Table 1 Role of T_h17 cells in murine models of IBD

Pathogenic role of IL-23	
Wiekowski *et al.*[23]	IL-23p19 transgenic mice spontaneously develop enterocolitis
Kullberg *et al.*[26]	No colitis in *Hh*-infected RAG-1$^{-/-}$×IL-23$^{-/-}$ mice transferred with CD4$^+$CD45RB^{high} T cells
Anti-IL-23 mAb suppresses colitis in anti-IL-10R-treated Hh*-infected mice*	
Yen *et al.*[24]	IL-23 administration exacerbates colitis in IL-10$^{-/-}$ mice, while anti-IL-23 mAb administration suppresses colitis in those mice
Elson *et al.*[25]	Anti-IL-23 mAb treatment suppresses the development of colitis in RAG-1$^{-/-}$ mice transferred with T_h17 cells from colitic C3Bir mice
Izcue *et al.*[27]	No colitis in RAG-1$^{-/-}$ x IL-23$^{-/-}$ mice transferred with CD4$^+$CD45RB^{high} T cells
Protective role of IL-23	
Becker *et al.*[29]	Severer TNBS-induced colitis in IL-23$^{-/-}$ mice
Pathogenic role of RORγt	
Leppkes *et al.*[28]	No colitis in RAG-1$^{-/-}$ mice transferred with RORγt$^{-/-}$CD4$^+$CD25$^-$ T cells
Pathogenic role of IL-17A	
Zhang *et al.*[30]	Milder TNBS-induced colitis in IL-17R$^{-/-}$ mice
Ito *et al.*[32]	Milder DSS-induced colitis in IL-17A$^{-/-}$ mice
Non-effective role of IL-17A	
Izcue *et al.*[27]	No change of colitis in RAG-1$^{-/-}$ mice transferred with IL-17A$^{-/-}$ CD4$^+$CD45RB^{high} T cells
Leppkes *et al.*[28]	Anti-IL-17A treatment does not affect colitis in RAG-1$^{-/-}$ mice transferred with CD4$^+$CD25$^-$ T cells
Yen *et al.*[24]	Anti-IL-17A treatment does not affect colitis in RAG-1$^{-/-}$ mice transferred with CD4$^+$CD45RB^{high} T cells (coadministration of anti-IL-17A and anti-IL-6 mAbs ameliorates colitis)
Protective role of IL-17A	
Ogawa *et al.*[31]	Anti-IL-17A administration exacerbates DSS-induced colitis
O'Connor *et al.*[33]	Exacerbation of colitis in RAG-1$^{-/-}$ mice transferred with IL-17A$^{-/-}$ or IL17R$^{-/-}$ CD4$^+$CD45RB^{high} T cells

DSS, dextran sulfate sodium; *Hh*, *Helicobacter hepaticus*; IBD, inflammatory bowel disease; IL, interleukin; mAb, monoclonal antibody; T_h, helper T cell; TNBS, 2,4,6-trinitrobenzene sulfonic acid.

a balance of cytokine interference, including the competition between T_h1 and T_h17 cells.

LINEAR DEVELOPMENTAL PATHWAY FROM T_H17 TO T_H1 CELLS IN THE DEVELOPMENT OF COLITIS

In the current immunology, the new T_h cell subsets, such as IL-10-producing Tr1 cells,[36] follicular helper T cells, which support antibody-producing B cells,[37] IL-9-producing Th9 cells,[38] and IL-22-producing T_h22 cells,[39] are emerging besides T_h1, T_h2, T_h17, and CD4$^+$CD25$^+$Foxp3$^+$ T regulatory (T_{reg}) cells,[40] and the plasticity between those cells is also now extensively under investigation.[7] Initially, it was thought that T_h1 and T_h17 cells are generated from naive CD4$^+$ T cells independently, and that terminally differentiated T_h cells seldom re-differentiate to other T_h subsets. Following the discovery of induced pluripotent stem cells,[41] which are reprogrammed from terminally differentiated cells, it became evident that many types of cells can re-differentiate to others; e.g., even protective T_{reg} cells can re-differentiate to pathogenic T_h1, T_h17, and follicular helper T cells.[42]

Against this background, Weaver's group recently reported that T_h1 cells are generated from IL-17F-expressing T_h17 cells in the late stages of colitis development.[43] They first showed that IL-17F reporter mice-derived IL-17F$^+$ (green fluorescent protein, GFP$^+$) IFN-γ$^-$ T_h17 cells generated in the presence of TGF-β and IL-6 converted to IL-17A$^-$/IFN-γ$^+$ "T_h1-like" cells in the absence of TGF-β and in the presence of IL-23. Then, the *in vitro*–manipulated IL-17F$^+$CD4$^+$ T_h17 cells were transferred into RAG-1$^{-/-}$ mice. In this setting, mice not only developed colitis but also retained IL-17A$^-$IFN-γ$^+$ T_h1-like cells in the inflamed mucosa, indicating that committed T_h17 cells give rise to progeny that lost IL-17A expression and upregulated IFN-γ expression in the late stage of colitis development. Although these findings explain why both T_h1 and T_h17 cells are

mandatory for the development of colitis by this linear pathway from T_h17 to T_h1 cells, they did not characterize the expression of RORγt and T-bet in the "T_h1-like" IL-17A$^-$IFN-γ$^+$ CD4$^+$ T cells in the inflamed mucosa of colitic RAG-1$^{-/-}$ mice. In addition, it remains unclear if *in vitro*–manipulated IL-17F$^+$CD4$^+$ T cells mirror the bona fide T_h17 cells that emerged in the early stage of colitis *in vivo*.

Previously, it was believed, although not experimentally proved, that both T_h1 and T_h17 cells are critically involved in the model of adoptive transfer of naive CD4$^+$ T cells into immunodeficient mice, and CD4$^+$CD25$^+$Foxp3$^+$ T_{reg} cells suppress development of this model, possibly by suppressing generation and maintenance of both colitogenic T_h1 and T_h17 cells.[44] We noticed that IL-17A$^+$IFN-γ$^+$ T_h17/T_h1 cells reside in only the inflamed mucosa of this model, but not in normal mucosa of WT mice. When RAG-2$^{-/-}$ mice were transferred with CD4$^+$CD45RB^{high} T cells derived from RORγt–GFP reporter mice, we first confirmed that they developed colitis, but only ~10% of CD3$^+$CD4$^+$ T cells were GFP$^+$ (RORγt$^+$), suggesting that this model would be T_h1 dominant as whole CD3$^+$CD4$^+$ T cells were CD44^{high}CD62L$^-$IL-7Rα^{high} effector-memory T cells. Thereafter, GFP$^+$ (RORγt$^+$) or GFP$^-$ (RORγt$^-$) CD4$^+$ T cells were isolated from inflamed mucosa of those mice using a fluorescence-activated cell sorter. The GFP$^-$ cells consisted of only IL-17A$^-$IFN-γ$^+$ T_h1 cells and did not include IL-17A$^+$IFN-γ$^-$ T_h17 and IL-17A$^+$IFN-γ$^+$ T_h17/T_h1 cells, while—surprisingly—GFP$^+$ cells not only consisted of T_h17 and T_h17/T_h1 subsets, but also had T_h1 cells in the highest ratio among these three subpopulations. Furthermore, both GFP$^-$ and GFP$^+$ cells expressed Tbx21, which is the gene product of *T-bet*, suggesting that GFP$^-$ cells were classical T_h1 cells, while GFP$^+$ cells included RORγt$^+$T-bet$^-$ T_h17, RORγt$^+$T-bet$^+$ T_h17/T_h1, and RORγt$^+$T-bet$^+$ T_h1-like cells, as the major subset of GFP$^+$ cells was IL-17A$^-$IFN-γ$^+$.

To further assess the roles of GFP$^-$ and GFP$^+$ cells in terms of colitogenecity, these cells were again transferred into new RAG-2$^{-/-}$ mice. In both cases, the re-transferred mice developed colitis to a similar extent. When the expression of GFP was examined after re-transfer, we found that LP CD4$^+$ T cells isolated from mice transferred with GFP$^-$ cells remained GFP-negative and preferentially produced IFN-γ, but not IL-17A, suggesting that re-differentiation from $T_h1 \rightarrow T_h17$ cells did not occur in this setting. In contrast, in mice transferred with GFP$^+$ cells, approximately half of cells lost GFP expression. Moreover, among GFP-retaining cells, most had IL-17$^-$IFN-γ$^+$ as the major subset, with IL-17A$^+$IFN-γ$^-$ T_h17 and IL-17A$^+$IFN-γ$^+$ T_h17/T_h1 cells as minor subsets, whereas almost all cells that lost GFP expression were IL-17A$^-$IFN-γ$^+$ T_h1 cells. Collectively, these results clearly showed that there is a distinct developmental pathway from $T_h17 \rightarrow T_h1$ cells, via possibly T_h17/T_h1 and T_h1-like cells during the development of colitis *in vivo* (**Figure 1**).[45] However, it is also possible that the lymphopenic host microenvironment favors transformation from T_h17 to alternative T_h1 cells, and this linear pathway may not occur under physiologically lymphosufficient conditions.

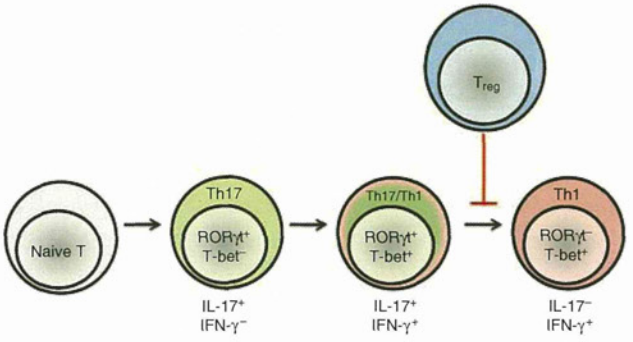

Figure 1 Alternative T_h1 cell differentiation from T_h17 cells during colitis development. T_{reg} cells suppress the path of $T_h17 \rightarrow$alternative T_h1 cells, resulting in a high proportion of T_h17 and T_h17/T_h1 cells in the presence of T_{reg} cells. IFN-γ, interferon-γ; IL-17, interleukin-17; T_h, helper T cell; T_{reg}, T regulatory cell.

Furthermore, recent work by Powrie's group nicely demonstrated that IL-23 promotes both accumulation of intestinal T_h17 cells, and emergence of a IL-17A$^+$IFN-γ$^+$ T_h17/T_h1 population; while blockage of IL-23/IL-23R axis suppresses the development of colitis induced by transfer of CD4$^+$CD45RB^{high} T cells into RAG-1$^{-/-}$ mice,[46] and particularly reduces the ratio of T_h17/T_h1 population. These results suggest that T_h17/T_h1 cells are the true pathological cells in this model. Our results, however, imply that IL-23 is critical to the linear developmental pathway of alternative T_h1 cells, and particularly promotes the path from T_h17 to T_h17/T_h1 cells. We also found that IL-17A-producing T_h17 cells did not decline more rapidly than IFN-γ-producing T_h1 cells during the *in vivo* competition between T_h1 and T_h17 cells after parabiosis between the two models of colitis and co-transfer experiment—not what one might expect from results showing that T_h17 cells transform into T_h1 cells. Further studies are warranted to resolve these interesting issues.

Is this linear developmental pathway from T_h17 to T_h1 cells the only one to mediate colitis? On the basis of the independent developmental pathway of T_h1 and T_h17 cells, it is likely that RAG-1$^{-/-}$ mice transferred with naive T cells from T-bet$^{-/-}$ or RORγt$^{-/-}$ mice can develop T_h17- or T_h1-mediated colitis, respectively. In this regard, however, RAG-1$^{-/-}$ mice transferred with naive T cells from T-bet$^{-/-}$ or RORγt$^{-/-}$ mice reportedly did not develop colitis at all.[28,47] This discrepancy suggests that the linear developmental pathway is essential for colitis development. We called colitogenic T_h1 cells that arise from T_h17 cells via such a linear pathway "alternative" T_h1 cells; they may be genuinely pathological T_h1 cells in colitis development. Interestingly, this hypothesis allows that the adoptive transfer model of colitis depends more on IL-23 than IL-12.[26] Further study is needed to determine whether classical T_h1 cells generated in RORγt-independent manner are truly involved in colitis development.

T_{REG} CELLS BLOCK THE DEVELOPMENTAL PATHWAY FROM T_H17 TO T_H1 CELLS

The next question arising is how CD4$^+$CD25$^+$Foxp3$^+$ T_{reg} cells affect the linear developmental pathway from T_h17 to

alternative T_h1 cells in the development of colitis. To examine this, we transferred Ly5.2$^+$CD4$^+$CD45RB^{high} T cells, with or without Ly5.1$^+$CD4$^+$CD25$^+$ T_{reg} cells, from RORγt–GFP reporter mice into RAG-2$^{-/-}$ mice. As expected, mice co-transferred with CD4$^+$CD45RB^{high} T cells and T_{reg} cells did not develop colitis, possibly due to the suppressive activity of T_{reg} cells, whereas mice transferred with CD4$^+$CD45RB^{high} T cells alone did develop colitis. Surprisingly, however, the proportions of RORγt-expressing IL-17A$^+$IFN-γ$^-$ T_h17 and IL-17A$^+$IFN-γ$^+$ T_h17/T_h1 cells in LP of the co-transferred mice were significantly higher than those of single-transferred mice, while, as expected, the proportion of RORγt-negative IL-17A$^-$IFN-γ$^+$ T_h1 cells in the co-transferred mice was significantly decreased compared with the single-transferred mice; this suggests that T_h17 and T_h17/T_h1 cells are non-colitogenic or pre-colitogenic, whereas T_h1 cells are colitogenic effector-memory T cells. Also, irrespective of GFP expression, LP CD3$^+$CD4$^+$ T cells in colitic mice transferred with CD4$^+$CD45RB^{high} T cells alone expressed Tbx21, whereas LP CD3$^+$CD4$^+$ T cells in non-colitic mice transferred with CD4$^+$CD45RB^{high} T cells and T_{reg} cells did not express Tbx21,[45] indicating that T_{reg} cells suppress not only the pathway between T_h17/T_h1 and T_h1 cells, but also the induction of Tbx21 in colitogenic effector-memory T cells (**Figure 1**). Furthermore, consistent with the results of Weaver's study showing that this transformation from T_h17 to T_h1 cells occurs in the absence of TGF-β and increases in mice treated with anti-IL-23R,[43] it is possible that TGF-β derived from T_{reg} cells suppresses the development from T_h1, T_h17/T_h1 to alternative T_h1 cells. Another possible mechanism of action of T_{reg} cells is shown in Chen and colleagues' recent demonstration that IL-2 consumption by IL-2Rα (CD25)-expressing T_{reg} cells increases T_h17 cell development in Foxp3.luciDTR mice, in which diphtheria toxin selectively depletes Foxp3$^+$ T_{reg} cells.[48] Pandiyan and colleagues reported a similar result.[49] In this regard, these two papers may explain the previous finding that IL-2 suppresses the development of T_h17 cells.[50]

RORγT-DEPENDENT INNATE LYMPHOID CELLS IN ANIMAL MODELS OF IBD PATHOGENESIS

Besides T_h17 lymphocytes, immune cells, such as natural killer (NK) cells, NKT cells, TCR γδ-expressing T cells, macrophages, and Paneth cells, can produce IL-17A.[51] Of particular importance, these more innate immune cells, unlike T_h17 cells, immediately secrete IL-17A in response to specific alert molecules such as pathogen-associated molecular patterns and cytokines such as IL-23. However, the involvement of these "non-Th17 cell IL-17A-producing cells" to colitis pathogenesis is not fully understood and are now an area of intense research. Furthermore, evidence is emerging of a new IL-17A-producing cell population, innate lymphoid cells (ILCs), that have crucial roles in not only the formation and maintenance of gut-associated lymphoid tissue (GALT), but also colitis pathogenesis in animals and humans.[52] There are various kinds of ILCs categorized by location, cytokine production, and expression of specific surface markers and intracellular transcription factors, including lymphoid tissue inducer (LTi) cells, which are involved in GALT formation during the fetal period. Similar LTi cells are also present in the gut and tonsils after birth, and are called adult LTi-like (hereafter described as LTi-like) cells.[53] These cells may have important roles in the generation of cryptopatches and isolated lymphoid follicles, and in the maintenance of all GALT after birth, but experimental proof for the role of LTi-like cells after birth is lacking. The LTi/LTi-like cells constitutively express RORγt; consistently RORγt$^{-/-}$ mice completely lack GALT. Therefore, RORγt is a master transcription factor for not only T_h17 cells, but also LTi/LTi-like cells. These cells also promptly produce IL-17A in response to IL-23, which may be induced in activated antigen-presenting cells such as macrophages and dendritic cells. Although it is known that LTi cells produce both IL-17A and IL-22, several groups have identified NK receptor (NKR)-expressing "LTi-like"-like cells that express RORγt and produce IL-22 but not IL-17A in the tonsils and intestines. These cells are now designated (not uniformly) as NK22, NKR-LTi, NCR-22, NKR$^+$RORγt$^+$ ILC, and ILC22 cells. These cells specifically express NKp46 but not NKp44 in mice, and in contrast, NKp44 but not NKp46 in humans.[54,55] Importantly, IL-22 is a IL-10 family cytokine, but its specific receptor seems to be expressed only on non-hematopoietic cells, such as epithelial cells, whereas IL-10R is specifically expressed on hematopoietic cells. Consistent with this, IL-22$^{-/-}$ mice and RORγt$^{-/-}$ mice develop more severe colitis with marked epithelial damage in the dextran sulfate sodium-induced colitis model than do control WT mice,[55,56] and IL-22$^{-/-}$ mice develop more severe intestinal epithelial damage in the *Citrobacter* infection colitis model,[57] suggesting that ILC22 (NK22) cells are critical to epithelial repair (**Figure 2**). However, it remains unclear whether ILC22 cells develop from LTi/LTi-like cells[55] or directly from RORγt$^+$ ILC precursor cells[58] that have not yet been identified. Nevertheless, it seems that LTi/LTi-like cells preferentially reside in GALT and have important roles in generating and maintaining GALT, while ILC22 cells diversely reside in intestinal LP to promote epithelial repair.

The next critical issue is whether ILC cells are pathologically involved in IBD development. In this regard, Powrie's group first described pathological ILC cells in two models of innate colitis (**Figure 2**).[59] In the first model of *Hh*-infected RAG-1$^{-/-}$ mice, the proportion and absolute number of unique Thy1^{high}Sca-1$^+$ ILC cells that produce not only IL-17A and IL-22 but also IFN-γ (ILC17 or ILC17/1 cells) were markedly increased in the colonic LP. Like physiological-condition LTi/LTi-like cells, ILC cells in colitic *Hh*-infected RAG-1$^{-/-}$ mice are Lin (CD11b, Gr1, B220)$^-$ RORγt$^+$NKp46$^-$IL-7Rα$^+$CCR6$^+$, but unlike LTi/LTi-like cells, they are Sca-1$^+$c-kit$^-$CD4$^-$. It is not known whether those pathological ILC cells are derived from physiological-condition LTi/LTi-like cells or are generated from RORγt$^+$ ILC precursor cells (**Figure 2**). Administration of anti-Thy1 mAb to target those Thy1^{high}Sca-1$^+$ ILC cells suppressed the development of *Hh*-induced innate colitis in RAG-1$^{-/-}$ mice, although it is unclear if anti-Thy1 treatment targets only the pathological ILC cells in this model, as Thy1 is broadly expressed on various cells (such as NK cells). Of note, pathological ILC cells expressed T-bet and RORγt, suggesting that they are counterparts of

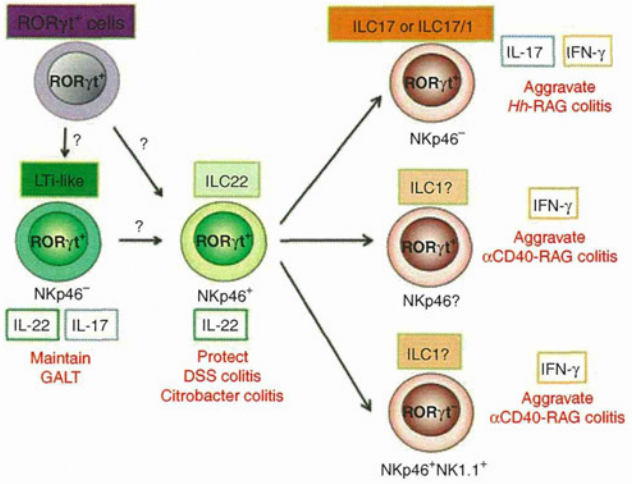

Figure 2 Role of newly identified RORγt-dependent ILC in the development of animal models of colitis. The various kinds of ILCs are categorized by location, cytokine production, and expression of specific surface markers and intracellular transcription factors; LTi cells and possibly adult LTi-like cells are involved in the formation and maintenance of GALT. ILC22 (NK22, NKR-LTi, NCR-22, or NKR$^+$ RORγt$^+$ ILC) cells may be involved in IL-22-mediated epithelial repair. However, it is unclear whether ILC22 cells develop from LTi/LTi-like cells[55] or directly from RORγt$^+$ ILC precursor cells.[58] Several studies described colitogenic ILC cells (ILC1, ILC17/1, or ILC17) in two models of innate colitis. GALT, gut-associated lymphoid tissue; IFN-γ, interferon-γ; IL, interleukin; ILC, innate lymphoid cell; LTi, lymphoid tissue inducer; NK22, natural killer-22; NKR-22, natural killer receptor-22; T_h, helper T cell; T_{reg}, T regulatory cell.

IL-17A/IFN-γ-double–producing T-bet$^+$RORγt$^+$ T_h1/T_h17 cells. This group attempted using another innate colitis model,[58] in which RAG-1$^{-/-}$ mice are administered anti-CD40 mAb to activate antigen-presenting cells. In this second model, increased Thy1^{high}Sca-1$^+$ ILC cells also emerged in inflamed colon mucosa, and anti-Thy1 mAb treatment blocked the development of colitis. Interestingly, unlike the model of *Hh*-infected RAG-1$^{-/-}$ mice, these ILC cells produced IFN-γ and IL-22, but not IL-17A, although they expressed RORγt. Therefore, this second type of ILC cells (possibly ILC1) that emerged in anti-CD40-administerred mice is not identical to the ILC17 or ILC17/1 in *Hh*-infected RAG-1$^{-/-}$ mice. Furthermore, RORγt$^{-/-}$× RAG-1$^{-/-}$ mice were totally resistant to this anti-CD40 mAb administration model, suggesting that RORγt is essential for the generation or maintenance of the second type of pathological ILC1 cells; i.e., ILC cells are essential for colitis development, following the activation of antigen-presenting cells via CD40 molecules to produce IL-23. Further studies should address the developmental pathway and plasticity of colitogenic ILC cells, their relationship to T-bet, the reason those two colitogenic ILC cells produce IL-22, and the distinct location of those cells.

Subsequently, Diefenbach's group elegantly showed a sequential developmental pathway for LTi-like cells→RORγt$^+$ NKR–ILC (NKp46$^+$)→IFN-γ-producing RORγt$^-$ NKR–ILC (NKp46$^+$) in the process of anti-CD40 mAb-induced innate colitis, using RORγt fate-map mice.[55] These colitogenic IFN-γ-producing RORγt$^-$ NKR–ILC cells differ from conventional NK (cNK) cells, as they could be depleted by anti-NK1.1 mAb, but not by anti-asialo GM1 mAb, while cNK cells were depleted by either anti-NK1.1 mAb or anti-asialo GM1 mAb. Consistent with this, administration of anti-NK1.1 mAb, but not anti-asialo GM1, ameliorated this colitis model.[54] It is not known whether RORγt$^-$ NKR–ILC cells in colitic mice after anti-CD40 mAb administration express NK1.1. Although the Diefenbach team showed that, before anti-CD40 mAb administration, half of the RORγt$^+$ NKR–ILC cells in fate-map mice expressed NK1.1, it would be interesting to know if the depletion of NKp46$^+$ cells affects development of this model. Other groups have shown that murine RORγt$^+$ ILC22 cells lose NK1.1 expression compared with cNK.[55] Otherwise NK1.1-expressing RORγt$^+$ NKR–ILC cells may be able to differentiate to colitogenic RORγt$^-$ NKR–ILC cells, or colitogenic RORγt$^-$ NKR–ILC cells may re-express NK1.1 after anti-CD40 mAb administration.

Apart from RORγt-expressing ILC cells, RORγt-independent ILC cells were recently identified by several groups. These ILC cells, such as natural helper cells, nuocytes, and innate helper type 2 (Ih2) cells, are called ILC2, as they produce T_h2 family cytokines, such as IL-5 and IL-13, in response to IL-25 or IL-33.[60–62] The natural helper cells reside in adipose tissue in the peritoneal cavity as "fat-associated lymphoid clusters", and are as dependent on common gamma (γc) receptors as LTi/LTi-like cells are, but—unlike LTi/LTi-like cells—are independent of RORγt, indicating that ILC2 subsets are generated from hematopoietic precursor cells in an RORγt-independent manner. However, it is unclear whether this new ILC2 subset is involved in the pathogenesis of human IBD and their animal models. The natural helper cells in fat-associated lymphoid cluster in the mesentery may have a pathological role in forming mesenteric fat lapping—a particular finding in CD. Interestingly, nuocytes[61] and Ih2 cells[62] are found in the small intestine and mesenteric lymph nodes, in addition to the spleen, suggesting the intestinal pathology involvement.

Accumulating evidence suggests that ILC cells are closer to innate immune cells than to acquired immune cells. Notably, various ILC subsets have corresponding partner T_h cells: IFN-γ-producing and RORγt-expressing (or previously expressing) ILC1 cells, IL-4/IL-5/IL-13-producing and GATA3-expressing ILC2 cells, IL-17A-expressing and RORγt-expressing ILC17 cells, and IL-22-expressing and RORγt-expressing ILC22 cells may correspond to NK cells, T_h1 cells, T_h2 cells, T_h17 cells, and T_h22 cells, respectively. Also, T_h17/T_h1 cells, which often emerge in pathological conditions, may be the counterparts of IL-17A/IFN-γ-double–producing and RORγt/T-bet-double–expressing ILC17/1 cells that increase in inflamed mucosa of *Hh*-infected RAG-1$^{-/-}$ mice. The Foxp3-expressing ILC cells may emerge as a type of regulatory ILC cell in the future.

RORγT-DEPENDENT ILCs IN HUMAN IBD PATHOGENESIS

A recent study shows that IL-17A-single, IFN-γ-single, or IL-17A/IFN-γ – double cells, which produce Lin$^-$CD45$^+$CD127$^+$ CD56$^-$ and are similar to LTi-like cells in terms of cell surface markers, are markedly increased in inflamed mucosa of CD, but not UC or non-IBD control[63] (**Figure 3**). These cells may be the counterparts of Thy1^{high}Sca-1$^+$ ILC1, ILC17 or ILC17/1 cells that emerge in inflamed mucosa of colitic *Hh*-infected

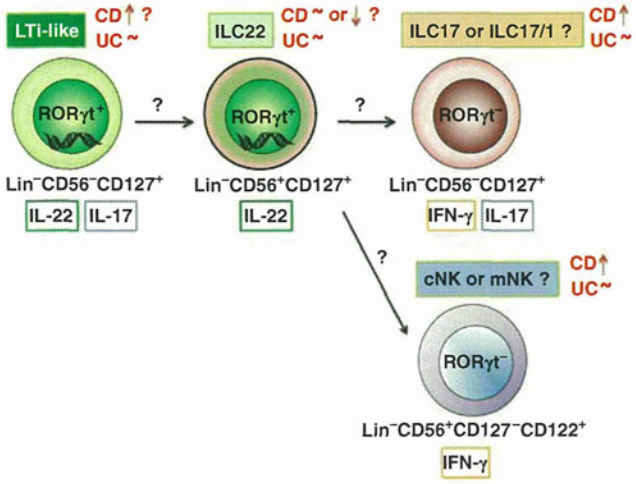

Figure 3 Role of LTi-like cells and NK cells in human IBD. IL-17A/IFN-γ-double–producing Lin$^-$CD45$^+$CD127$^+$CD56$^-$ cells are markedly increased in inflamed CD mucosa.[63] However, whether LTi-like cells and ILC22 cells are increased or decreased in inflamed IBD mucosa is still debatable;[63,64] and the origin of mNK cells is still undetermined. CD, Crohn's disease; ILC, innate lymphoid cells; LTi, lymphoid tissue inducer; UC, ulcerative colitis.

RAG-1$^{-/-}$ mice and/or anti-CD40 mAb-injected mice, rather than IL-22/IL-17A-producing LTi-like cells that reside in the intestine in physiological condition. However, as the absolute numbers of these inflammatory ILC subpopulations are very small in inflamed IBD lesions, it is largely unclear if those cells are critically involved in human IBD pathogenesis, and further studies are needed to determine their exact role. According to this same group, the proportion of Lin$^-$CD45$^+$CD127$^+$CD56$^+$ cells (analogous to human ILC22 cells) was comparable among CD, UC, and controls.[63] However, we previously found two types of NK cells with different surface markers residing in the human intestinal mucosa: NKp44$^+$NKp46$^-$CD56$^+$CD122$^-$CD127$^+$ and NKp44$^-$NKp46$^+$CD56$^+$CD122$^+$CD127$^-$. The former NKp44$^+$ cells express RORC and IL-22, whereas the latter NKp46$^+$ cells express IFN-γ, suggesting that NKp44$^+$ and NKp46$^+$ cells are IL-22-producing ILC22 cells and IFN-γ-producing cNK cells, respectively.[64] Interestingly, the proportion of NKp44$^+$ cNK cells in inflamed CD mucosa was significantly decreased compared with controls, whereas the proportion of NKp46$^+$ ILC22 cells was conversely increased compared with controls, indicating that the imbalance between protective NKp44$^+$ ILC22 cells and colitogenic NKp46$^+$ cNK cells in intestinal mucosa is involved in CD pathogenesis in at least one critical pathological pathway. Surprisingly, however, the expression of IL-23Rα on NKp46$^+$ cells was significantly higher than that on NKp44$^+$ cells, and NKp46$^+$ cells strongly responded to IL-23 to produce IFN-γ, suggesting that NKp46$^+$ cNK cells in the intestinal sites are differentiated from NKp44$^+$ ILC22 cells. In this regard, we previously identified Lin$^-$c-kit$^+$CD127$^+$CD25$^+$Id2$^+$LTα$^+$ cells, which are similar to LTi-like cells, in human intestinal mucosa, and these cells differentiate into IFN-γ-producing NK cells.[65] Therefore, unlike cNK cells in extraintestinal locations, mucosal NK cells in humans may be independently generated from self-renewal NK precursor cells in intestinal mucosa to LTi-like cells, ILC22 cells, and IFN-γ-producing NK cells[52] (**Figure 3**), although it remains largely unknown why those mucosal IL-23R-expressing ILC-like NKp44$^+$ cNK cells expressed CD122 but not CD127.

CONCLUSIONS

- Although IL-17A is thought to be preferentially produced by T_h17 cells and to have a critical role in autoimmune disease development, it seems to inhibit the development of T_h1 cells in some circumstances.
- Colitogenic T_h1 and T_h17 cells seem to interfere with each other *in vivo* under inflammatory conditions. At least in inflammatory conditions, colitogenic T_h1 and T_h17 cells are not independently generated; rather the linear sequential developmental pathway from T_h17 to T_h1 cells seems to dominate. Further study is needed to determine whether the classical T_h1 cells directly generated from naive T cells in an RORγt-independent manner are also involved in IBD pathogenesis.
- The pathway from T_h17/T_h1 cells to alternative T_h1 cells is suppressed by T_{reg} cells, resulting in the accumulation of T_h17 cells by T_{reg} cells.
- A newly identified immune cell subset, ILC, may be involved in both the generation and maintenance of GALT, and pathogenesis of chronic intestinal inflammation.

Much new evidence about IL-17A-producing immune cells will likely soon emerge. We can be cautiously optimistic that these new findings will solve many puzzles of IBD pathogenesis, and generate novel therapeutic strategies for these diseases.

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DISCLOSURE

The authors declared no conflict of interest.

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今月のテーマ　クローン病診療のトピックス

インフリキシマブ二次無効の機序と対策，治療方針

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要旨：インフリキシマブ治療はクローン病治療に革命をもたらしたといっても過言ではない．しかし，治療経過中に効果が減弱するいわゆる二次無効について国内外で活発に議論がなされている．本邦でも，2011年，インフリキシマブの10mg/kgへの増量が承認され，二次無効症例に対して直接的な対処が可能になった．しかし，10mg/kg増量ですべての二次無効症例が再び8週間隔の維持治療で寛解を維持できるまで効果が回復するとは限らない．また，本邦では2番目に登場したアダリムマブとの治療優先に関する議論，アダリムマブ増量の議論，さらには本邦オリジナルな白血球除去療法，栄養療法との併用など，長期の寛解維持を達成させるための適切な薬剤選択，適切な増量のタイミングを明らかにすることが重要である．

索引用語：生物学的製剤，抗体医薬，炎症性腸疾患，クローン病，二次無効

はじめに

2002年5月，本邦でインフリキシマブ（レミケード®）がクローン病治療薬として承認されて以降，現在まで多くの患者に投与され，すでにクローン病内科治療の中心的な役割を担っているのが実情である．インフリキシマブは高い寛解導入，維持効果を示すだけでなく，粘膜治癒（mucosal healing）効果や入院率/手術率を減少させる効果を有し，クローン病の自然史を変えたといっても過言ではない．しかし，5mg/kg，8週間隔の維持療法中に効果が減弱する，いわゆる“二次無効”症例が存在し，長期寛解維持においてその対策が課題となっている．本稿では，これまで報告されたエビデンスならびに使用経験をもとに，インフリキシマブの二次無効症例の現状とその対応策について概説する．

I　二次無効発現率

北米，欧州を中心に実施された活動性クローン病患者を対象に実施したACCENT I試験[1)]において，インフリキシマブ5mg/kgを0週，2週，6週，その後8週間隔の維持投与を行ったところ，2週後に効果を示した症例においては35.3%（40例/113例）が，すべての症例においては30.2%（58例/192例）が，54週までに効果が減弱した[2)]．瘻孔を有するクローン病患者を対象にインフリキシマブの同一の投与スケジュールで実施した

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Mechanism and therapeutic strategy of second failure of infliximab treatment for Crohn's disease

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ACCENT II試験における54週までの効果減弱率は，10週後に効果を示した症例においては29.2%（28例/96例），すべての症例においては25.2%（35例/139例）であった[3]．

一方，2002年から2005年までピッツバーグ大学インフュージョンセンターにて少なくともインフリキシマブを8回以上投与された患者（108例）の中で，30カ月以上の間で54.3%が効果減弱によるインフリキシマブの短縮and/or増量を必要とした[4]．また，Rudolphらの報告によると，インフリキシマブによる維持療法を少なくとも30週以上受けたクローン病患者（198例）において，non-response（24例），有害事象（23例），効果減弱（16例）などを理由にインフリキシマブの投与が中止されており，初回治療有効症例における72カ月後の効果持続率は66.4%であった[5]．さらに，1994年から2007年までにルーベン大学にてインフリキシマブ治療を受けた炎症性腸疾患患者614例を対象に長期使用成績を検討した結果，初回治療有効例中50.1%（274例/547例）が5mg/kg，8週間隔の投与では効果が持続せず，インフリキシマブの増量や投与期間短縮など何らかの対応を必要としていた[6]．本結果は実地診療科でのcohort研究から得られたもので，エピソディック投与や，エピソディックからメンテナンス投与に切り替えた症例が多数含まれていたため，他の報告に比べて効果減弱率が高めになったと考えられる．以上，これまでの報告をまとめると，インフリキシマブ5mg/kg，8週間隔維持投与における二次無効の発現率は，約25～50%であると想定される．

II 二次無効の機序

インフリキシマブによる維持療法における有効性は，インフリキシマブの血中トラフ濃度に依存することが報告されている[7]．さらにHibiらは，インフリキシマブの血中トラフ濃度（中央値）が1μg/mL以上の症例でclinical response率が高いことに加え，効果が減少する原因の1つが血中インフリキシマブトラフ濃度の低下にあることを明らかにした[8]．すなわち，インフリキシマブ5mg/kg，8週間隔投与の維持療法中に効果が減弱した症例の血中インフリキシマブトラフ濃度は0.8μg/mLであり，それらの症例に対して投与間隔を4週に短縮したところ，血中インフリキシマブトラフ濃度が1μg/mLに上昇し，効果が回復した．また，血中インフリキシマブトラフ濃度が検出限界以下の炎症性腸疾患患者に対してインフリキシマブを増量したところ，86%の患者にclinical responseが得られたという報告もある[9]．以上のことから，二次無効の主な原因は血中インフリキシマブ濃度の低下であると考えられた．次に，インフリキシマブの濃度を低下させる要因について整理してみたい．

1. 抗インフリキシマブ抗体

インフリキシマブによる治療中に，抗インフリキシマブ抗体（antibody to infliximab；ATI）が出現する場合がある．ATIの臨床効果に対する影響を検討したACCENT I試験のサブ解析結果では，ATIの有無による54週後のclinical response率およびremission率に差は認められなかった[10]．一方，ATI濃度と血中インフリキシマブトラフ濃度および効果持続時間には相関が認められ，ATI濃度が高い症例では低い症例に比べて血中インフリキシマブ濃度が低下し，効果持続時間が有意に短いという報告もある[11]．これらのことから，ATIの出現はインフリキシマブの血中濃度の維持を妨げ，効果を減弱させる要因の1つと考えられた．特に，血中からインフリキシマブが消失している状態でインフリキシマブ投与を行う，いわゆるエピソディック投与では，それが免疫源として作用し，ATIが発現しやすくなる可能性がある．ATI産生を最小化させるためには血中濃度を保つことが重要であると考えられる[7]．

2. インフリキシマブ治療前の血中TNF-α濃度

インフリキシマブ治療前の血中TNF-α濃度が血中インフリキシマブ濃度に与える影響については，関節リウマチ患者を対象にしたRISING試験で詳細に検討されている[12]．この試験では，3mg/kgのインフリキシマブを0週，2週，6週に投与した後，3，6，および10mg/kg投与群の3群に

割り付け，46週まで8週間隔で投与し，54週後の臨床効果を比較した．その結果，血中TNF-α濃度が低値の症例では，いずれの投与量群でも1μg/mL以上の血中インフリキシマブトラフ濃度が維持され，54週後のclinical response率に差はなかった．一方，血中TNF-α濃度が高値の症例では，3および6mg/kg投与群の血中インフリキシマブ濃度は検出限界未満に低下し，2μg/mL以上の血中インフリキシマブトラフ濃度が維持された10mg/kg投与群に比べて54週後のclinical response率が有意に低かった．これらのことから，インフリキシマブの標的分子であるTNF-α濃度も，インフリキシマブの血中濃度に影響する因子であることが示唆された．クローン病においては，投与開始前の血中TNF-α濃度がインフリキシマブ維持療法における効果減弱に影響することが報告されているものの[13]，TNF-α濃度と血中インフリキシマブ濃度の関連は明らかにされておらず，今後の検討が望まれる．

3. Fcγ受容体の遺伝子多型

インフリキシマブは，ヒトIgG1由来のFc領域を有する．したがって，インフリキシマブがTNF-αと結合して免疫複合体を形成すると，Fcγ受容体（FcγR）を介して除去・排泄されると考えられている．Nishioらは，少ない投与量でも十分な血中インフリキシマブ濃度が維持され症状がコントロールされていた関節リウマチ患者について，FcγRの遺伝子解析を行った[14]．その結果，FcγRの遺伝子多型がインフリキシマブのクリアランスに関係することが示唆された．クローン病においても，インフリキシマブによるclinical responseがFcγR IIIBの遺伝子多型に関与することが報告されている[15]．これらのことから，FcγRの遺伝子多型もインフリキシマブのクリアランスに影響を及ぼすことが考えられた．

III 二次無効の予測因子

Chaparroら[16]は，インフリキシマブの寛解導入治療に効果を示した309例のクローン病患者の長期的な効果持続率および効果減弱例の予測因子を検討した．多変量解析の結果，免疫調節剤の併用は効果減弱のリスクを低下させ[hazard ratio＝0.37，p＝0.02]，診断時の喫煙歴のある患者はリスクを上昇させる[hazard ratio＝2.05，p＝0.019]ことがわかった．一方，免疫調節剤の併用に関しては，Van Asscheらが報告したIMID試験[17]ではインフリキシマブとの6カ月間以上の併用有無で有効率・治療継続率に差がみられず，一定の見解が得られていない．さらなる長期的データの蓄積が必要と考えられる．

IV 二次無効に対する対策

1. インフリキシマブ10mg/kg増量投与

二次無効の主な原因は血中インフリキシマブ濃度の低下であることから，インフリキシマブによる維持療法において効果減弱を回避するためには，インフリキシマブの血中濃度を維持させることが重要であると考えられる．ACCENT I試験において，インフリキシマブ5mg/kgを単回投与し2週後に効果を示した症例中，54週までに効果が減弱した40例に対し，10mg/kgへの増量を行った結果，36例（90%）が効果を回復した[2]．また，ルーベン大学のcohort研究の結果によると，二次無効症例中，26.3%に10mg/kgへの増量が行われ，それらの症例では効果が回復するだけでなく，興味深いことに71.5%の患者で再び通常用量である5mg/kgへ戻すことが可能であった．以上，海外では二次無効に対する10mg/kg増量の良好な治療効果が確認されており，国内でも増量効果を確認するための臨床試験が実施された[18]．インフリキシマブ5mg/kg，8週間隔投与による維持療法を受けているにもかかわらず，投与8週後のCDAIが175以上，かつ投与4週のCDAIと比較して50ポイント以上増加している効果減弱患者39例を対象とし，10mg/kgへの増量を行い，8週間隔投与で40週まで評価を行った．その結果，8週後の評価対象（33例）におけるCDAIスコアの変化量（中央値）は95ポイント，寛解率（CDAI＞150）は39.4%であった．また，40週後の寛解率は50%であり，8週間隔で投与を継続することにより効果が維持された．増量前の血中インフリキシマブトラフ濃度は0.3μg/mLであったが，10mg/kgに増量することで1.29μg/mLに上昇し，臨床効果を得るための

必要であると考えられる1μg/mL以上に到達することも確認された.

2. インフリシキマブ5mg/kg短縮投与

Hibiらは，5mg/kg，8週間隔維持投与中にCDAIが175以上かつ，投与10週までに最も低値を示した時のCDAIと比較して35%以上かつ70ポイント以上増加した場合，4週間隔投与へ移行することにより，8週後に約40%の寛解率が得られることを報告した[8]．またKatzらは，インフリキシマブ10mg/kg，8週間隔投与（増量群）または，5mg/kg，4週間隔投与（短縮群）を受けていた二次無効の患者をレトロスペクティブに解析した．その結果，増量もしくは短縮介入直後の有効性は増量群（112例）で77%，短縮群（56例）で66%と同等の効果を示し，その傾向は12カ月後においても同様であった[19]．以上のように，インフリキシマブの増量，投与間隔短縮のどちらの介入法においても，有効性を示すために必要な血中インフリキシマブトラフ濃度が維持され，ほぼ同等の臨床効果が得られることが期待される．本邦では，承認用量である10mg/kgへの増量で二次無効症例の多くは対応可能であると考えられる．

以上，インフリキシマブの有効性は血中濃度依存的であり，効果が減弱した症例の多くは血中インフリキシマブトラフ濃度が有効性を示す閾値未満であったことから，血中インフリキシマブ濃度のモニタリングが長期の効果維持に有用であることが示唆されている[8]．しかし，日常診療において，血中インフリキシマブ濃度をモニタリングすることは困難であり，そのサロゲートマーカーとなる簡便なマーカーの探索が望まれる．血中インフリキシマブ濃度と炎症マーカーであるCRPとの相関関係がMaserら[7]によって報告されていることから，CRPをサロゲートマーカーとして活用できるかもしれない．

V 他の生物学的製剤への切り替え

インフリキシマブの効果減弱もしくは不忍容であった中等度～重度のクローン病患者（159例）に対してアダリムマブへの切り替えを検討したGAIN試験[20]の結果によると，アダリムマブ160mg，80mgの隔週投与によりCDAI（中央値）は切り替え前313であったものが，4週後には226に減少していた．4週時の寛解導入率は21%であった．インフリキシマブ10mg/kg増量後8週の寛解率は39.4%であり[18]，効果減弱症例に対してインフリキシマブの10mg/kgへの増量あるいはアダリムマブへの切り替えのどちらが良いかについての議論は残るが，ECCO（The European Crohn's and Colitis Organisation）ガイドライン[19]では，「抗TNF-α治療中に二次無効が認められた場合，疾患活動性の再確認，合併症を検査し除外，および外科的治療の選択についての患者との相談がなされるべきである．活動性が認められた場合，薬剤のスイッチを行う前の治療戦略として投与間隔の短縮や投与量の増量を行うのが適切である．また，薬剤のスイッチも有効な治療ではあるが，将来的な治療オプションを減らしてしまうため，忍容性がない場合や特に重症な場合に他の抗TNF製剤に変更すべきといえる．（ECCO Statement 5J）」と記述されている．

欧米では，生物学的製剤としてインフリキシマブ，アダリムマブに加え，セルトリズマブ，ナタリズマブがクローン病治療薬として使用されているが，本邦はインフリキシマブ，アダリムマブの2剤のみであることから，ECCOガイドラインに記述されているように，現在使用している生物学的製剤の効果を最大化させる前に安易なスイッチを行うことはできる限り避けることが望ましい．

VI 当院1施設における二次無効の現状とその対策

当院でインフリキシマブを導入し，維持投与を行っているクローン病患者148名を対象として検討したところ，8週間隔を維持しているのはインフリキシマブ開始30週時点では55%と約半数であった．8週間隔継続例と短縮例を比較してみると，短縮例において罹病期間が有意に長いことがわかった．また，免疫調節剤の有無では両群間で有意差は認めなかったが，インフリキシマブ投与前に免疫調節剤の投与を受けていなかった症例に限って解析すると，免疫調節剤の併用によって有意に8週間隔維持率が高かった．これらは，難治

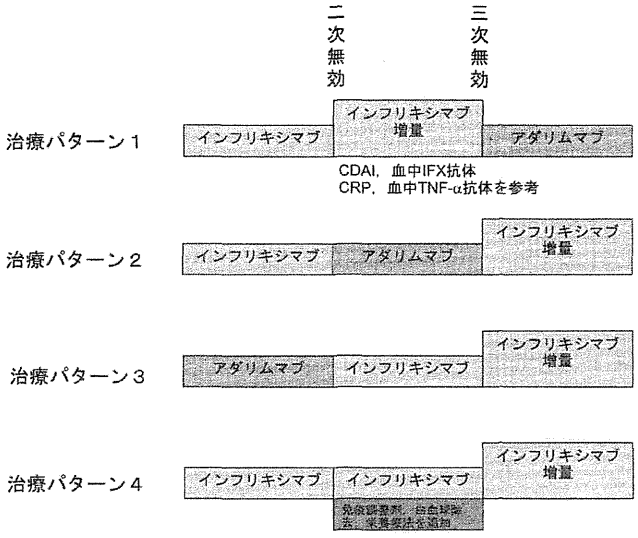

Figure 1. 本邦における二次無効の治療ストラテジー．三次無効とは二次無効後に実施した治療後に再び無効になった症例と定義した．IFX，インフリキシマブ．

例や予後不良因子の多い症例においては，できるだけ速やかに免疫調節剤併用でインフリキシマブを導入することが，インフリキシマブ治療成功のためには重要であることを示す結果と考えている．

当院では，保険適応の問題はあるが，二次無効に至った症例に対してはインフリキシマブの投与期間短縮を中心に対処してきた（10mg/kg投与認可前）．投与期間短縮を行った26例を検討してみると，CRPが投与期間短縮によって低下した症例は10例（38.5%）と，約40%の症例で有効性を認めた．次に，インフリキシマブ二次無効でアダリムマブに移行した症例は12例であり，そのうち6例（50%）は効果不十分のためインフリキシマブ投与に戻っているが，半数の症例では切り替えで経過良好である．インフリキシマブの10mg/kgへの増量は2011年8月に認可されたばかりで経験が少なく，実際の効果はまだわからない．今後は，インフリキシマブ増量や短縮，またはアダリムマブへのスイッチングのいずれが最も効果的であるのかを，個々の症例で判別できるようなマーカーの開発が急務と考えられる．

おわりに

2011年8月に本邦でもようやくインフリキシマブの10mg/kgへの増量が承認され，二次無効に対して直接的な対処が可能になった．しかし，10mg/kg増量ですべての二次無効症例が再び8週間隔の維持治療で寛解を維持できるまで効果が回復するとは限らない．本邦では2番目に登場したアダリムマブとの治療優先に関する議論，すなわち，インフリキシマブの二次無効，アダリムマブの二次無効の問題も解決する必要がある．また，アダリムマブの増量の意義や本邦オリジナルな治療法である白血球除去療法，栄養療法との併用など，今後，さらなるデータの蓄積と新たな臨床治験を展開し，長期の寛解維持を達成させるための適切な薬剤選択，適切な増量のタイミング（Figure 1）を明らかにすることが重要であると考える．

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Functional engraftment of colon epithelium expanded *in vitro* from a single adult Lgr5$^+$ stem cell

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Adult stem-cell therapy holds promise for the treatment of gastrointestinal diseases. Here we describe methods for long-term expansion of colonic stem cells positive for leucine-rich repeat containing G protein-coupled receptor 5 (Lgr5$^+$ cells) in culture. To test the transplantability of these cells, we reintroduced cultured GFP$^+$ colon organoids into superficially damaged mouse colon. The transplanted donor cells readily integrated into the mouse colon, covering the area that lacked epithelium as a result of the introduced damage in recipient mice. At 4 weeks after transplantation, the donor-derived cells constituted a single-layered epithelium, which formed self-renewing crypts that were functionally and histologically normal. Moreover, we observed long-term (>6 months) engraftment with transplantation of organoids derived from a single Lgr5$^+$ colon stem cell after extensive *in vitro* expansion. These data show the feasibility of colon stem-cell therapy based on the *in vitro* expansion of a single adult colonic stem cell.

Epithelial stem cells maintain tissue homeostasis throughout the gastrointestinal tract[1–3]. The Wnt, bone morphogenetic protein (BMP) and Notch cascades function together to regulate stem-cell maintenance[4,5]. *Lgr5* marks stem cells in small intestinal and colonic crypts[6] and in gastric units[7]. *Bmi1* may mark distinct stem cells in the proximal small intestine[8]. It has been shown that freshly isolated intestinal epithelium can be transplanted in rodents after resident epithelium has been surgically removed[9,10]. We previously developed a three-dimensional culture technique that allows expansion of single Lgr5$^+$ stem cells from small intestine[11], stomach[7] and colon[12]. The resulting organoids then expand and self organize into an epithelial architecture that is reminiscent of that seen in *in vivo* histology. Moreover, the growing organoids maintain their tissue identity even after prolonged culture. Here we sought to evaluate whether the cultured Lgr5$^+$ cells faithfully represent the tissue-resident Lgr5$^+$ stem cells and, thus, are able to regenerate epithelial tissue *in vivo*. Considering that the colon is very vulnerable to disease in humans, we focused on colonic stem cells in our analyses.

RESULTS

Long-term, serum-free culture system for colonic organoids

We subjected the colons of adult mice to a combination of enzymes[13], reducing agents[14] and mechanical disruption. The resulting crypt fragments were mostly devoid of α smooth muscle actin gene (*Acta2*)-expression–positive non-epithelial components and consisted of a mix of cadherin 1, type 1, E-cadherin (*Cdh1*)$^+$ cells expressing terminal differentiation marker genes (*Muc2*, *CA2* and *ChgA*) and Lgr5$^+$ stem cells (**Supplementary Fig. 1a,b**).

The addition of R-spondin 1 (Rspo1), Noggin and epidermal growth factor (EGF), which are all essential to small intestine culture[11], did not maintain the growth of colonic crypts. We therefore developed the following 'TMDU (Tokyo Medical and Dental University) protocol': we embedded crypts in type I collagen in serum-free medium with Wnt3a, hepatocyte growth factor (HGF)[15,16] and BSA, in addition to Rspo1, Noggin and EGF (**Supplementary Fig. 1c**). Sequential imaging of the cultures revealed rapid growth of cystic structures (**Fig. 1a**). Wnt3a, Rspo1 and BSA were essential to this growth (**Supplementary Fig. 1d**). As predicted by previous results[17,18], Rspo1 could be substituted with Wnt3a (data not shown). Although Noggin, EGF and HGF were not essential for growth of the colonic crypts, each enhanced their growth (**Supplementary Fig. 1e**). The colonic organoids rarely had buds (**Fig. 1a**, **Supplementary Fig. 2a** and **Supplementary Video 1**). Of note, small intestinal organoids also generate cystic structures when Wnt3a is added to them[19].

The colonic organoids were single layered (**Supplementary Fig. 2b**), and all the cells within were positive for Cdh1 expression (**Fig. 1b**). The basal membranes of the organoids faced outward (**Fig. 1b**). Ki67$^+$ cells were present in the colonic organoids (**Fig. 1b**), as were alcian blue–positive goblet cells, chromogranin A (ChgA)$^+$ enteroendocrine cells, carbonic anhydrase II (CA2)$^+$ colonocytes and cytochrome c oxidase subunit I (COX1)$^+$ tuft cells[20] (**Fig. 1b**). Transmission electron microscopy revealed epithelial characteristics such as microvilli (**Fig. 1c**) and junctional complexes (**Fig. 1d**) in the organoids. However, stromal cells were absent (**Supplementary Fig. 2c**). Mitotic cells with condensed chromosomes were present in the organoids (**Fig. 1e**), and goblet cells (**Fig. 1f**) and enteroendocrine cells (**Fig. 1g**) could also be clearly detected.

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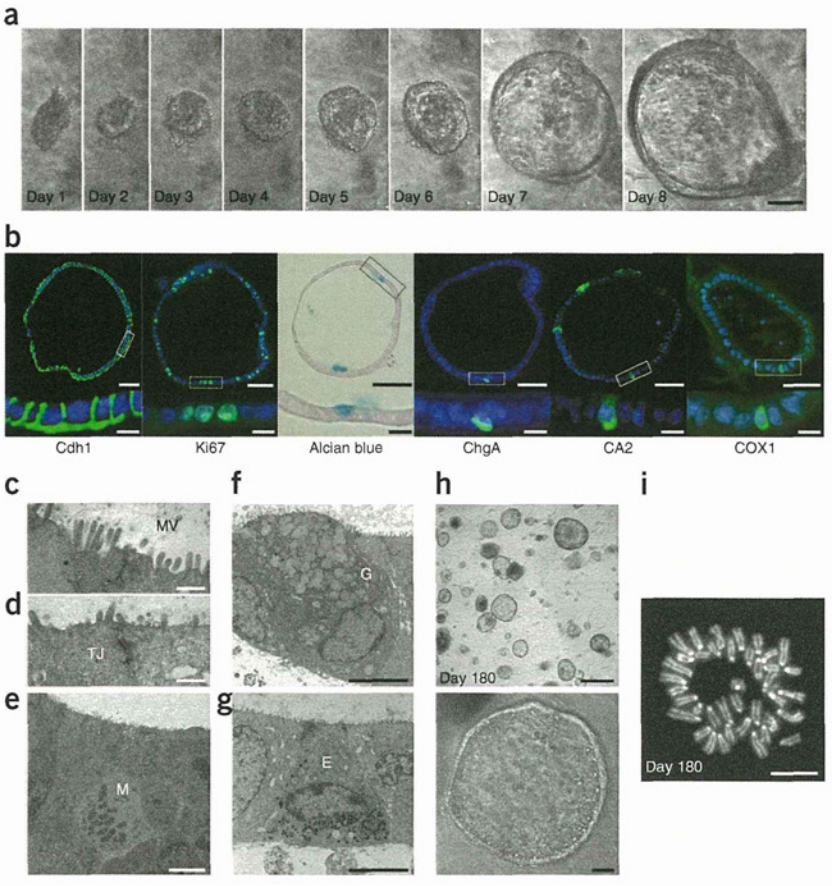

Figure 1 Long-term, serum-free culture of colonic epithelial cells. (**a**) A representative colonic crypt growing as a cystic structure. Scale bar, 50 μm. Time-lapse images of another colonic crypt are shown in **Supplementary Figure 2a** and **Supplementary Video 1**. (**b**) Histology of the colonic organoids at day 8 of culture. Cdh1$^+$ cells, actively proliferating Ki67$^+$ cells (green) and terminally differentiated cells stained with alcian blue (blue, goblet cells) or immunostained with ChgA (green, enteroendocrine cells), CA2 (green, colonocytes) or COX1 (green, tuft cells) are shown. Higher magnification views of the boxed areas are shown at the bottom. DAPI staining was performed, except for the experiments in which we performed alcian blue staining. Scale bars, top, 50 μm; bottom, 10 μm. (**c**–**g**) Transmission electron microscopy analysis for organoids at day 8. (**c**,**d**) Microvilli (MV) and intracellular tight junctions (TJ) are shown. (**e**) Mitotic (M) cells showing chromatin condensation. (**f**,**g**) Goblet cells (G) with mucus granules (**f**) and enteroendocrine cells (E) with electron dense granules (**g**) are shown. Scale bars: **c**,**d**, 0.5 μm; **e**–**g**, 5 μm. Low-power views of **f** and **g** are also shown in **Supplementary Figure 2c**. (**h**) The culture at day 180 (top) and its representative organoid (bottom). Scale bars, top, 500 μm; bottom, 50 μm. Images of the growth of a single cell after passage are shown in **Supplementary Figure 3** and **Supplementary Video 2**. (**i**) Metaphase spread of a cell at day 180 shows a normal karyotype ($2n = 40$). Scale bar, 10 μm.

The organoids could be passaged weekly at a 1:2 ratio (**Supplementary Fig. 3** and **Supplementary Video 2**). Addition of the Rho kinase inhibitor Y-27632 (ref. 21) improved the replating efficiency of the organoids[11]. We successfully propagated organoids for more than 6 months without clear alterations of morphology (**Fig. 1h**) or karyotype (**Fig. 1i**).

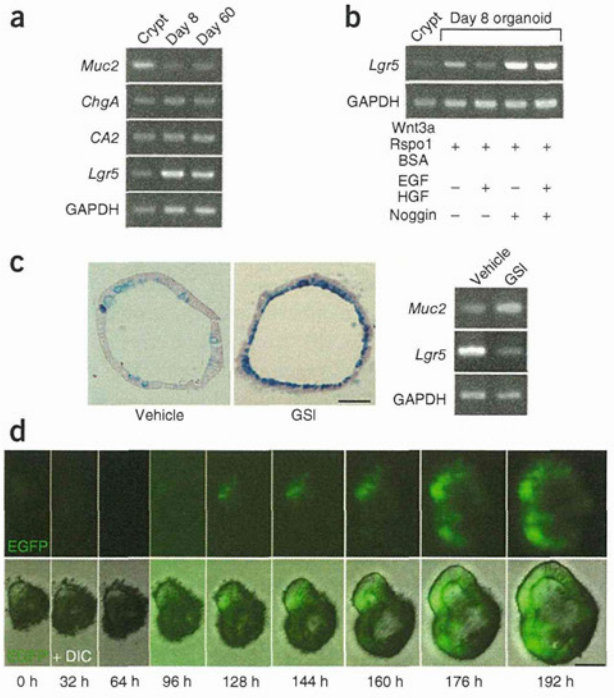

Figure 2 Lgr5$^+$ stem cells are enriched in cultured organoids. (**a**) RT-PCR analysis of the colonic crypts immediately after isolation (crypt) or organoids cultured for 8 or 60 d. *Lgr5* was upregulated and stayed constant thereafter. Differentiation marker genes (*Muc2*, *ChgA* and *CA2*) were expressed over 60 d. The primers used are listed in **Supplementary Table 1**. (**b**) RT-PCR shows that *Lgr5* upregulation is mediated by a combination of minimum factors (Wnt3a, Rspo1 and BSA) and Noggin but not by EGF and HGF. (**c**) Notch signal-mediated cell fate determination *in vitro*. Cultured organoids were treated with GSI, LY-411575 or vehicle alone from day 4 to day 8. Organoids stained with alcian blue are shown (left). Scale bar, 50 μm. RT-PCR shows that the expression of *Muc2* is upregulated, whereas the expression of *Lgr5* is reciprocally downregulated in organoids treated with LY-411575 (GSI, right). Similar results were obtained in three independent experiments, and representative data are shown. (**d**) A time-lapse imaging of a growing colonic crypt obtained from an *Lgr5-EGFP-ires-CreERT2* mouse over 192 h. The top panel shows EGFP and the bottom panel shows merged images of EGFP and differential interference contrast (DIC). Scale bar, 50 μm. The corresponding video (**Supplementary Video 3**) and similar results from another example are available as **Supplementary Figure 4a** and **Supplementary Video 4**.

Lgr5$^+$ cells are enriched in colonic organoids

We tracked the expression of *Lgr5* over 60 d and found a substantial elevation during the first 8 d of observation (**Fig. 2a**). We found no change in the expression of *ChgA* and *CA2*, whereas *Muc2* expression was repressed in the first 8 d (**Fig. 2a**). Addition of a combination of Wnt3a, Rspo1 and BSA induced *Lgr5* expression (**Fig. 2b**). *Lgr5* expression was further upregulated by the addition of Noggin, which is an antagonist of BMP[22] (**Fig. 2b**). The Notch pathway suppresses the

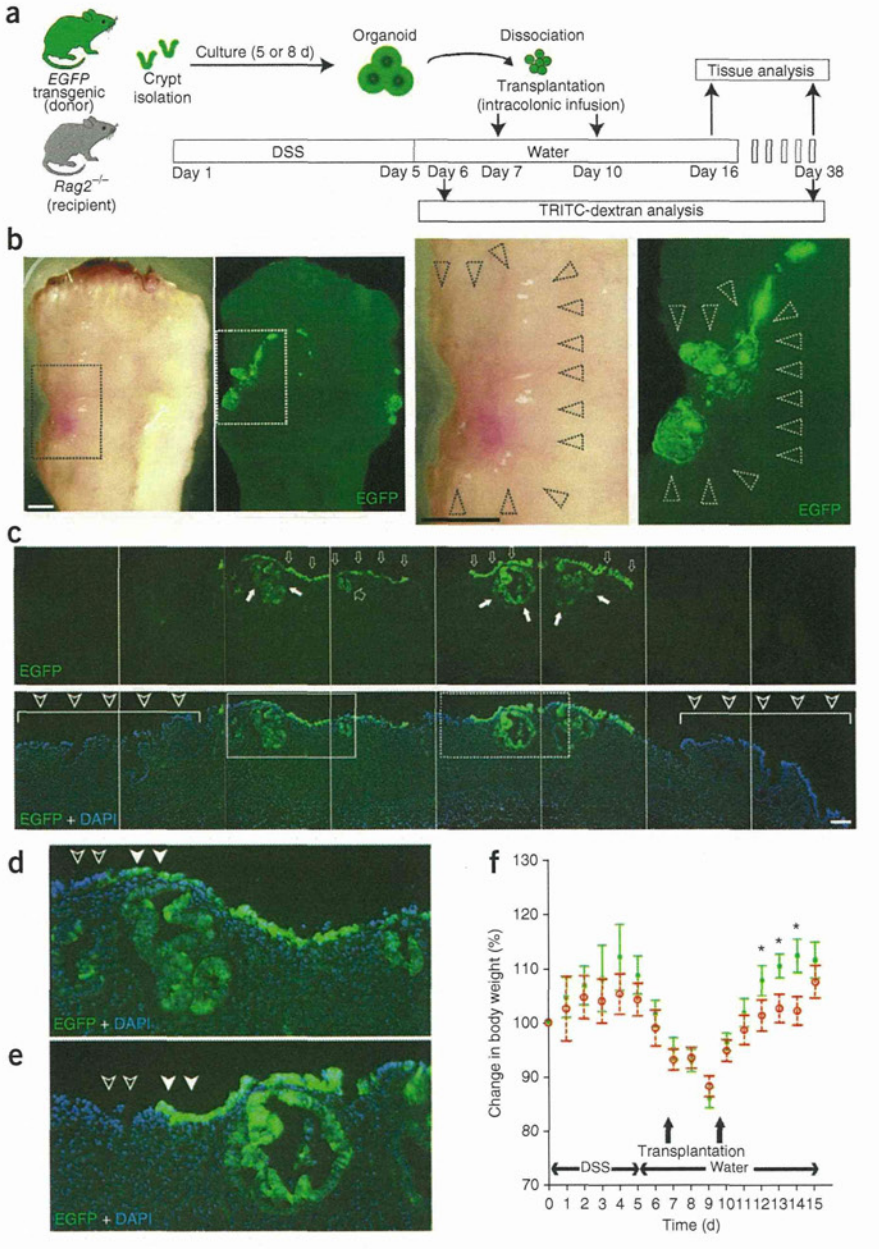

Figure 3 Transplantation of cultured cells improves acute colitis. (**a**) Experimental protocols. (**b**) Recipient colon at 6 d after transplantation. Low-power views (stereoscopic and fluorescent images) are shown on the left. High-power views of the areas in the dotted squares are shown in the right. The black dotted arrowheads show a depressed area surrounded by edematous mucosa. EGFP$^+$ areas overlapping the damaged region (white dotted arrowheads) are also shown. Note that the outline of the tissue is not precisely the same in the stereoscopic and fluorescent images, as they were acquired on different microscopes. Scale bars, 1 mm. (**c**) Histology of the EGFP$^+$ area shown in **b**. EGFP (top) and the merged image with DAPI staining (bottom). EGFP$^+$ cells cover the damaged mucosa that intervene separate areas preserving crypt structures (bottom, arrowheads). EGFP$^+$ cells constitute flat linings (top, narrow open arrow) or an invagination (top, wide open arrows), the latter of which is reminiscent of crypts. EGFP$^+$ cystic structures were also observed in the EGFP$^+$ cells (top, filled white arrows). The regions in the solid- and dotted-line boxes are shown at higher magnification in **d** and **e**, respectively. Scale bar, 100 μm. (**d**) High-power view of the solid box in **c**. (**e**) High-power view of the dotted box in **c**. (**f**) *Rag2*$^{-/-}$ mice were given DSS for 5 d, and then transplantation ($n = 6$) or sham-transplantion ($n = 6$) was performed. On day 16, the presence of engraftment was retrospectively assessed after the mice were killed. The body weights of the mice with EGFP$^+$ engraftment (green squares, $n = 4$) and sham-transplanted controls (red open circles, $n = 6$) are presented as a percentage of their initial weight. Error bars, s.e.m. $^*P < 0.05$ (Student's *t* test).

differentiation of progenitors[23,24] and stem cells[25] toward secretory lineages. We treated the colonic organoids with LY-411575, a γ-secretase inhibitor (GSI) that is capable of inhibiting Notch signaling[26,27]. Notch inhibition induced a goblet-cell phenotype with an increased level of *Muc2* mRNA and a reciprocal decrease in the expression of *Lgr5* (**Fig. 2c**).

We next performed live imaging of colonic organoids obtained from *Lgr5-EGFP-internal ribosome entry site (ires)-CreERT2* mice[6] in which an enhanced GFP (EGFP) and tamoxifen-inducible Cre recombinase cassette is integrated into the *Lgr5* locus. The *Lgr5*-promoter–driven EGFP expression initially stayed at a marginal level but then increased beginning at day 5 (**Fig. 2d**, **Supplementary Fig. 4a** and **Supplementary Videos 3** and **4**). We confirmed the expansion of Lgr5$^+$ cells at a single-cell resolution (**Supplementary Fig. 4b**). Over multiple passages, the *Lgr5-EGFP* locus tended to become silenced, whereas the wild-type *Lgr5* allele remained active (**Fig. 2a**,**b**). Taken together, colonic Lgr5$^+$ stem cells were able to self renew and expand *in vitro*.

Cultured colonic organoids rescue damaged epithelium

We next tested the transplantability of the cultured organoids (**Fig. 3a**). We induced colonic mucosal damage by providing immunocompromised *Rag2*$^{-/-}$ mice with colitis-inducing dextran sulfate sodium (DSS)[28] for 5 d. Most of the mice developed acute colitis characterized by weight loss, bloody stool, diarrhea and epithelial injury in the distal colon. At 7 and 10 d after initiating DSS administration, we dissociated the organoids cultured from *EGFP* transgenic mice[29] into small fragments, suspended them in a Matrigel-containing PBS and instilled them by enema in recipient mice.

At 16 d after the start of DSS administration, the recipient colons showed varying degrees of recovery. Multiple EGFP$^+$ areas appeared as well-demarcated patches in the treated colons (**Fig. 3b**). We did not observe any EGFP$^+$ areas in colons not treated with DSS (data not shown). Histologically, the EGFP$^+$ cells covered the submucosa and were located between the less damaged recipient tissues (**Fig. 3c**). The EGFP$^+$ cells formed flat or slightly invaginated linings (**Fig. 3c**). We also observed large cystic EGFP$^+$ structures below the surface of the treated colons (**Fig. 3c**). Some of the EGFP$^+$ areas connected to the recipients' epithelium (**Fig. 3d**), whereas others repopulated areas that were devoid of recipient epithelium (**Fig. 3e**). Notably, the body weights of the mice with engraftment were

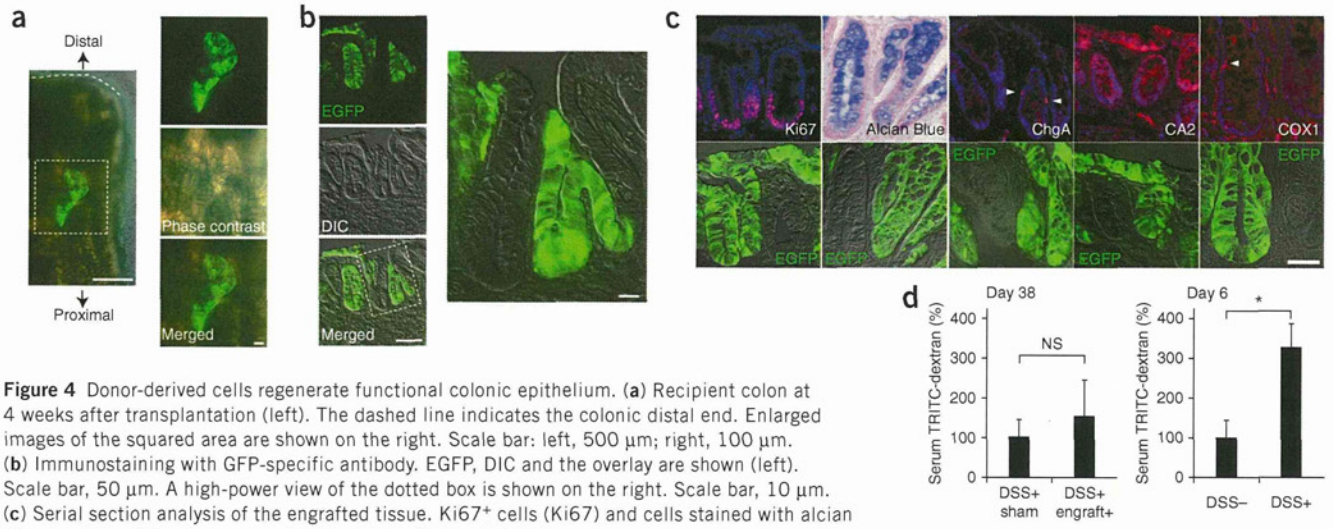

Figure 4 Donor-derived cells regenerate functional colonic epithelium. (**a**) Recipient colon at 4 weeks after transplantation (left). The dashed line indicates the colonic distal end. Enlarged images of the squared area are shown on the right. Scale bar: left, 500 μm; right, 100 μm. (**b**) Immunostaining with GFP-specific antibody. EGFP, DIC and the overlay are shown (left). Scale bar, 50 μm. A high-power view of the dotted box is shown on the right. Scale bar, 10 μm. (**c**) Serial section analysis of the engrafted tissue. Ki67$^+$ cells (Ki67) and cells stained with alcian blue (goblet cells) or immunostained for ChgA, CA2 and COX1 are shown. Images are shown with or without DAPI staining. The bottom row shows neighboring sections stained for GFP. Arrowheads point to ChgA$^+$ or COX1$^+$ cells. Scale bar, 50 μm. (**d**) After DSS colitis induction, transplantation ($n = 6$) or sham transplantation ($n = 6$) was performed. Mice were administered TRITC-dextran by gavage before killing on day 38. Four out of six colons in the transplanted group had EGFP$^+$ engraftment, and the serum TRITC concentration in these mice is shown (DSS+ engraft+; $n = 4$) as a percentage of that in the sham-transplanted group (DSS+ sham; $n = 6$). As a control, DSS colitis was induced (DSS+; $n = 6$) or uninduced (DSS–; $n = 6$) in *Rag2*$^{-/-}$ mice, and these mice were subjected for the same assay on day 6 without transplantation. Data are shown as a percentage of the concentrations in uninduced mice. Error bars, s.e.m. $^*P < 0.05$, NS, not significant (Student's *t* test).

higher than those of sham-transplanted mice (**Fig. 3f**; with statistically significant results at days 12, 13 and 14, $P < 0.05$).

At 4 weeks after transplantation, tube-like EGFP$^+$ crypts appeared in the distal colon (**Fig. 4a**) that were morphologically indistinguishable from the surrounding EGFP$^-$ epithelium (**Fig. 4b**). Notably, the engrafted crypts were entirely EGFP$^+$, indicating the presence of EGFP$^+$ stem cells (**Fig. 4b**). Cells in the lower part of the EGFP$^+$ crypts were normally Ki67$^+$, and the EGFP$^+$ crypts contained all

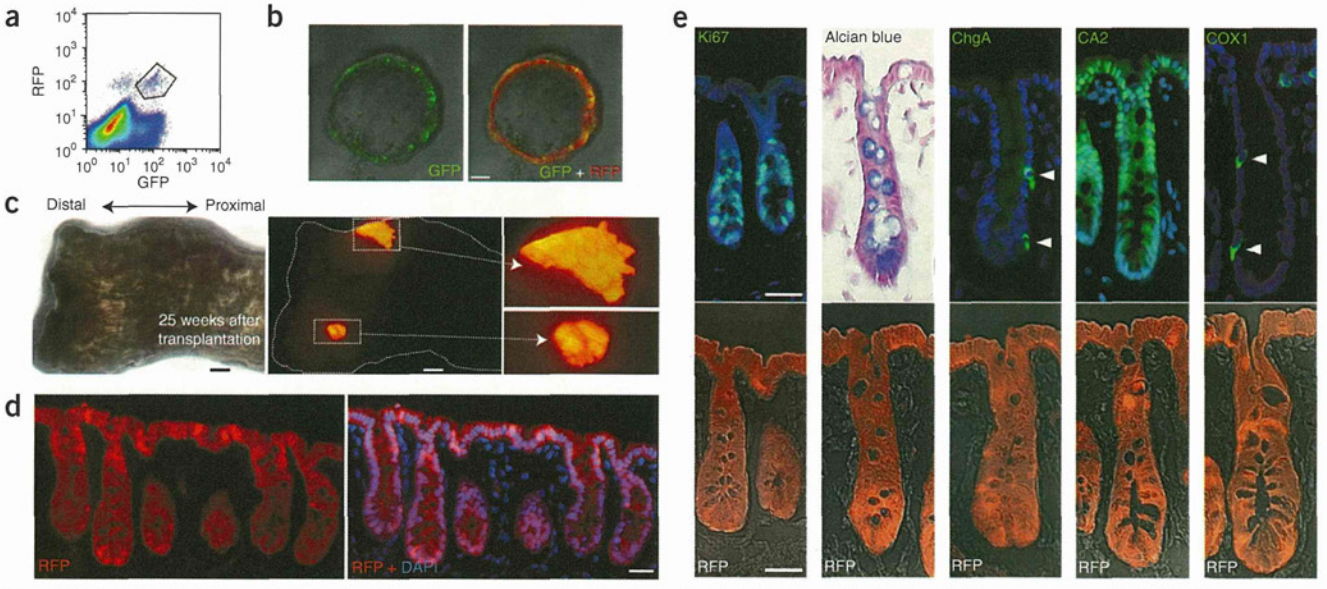

Figure 5 Single Lgr5$^+$ stem-cell–derived cultured cells serve as long-lived, multipotential stem cells *in vivo*. (**a**) Fluorescence-activated cell sorting analysis of colonic cells of *R26R-Confetti* mice crossed with *Lgr5-EGFP-ires-CreERT2* mice 3 d after Cre induction. The EGFP$^+$ and RFP$^+$ populations located in the box were sorted and cultured. (**b**) Images are shown of one out of four organoids grown from sorted single Lgr5$^+$ cells at day 6. EGFP$^+$ stem cells are scattered in the organoid (left), with all the offspring being positive for RFP (right). Scale bar, 50 μm. (**c**) Images of the recipient colon at 25 weeks after transplantation. The phase-contrast view of the recipient colon is shown on the left. The fluorescent image shows the tissue contains RFP$^+$ grafts (right). Scale bar, 1 mm. Enlarged images of the boxed areas are also shown (2.7-fold magnification). (**d**) Immunostaining of the RFP$^+$ engraft at 25 weeks after transplantation. An image of RFP-specific antibody staining (left) and an image of RFP staining merged with DAPI staining (right) are shown. Scale bar, 50 μm. (**e**) Serial section analysis of the engrafted RFP$^+$ tissue at 25 weeks after transplantation. The top panels show Ki67, alcian blue, ChgA, CA2 or COX1 staining with or without nuclei stained by DAPI. The bottom panels show the adjacent sections stained for RFP. Arrowheads point to ChgA$^+$ enteroendocrine cells and COX1$^+$ tuft cells. Scale bars, 50 μm.

terminally differentiated cell types (**Fig. 4c**). We probed the epithelial permeability of the engrafts using tetramethylrhodamine isothiocyanate (TRITC)-conjugated dextran (TRITC-dextran). Blood TRITC concentrations in transplanted mice were comparable to those in control mice, indicating a maintenance of epithelial barrier function in these engrafts (**Fig. 4d**). Notably, transplantation was less successful with freshly isolated donor cells ($P < 0.05$, Mann-Whitney U test; **Supplementary Fig. 5**), suggesting that the expansion of stem cells during the culture is associated with a higher success rate of transplantation. In addition, Matrigel-containing organoid suspensions transplanted better than organoids suspended in PBS (**Supplementary Fig. 5**; $P < 0.05$, Mann-Whitney U test), proposing a role for the simultaneous supply of extracellular matrix in successful transplantation.

Engraftment of organoids derived from a single Lgr5$^+$ cell

We next sought to initiate the protocol described above from a single stem cell (**Supplementary Fig. 6a**). We crossed *Lgr5-EGFP-ires-CreERT2* mice with *R26R-Confetti* reporter mice[30]. In the resulting offspring, tamoxifen-induced Cre activation resulted in Cre-mediated recombination at the *Rosa26* locus in individual Lgr5$^+$ stem cells, leading to stochastically selected expression of one out of four fluorescent proteins: red fluorescent protein (RFP), cyan fluorescent protein (CFP), GFP or yellow fluorescent protein (YFP). At 3 d after Cre activation, we sorted cells double positive for Lgr5-EGFP and Confetti-RFP, which consisted of ~0.02% of the total cells (**Fig. 5a**), equivalent to ~100 cells per mouse.

We cultured the sorted cells after a limiting dilution (100 cells per 96 well) using the Hubrecht protocol (Online Methods; protocol described previously[12] with addition of Y-27632 in the first 2 d). Four stem cells double positive for Lgr5-EGFP and Confetti-RFP grew out, which was comparable to the culture efficiency of small intestinal stem cells[11] (**Fig. 5b**). Organoids were expanded to more than 100 wells in >10 weeks, frozen and shipped. After thawing, we recovered the cells under the TMDU protocol. We transplanted ~500 organoids per recipient mouse, as described above. Analyses at 4, 17, 21 and 25 weeks after transplantation revealed the presence of grafts in these mice (**Fig. 5c** and **Supplementary Figs. 6b,c** and **7a**). At 25 weeks after transplantation, RFP$^+$ cells still generated a single-layered epithelium. We noted no sign of adenomatous or dysplastic change in any of the transplanted areas (**Fig. 5d**). Again, all differentiated cell types, as well as Ki67$^+$ proliferating cells, were present at normal ratios (**Fig. 5e** and **Supplementary Fig. 7b**).

DISCUSSION

Here we describe methodologies to isolate, culture and transplant Lgr5$^+$ colon stem cells. Our observations confirm that *Lgr5* marks genuine stem cells that retain their self-renewal and multilineage-differentiation properties even after prolonged culture. A major difference between small-intestinal and colon-culture conditions is in the latter's requirement for Wnt. Although Wnt factors can initiate Wnt signals on their own, R-spondins (such as Rspo1) can only augment preexisting Wnt signals[31]. Because Paneth cells produce Wnt3, they serve as the center of organization of the stem cell niche[19]. At the colon crypt bottoms, secretory cells are located between the Lgr5$^+$ stem cells that—like Paneth cells—express CD24 (ref. 19). However, these CD24$^+$ secretory cells do not produce a sufficient amount of Wnt proteins *in vitro* (data not shown). Therefore, colon organoids cannot grow from Rspo1 alone but, rather, also require exogenous Wnt.

This study provides proof of principle that cultured Lgr5$^+$ cells can be used for stem-cell therapy to repair damaged epithelium. Transplanted cells adhere to and cover superficially damaged tissue. Further, engrafted recipient mice had higher body weights than ungrafted controls, implying a beneficial role for the donor cells in DSS-induced acute colitis. Although further optimization is clearly needed, the current study implies that *in vitro* expansion and transplantation of gastrointestinal stem cells may be a promising option for patients with severe gastrointestinal epithelial injuries.

Lgr5$^+$ stem cells divide once every day *in vivo*[6], thus defying the Hayflick limit[32]. They appear similarly unrestricted in their proliferative capacity *in vitro*, while they retain their original tissue identity. It is of interest that the Lgr5 protein is now known to reside in the Wnt receptor complex to function as a receptor for Rspo1 (refs. 33,34), which is a crucial component of long-term organoid culture systems that we have developed. As the resulting organoids have now been proven to be transplantable, the Lgr5$^+$ stem cell isolation and expansion technology may provide a simple and safe avenue for the development of new regenerative and gene-therapy strategies.

METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/naturemedicine/.

Note: Supplementary information is available on the Nature Medicine website.

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AUTHOR CONTRIBUTIONS

T. Nakamura, H.C. and M.W. designed the study. S.Y., T. Nakamura and T.S. performed experiments and analyzed data. T. Nakamura, T.S. and H.C. wrote the paper. Y.N., T. Nagaishi and K.T. assisted in transplantation experiments. T.M., X.Z. and K.T. gave support in gene analysis. R.O. helped with the immunohistochemistry. S.I. advised on the electron microscopy. H.C. and M.W. gave conceptual advice and supervised the project.

COMPETING FINANCIAL INTERESTS

The authors declare competing financial interests: details accompany the full-text HTML version of the paper at http://www.nature.com/naturemedicine/.

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ONLINE METHODS

Mice. *Rag2*$^{-/-}$ mice were from Taconic Farms and Central Laboratories for Experimental Animals. *EGFP* transgenic mice[29], *Lgr5-EGFP-ires-CreERT2* mice[6] and *R26R-Confetti* mice[30] are described elsewhere. Male and female mice were randomly used for all experiments. All animal experiments were performed with the approval of the Institutional Animal Care and Use Committee of TMDU.

TMDU protocol for crypt isolation and three-dimensional culture. The colonic tissue was minced and digested. The crypts were further purified by mechanical disruption and density gradient centrifugation. A total of 2,000 crypts were suspended in 200 µl of the collagen type I solution (Nitta Gelatin Inc.) and placed in 48-well plates. After polymerization, 500 µl of Advanced DMEM/F12 containing BSA (Sigma), mouse EGF (mEGF) (PeproTech), mWnt3a, mRspo1, mHGF and mNoggin (all from R&D Systems) was added (TMDU medium). For passage, the gel was digested, and then the organoids were disaggregated with EDTA. The dissociated organoids were mixed in type I collagen solution and used for culture. A Rho kinase inhibitor, Y-27632, was added for the first 2 d after the cells were propagated. Where indicated, to induce goblet cell differentiation, organoids were treated with LY-411575, a GSI. See details in the **Supplementary Methods**.

Chromosome analysis. Chromosome karyotyping was performed according to a standard protocol as detailed in the **Supplementary Methods**.

Stereomicroscopy, phase-contrast imaging and histology. Images were acquired on either a fluorescence microscope equipped with phase-contrast setting (BZ-8000, KEYENCE), a fluorescent stereomicroscope system MVX10 (Olympus) or a fluorescence microscope DeltaVision system (Applied Precision). For histology and immunohistochemistry, tissues and organoids were fixed, sequentially dehydrated in sucrose in PBS, and frozen in OCT compound (Tissue Tek). Cryosections were examined by conventional H&E, alcian blue staining and a spectrum of immunohistochemical reactions, as detailed in the **Supplementary Methods**.

Transmission electron microscopy. Transmission electron microscopy was performed in a standard fashion and is detailed in the **Supplementary Methods**.

Live imaging. Live imaging was performed on the DeltaVision system. A culture dish placed on the microscope stage was covered with a chamber in which a humidified premixed gas consisting of 5% CO_2 and 95% air was infused, and the whole setup was set at 37 °C. DIC and fluorescent images were acquired at 20-min intervals. The data were processed using Softworx (Applied Precision) and, if necessary, image editing was performed using Adobe Photoshop Elements 7.0.

Semi-quantitative RT-PCR. Semi-quantitative RT-PCR was performed in standard fashion. The primer sequences used are listed in **Supplementary Table 1**. PCR products were separated on agarose gels and visualized using ImageQuant TL system (GE Healthcare).

Sorting and Hubrecht-protocol culture for single Lgr5$^+$ cells. Tamoxifen was injected into *R26R-Confetti* mice crossed with *Lgr5-EGFP-ires-CreERT2* mice, and the colonic crypts from the resulting mice were isolated 3 d later. Epithelial cells were dissociated with TrypLE express (Invitrogen) and analyzed by MoFlo (DakoCytomation). Viable single cells were gated, and then the cells doubly positive for EGFP and RFP were sorted and embedded in Matrigel (BD Bioscience) on 96-well plates. An Advanced DMEM/F12 culture medium supplemented with penicillin and streptomycin, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), glutamax, N2, B27 (all from Invitrogen) and growth factors (EGF, noggin and R-spondin) was diluted 1:1 with Wnt3a-conditioned medium and used as Hubrecht medium. Y-27632 was included for the first 2 d to avoid anoikis. Growth factors were added every other day, and the entire medium was changed every 4 d. See the **Supplementary Methods** for additional details.

Transplantation experiments. For the EGFP$^+$ cell transplantations, cells isolated from colon tissues were cultured for 5 or 8 d according to the TMDU protocol and used as donor cells. For single Lgr5$^+$-cell–derived organoid transplantation, cells were expanded based on the Hubrecht protocol and then cryopreserved. The cells were then shipped, thawed and further cultured. Acute colitis was induced by feeding 6-week-old *Rag2*$^{-/-}$ mice with 3.0% DSS (molecular weight 10,000; Ensuiko Sugar Refining Co.) dissolved in drinking water for 5 d (days 1–5). At 7 and 10 d after the start of DSS administration, donor cells equivalent to those from ~500 organoids were instilled into colonic lumen as a suspension. After infusion, the anal verge was glued for 6 h. After the transplantation on day 10, mice were maintained as usual before they were killed and analyzed. See the **Supplementary Methods** for additional details.

TRITC-dextran permeability assay. Intestinal permeability was assessed by enteral administration of TRITC-dextran (molecular mass 4.4 kDa; Sigma). Transplanted or sham-transplanted mice were gavaged with TRITC-dextran 4 h before killing on day 38. Whole blood was obtained at the time of killing, and then the colonic tissues were examined for whether the EGFP$^+$ engrafts were present. TRITC-dextran measurements were performed on an ARVO MX (PerkinElmer), with serial dilutions of TRITC-dextran used as a standard curve.

Statistical analyses. Data are shown as means ± s.e.m. Data for **Figures 3f**, **4d** and **Supplementary Figure 7b** were statistically analyzed by the two-sample Student's *t* test. The data for **Supplementary Figure 5** showed non-normal distributions and were analyzed by Mann-Whitney *U* test. Statistical significance for comparisons was assigned at $P < 0.05$.

Additional methods. Detailed methodology is described in the **Supplementary Methods**.

Supplementary Information

Functional engraftment of colon epithelium expanded *in vitro* from a single adult Lgr5^{+} stem cell

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