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 $CD44^{high}CD62L^-CD4^+IL\text{-}7R\alpha^{high}$ 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° 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° 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° 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° colitic $CD4^+$, >7° colitic $CD4^+$, and >7° 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° colitic, the >7° colitic, and the >7° 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° non-colitic $CD4^+$ T cells than in mice transferred with the 1° colitic or >7° colitic $CD4^+$ T cells.

Cell surface markers of the 1° colitic, >7° colitic, and >7° non-colitic SP and LP $CD4^+$ T cells had a phenotype of $CD44^{high}CD62L^-IL\text{-}7R\alpha^{high}$ T_{EM}-like cells (Fig. 4A). An activation marker, CD69, was expressed on approximately two thirds of the 1° colitic, >7° colitic, and >7° non-colitic LP $CD4^+$ T cells and on one third of the 1° colitic and >7° colitic SP $CD4^+$ T cells, but was markedly down-modulated on the >7° non-colitic SP $CD4^+$ T cells (Fig. 4A), indicating that >7° 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° non-colitic $CD4^+$ T cells both in the LP and SP as compared with the paired 1° colitic and >7° colitic $CD4^+$ T cells (Fig. 4A). In contrast, no difference in CD28 expression was observed among the 1° colitic, >7° colitic, and >7° 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.

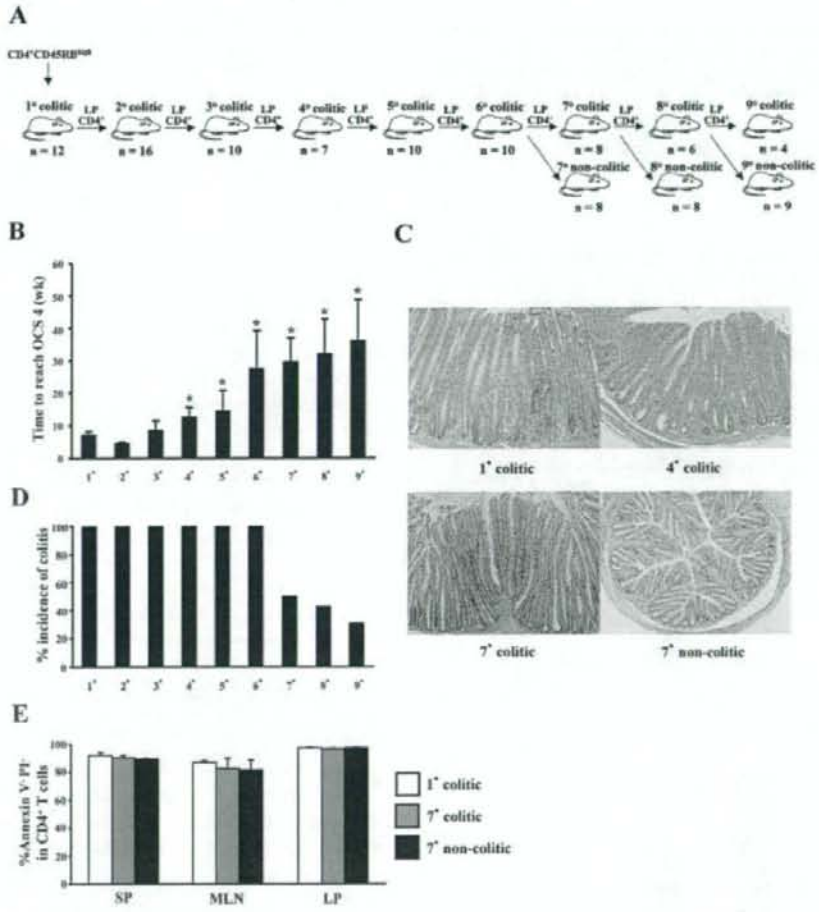

Figure 1. Incidence of colitis induced by adoptive transfer of colitic LP $CD4^+$ T cells gradually decreased as the transfers progress. (A) Schematic transfer protocol. C.B-17 SCID mice were transferred with BALB/c $CD4^+CD45RB^{high}$ T cells (1° colitic). When they reached an ongoing clinical score 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.

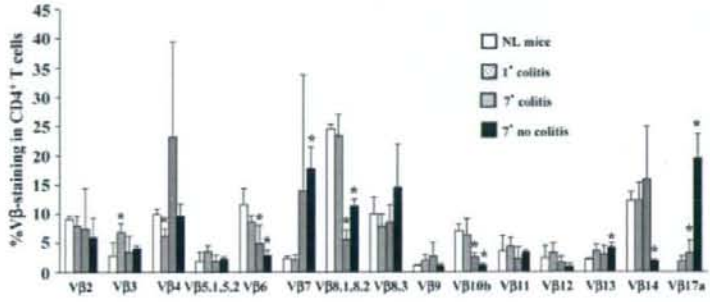

Figure 2. Flow cytometric analysis of the expression of Vβ families on the surface of the splenic normal, 1° colitic, >7° colitic, or >7° non-colitic CD4+ T cells. To analyze the TCR Vβ family repertoire, splenic cells were triple-stained with PECy5-conjugated anti-CD4 mAb and the following a panel of 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).

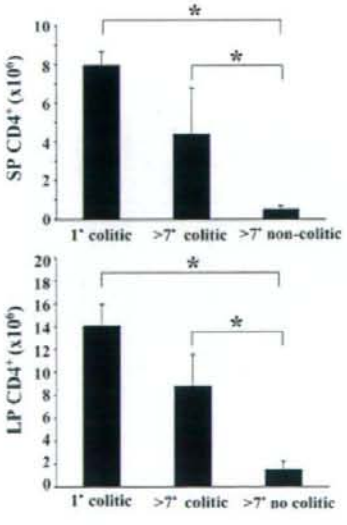

Figure 3. Expansion of $CD4^+$ T cells in the >7° non-colitic mice was significantly decreased. SP and LP $CD4^+$ T cells were isolated from colons when the colitic mice reached an ongoing clinical score of four, or the non-colitic mice lived up to 40 weeks post transfer. The number of $CD4^+$ T cells 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

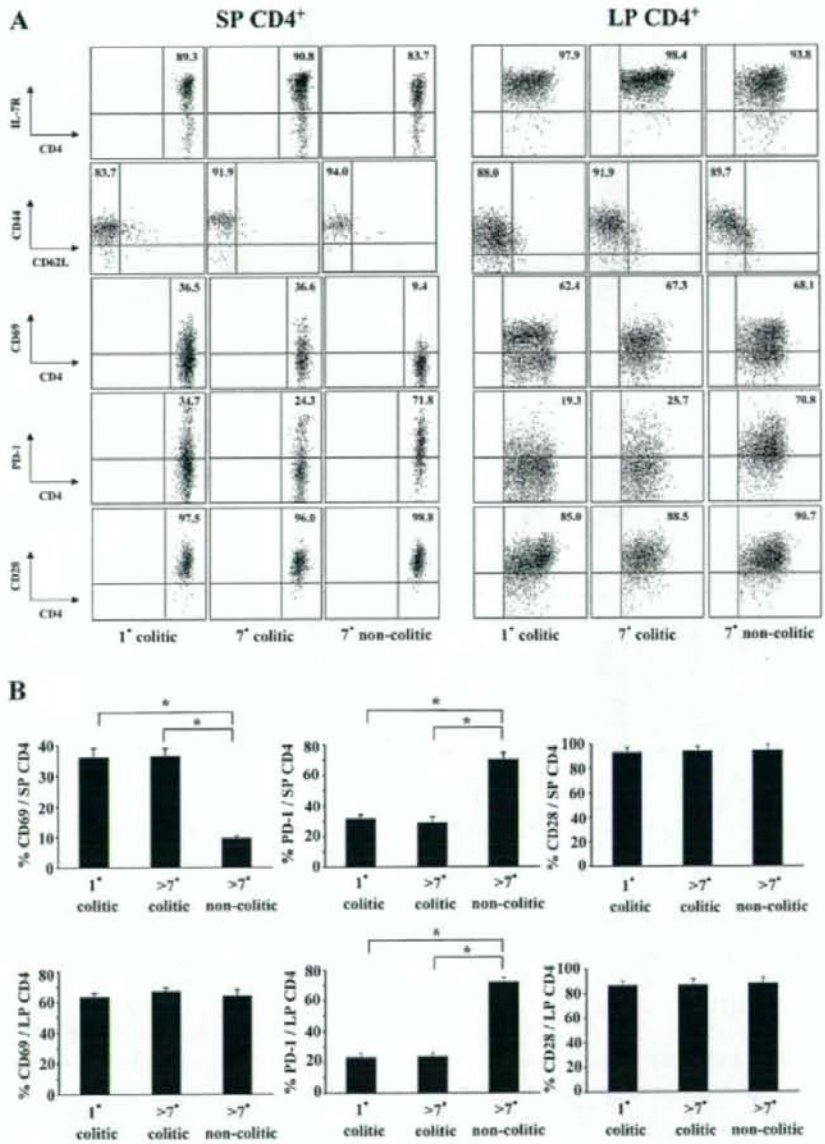

Figure 4. Expression of various cell surface markers on freshly isolated SP and LP $CD4^+$ T cells from 1° colitic, >7°colitic, and >7° non-colitic mice. (A) Representative analysis of IL-7Rα, CD62L, CD69, PD-1, and CD28 expression on SP or LP $CD4^+$ cells from 1° colitic, >7° colitic, and >7° non-colitic 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. *$p<0.05$.

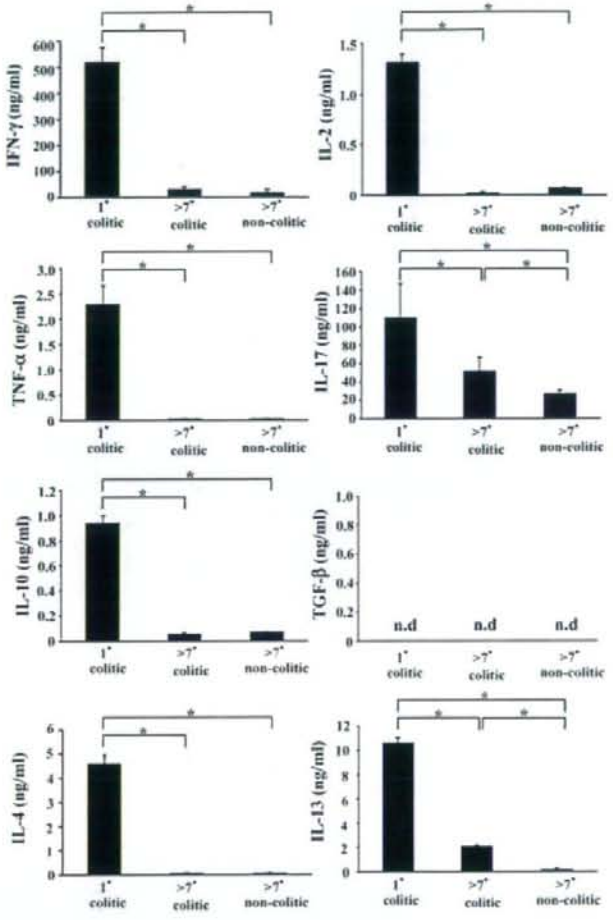

Figure 5. Cytokine production by LP CD4$^+$ T cells from 1° colitic, >7° colitic, and >7° non-colitic mice. LP CD4$^+$ T cells were isolated, and stimulated with anti-CD3/CD28 mAb for 48 h. The indicated cytokines in these supernatants were measured by ELISA. Data are shown as mean ± SD of seven mice 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

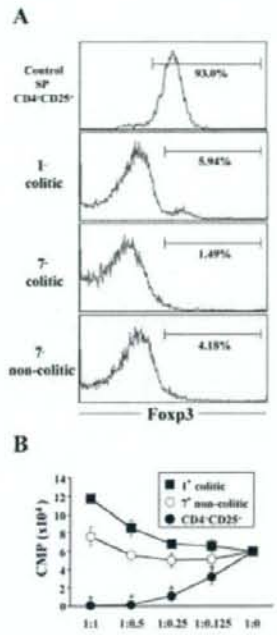

Figure 6. $CD4^+$ T cells obtained from non-colitic mice after seven or more transfers did not have a regulatory character *in vitro*. (A) Expression of Foxp3 in the indicated subpopulations was determined by flow cytometry as described in *Materials and methods*. (B) Suppressive activity of the indicated 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 $>7^o$ 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^o non-colitic $CD4^+$ T cells (group 3). Collectively, these results indicated that, at least *in vivo*, the $>7^o$ 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-$7R\alpha^{high}$ 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-$7R\alpha^{high}$ 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

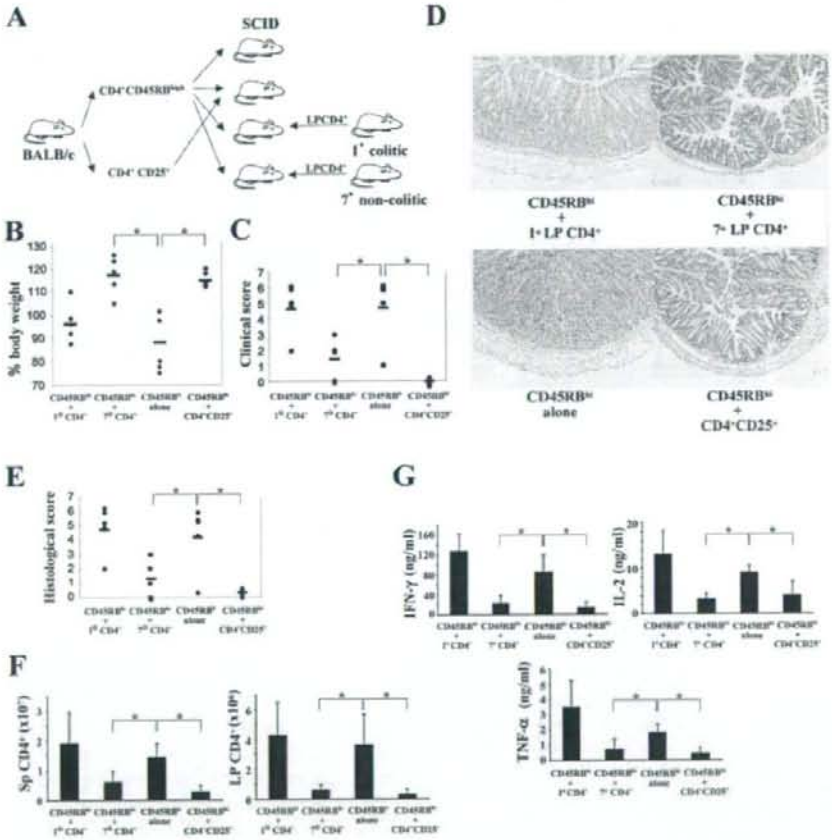

Figure 7. Cotransfer of LP $CD4^+$ T cells from >7° non-colitic mice prevents the development of colitis induced by adoptive transfer of $CD4^+CD45RB^{high}$ T cells into SCID mice. (A) New SCID mice were divided into four groups: mice transferred with $CD4^+CD45RB^{high}$ cells (3×10^5 per mouse) alone 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 $CD4^+PD\text{-}1^+$ 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-I 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

Full correspondence: Takanori Kanai, Department of Gastroenterology and Hepatology, Tokyo Medical and Dental University, 1-5-45 Yushima, Bunkyo-ku, Tokyo 113–8519, Japan
Fax: +81-3-5803-0268
e-mail: taka.gast@tmd.ac.jp

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Inhibition of intracellular hepatitis C virus replication by nelfinavir and synergistic effect with interferon-α.

Satoshi Toma[1], Tsuyoshi Yamashiro[2*], Shingo Arakaki[1], Joji Shiroma[1], Tatsuji Maeshiro [1], Kenji Hibiya[1], Naoya Sakamoto [3], Fukunori Kinjo[4], Masao Tateyama[1], and Jiro Fujita[1]

[1]First Department of Internal Medicine, School of Medicine, University of the Ryukyus, Okinawa, Japan

[2]Department of Blood Transfusion Medicine, Ryukyu University Hospital, Okinawa, Japan

[3]Department of Gastroenterology and Hepatology, Tokyo Medical and Dental University, Tokyo, Japan

[4]Department of Endoscopy, Ryukyu University Hospital, Okinawa, Japan

Running title: Nelfinavir inhibits HCV replication

*Correspondence to: Tsuyoshi Yamashiro, M.D., Ph.D.
Department of Blood Transfusion Medicine
Ryukyu University Hospital, Okinawa, Japan
207 Uehara, Nishihara-cho, Okinawa 903-0215, Japan.
Phone +81 98-895-1144 Fax +81 98-895-1414
E-mail: tsuyo-y@med.u-ryukyu.ac.jp

Key words: hepatitis C virus, human immunodeficiency virus, nelfinavir

Abbreviations: HCV, hepatitis C virus; HIV, human immunodeficiency virus; IFN, interferon; HAART, highly active anti-retroviral therapy; LDH, Lactate dehydrogenase; CI, combination index; TUNEL, terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate-biotin nick end-labeling; MTS, 5-(3-carboxymethoxyphenyl)-2-(4,5-dimethylthiazoly)-3-(4-sulfophenyl) tetrazolium inner salt

Abstract

Liver diseases associated with hepatitis C virus (HCV) infection have become the major cause of mortality in patients with human immunodeficiency virus (HIV) infection since the introduction of highly active anti-retroviral therapy (HAART). HCV-related liver disease is more severe in HIV-infected patients than in non-HIV-infected patients, but the standard therapies used to treat chronic hepatitis C in HCV/HIV coinfected patients are the same as those for patients infected with HCV alone. HIV protease inhibitor might have potential to down-regulate HCV load of HCV/HIV-coinfected patients. In this study, we evaluated the effects of nelfinavir on intracellular HCV replication using the HCV replicon system. We constructed an HCV replicon expressing a neomycin-selectable chimeric firefly luciferase reporter protein. Cytotoxicity and apoptosis induced by nelfinavir were assessed and synergism between nelfinavir and interferon (IFN) was calculated using CalcuSyn analysis. Nelfinavir dose-dependently repressed HCV replication at low concentrations (IC_{50}, 9.88 μmol/l). Nelfinavir failed to induce cytotoxicity or apoptosis at concentrations that inhibited HCV replication. Clinical concentrations of nelfinavir (5 μmol/l) combined with IFN showed synergistic inhibition of HCV replication. Our results suggest that the direct effects of nelfinavir on the HCV subgenome and its synergism with IFN could improve clinical responses to IFN therapy in HCV/HIV coinfected patients.

Introduction

Patients with human immunodeficiency virus (HIV) infection are frequently coinfected with hepatitis C virus (HCV), because these viruses have similar routes of transmission, including blood transfusion, intravenous drug use, and sexual contact (1, 2). The optimal therapy for HIV infection is highly active anti-retroviral therapy (HAART), which combines HIV reverse transcriptase inhibitors, often with HIV protease inhibitors. Since the introduction of HAART, the morbidity and mortality associated with HIV infection have declined. This reduction in mortality due to opportunistic infections has made HCV-associated liver disease the leading causes of mortality (3).

Several studies have reported that HCV-related liver disease is more severe in HIV-infected patients than in non-HIV-infected patients (4, 5). The severity of liver disease increases as the immunodeficiency progresses and HIV seropositivity accelerates the progression of liver fibrosis related to chronic hepatitis C (6, 7). However, the standard therapies used to treat chronic hepatitis C in HCV/HIV coinfected patients are the same as those for patients infected with HCV alone (8).

HAART has been reported to reduce serum HCV RNA levels accompanied by immune improvement (9), but the decrease in HIV viral load was associated with a persistent and significant increase in HCV viral load (10). While the association between HAART and HCV infection remains controversial (11), Trimoulet et al. showed that patients treated with HAART that included protease inhibitors had significantly lower intrahepatic HCV loads than those treated with HAART without protease inhibitors (12).

HIV protease is a small, dimeric aspartyl protease that specifically cleaves the polyprotein precursors encoding the structural proteins and enzymes of the virus. This proteolytic activity is absolutely required for the production of mature, infectious virions. HIV protease inhibitors block HIV maturation and show remarkable antiviral potency (13). It has

recently been reported that HIV protease inhibitors also have non-viral effects on the host cells, beyond their effect of blocking HIV protease enzymatic activity (14). NF-κB is central to the overall immune response through its ability to activate genes coding for regulators of apoptosis and cell proliferation (15). The HIV protease inhibitor nelfinavir has been shown to regulate NF-κB activation (16).

In the present study, we investigated the action of nelfinavir alone, or in combination with interferon (IFN), on HCV replication using the replicon system.

Materials and methods

Cell culture. The human hepatoma cell line, Huh7, was maintained in Dulbecco's modified minimal essential medium (Sigma, St. Louis, MO) supplemented with 2mM L-glutamine and 10% fetal calf serum at 37°C under 5% CO_2. Huh7 cells expressing the HCV replicon were cultured in medium containing 500 μg/ml G418 (Nakalai Tesque, Kyoto, Japan).

HCV replicon constructs and transfected cell lines. An HCV subgenomic replicon plasmid, pHCVIbneo-delS (designated pRep-N), was derived from an infectious HCV clone, HCV-N, genotype Ib (17). The replicon, pRep-N was reconstructed by substituting the neomycin phosphotransferase gene with a fusion gene comprising firefly luciferase and neomycin phosphotransferase (pRep-Feo) (18-20). RNA was synthesized from pRep-Feo using T7-polymerase (Promega, Madison, WI) and transfected into Huh7 cells. After culture in the presence of G418, cell lines stably expressing the replicon were established. We have previously reported that firefly luciferase activities of Feo replicon-expressing cells correlated well with HCV NS3, NS4A, and NS5A protein expression levels and with replicon RNA expression levels (18).

Treatment with IFN and nelfinavir. Recombinant human IFN-α-2b (Schering-Plough, Kenilworth, NJ) and purified nelfinavir (Japan Tobacco Inc., Tokyo, Japan) were used. IFN and nelfinavir treatment schedules were as described in the results.

Luciferase assays. Luciferase activity was quantified using a luminometer (Lumat LB9501; Promega, Madison, WI) and the Bright-Glo Luciferase Assay System (Promega). Typically, 5×10^3 cells/well, plated onto 24-well plates and cultured for 48 h, were lysed with 100 μl 1× Glo luciferase Buffer (Promega), and the luciferase activity in 100 μl of the lysate was measured by adding an equal volume of Bright-Glo Luciferase Assay Reagent (Promega).

Assays were performed in triplicate, and the results were expressed as mean ± SD relative light units.

Western blot analysis. Cells were lysed in buffer containing 62.5μM Tris-HCl (pH 6.8), 2% sodium dodecyl sulfate, 10% glycerol, 6% 2-mercaptoethanol, and 0.01% bromophenol blue. Equal amounts of protein (10 μg) were subjected to electrophoresis on sodium dodecyl sulfate-polyacrylamide gels (Invitrogen, Carlsbad, CA), followed by transfer to a polyvinylidene difluoride membrane (Roche, Basle, Switzerland) and sequential probing with a monoclonal anti-NS5A antibody (VIROGEN, Watertown, MA) and β-actin antibody (Thermo Fisher Scientific, Fremont, CA), respectively. The bands were visualized using an enhanced chemiluminescence kit (Amersham Biosciences, Piscataway, NJ).

Cytotoxicity assay. Lactate dehydrogenase (LDH) tests and 5-(3-carboxymethoxyphenyl)-2-(4,5-dimethylthiazoly)-3-(4-sulfophenyl) tetrazolium inner salt (MTS) reduction assays were performed to investigate cytotoxicity and cell viability. LDH levels were measured in the supernatants using the LDH-Cytotoxic Test (Wako Pure Chemical Industries, Osaka, Japan), according to the manufacturer's instructions. The level of specific cytotoxicity was calculated using the following formula: % of specific LDH release = ([experimental LDH release - the mean of negative control release]/[the mean of positive control release - the mean of negative control release]) × 100. LDH release from cells treated with 0.2% Tween 20 was used as a positive control, while LDH release from non-treated cells was used as a negative control. Viable cell growth was determined by MTS assay using the CellTiter 96 Aqueous One Solution Cell Proliferation Assay (Promega), according to the manufacturer's instructions.

TUNEL method. Terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate-biotin nick end-labeling (TUNEL) was used to detect DNA fragmentation of nuclei. Using 24-well plates, 5×10^3 cells/well were plated with 5.0μM nelfinavir. After

incubation for 2 days, the glass coverslips were harvested, fixed with 4% paraformaldehyde and washed with phosphate buffered saline. The cells were permeabilized with 0.5% Tween 20 and treated with MEBSTAIN Apoptosis Kit Direct (Medical and Biological Laboratories Co., Nagoya, Japan). Cells were then treated with RNase and propidium iodide. The nick end-labeling was analyzed using a confocal laser scanning microscope (Fluorview, Olympus, Tokyo, Japan).

Analysis of drug synergism. The effects of treatment of Huh7/Rep-Feo cells with nelfinavir and IFN, alone and in combination, were analyzed using isobologram analysis. Dose-inhibition curves were drawn for IFN and nelfinavir, used alone or in combination. For each drug combination, the 50% inhibitory concentration (IC_{50}) values were plotted against the fractional concentration of IFN and nelfinavir on the x axis and y axis, respectively. IC_{50}, IC_{20} and IC_{80} values ware determined using the Calcusyn™ software package (Biosoft, Cambridge, UK), which performs single and multiple drug dose-effect calculations and determines the presence of antagonism, additivity or synergism. Using the median effect equation, we used this program to plot dose-effect curves for each drug and combination of drugs. The x intercept of the median effect equation gives the ID_{50} for each drug. The median effect plot also gives information on the slope of the dose-effect curve. This information can then be used to calculate the combination index (CI). CI >1 denotes antagonism, CI = 1 denotes additivity, and CI < 1 denotes synergism.

Statistical analysis. Statistical analysis was performed using the Student's t test. $P < 0.05$ was considered to be statistically significant.