that maintain the integrity of many organs containing epithelia.[1,5,26,27] Nevertheless, the details of γδ T-cell functions are still not well understood as compared with those of αβ T cells. γδ T cells might play a defensive role against infections by various pathogenic microorganisms because exaggerated and severe infectious diseases occur in $\delta^{-/-}$ mice.[28–33] However, the same $\delta^{-/-}$ mice have also been demonstrated to display an increased host resistance to infection.[34,35] With regard to this, it is noteworthy that $V\gamma1^{+}$ γδ T cells are reported to eliminate the

Table 1. Real-Time RT-PCR Analysis and ELISA Assay of Cytokines in the Colonic Tissues

	Cytokine							
Mice (n)	TNF-α	IL-1β	IL-6	TGF-β	IFN-γ	IL-7	IL-10	IL-12
RT-PCR (copies per 10^3 HPRT)								
WT (5)	11.4 ± 0.53	6.86 ± 0.55	2.45 ± 0.64	123 ± 14.6	ND	11.7 ± 1.03	2.63 ± 0.28	0.82 ± 0.21
$\gamma^{-/-}$ (5)	9.20 ± 0.76	6.01 ± 0.61	2.14 ± 0.88	106 ± 10.7	ND	10.2 ± 0.78	2.09 ± 0.31	0.33 ± 0.09
$\alpha^{-/-}$ (7)	457 ± 35.7**	260 ± 18.6***	7.06 ± 1.04*	538 ± 48.6*	64.4 ± 8.90	11.0 ± 0.89	4.99 ± 0.72	9.58 ± 1.67
$\alpha\gamma^{-/-}$ (6)	205 ± 51.8**	98.5 ± 21.4***	2.93 ± 0.73*	319 ± 57.4*	45.0 ± 15.6	9.29 ± 1.11	6.42 ± 2.11	6.63 ± 1.01
ELISA (pg/mg protein)								
$\alpha^{-/-}$ (7)	100 ± 12.2**	975 ± 70.1**	8.24 ± 1.39**	2.09 ± 0.11	8.34 ± 1.50			
$\alpha\gamma^{-/-}$ (6)	27.0 ± 8.28**	647 ± 24.6**	2.92 ± 0.52**	2.57 ± 0.31	5.92 ± 2.56			

NOTE. All results are expressed as mean ± SE.
*$P < .05$.
**$P < .01$.
***$P < .001$.
ND, not detected

Table 2. Real-Time RT-PCR Analysis and ELISA Assay of Chemokines in the Colonic Tissues

	Chemokine					
Mice (n)	KC	MIP-2	GCP-2	MCP-1	MIP-1α	MIP-1β
RT-PCR (copies per 10^3 HPRT)						
WT (5)	6.05 ± 1.38	0.23 ± 0.01	12.3 ± 4.11	1.67 ± 0.17	0.78 ± 0.07	1.36 ± 0.22
$\gamma^{-/-}$ (5)	11.8 ± 1.19	0.22 ± 0.03	4.03 ± 1.49	1.77 ± 0.26	0.80 ± 0.04	1.42 ± 0.11
$\alpha^{-/-}$ (7)	382 ± 88.6*	101 ± 12.8**	721 ± 136	23.3 ± 4.37	35.0 ± 3.01	19.8 ± 0.88
$\alpha\gamma^{-/-}$ (6)	65.2 ± 17.4*	28.0 ± 9.80**	307 ± 168	16.0 ± 4.11	23.9 ± 7.34	14.2 ± 3.97
ELISA (pg/mg protein)						
$\alpha^{-/-}$ (7)	144 ± 22.4**	113 ± 21.2*	737 ± 139			
$\alpha\gamma^{-/-}$ (6)	32.4 ± 13.4**	44.9 ± 14.0*	414 ± 219			

NOTE. All results are expressed as mean ± SE.
*$P < .05$.
**$P < .01$.

macrophages infected with *Listeria monocytogenes*, whereas γδ T cells using Vγ elements other than Vγ1 gene appear to lack the ability to control macrophages but possess the ability to protect hosts from the infection-induced tissue injury.[36,37] In contrast to the beneficial function of γδ T cells by virtue of the fact that they can maintain the homeostasis of different types of organs,[1-5,8,27] a deleterious effect of γδ T cells on the regulation of neutrophil-mediated tissue damage after thermal (postburn) injury has been reported.[38] In various chronic and/or autoimmune inflammatory diseases, such as collagen-induced arthritis in mice[39] and murine insulin-dependent diabetes,[40] γδ T cells have been shown to exert a protective effect. Conversely, γδ T cells may directly contribute to autoimmune pathology of murine experimental allergic encephalomyelitis[41] as well as lupus in MRL/lpr mice.[42] Overall, both the beneficial and detrimental roles of γδ T cells in inflammatory process are evident.[43]

In chemically induced acute intestinal inflammation models (2,4,6-trinitrobenzene sulfonic acid- or dextran sulfate sodium-induced colitis), γδ T cells have been reported to play a protective role.[11-14] Depletion of γδ T cells by administration of anti-TCRγδ mAb into $TNF^{\Delta ARE}$ mice with a high frequency of spontaneous ileitis[44] did not lead to any histologic changes of ileitis.[45] However, transfer of bone marrow-derived γδ T cells has been shown to induce CD-like colitis in the bone marrow transplanted CD3εtg colitis model.[46] Although the role of γδ T cells in spontaneous chronic colitis remains to be explored to date, the results of the present study demonstrate the exacerbating effect of γδ T cells on the UC-like chronic colitis in $\alpha^{-/-}$ mice (Figures 3 and 4 and Tables 1 and 2). Interestingly, approximately 20% of $\alpha^{-/-}$ mice during 20 to 60 weeks of age suffered from ARP, whereas none of age-matched $\alpha\gamma^{-/-}$ mice showed ARP (Figure 3*D*). Of note, there were no differences in the age of onset of colitis and in the incidence of colitis (~80%) among 20- to 32-week-old $\alpha^{-/-}$ and $\alpha\gamma^{-/-}$ mice, but much more severe colitis was observed in $\alpha^{-/-}$ mice as compared with $\alpha\gamma^{-/-}$ mice. Therefore, it is possible that ARP may reflect increased severity of colitis and that γδ T cells may participate in the development of ARP.

Absence of γδ T cells in $\alpha\gamma^{-/-}$ mice leads to a significantly reduced production of TNF-α, IL-1β, and IL-6 proteins in the colonic tissues. These findings are consistent with our previous results[24] showing the involvement of TNF-α, IL-1β, and IL-6 in the perpetuation of inflammatory process in $\alpha^{-/-}$ mice. These inflammatory mediators have been shown to be important for host defense and wound repair.[47] Both KC and MIP-2 attract neutrophils to inflamed sites, and, in certain microbial infection, the collection of neutrophils leads to suppuration reflecting an active and vigorous host response against microbes. We also confirmed that colonic extracts from $\alpha\gamma^{-/-}$ mice exhibited the significantly weaker chemotactic activity to neutrophils than those from $\alpha^{-/-}$ mice. KC and MIP-2 mRNA expressions were lower in all purified cell subsets ($Gr\text{-}1^+$, $F4/80^+$, $CD11c^+$, and $Gr\text{-}1^-F4/80^-CD11c^-$ cells) from $\alpha\gamma^{-/-}$ mice than those from $\alpha^{-/-}$ mice. Therefore, in the presence of γδ T cells, many types of immune cells may be triggered to produce more chemokines, followed by infiltration of neutrophils into the colonic mucosa in $\alpha^{-/-}$ mice. γδ T-cell responsiveness that is manifested by recruitment and activation of inflammatory cells in which neutrophils predominate has also been reported.[1,31] In this context, it is of importance to note that the activity and severity of UC patients with increase in γδ T cells in the inflamed mucosa[9,10] (Figure 1) can be judged by the activation state of neutrophils in circulation[48] as well as by regional accumulation of neutrophils in the colonic crypt walls (cryptitis) or in the lumen of crypts (crypt abscess).[49]

The suppressive role of B cells[50] and the aggravating role of $TCR\beta^{dim}$ T cells[22,51] in the pathogenesis of colitis in $\alpha^{-/-}$ mice have been reported. Therefore, it is possible that γδ T cells may contribute to the exacerbation of this colitis by dampening regulatory B-cell function or by cooperating the colitogenic $TCR\beta^{dim}$ T cells. The possible complicated mechanism remains to be explored in the future. Levels of TNF-α and IL-1β mRNA in $F4/80^+$ cells

are higher in $\alpha^{-/-}$ mice compared with $\alpha\gamma^{-/-}$ mice (see Supplementary Figure 1 online at www.gastrojournal.org), suggesting that γδ T cells may activate macrophages to secrete large amounts of proinflammatory cytokines.

In conclusion, although γδ T cells at the inflamed colonic LP of $\alpha^{-/-}$ mice may protect intestinal epithelial injury, proinflammatory cytokines and neutrophil- and/or monocyte-chemoattractant chemokines induced by γδ T cells may directly and/or indirectly contribute to increased severity of UC-like chronic colitis in $\alpha^{-/-}$ mice. Further understanding of the molecular mechanisms of γδ T cell-mediated exacerbation of colitis in $\alpha^{-/-}$ mice will lead us to work out better therapeutic strategies for human UC.

Supplementary Data

Note: To access the supplementary material accompanying this article, visit the online version of *Gastroenterology* at www.gastrojournal.org, and at doi: 10.1053/j.gastro.2007.11.056.

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Immunosenescent colitogenic CD4$^+$ T cells convert to regulatory cells and suppress colitis

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Inflammatory bowel diseases progress steadily by the expansion of colitogenic CD4$^+$ cells. However, it remains unknown whether colitogenic CD4$^+$ cells are long-living like memory cells or exhausted like effector cells. To assess the longevity of colitogenic lamina propria (LP) CD4$^+$ cells, we performed sequential transfers of LP CD4$^+$ cells from colitic CD4$^+$CD45RB^{high} cell-transferred SCID mice into new SCID mice. Although SCID mice transferred with colitic LP CD4$^+$ cells stably developed colitis until at least the sixth transfer, the interval to the development of colitis gradually lengthened as the number of transfers increased. The incidence of colitis gradually decreased after the seventh transfer. Furthermore, non-colitic LP CD4$^+$ cells from mice transferred over seven times expressed significantly higher levels of PD-1 and produced significantly lower amounts of IFN-γ, TNF-α, and IL-17 than colitic LP CD4$^+$ cells recovered after the first transfer. Most notably, we found that re-transfer of non-colitic LP CD4$^+$ cells recovered after multiple transfers prevented the development of colitis in SCID mice co-transferred with CD4$^+$CD45RB^{high} cells. Thus, colitogenic LP CD4$^+$ cells may be exhausted over time, become non-functional, convert to regulatory cells, and finally suppress colitis in the process of immunosenescence.

Key words: Animal models · CD4$^+$ T cells · Intestinal immunity · Mucosal immunity

Introduction

Inflammatory bowel diseases (IBD), such as Crohn's disease and ulcerative colitis, are thought to result from the inappropriate activation and expansion of colitogenic CD4$^+$ T cells, which are driven by activated macrophages and dendritic cells. Antigens derived from the persistently present commensal bacteria continuously stimulate such cells, and this is presumably required for the induction of colitis. In general, IBD progresses steadily or with transient remissions throughout life [1, 2]. Importantly, the recurrent disease shows similar clinical features to the previous disease episode, and it is extremely uncommon that a patient with Crohn's disease relapses with another form of disease, such as ulcerative colitis [3–6]. Thus, it is conceivable that the sequential disease episodes are driven by a group of disease-specific colitogenic CD4$^+$ memory T cells, which may be designated as 'memory stem cells' [7] of the disease. In this scenario, colitogenic CD4$^+$ effector T cells established in the initial attack seem to arise repeatedly from the colitogenic CD4$^+$ memory T cells, but are presumably suppressed by regulatory T (Treg) cells during remission [8].

Although it appears that memory T cells are generally long living [9, 10] as shown by a series of successful vaccine programs, the details of their longevity are still unknown. In fact, it has been reported that the number of memory CD4$^+$ T cells declines over time in mice infected with lymphocytic choriomeningitis virus (LCMV) [11]. Furthermore, decline of the immune function due to the immunosenescence may affect the maintenance of memory CD4$^+$ T cells [12–14]. To evaluate this unsolved issue, we assessed the longevity of colitogenic lamina propria (LP) CD4$^+$ T cells and

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the characteristics of immunosenescent colitogenic LP CD4$^+$ T cells in a murine model of chronic colitis induced by adoptive transfer of CD4$^+$CD45RB^{high} T cells [15]. The present model is useful for this purpose, because the primarily transferred CD4$^+$CD45RB^{high} T cells in the recipient can be tracked over time, thereby allowing us to exclude the impact of new naive CD4$^+$ T cells that are continuously supplied from the thymus. Furthermore, we performed sequential adoptive transfers of the colitic LP CD4$^+$ T cells after developing CD4$^+$CD45RB^{high} T cell- or colitic LP CD4$^+$ T cell-transferred colitis in SCID mice. This model is also very useful to induce the extremely rapid proliferation of colitogenic LP CD4$^+$ T cells, which presumably respond to commensal bacterial-driven or autogenous antigens by lymphopenia-driven proliferation [9]. Using this unique sequential adoptive transfer model of colitogenic LP CD4$^+$ cells in SCID mice, we assessed the characteristics of immunosenescent colitogenic LP CD4$^+$ T cells that were generated by repeated transfers into lymphopenic host mice.

Results

Incidence of colitis is gradually decreased by repeated transfers of colitic LP CD4$^+$ T cells

We previously demonstrated that LP CD4$^+$ T cells obtained from colitic SCID mice that received adoptive transfer of CD4$^+$CD45RB^{high} T cells (the first transfer) are colitogenic CD44^{high}CD62L$^-$CD4$^+$IL-7Rα^{high} effector-memory (T_{EM})-like cells [16]. SCID mice transferred with such colitic LP CD4$^+$ T cells (the second transfer) develop colitis similar to the original CD4$^+$CD45RB^{high} T cell-transferred colitis in an IL-7-dependent manner [17]. This adoptive transfer model is also characterized by the rapid proliferation of donor CD4$^+$ T cells by lymphopenia-driven proliferation [9, 17], which provides an advantageous tool to assess the longevity and change in characteristics of these colitic LP CD4$^+$ T cells during repetitive transfer into SCID mice (Fig. 1A). As a rule of the current protocol, each mouse was killed when it reached over four points of the ongoing clinical score (see *Materials and methods*) within 40 weeks from transfer. Isolated LP CD4$^+$ T cells were then transferred into new SCID mice, and the procedure was repeated until the recipient mice failed to develop colitis within 40 weeks from transfer. Recipient mice that did not develop colitis within 40 wk from transfer were judged to be non-colitic, and were sacrificed for further assessment (Fig. 1A).

Although the interval between transfers gradually lengthened with the increase in number of transfers after the second (Fig. 1B), all the recipient mice examined until the sixth transfer stably developed wasting disease with colitis within 40 weeks from transfer. After the seventh transfer, however, some mice showed no sign of colitis up to 40 weeks from transfer as assessed by the ongoing clinical score (Fig. 1C), and the incidence of colitis development decreased (Fig. 1D). To further assess whether the cell viability of the transferred cells affected the present results, we performed Annexin V/PI staining of cells directly isolated from LP of mice by flow cytometry. As shown in Fig. 1E, there were no differences in the ratio of viable Annexin$^-$/PI$^-$ cells among LP CD4$^+$ T cells obtained from original CD4$^+$CD45RB^{high} T cell-transferred colitic mice (1^o colitic CD4$^+$), LP CD4$^+$ T cells obtained from colitic mice transferred with colitic LP CD4$^+$ T cells that were sequentially transferred over seven times (>7^o colitic CD4$^+$), and LP CD4$^+$ T cells from non-colitic mice transferred with colitic LP CD4$^+$ T cells that were sequentially transferred over seven times (>7^o non-colitic CD4$^+$).

Pattern of TCR Vβ are equivalent irrespective of the number of transfer

One reason why repeated transfer of colitic LP CD4$^+$ T cells leads to delayed onset and decreased incidence of the murine colitis may be that extensively proliferating colitogenic CD4$^+$ T cell clones are selectively depleted over time. Thus, we next checked TCR Vβ repertoire patterns of the 1^o colitic CD4$^+$, >7^o colitic CD4$^+$, and >7^o non-colitic CD4$^+$ T cells by flow cytometry. As depicted in Fig. 2, although the patterns of TCR Vβ repertoire were actually skewed into some group of TCR Vβ repertoire after both single adoptive and multiple adoptive transfers compared to those before transfer, they never integrated into a single specific TCR Vβ repertoire.

Non-colitic LP CD4$^+$ T cells generated by repeated transfer are inactivated

We next compared the immunological phenotypes of the 1^o colitic, the >7^o colitic, and the >7^o non-colitic CD4$^+$ T cells. We first compared the phenotypic composition of these cells in the colonic LP and in the spleen (SP) of mice transferred with the corresponding cells. As shown in Fig. 3, the number of cells recovered from LP or SP was, as expected, significantly lower in mice transferred with the >7^o non-colitic CD4$^+$ T cells than in mice transferred with the 1^o colitic or >7^o colitic CD4$^+$ T cells.

Cell surface markers of the 1^o colitic, >7^o colitic, and >7^o non-colitic SP and LP CD4$^+$ T cells had a phenotype of CD44^{high}CD62L$^-$IL-7Rα^{high} T_{FM}-like cells (Fig. 4A). An activation marker, CD69, was expressed on approximately two thirds of the 1^o colitic, >7^o colitic, and >7^o non-colitic LP CD4$^+$ T cells and on one third of the 1^o colitic and >7^o colitic SP CD4$^+$ T cells, but was markedly down-modulated on the >7^o non-colitic SP CD4$^+$ T cells (Fig. 4A), indicating that >7^o non-colitic SP CD4$^+$ T cells were inactivated. Since it has recently been suggested that a costimulatory molecule, PD-1, might serve as a useful marker to indicate the degree of non-functional T cell exhaustion on virus-specific CD8$^+$ and CD4$^+$ T cells [18–20], we assessed the expression of this molecule on our cells. As expected, PD-1 expression was significantly up-regulated on the >7^o non-colitic CD4$^+$ T cells both in the LP and SP as compared with the paired 1^o colitic and >7^o colitic CD4$^+$ T cells (Fig. 4A). In contrast, no difference in CD28 expression was observed among the 1^o colitic, >7^o colitic, and >7^o non-colitic

$CD4^+$ T cells (Fig. 3A). These results were also confirmed by statistical analysis (Fig. 4B).

We next determined cytokine production by anti-CD3/CD28 mAb-stimulated 1° colitic, >7° colitic, and >7° non-colitic $CD4^+$ T cells. As shown in Fig. 5, the >7° $CD4^+$ T cells, whether colitic or non-colitic, produced markedly less IFN-γ, IL-2, TNF-α, IL-10, IL-4, and IL-13 than 1° colitic $CD4^+$ T cells. The production of IL-17 by the >7° colitic and >7° non-colitic $CD4^+$ T cells was significantly lower than that by the 1° colitic $CD4^+$ T cells, although it was not completely abolished. Notably, the production of IL-17 by the >7° non-colitic $CD4^+$ T cells was significantly lower than that by the >7° colitic $CD4^+$ T cells. None of the cells produced TGF-β upon the present *in vitro* stimulation. These results suggested that the >7° non-colitic $CD4^+$ T cells have a functional defect in both activation and cytokine production, presumably due to immunosenescence or exhaustion induced by lymphopenia-induced proliferation for over 2 years.

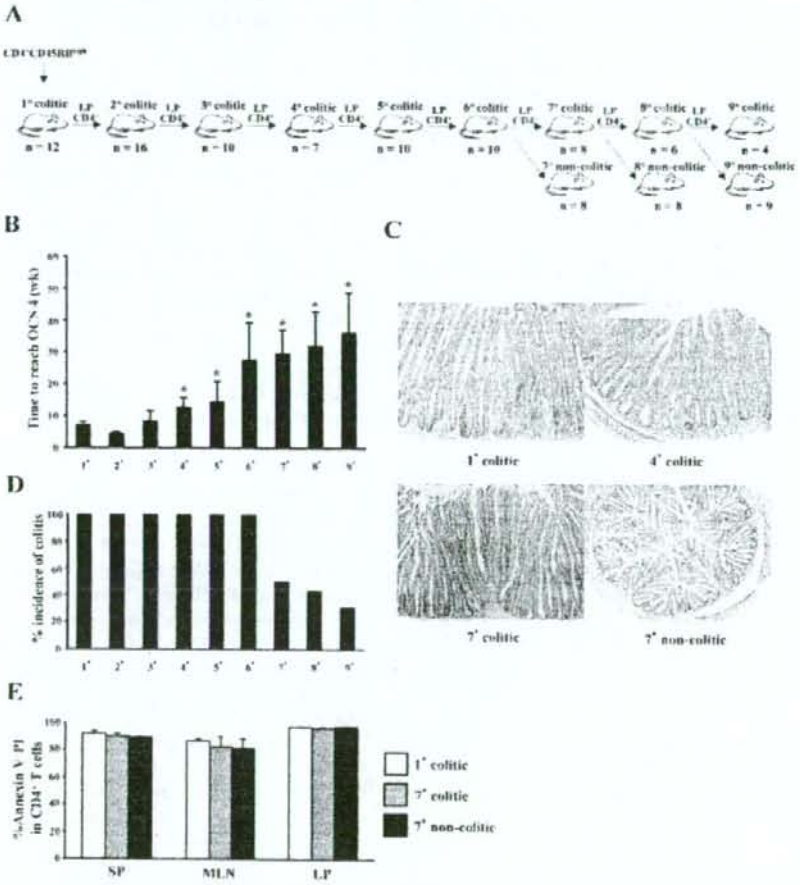

Figure 1. Incidence of colitis induced by adoptive transfer of colitic LP $CD4^+$ T cells gradually decreased as the transfers progress. (A) Schematic transfer protocol. C.B-17 SCID mice were transferred with BALB/c $CD4^+CD45RB^{high}$ T cells (1° colitic). When they reached an ongoing clinical score of four (see *Materials and methods*), LP $CD4^+$ T cells were isolated, and transferred into new SCID mice. Transfer of LP $CD4^+$ T cells was repeated up to nine times (2°–9° colitic), but was terminated when colitis did not develop within 40 weeks from transfer (7°–9° non-colitic). (B) The mean interval between transfer and establishment of colitis with an ongoing clinical score of four. Mice that did not develop colitis within 40 weeks from transfer were excluded for this index and judged to be non-colitic. OCS, ongoing clinical score. *$p < 0.05$ *vs.* the 2° transfer. (C) Histopathological findings of the colon. Original magnification, ×100. (D) The mean incidence rate of colitis in each transfer group. (E) Viability of cells directly isolated from LP of 1° colitic, >7° colitic, or >7° non-colitic mice. The number of viable cells (Annexin V^-/PI^-) was determined by a flow cytometry. Data are presented as mean ± SEM of % PI^- Annexin V^-/PI^- cells from four mice in each group. NS, not significantly different.

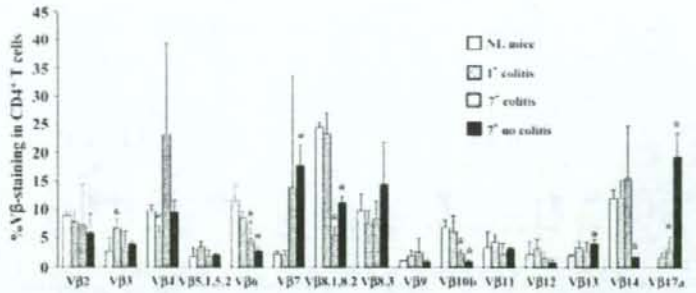

Figure 2. Flow cytometric analysis of the expression of Vβ families on the surface of the splenic normal, 1° colitic, >7° colitic, or >7° non-colitic CD4+ T cells. To analyze the TCR Vβ family repertoire, splenic cells were triple-stained with PECy5-conjugated anti-CD4 mAb and the following a panel of 15 FITC-conjugated Vβ mAb. Each percentage value indicates the frequency of each Vβ pooled from three independent experiments (each transfer; n=6). *$p<0.05$ *vs.* normal BALB/c mice.

Non-colitic LP CD4+ T cells have no characteristics of CD4+Foxp3+ Treg cells

Vukmanvic-Stejic and colleagues [21] have recently reported that a proportion of peripheral $CD4^+CD25^{high}Foxp3^+$ Treg cells in humans are generated from rapidly dividing memory $CD4^+CD45RO^+$ T cells in addition to thymus-derived classical $CD4^+CD25^{high}Foxp3^+$ Treg cells. Furthermore, colitogenic $CD4^+$ T cells in this colitis model proliferate and expand in response to foreign antigens more rapidly in immunodeficient SCID mice than do slow-dividing antigen-specific 'true' memory T cells [17]. We thus hypothesized that the colitogenic LP $CD4^+$ T cells gradually convert after multiple transfers to cytokine-non-producing $CD4^+$ Treg cells that have not only lost the ability to induce colitis but, conversely, gained the ability to suppress colitis. To assess this possibility, we next explored whether the >7° non-colitic $CD4^+$ T cells retain Treg cell activity *in vitro*. Since it has been shown that resting $CD4^+$ Treg cells express Foxp3 [22], we first analyzed the expression of Foxp3 in the 1° colitic, >7° colitic, and >7° non-colitic $CD4^+$ T cells, with splenic $CD4^+CD25^+$ Treg cells serving as a positive control. Unexpectedly, intracellular Foxp3 expression in the >7° non-colitic $CD4^+$ T cells was slight, and was not significantly higher than that in the 1° colitic and >7° colitic $CD4^+$ T cells, while the splenic $CD4^+CD25^+$ Treg cells expressed Foxp3 at a high level (Fig. 6A).

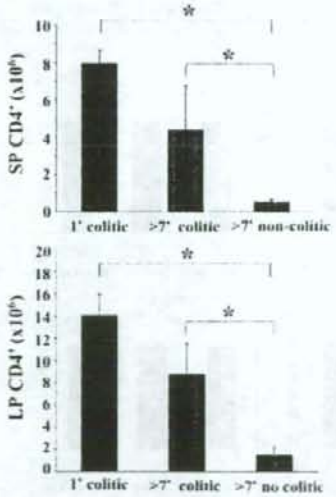

Figure 3. Expansion of $CD4^+$ T cells in the >7° non-colitic mice was significantly decreased. SP and LP $CD4^+$ T cells were isolated from colons when the colitic mice reached an ongoing clinical score of four, or the non-colitic mice lived up to 40 weeks post transfer. The number of $CD4^+$ T cells was determined by flow cytometry. Data are indicated as mean ± SEM of six mice in each group. *$p<0.05$ *vs.* the 1° coltic mice.

To further assess the possibility that the >7° non-colitic $CD4^+$ T cells may function as Treg cells, we examined whether these cells could suppress the proliferation of $CD4^+$ responder T cells in *in vitro* co-culture assay. Although splenic $CD4^+CD25^+$ Treg cells were able to suppress the proliferation of splenic $CD4^+CD25^-$ responder cells at a ratio of 1:1 to 1:0.125 of responder/Treg cells in the presence of mitomycin-C (MMC)-treated $CD4^-$ APC and soluble anti-CD3 mAb, the >7° non-colitic $CD4^+$ T cells and 1° colitic $CD4^+$ T cells could not suppress the proliferation at any ratio (Fig. 6B). Thus, at least in *in vitro* analysis, the >7° non-colitic $CD4^+$ T cells were a completely distinct cell population from peripherally induced $CD4^+Foxp3^+$ Treg cells, IL-10-producing Tr1 cells (Fig. 5) [23], and TGF-β-producing Th3 cells (Fig. 5) [24].

Co-transfer of non-colitic LP CD4+ T cells suppresses the development of colitis

Although the >7° non-colitic LP $CD4^+$ T cells did not show a regulatory function in the *in vitro* co-culture assay, such assays do not always represent *in vivo* function. To assess whether the >7° non-colitic LP $CD4^+$ T cells have characteristics of Treg cells and

can suppress colitis *in vivo*, we performed an *in vivo* adoptive transfer experiment with four groups of SCID mice: group 1, new SCID mice transferred with $CD4^+CD45RB^{high}$ T cells alone (3×10^5) as a positive control; group 2, SCID mice transferred with $CD4^+CD45RB^{high}$ T cells (3×10^5) and $CD4^+CD25^+$ Treg cells (1×10^5) as a negative control; group 3, SCID mice

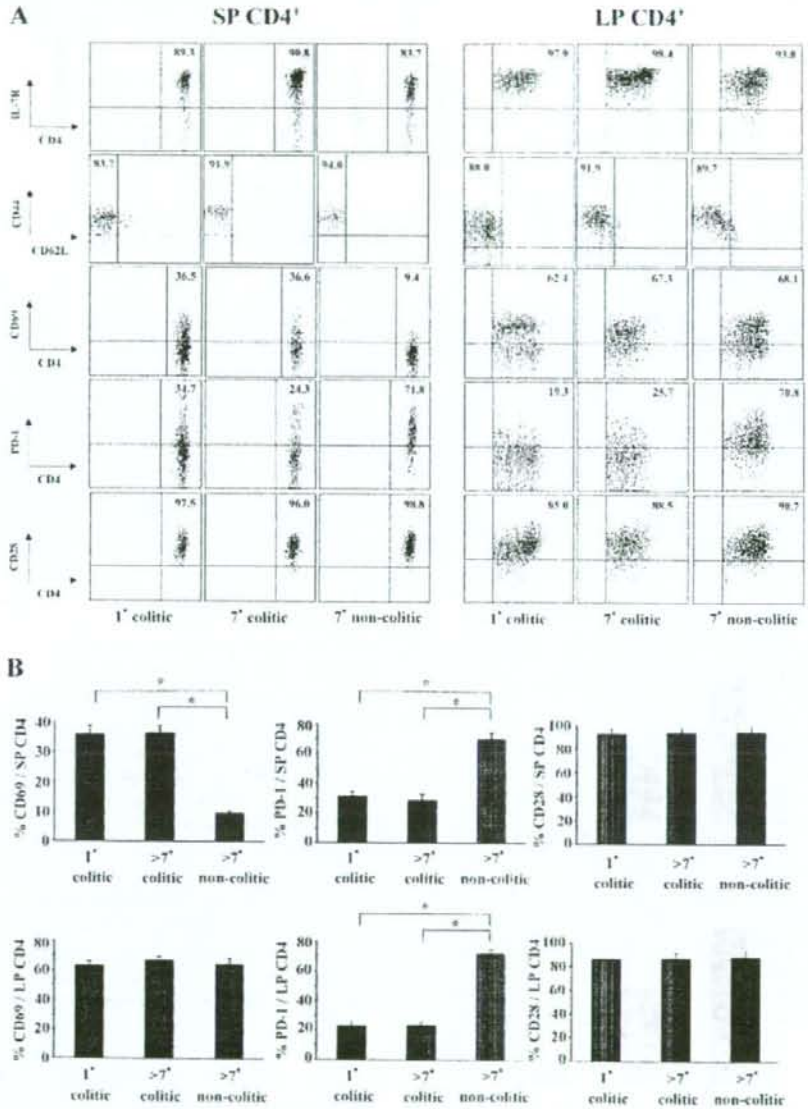

Figure 4. Expression of various cell surface markers on freshly isolated SP and LP $CD4^+$ T cells from 1° colitic, >7° colitic, and >7° non-colitic mice. (A) Representative analysis of IL-7Rα, CD62L, CD69, PD-1, and CD28 expression on SP or LP $CD4^+$ cells from 1° colitic, >7° colitic, and >7° non-colitic mice. Cells were stained with either FITC-conjugated anti-CD4, and the indicated biotinylated mAb, followed by PE-conjugated streptavidin or with fluorochrome-conjugated control Ig (not shown). (B) Percent positive cells of CD69, PD-1, and CD28 expression among SP or LP $CD4^+$ cells from 1° colitic, >7° colitic, and >7° non-colitic mice were determined by flow cytometry. Data are indicated as mean ± SEM of seven mice in each group. *$p < 0.05$.

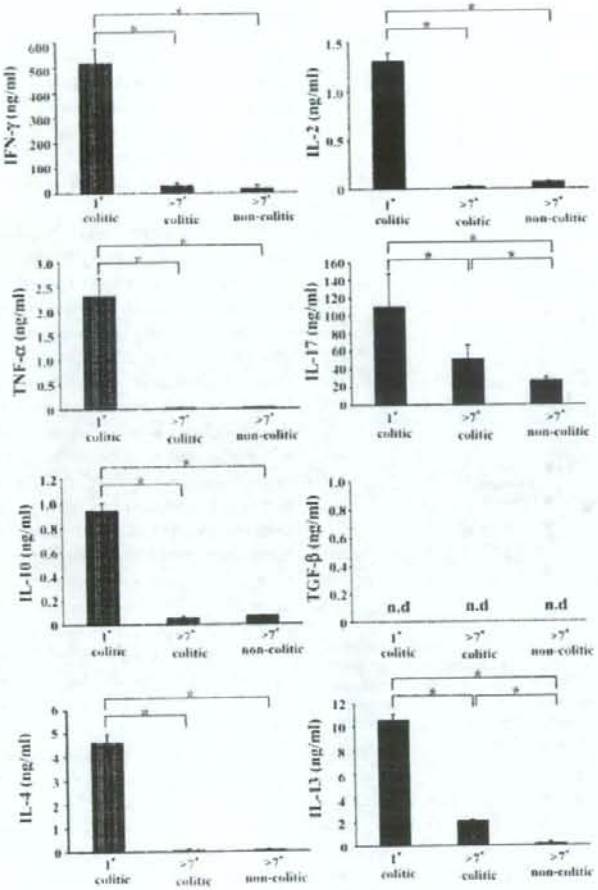

Figure 5. Cytokine production by LP CD4$^+$ T cells from 1° colitic, >7° colitic, and >7° non-colitic mice. LP CD4$^+$ T cells were isolated, and stimulated with anti-CD3/CD28 mAb for 48 h. The indicated cytokines in these supernatants were measured by ELISA. Data are shown as mean ± SD of seven mice in each group. *$p<0.05$ *vs.* the 1° colitic mice. n.d., not detected.

transferred with CD4$^+$CD45RB^{high} T cells (3×10^5) and 1° colitic LP CD4$^+$ T cells (1×10^5); and group 4, SCID mice transferred with CD4$^+$CD45RB^{high} T cells (3×10^5) and >7° non-colitic CD4$^+$ LP T cells (1×10^5) (Fig. 7A). Mice were killed 6 weeks after transfer. Surprisingly, the >7° non-colitic LP CD4$^+$ T cell fraction, like the control CD4$^+$CD25$^+$ Treg fraction, clearly showed a regulatory function toward intestinal inflammation, as these cell types both significantly inhibited the development of both wasting disease and colitis, when co-transfer with CD4$^+$CD45RB^{high} T cells (Fig. 7B–E). Colons of group 4 mice exhibited no pathological changes and were indistinguishable from the colons of group 2 mice (negative control) (Fig. 7B and D). In contrast, group 1 mice (positive control) and group 3 mice both developed wasting disease with severe colitis (Fig. 7B and D). The clinical and histological scorings also statistically confirmed these results (Fig. 7C and E).

A further quantitative evaluation of CD4$^+$ T cell infiltration was made by isolating the SP and LP CD4$^+$ T cells. As depicted in Fig. 7F, significantly fewer CD4$^+$ T cells were recovered from the SP and LP of mice reconstituted with CD4$^+$CD45RB^{high} and >7° non-colitic CD4$^+$ T cells (group 4) or CD4$^+$CD45RB^{high} + CD4$^+$CD25$^+$ T_R cells (group 2) as compared with mice reconstituted with CD4$^+$CD45RB^{high} cells alone (group 1) or CD4$^+$CD45RB^{high} cells + 1° colitic CD4$^+$ T cells (group 3).

To determine the effect of >7° non-colitic CD4$^+$ T cells on Th1 development, we measured IFN-γ, IL-2, and TNF-α production by anti-CD3/CD28-stimulated CD4$^+$ LP T cells. As shown in Fig. 7G, the production of IFN-γ, IL-2 and TNF-α was significantly reduced

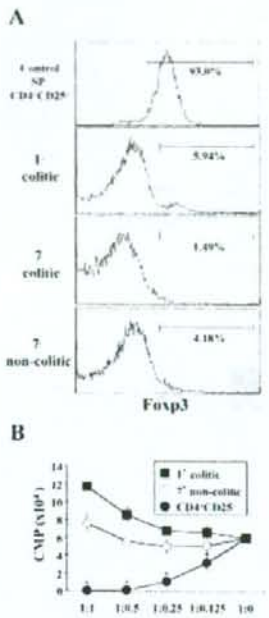

Figure 6. CD4$^+$ T cells obtained from non-colitic mice after seven or more transfers did not have a regulatory character *in vitro*. (A) Expression of Foxp3 in the indicated subpopulations was determined by flow cytometry as described in *Materials and methods*. (B) Suppressive activity of the indicated subpopulations was determined at a responder/Treg ratio of 1:0, 1:0.125, 1: 0.25, 1:0.5, or 1:1. $^*p < 0.05$.

by the co-transfer of CD4$^+$CD45RB^{high} and >7° non-colitic CD4$^+$ T cells (group 4) or CD4$^+$CD45RB^{high} and CD4$^+$CD25$^+$ T_R cells (group 2) as compared with that of CD4$^+$CD45RB^{high} T cells alone (group 1) or CD4$^+$CD45RB^{high} and 1° non-colitic CD4$^+$ T cells (group 3). Collectively, these results indicated that, at least *in vivo*, the >7° non-colitic CD4$^+$ T cells act as Treg cells to suppress the development of Th1-mediated colitis in a comparable manner to the control CD4$^+$CD25$^+$ Treg cells.

Discussion

In the present study, we performed seven or more sequential adoptive transfers of colitogenic LP CD4$^+$ T cells obtained from colitic SCID mice into new SCID mice. The SCID mice transferred with colitic LP CD4$^+$ T cells stably developed colitis, but interestingly the interval between transfer and development of colitis gradually lengthened as the number of transfers increased. Furthermore, the incidence of colitis gradually decreased after seven sequential transfers, accompanied by markedly increased expression of PD-1 but decreased production of various cytokines by the LP CD4$^+$ T cells. Importantly, transfer of non-colitic LP CD4$^+$ T cells that were recovered after seven or more transfers suppressed the development of colitis in SCID mice, which should have been induced by the transfer of CD4$^+$CD45RB^{high} T cells. Collectively, LP CD4$^+$ T cells that are colitogenic in origin may differentiate into CD4$^+$ Treg cells through the process of immunological exhaustion caused by lymphopenia-driven rapid proliferation [9], and gain the ability to suppress colitis. These findings have important implications for our understanding of the nature of colitogenic CD4$^+$ T cells as well as the natural course of IBD.

To exclude the possibility that changes of the bacterial flora in the examined mice affected the incidence of colitis in the present study, we routinely checked whether the examined mice might have been infected by pathological bacteria such as *Helicobacter hepaticus*, but found no evidence of contaminating bacteria throughout the experimental period for over 3 years (data not shown). In addition, we confirmed that colitis could be stably induced in SCID mice by transfer of CD4$^+$CD45RB^{high} T cells throughout this period, including the time when colitis did not develop in some SCID mice that were co-transferred with immunosenescent LP CD4$^+$ cells. Nevertheless, further study will be needed to address this issue, since we could not evaluate the components of non-pathological commensal bacteria using a comparative 16S-rRNA-gene-sequence survey in the examined mice. Also it has recently been reported that the bacterial flora of diseased and non-diseased animals are apparently distinct, even if they appear to be in the same environment [25].

Although accumulating evidence from the models of acute virus infection suggests that memory T cells, especially CD8$^+$ memory T cells, are long living [9, 10], it remains controversial whether this is also the case with CD4$^+$ memory T cells [26]. Furthermore, it is believed that "true" memory T cells, especially CD8$^+$ memory T cells, are established after the first clearance of the corresponding antigens [27], but this is also doubtful in the case of CD4$^+$ memory T cells. Conversely, recent reports suggest that both homeostatic stimulation by IL-7 and antigenic stimulation are needed for the full maintenance of CD4$^+$ memory T cells [28]. In fact, we showed here that the colitic CD4$^+$IL-7Rα^{high} T cells were stably transferable to new SCID mice, and that they continued to induce colitis in the presence of commensal bacteria through at least six transfers over a period of more than 2 years without additional supply of naive CD4$^+$ T cells.

We believe that the immunological memory of antigens is not related to the requirement of antigen clearance from the host body, because antigen-specific effector or memory T cells are inevitably separated from antigen-loading dendritic cells residing at draining lymph nodes, which leave there regardless of the presence of a corresponding antigen in the body [29]. It seems, however, that the interval before antigen-specific effector or memory T cells re-encounter the same antigen will be shorter. In fact, we previously demonstrated that colitogenic CD4$^+$CD44^{high}IL-7Rα^{high} T cells reside within the SP and bone marrow [30], which lack commensal bacterial antigens. Thus, it is possible and also reasonable that separation from sites where the corresponding antigen resides, rather than complete removal of antigens, is important for the generation of memory T cells. Furthermore,

accumulating evidence suggests that IL-7 dependency is a reliable assumption for CD4$^+$ memory T cells *in vivo* [31]. Consistently, we previously demonstrated that colitic LP CD4$^+$ T cells from colitic CD4$^+$CD45RB^{high} T cell-transferred RAG-2$^{-/-}$ mice express representative cell surface markers of memory T cells such as IL-7Rα and CD44 at a high level, and that the IL-7$^{-/-}$ × RAG-1$^{-/-}$ recipient mice transferred with CD4$^+$CD45RB^{high} T cells or colitic LP CD4$^+$ T cells never develop colitis [17].

However, even if the colitogenic CD4$^+$ T cells found in the CD4$^+$CD45RB^{high} T cell-transferred colitic mice can be called memory T cells, or 'persistent antigen-specific T cells' [27], their longevity and how their characteristics change with immunosenescence through multiple rounds of cell division. To evaluate this issue, we conducted sequential adoptive transfers of colitic LP CD4$^+$ T cells into new SCID mice. Although this method may be artificial, it is quite useful to examine colitic LP CD4$^+$ T cells that

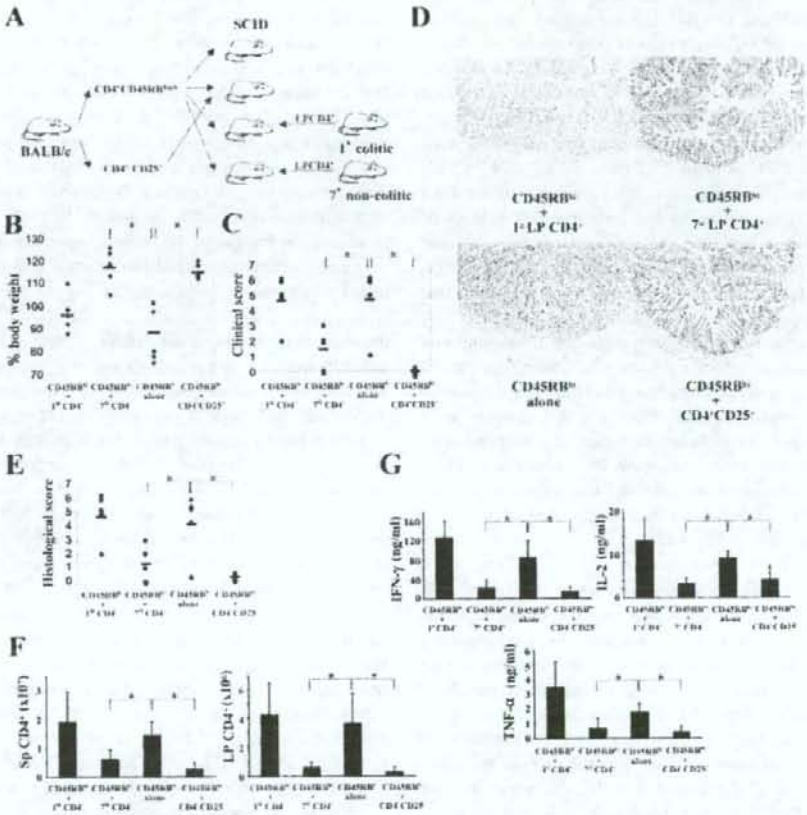

Figure 7. Cotransfer of LP CD4$^+$ T cells from 7° non-colitic mice prevents the development of colitis induced by adoptive transfer of CD4$^+$CD45RB^{high} T cells into SCID mice. (A) New SCID mice were divided into four groups: mice transferred with CD4$^+$CD45RB^{high} cells (3×10^5 per mouse) alone as a positive control, mice transferred with CD4$^+$CD45RB^{high} cells (3×10^5) and CD4$^+$CD25$^+$ cells (1×10^5) as a negative control, mice transferred with CD4$^+$CD45RB^{high} cells (3×10^5) and 1° colitic LP CD4$^+$ cells (1×10^5), and mice transferred with CD4$^+$CD45RB^{high} cells (3×10^5) and 7° non-colitic LP CD4$^+$ cells (1×10^5). Mice were killed six weeks after transfer. Each experiment was performed with groups of three mice each. The data are the sum of three independent experiments (n=9). (B) Change in body weight over time is expressed as percent of the original weight. Data are represented as mean ± SEM of nine mice in each group. *$p<0.05$ compared to mice transferred with CD4$^+$CD45RB^{high} cells alone. (C) Clinical scores were determined at 6 weeks after transfer. Data are indicated as mean + SEM of nine mice in each group. *$p<0.05$ compared to mice transferred with CD4$^+$CD45RB^{high} cells alone. (D) Histological examination of the colon from each group of mice at 6 weeks after transfer. Original magnification, ×100. (E) Histological scores were determined at 6 weeks after transfer. Data are indicated as the mean + SEM of nine mice in each group. *$p<0.05$ compared to mice transferred with CD4$^+$CD45RB^{high} cells alone. (F) SP and LP mononuclear cells were isolated from the colon at 6 weeks after transfer, and the number of CD4$^+$ cells was determined by flow cytometry. Data are indicated as mean ± SEM of nine mice in each group. *$p<0.05$ compared to mice transferred with CD4$^+$CD45RB^{high} cells alone. (G) Cytokine production by LP CD4$^+$ T cells. LP CD4$^+$ T cells were prepared from the colons at 6 weeks after transfer and stimulated with anti-CD3/CD28 mAbs for 48 h. The indicated cytokines in these supernatants were measured by ELISA. Data are indicated as mean + SEM of six mice in each group, which was selected from the first two *in vivo* experiments. *$p<0.05$ compared to mice transferred with CD4$^+$CD45RB^{high} cells alone.

have undergone multiple rounds of cell division. In this study, we found that (i) the interval between transfer and development of colitis gradually lengthened as the number of transfers increased, (ii) a certain population of the recipient SCID mice did not develop colitis after seven or more transfers, and (iii) the production of IFN-γ, TNF-α and IL-17 by LP CD4$^+$ T cells recovered after seven or more transfers was significantly impaired. These data clearly indicate that colitogenic CD4$^+$ T cells are gradually exhausted over time and finally lose the ability to induce colitis. However, it remains unclear whether LP CD4$^+$ T cells recovered from recipient mice and maintained for over 40 weeks post transfer are able to induce colitis, since the designation of mice that did not develop colitis within 40 weeks post transfer as non-colitic was made arbitrarily. Further study will be needed to address this issue.

Most notably, however, we also found that new SCID mice transferred with CD4$^+$CD45RB^{high} T cells and LP CD4$^+$ T cells obtained from non-colitic SCID mice after seven transfers did not develop colitis. This finding further indicates that colitogenic CD4$^+$ T cells not only lose their colitogenicity over time, but also gain a regulatory function like CD4$^+$ Treg cells through the process of immunosenescence, and suppress colitis. It is also possible that LP CD4$^+$ T cells obtained from non-colitic SCID recipient mice may simply delay, but not completely suppress, the development of colitis through competition for cytokines (cytokine deprivation) [32] between newly recruited effector cells. However, our findings may also correlate with the clinical nature of IBD, as the majority of patients actually run a chronic or relapsing course, whereas patients with severe symptoms show diminishing severity of symptoms over time, presumably through the immunosenescence of colitogenic CD4$^+$ T cells along with the decrease of new naive T cell supply from the thymus [33].

At the moment, it is largely unknown which type of Treg cells is closely associated with the immunosenescent LP CD4$^+$ T cells described in the current study, which were colitogenic in origin, but acquired regulatory activity to suppress the development of colitis. A recent publication by Vukmanovic-Stejic and colleagues [21] demonstrated that a substantial proportion of peripheral human CD4$^+$CD25^{high}Foxp3$^+$ Treg cells is generated from rapidly dividing, highly differentiated CD4$^+$ memory T cells in addition to the cells of same phenotype derived from the thymus. Moreover, Liu and colleagues [34] reported that the interaction between neurons and CD4$^+$ T cells results in the conversion of encephalogenic CD4$^+$ T cells to CD4$^+$CD25$^+$Foxp3$^+$ T_R cells in a murine model of experimental autoimmune encephalomyelitis (EAE). Although peripherally inducible CD4$^+$CD25$^+$Foxp3$^+$ Treg cells appear quite similar to our immunosenescent LP CD4$^+$ T cells in that they are generated by continuous stimulation of antigens in the periphery, we could not detect the up-regulation of Foxp3 in the immunosenescent LP CD4$^+$ T cells.

It has recently been reported that PD-1 is a marker for exhausted CD8$^+$ and CD4$^+$ T cells in chronic lymphocytic choriomeningitis virus (LCMV) and HIV infections [18–20]. Consistent with this, we demonstrated that LP CD4$^+$ T cells obtained from non-colitic mice after over seven or more transfers expressed significantly higher levels of PD-1. In addition, we previously showed that peripheral CD4$^+$PD-1$^+$ T cells in normal mice possess a regulatory function both *in vivo* and *in vitro*, regardless of the expression of CD25 [35]. Thus, it is possible that the PD-1/PD-L1 pathway is required for immunosenescent LP CD4$^+$ T cells to function *in vivo* as Treg cells, although further study using mAb to block the PD-1/PD-L signal pathway is needed to address this issue. Apart from Treg cells, it is also noteworthy that dying exhausted cells (apoptotic cells) are frequently associated with an immunosuppressive activity against other immune cells including CD4$^+$ T cells [36, 37]. This mechanism involves anti-inflammatory TGF-β released by macrophages, which phagocyte the apoptotic cells. Although we could not detect decreased viability (Fig. 1) or increased TGF-β activity (Fig. 5) of the non-colitic LP CD4$^+$ T cells after seven transfers, it is conceivable that exhausted LP CD4$^+$ T cells are phagocytosed by surrounding macrophages, and the production of anti-inflammatory cytokines by such cells may be involved in the induction of immunosuppression. Further studies will be needed to address the regulatory mechanism of the immunosenescent LP CD4$^+$ T cells.

Finally, our results should be discussed in connection with a recent publication by Abadia-Molina and colleagues [38], which reported serial adoptive transfer of colitic CD4$^+$ T cells residing in the mesenteric lymph nodes (MLN) of their original model of colitis induced by the transplantation of wild-type bone marrow into adult tgε26 mice (called BM→tgε). They demonstrated that the isolated MLN CD4$^+$ T cells in colitic BM→tgε mice not only maintained colitogenicity with a dominant Th1 phenotype after over eight or more transfers, but also converged into a single TCR Vβ usage (Vβ8.1/2, Vβ8.3, Vβ10b or Vβ14) of up to 90% in a certain line of colitic mice, leading to a novel method for cloning colitogenic CD4$^+$ cells through serial adoptive transfers. In our system using LP cells as donor cells, however, we could not detect any convergence of TCR Vβ usage in Vβ8.1/2, Vβ8.3, Vβ10b and Vβ14 (Fig. 2). Furthermore, we found that the ability to reproduce colitis upon sequential transfer gradually decreased in terms of the interval between transfers and the incidence of colitis. These discrepancies would be explained by differences in the model of colitis; differences in the presence of NK and B cells, and differences in the type of donor cells, those from MLN being rich in central memory T (T_{CM}) cells, and those from LP being rich in T_{EM} cells [39]. Further study will be needed to address this issue.

In summary, we demonstrated through our unique model of sequential adoptive transfers into lymphopenic SCID mice that colitogenic CD4$^+$ T_{EM}-like cells in colitic mice are exhausted over time and are finally converted into cytokine-non-producing Treg cells that suppress the development of colitis. Thus, our current study may provide a new approach for the treatment of IBD by transfer of immunosenescent CD4$^+$ T cells generated from colitogenic CD4$^+$ T cells by *in vitro* acceleration of cell divisions to promote their regulatory function.

Materials and methods

Animals

Female BALB/c and C.B-17 SCID mice were purchased from Japan Clea (Tokyo, Japan). Mice were maintained under specific pathogen-free (SPF) conditions in the Animal Care Facility of Tokyo Medical and Dental University. Donors and littermate recipients were used at 6–12 weeks of age. All experiments were approved by the regional animal study committees and were done according to institutional guidelines and Home Office regulations.

Antibodies

The following mAb except biotin-conjugated anti-mouse IL-7Rα (A7R34; eBioscience, San Diego) were obtained from BD Pharmingen (San Diego, CA) for purification of cell populations and flow cytometry analysis: 145-2C11, FITC-conjugated anti-mouse CD3; RM4-5, FITC- or PE-conjugated anti-mouse CD4; 16A, FITC-conjugated anti-mouse CD45RB; 7D4, FITC-conjugated anti-mouse CD25; IM7, PE-conjugated anti-mouse CD44; MEL-14, FITC- or PE-conjugated anti-mouse CD62L; H1.2F3, FITC-conjugated anti-mouse CD69; 37.51, PE-conjugated anti-mouse CD28; J43, PE-conjugated anti-mouse PD-1 and the following FITC-conjugated antibodies; Vβ2 (B20.6), Vβ3 (KJ25), Vβ4 (KT4), Vβ5 (MR9–4), Vβ6 (RR4–7), Vβ7 (TR310), Vβ8.1/2 (MR5–2), Vβ8.3 (B21.14), Vβ9 (MR10–2), Vβ10b (B21.5), Vβ11 (RR3–15), Vβ12 (MR11–1), Vβ13 (IN12.3), Vβ14 (14.2), and Vβ17 (KJ23). Biotinylated antibodies were detected with PE- or Cy-Chrome™-streptavidin (BD Pharmingen).

T cell preparation

SP and LP $CD4^+$ T cells were isolated from mice as previously described [15]. The resultant cells contained > 94% $CD4^+$ cells when analyzed by FACSCalibur. SP $CD4^+$ T cells were then labeled with PE-conjugated anti-mouse CD4 mAb and FITC-conjugated anti-CD45RB mAb, and then sorted to yield the $CD45RB^{high}$ (highest staining 30%) fraction by the FACS Vantage SE (Becton Dickinson, Sunnyvale, CA).

Sequential adoptive transfer experiments

Colitis was induced in C.B-17 SCID mice by adoptive transfer of $CD4^+CD45RB^{high}$ T cells [15]. Each SCID mouse was injected intraperitoneally with 3×10^5 $CD4^+CD45RB^{high}$ T cells. All the recipient mice were weighed initially, and three times per week after transfer. Mice were killed when their ongoing clinical score after transfer reached four points or more as mentioned below, and LP $CD4^+$ T cells were isolated for the next transfer. The isolated colitic LP $CD4^+$ T cells (3×10^5/mouse) were then transferred into new SCID mice [16]. After seven sequential transfers, we found that some mice failed to develop colitis within 40 weeks from transfer. To characterize these non-colitic LP $CD4^+$ T cells, we transferred $CD4^+CD45RB^{high}$ T cells (3×10^5) alone (group 1), $CD4^+CD45RB^{high}$ T cells (3×10^5) and LP $CD4^+$ T cells obtained from colitic mice originally transferred with $CD4^+CD45RB^{high}$ T cells (1×10^5) (group 2), or $CD4^+CD45RB^{high}$ T cells (3×10^5) and LP $CD4^+$ T cells obtained from the non-colitic mice after seven transfers (1×10^5) (group 3) into new SCID mice. Mice were killed 6 weeks after transfer.

Clinical and histological scorings

The recipient mice were weighed initially, then three times per week after transfer. They were observed for clinical signs of illness [40]: hunched over appearance, piloerection of the coat, diarrhea, and blood in the stool. When mice were killed at a predetermined time point, their clinical score was assessed as the sum (0–8 points) of four parameters: hunching and wasting, 0 or 1; colon thickening, 0–3 (0, no colon thickening; 1, mild thickening; 2, moderate thickening; 3, extensive thickening); stool consistency, 0–3 (0, normal beaded stool; 1, soft stool; 2, diarrhea); and gross blood, 0 or 1 [17]. For the sequential adoptive transfers, we monitored the clinical signs during the observation period, and the ongoing clinical score was defined as the sum (0–5 points) of the above-parameters other than colon thickening [40]. Mice were killed when their ongoing clinical score reached four points or more, and isolated LP $CD4^+$ T cells were transferred into new SCID mice. Transfers were repeated as long as the mice continued to develop colitis within 40 weeks post transfer. We judged recipient mice to be 'non-colitic' when they did not develop colitis within 40 weeks post transfer, and killed them at this time for further analysis. Tissue samples were fixed in PBS containing 10% neutral-buffered formalin. Paraffin-embedded sections (5 μm) were stained with H&E. The mean degree of inflammation in the colon was calculated as previously described [15].

Flow cytometry

To detect surface expression of various molecules, isolated splenocytes or LP mononuclear cells (LPMC) were preincubated with an FcγR-blocking mAb (CD16/32; 2.4G2, BD PharMingen) for 15 min, then incubated with specific FITC-, PE-, PECy5- or biotin-labeled antibodies for 20 min on ice. Biotinylated antibodies were detected with PE- or Cy-Chrome™-streptavidin. Intracellular Foxp3 staining was performed with the PE-anti-mouse Foxp3 staining set (eBioscience) according to the manufacturer's instructions. Standard two- or three-color flow cytometric analyses were obtained using the FACSCalibur equipped with CellQuest software. Background fluorescence was assessed by staining of the control irrelevant isotype-matched mAb.

Cytokine ELISA

To measure cytokine production, 1×10^5 LP $CD4^+$ T cells were cultured in 200 μL culture medium at 37°C in a humidified atmosphere containing 5% CO_2 in 96-well plates (Costar, Cambridge, MA) pre-coated with 5 μg/mL hamster anti-mouse CD3ε mAb (145-2C11, BD PharMingen) and 2 μg/mL 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 per the manufacturer's recommendation (R&D, Minneapolis, MN).

In vitro functional analysis for Treg cells

$CD4^-$ cells were prepared from BALB/c splenocytes as APC by depleting $CD4^+$ cells with anti-CD4 MACS and treatment with 50 μg/mL MMC for 45 min at 37°C. In co-culture experiments, $CD4^+CD25^+$ T cells ($0–1 \times 10^4$ as Treg) or isolated LP $CD4^+$ T cells ($0–1 \times 10^4$) were cultured with $CD4^+CD25^-$ responder cells (1×10^4) and MMC-treated $CD4^-$ cells (5×10^5) in the presence of anti-CD3 mAb (1 μg/mL). To determine proliferation, each well was pulsed with 1 μCi [^{3}H]thymidine (NEN, Boston, MA) for the last 9 h of 72-h culture.

Statistical analysis

The results were expressed as the mean ± SEM. Groups of data were compared by the Mann-Whitney U test. Differences were considered to be statistically significant when $p<0.05$.

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Abbreviations: **BM-DC:** bone marrow-derived DC · **LSEC:** liver sinusoidal endothelial cells · **MSC:** mesenchymal stem cell

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Musashi-1 suppresses expression of Paneth cell-specific genes in human intestinal epithelial cells

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Background. Musashi-1 (Msi-1) is a RNA-binding protein, known as a putative marker of intestinal stem cells (ISCs). However, little is known about the function of Msi-1 within human intestinal epithelial cells (IECs). Thus, the present study aimed to clarify the role of Msi-1 in differentiation and proliferation of IECs. ***Methods.*** A human intestinal epithelial cell line stably expressing Msi-1 was established. Proliferation of the established cell lines was measured by bromodeoxyuridine incorporation, whereas differentiation were assessed by reverse transcriptase-polymerase chain reaction (RT-PCR) analysis of lineage-specific genes. Activities of the Notch and Wnt pathways were examined either by reporter assays or expression of downstream target genes. The distribution of Msi-1 and PLA2G2A expression in vivo was determined by immunohistochemistry. ***Results.*** Constitutive expression of Msi-1 in IECs had no significant effect on cell proliferation, but suppressed expression of Paneth cell-specific genes, including *PLA2G2A*. Msi-1 appeared to suppress expression of the *PLA2G2A* gene at the mRNA level. Analysis of Notch and Wnt pathway activity, however, revealed no significant change upon Msi-1 expression. The expression of Msi-1 and PLA2G2A in vivo was restricted to IECs residing at the lowest part of the human intestinal crypt, but was clearly separated to within basal columnar cells or mature Paneth cells, respectively. ***Conclusions.*** Msi-1 suppresses expression of Paneth cell-specific genes in IECs, presumably through a pathway independent from Notch or Wnt. These findings suggest Msi-1 is a negative regulator of Paneth cell differentiation, an may contribute to maintain the undifferentiated phenotype of ISCs.

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Introduction

The rapid and continuous renewal of the intestinal epithelium is maintained by the regulated supply of newborn cells that arise from a common progenitor cell called the intestinal stem cell (ISC).[1] Such tissue-specific stem cells share common potentials to self renew and also to give rise to all cell lineages composing the residing tissue.[2] Such properties of stem cells are maintained by a complex interaction of various cell-signaling pathways.[3] Among such signaling pathways, Wnt and Notch represent the core molecular pathways that play crucial roles in maintaining stem cell properties.[4] Indeed, both Wnt and Notch signaling have been shown to function in intestinal crypt epithelial cells, including the ISCs.[5] A recent study identified Lgr-5, a direct target of the canonical Wnt pathway, as a definite marker of murine ISCs.[6] This study further emphasized the dominant role of the canonical Wnt pathway in maintaining cell proliferation and multipotency of ISCs. It is, however, known that activation of the canonical Wnt pathway is present not only in ISCs but also in Paneth cells residing just adjacent to ISCs, where it promotes maturation and restricts the cell position of such cells.[7] Thus, activation of the canonical Wnt pathway appears to have a completely different function in ISCs and Paneth cells, presumably depending on the cell context determined by other molecular factors. However, the molecular mechanism regulating such lineage-specific functions of canonical Wnt signaling in IECs remains largely unknown.

Musashi-1 (Msi-1) is an RNA-binding protein, and its gene was formerly reported as another candidate marker gene for ISCs.[8,9] Its molecular function has been deter-