Abstract: Colitogenic memory CD4<SUP>+</SUP> T cells are important in the pathogenesis of inflammatory bowel disease (IBD). Although memory stem cells with high survival and self-renewal capacity were recently identified in both mice and humans, it is unclear whether a similar subset is present in chronic colitis mice. We sought to identify and purify a long-lived subset of colitogenic memory CD4<SUP>+</SUP> T cells, which may be targets for treatment of IBD. A long-lived subset of colitogenic memory CD4<SUP>+</SUP> T cells was purified using a long-term culture system. The characteristics of these cells were assessed. Interleukin (IL)-7 promoted the in vitro survival for >8 weeks of lamina propria (LP) CD4<SUP>+</SUP> T cells from colitic SCID mice previously injected with CD4<SUP>+</SUP>CD45RB<SUP>high</SUP> T cells. These cells were in a quiescent state and divided a maximum of 5 times in 4 weeks. LP CD4<SUP>+</SUP> T cells expressed higher levels of Bcl-2, integrin-α4β7, CXCR3 and CD25 after than before culture, as well as secreting high concentrations of IL-2 and low concentrations of IFN-γ and IL-17 in response to intestinal bacterial antigens. LP CD4<SUP>+</SUP> T cells from colitic mice cultured with IL-7 for 8 weeks induced more severe colitis than LP CD4<SUP>+</SUP> T cells cultured for 4 weeks. We developed a novel culture system to purify a long-lived, highly pathogenic memory subset from activated LP CD4<SUP>+</SUP> T cells. IL-7 promoted long-term in vitro survival of this subset in a quiescent state. This subset will be a novel, effective target for the treatment of IBD.
Aug 19th, 2013

Editor-in-Chief

*Immunology Letter*

Dr. Vito Pistoia

Thank you very much for your kind letter on September 9th, with regard to our manuscript entitled "IL-7 promotes long-term in vitro survival of unique long-lived memory subset generated from mucosal effector memory CD4\(^+\) T cells in chronic colitis mice" (manuscript reference # IMLET-D-13-00143) together with the comments from the Associate Editor and the reviewers.

We appreciate the editors and reviewers of our manuscript for their careful and helpful reviews. We believe that their suggestions have enabled us to improve our manuscript markedly. We have revised our manuscript in response to these suggestions, and trust we have addressed all of the reviewers' concerns. The following is a point-by-point response to these suggestions.

We agree to pay all costs for each color page, involved in printing the color photographs for our paper.

Looking forward to hearing from you soon.

Sincerely,

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Point by point response

We appreciate all reviewers and board members for the careful review of our manuscript. Now, with their accurate suggestions, we believe that our manuscript and figures have been improved dramatically. Concerns raised by the reviewers were addressed as following, and we were glad to correct these points in the revised manuscript.

Reviewer #1: Takahara et al.

This manuscript describes the long-term culture of colitogenic effector memory T cells. Unlike other homeostatic or diseased states, so-called T memory stem cells were not found in mice with chronic colitis. Instead, long-lived effector memory CD4 T cells were identified. These cells survived in vitro in the presence of IL-7 but did not proliferate extensively. The results are clear and interesting and the experiments performed are believable and well done.

Points:
1. There are a few abbreviations that aren't defined although I could guess what they were (SP, BM). Also, a few spelling errors (SCID is spelled SICD on p12).
   → We corrected these points in the revised manuscript.
2. Figure 3 is busy and confusing. It is mainly descriptive and the text does not describe what the results mean at all? Focusing on the important or differently expressed markers would help with clarity.
   → As you suggested, we focused only important markers on cultured-LP CD4+ T cells in revised figure 3.
3. Why are the results from 4wk cultured cells so different in Fig 5A and Fig 7B? In 5A, the cells induce on-going pathology at week 3 while in 7B it does not appear until week 5. If the mice from fig 5A were used in 7B, there would be no difference. Is 7B an anomaly or is 5A the outlier?
   → The adoptive transfer experiment in Figure 5 is different from those in Figure 7. We performed each experiments separately. Although we uniformed the condition of donor cells and recipient mice as much as possible, there is some difference in strength of colitis among each experiment in this model.

Reviewer #2: The manuscript entitled "IL-7 promotes long-term in vitro survival of unique long-lived memory subset generated from mucosal effector memory CD4+ T cells in chronic colitis
"mice" by Watanabe and colleagues characterized an in vitro cultured long-lived "memory" CD4+ T cell population that could induce colitis in vivo. While the manuscript is clearly written, several concerns are associated with this model.

**Major comments:**

It is clear that the cultured LP CD4+ T cells are pathogenic, and phenotypically different from primary LP CD4+ T cells. However, to exclude any potential artifact of in vitro culture system, and to conclude that "the in vitro cultured CD4+ T cell subset will be a novel, effective target for the treatment of IBD", the authors need to justify that this "unique" cell population does exist in vivo.

To exclude the potential artifact of in vitro system, we checked whether there is a population similar to cultured-LP CD4+ T cells in vivo or not. As shown in revised figure 8, in the colitic mice, LP CD4+CXCR3+CD25+ cells expressed higher level of Bcl-2, integrin-a4b7 and IL-2 than the other subsets, which means that this subset is similar to cultured-LP CD4+ T cells.

The authors need to discuss why cultured-LP CD4+ T cells express higher levels of gut homing molecules but end up accumulating in the spleen.

In the experiment in Figure 6, we analyzed colitic mice 6 weeks after the transfer. Primary-LP CD4+ T cell are gut tropic, so most of them migrate to MLN of LP, while cultured-LP CD4+ T cells might acquire the ability to recirculate to SP. In the early time point, larger number of cultured-LP CD4+ T cells might accumulate to LP than primary-LP CD4+ T cells. However, after the development of colitis, in the effector site, LP, primary LP CD4+ T cells have already expanded dramatically, while cultured-LP CD4+ T cells are quiescent and they have not expanded as same as primary LP CD4+ T cells. We added this point in discussion part of revised manuscript.

**Minor comments:**

For cell surface marker staining (figure 3), the authors need to show isotype/negative control for gating.

We added negative controls in revised figure 3.

The authors need to provide for each experiment how many repeats were done.

We added this information in revised manuscript.
Highlights

1. Hierarchy of memory T cells previously reported cannot apply to inflammatory bowel diseases (IBD) model mice, and that $T_{EM}$ in colitic mice are not short-lived cells but include long-lived cells.

2. We developed for the first time long-term culture system to purify long-lived and highly pathogenic memory subset from colitogenic memory CD4$^+$ T cells.

3. LP CD4$^+$ T cells expressed higher levels of Bcl-2, integrin-$\alpha 4\beta 7$, CXCR3 and CD25 after than before culture, as well as secreting high concentrations of IL-2 and low concentrations of IFN-$\gamma$ and IL-17 in response to intestinal bacterial antigens.

4. LP CD4$^+$ T cells from colitic mice cultured for 8 weeks induced more severe colitis than LP CD4$^+$ T cells cultured for 4 weeks.

5. This subset will be a novel, effective target for the treatment of IBD. This long-term culture system will be a useful tool to investigate the maintenance system of long-lived memory CD4$^+$ T cells in chronic inflammatory diseases.
IL-7 promotes long-term in vitro survival of unique long-lived memory subset generated from mucosal effector memory CD4\(^+\) T cells in chronic colitis mice

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Nonstandard abbreviations used: IBD: inflammatory bowel disease; LP: lamina propria; MLNs: mesenteric lymph nodes; SP: spleen; BM: bone marrow; T\(_{\text{EM}}\): effector memory T (cells); T\(_{\text{CM}}\): central memory T (cells); T\(_{\text{reg}}\): regulatory T (cells)

Keywords: inflammatory bowel disease, memory T cells, CD4, IL-7, cell culture
Abstract

Colitogenic memory CD4+ T cells are important in the pathogenesis of inflammatory bowel disease (IBD). Although memory stem cells with high survival and self-renewal capacity were recently identified in both mice and humans, it is unclear whether a similar subset is present in chronic colitis mice. We sought to identify and purify a long-lived subset of colitogenic memory CD4+ T cells, which may be targets for treatment of IBD. A long-lived subset of colitogenic memory CD4+ T cells was purified using a long-term culture system. The characteristics of these cells were assessed. Interleukin (IL)-7 promoted the in vitro survival for >8 weeks of lamina propria (LP) CD4+ T cells from colitic SCID mice previously injected with CD4+CD45RBhi T cells. These cells were in a quiescent state and divided a maximum of 5 times in 4 weeks. LP CD4+ T cells expressed higher levels of Bcl-2, integrin-αβ7, CXCR3 and CD25 after than before culture, as well as secreting high concentrations of IL-2 and low concentrations of IFN-γ and IL-17 in response to intestinal bacterial antigens. LP CD4+ T cells from colitic mice cultured with IL-7 for 8 weeks induced more severe colitis than LP CD4+ T cells cultured for 4 weeks. We developed a novel culture system to purify a long-lived, highly pathogenic memory subset from activated LP CD4+ T cells. IL-7 promoted long-term in vitro survival of this subset in a quiescent state. This subset will be a novel, effective target for the treatment of IBD.
1. Introduction

Inflammatory bowel disease (IBD) is a chronic inflammation of the gastrointestinal tract, occurring primarily in younger individuals and usually persisting over long periods of time (1). Monoclonal antibodies against TNF-α have been shown effective in patients with both Crohn’s disease (CD) and ulcerative colitis (UC), changing the natural history of these disorders (2). However, even if these treatments induce remission, these patients require life-long maintenance therapy because of the risk of IBD recurrence.

Pathogenic immunological memory is the primary cause of IBD persistence. Memory T cells can survive in a quiescent state, even in patients in life-long remission, with anti-effector therapy unable to remove these cells. For example, when bacteria translocate from the intestinal lumen to the blood circulation as a result of acute infectious colitis, memory T cells immediately differentiate to effector cells and exert effector function, leading to recurrence.

We have shown the importance of memory CD4+ T cells in the pathogenesis of chronic colitis (3). Using a mouse model of colitis, in which SCID mice were injected with CD4+CD45RB<sup>High</sup> T cells, we found that large numbers of CD4+CD44<sup>+ </sup>CD62L<sup>-</sup> memory T cells had infiltrated the colonic lamina propria (LP). These cells expressed high levels of the memory cell markers interleukin (IL)-7Rα and Bel-2, reproducing colitis upon transfer to new SCID mice for up to five transfers. These LP CD4<sup>+</sup> cells of colitic mice have therefore been described as “colitogenic memory T cells” (3).

Colitogenic memory T cells may be attractive targets for radical treatment of IBD without the need for maintenance therapy. Although bone marrow transplantation after strong chemotherapy to ablate the recipient’s hematopoietic cells was found to induce remission in CD patients without the need for maintenance therapy (4), these treatments
can cause severe infection, even death, because these treatments strongly suppress not only pathogenic memory T cells, but also protective T cells.

Memory T cells are heterogeneous and can be divided into two subpopulations. Central memory T cells (T_{CM}) reside mainly in the secondary lymphoid organs and are able to proliferate, whereas effector memory T cells (T_{EM}) reside mainly in effector sites such as intestinal LP and have immediate effector functions (5). Recently, memory stem cells (T_{SCM}), with high potential for survival, self-renewal and multi-differentiation, were identified in both mice and humans (6). These cells are defined primarily by their surface markers, including CD44, a memory and effector marker, and CD62L and CCR7, both of which are homing receptors to lymph nodes. The pathway of T cell differentiation is thought to consist of T_{naive} \rightarrow T_{SCM} \rightarrow T_{CM} \rightarrow T_{EM} \rightarrow T_{effector}. T_{SCM} are the least differentiated memory cells, have the best proliferative potential and are long-lived, whereas T_{EM} are the most differentiated memory cells, have the poorest proliferative potential, and are short-lived (7, 8). However, it remains unclear whether this pathway is active in IBDs and autoimmune diseases.

Identifying a key subset of colitogenic memory T cells, i.e. memory stem cells, may help develop more effective and safer treatments for IBD. We therefore sought to clarify the roles of each subset of memory CD4^{+} T cells, and to identify the most effective fraction to target during radical treatment of IBD.
2. Materials and Methods

2-1. Animals. C57BL/6, Balb/c, CB17-icr SCID mice were purchased from Japan CLEA (Tokyo, Japan). RAG-2 deficient mice (RAG-2<sup>−/−</sup>) were obtained from Taconic Laboratory (Hudson, NY) and Central Laboratories for Experimental Animals (Kawasaki, Japan). Mice were intercrossed and maintained under specific pathogen-free conditions in the Animal Care Facility of Tokyo Medical and Dental University (TMDU). Donors and recipients were used at 6–12 weeks of age. All experiments were approved by the regional animal study committees and were performed according to institutional guidelines and Home Office regulations.

2-2. Antibodies. The following mAbs were obtained from BD PharMingen (San Diego, CA) and used for purification of cell populations and flow cytometric analysis: FITC-, PE-, PerCP-, and APC-conjugated anti-mouse CD4 (RM4-5); FITC-, PerCP-conjugated anti-mouse CD3 (145-2C11); PE-conjugated anti-mouse CD44 (IM7); FITC-conjugated anti-mouse CD62L (MEL-14); PE-conjugated anti-mouse IL-17A (TC11-18H10); FITC-conjugated anti-mouse IFN-γXMG1.2; FITC-, PE-conjugated anti-mouse Bel-2 (3F11); PE-conjugated anti-mouse integrin-α4β7 (DATK32); FITC-conjugated anti-mouse VβTCR Screening Panel; FITC-conjugated anti-mouse CD69 (H1.2F3); FITC-conjugated anti-mouse CD45RB (16A); PE-conjugated anti-mouse CD25 (PC61); FITC-conjugated anti-mouse CD103 (M292); PE-conjugated anti-mouse CD117 (2B8); FITC-, PE-conjugated streptavidin. The following mAbs were obtained from eBioscience (San Diego, CA): biotin-conjugated anti-mouse IL-7Rα (A7R34); PerCP-Cy5.5-conjugated anti-mouse CCR7 (4B12); APC-conjugated anti-mouse Sca-1(D7). The following mAbs were obtained from Biolegend (San Diego, CA): PE/Cy7-conjugated anti-mouse CD3 (145-2C11); PE-, Brilliant Violet 421<sup>TM</sup>-conjugated anti-mouse
CXCR3(173); Biotin-conjugated anti-mouse integrin-α4β7 (DATK32); FITC-conjugated anti-mouse IL-2(JES6-5H4); PE/Cy7-conjugated anti-mouse IL-17A (TC11-18H10). The following mAbs was obtained from R&D Systems (Minneapolis, MN): PE-conjugated anti-mouse CCR9 (242503).

2-3, Induction of CD4⁺CD45RB<sup>high</sup> T cells-transfer colitis. CD4⁺ T cells were isolated from the spleens of C57BL/6 and Balb/c mice using the anti-CD4 (L3T4)-MACS system (Miltenyi Biotec, Auburn, CA) according to the manufacturer’s instructions. Enriched CD4⁺ T cells were labeled with PE-conjugated anti-mouse CD4 (RM4-5; BD Pharmingen, San Diego, CA) and FITC-conjugated anti-CD45RB (16A; BD Pharmingen). CD4⁺CD45RB<sup>high</sup> T cells were isolated using FACS Aria II (Becton Dickinson); these cells were 98.0-100% pure on reanalysis. Recipient mice, RAG-2⁻/⁻ mice for C57BL/6J donor cells and CB17-icr SCID mice for Balb/c donors, were intraperitoneally injected with 3 x 10⁵ CD4⁺CD45RB<sup>high</sup> T cells. All mice were monitored for clinical signs, including hunched posture, piloerection, diarrhea, and blood in the stool. At autopsy, their clinical scores were assessed as the sum of three parameters: hunching and wasting, 0 or 1; colon thickening, 0–3 (0, none; 1, mild; 2, moderate; or 3, extensive); and stool consistency, 0–3 (0, normal beaded stool; 1, soft stool; 2, diarrhea; 3, bloody stool) (3). In some experiments, on going colitis score (0–4 points) was defined as the sum of hunching and wasting (0 or 1) and stool consistency (0, normal beaded stool; 1, soft stool; 2, diarrhea) scores.

2-4, Histological examination. Tissue samples were fixed in PBS containing 6% neutral-buffered formalin. Paraffin-embedded sections (5 μm) were stained with H&E. Three tissue samples from the proximal, middle, and distal parts of the colon were prepared. The sections were analyzed without prior knowledge of the type of T cell
reconstitution or treatment. The mean degree of inflammation in the colon was calculated using a modification of a previously described scoring system (3), consisting of the sum of three parameters: crypt elongation, 0-3; mononuclear cell infiltration, 0-3; and frequency of crypt abscesses, 0-3.

2-5, Isolation of mononuclear cells from murine organs. Single cell suspensions were prepared from the SP, MLN, LP, and BM as described (3). To isolate LP CD4+ T cells, the entire length of colon was opened longitudinally, washed with PBS, and cut into small pieces. The dissected mucosae were incubated for 45 min with Ca2+-, Mg2+-free Hanks’ balanced salt solution containing 1 mM DTT (Sigma-Aldrich) to remove mucus and then treated with 3.0 mg/ml collagenase (Roche Diagnostics GmbH, Germany) and 0.01% DNase (Worthington Biomedical Co., Freehold, NJ) for 2 h. The cells were pelleted twice through a 40% isotonic Percoll solution and then subjected to Ficoll–Hypaque density gradient centrifugation (40%/75%). Enriched LP CD4+ T cells were obtained by positive selection using anti-CD4 (L3T4) MACS magnetic beads. Of the resultant cells, >95% were CD4+ cells, as determined by flow cytometry. BM cells were obtained by flushing two femurs with cold RPMI-1640.

2-6, Flow cytometry. To detect the surface expression of a variety of molecules, isolated SP, BM, MLN and LP mononuclear cells were incubated with specific FITC-, PE-, PerCP-, APC- and/or biotin-labeled antibodies for 20 min at 4°C. Standard two-, three-, or four-color flow cytometric analyses were performed using FACS Calibur flow cytometer and CellQuest software or using FACS Canto-II and Diva software.
2-7, **Cytokine ELISA.** Cytokine production was measured by culturing $1 \times 10^5$ LP CD4$^+$ T cells at 37°C in a humidified atmosphere containing 5% CO$_2$ in 200 μl culture medium containing 2 mg/ml hamster anti-mouse CD28 mAb (37.51, BD Pharmingen) in 96-well plates (Costar, Cambridge, MA) that had been pre-coated with 5 mg/ml hamster anti-mouse CD3e mAb (145-2C11, BD Pharmingen) for 3 hours at 37°C. Supernatants were collected 48 hour later, and cytokine concentrations in the supernatants were measured by ELISA, according to the manufacturer’s recommendation (R&D Systems).

2-8, **Intracellular staining of cytokines** Isolated CD4$^+$ T cells were cultured for 12 hours with ionomycine (500 ng/ml), PMA (50ng/ml) and BD GolgiPlug (1μl/ml BD Pharmingen) and collected and their surface molecules were stained. Following cell fixation using a Cytofix/Cytoperm Kit (BD Pharmingen), the cells were incubated with PE-conjugated anti-IL-17A mAb (TC11-18H10: BD Pharmingen), PE/Cy7-conjugated anti-mouse IL-17A (TC11-18H10: Biolegend) and FITC-conjugated anti-IFN-γ mAb (XMG1.2: BD Pharmingen), FITC- conjugated anti-mouse IL-2(JES6-5H4: Biolegend) for 20 min.

2-9, **Transfer of T$_{CM}$ and T$_{EM}$ from colitic mice to SCID recipients** SP and MLN CD4$^+$ T cells from colitic SCID mice previously injected with CD4$^+$CD45RB$^{high}$ T cells were isolated using the anti-CD4 (L3T4)-MACS system described above. The cells were labeled with PerCP-conjugated anti-mouse CD3, APC-conjugated anti-mouse CD4, PE-conjugated anti-mouse CD44 and FITC-conjugated anti-CD62L. CD3$^+$CD4$^+$CD44$^+$CD62L$^-$ T$_{EM}$ and CD3$^+$CD4$^+$CD44$^+$CD62L$^+$ T$_{CM}$ cells from the SP
and MLN were sorted using FACS Aria II, and 1 x 10^4 of each were injected intraperitoneally into new SCID mice (n=4). Mice were monitored for up to 16 weeks, sacrificed and analyzed.

2-10, Long-term culture of LP CD4^+ T cells from colitic SCID mice with anti-CD3e antibody, IL-2, IL-15 or IL-7. LP CD4^+ T cells were isolated from colitic SCID mice previously injected with CD4^+CD45RB^{high} T cells using the anti-CD4 (L3T4)-MACS system and labeled with 2.5 μM CFSE (Invitrogen) according to the manufacturer’s instructions. These cells (3 x 10^5/well) were cultured in 6 well plates in RPMI 1640 medium (Sigma) containing 10% FBS, HEPES, 50μM 2-ME and penicillin/streptomycin/L-glutamine, as well as anti-CD3e antibody (5 μg/mL, BD Pharmingen), human recombinant IL-2(10 U/mL, 100 U/mL, or 1000 U/mL; Shionogi), mouse recombinant IL-15 (1 ng/mL, 10 ng/mL, or 100 ng/mL; PeproTech) or human recombinant IL-7 (1 ng/mL, 10 ng/mL, or 100 ng/mL; PeproTech). Fresh medium was added once a week. Four weeks later, the CD4^+ T cells were collected and analyzed by flow cytometry.

2-11, Long-term culture of SP, BM and LP CD4^+ T cells from colitic SCID and normal Balb/c mice with IL-7. SP, BM and LP CD4^+ T cells isolated from colitic SCID mice previously injected with CD4^+CD45RB^{high} T cells and from normal mice using the anti-CD4 (L3T4)-MACS system were labeled with 2.5 μM CFSE and cultured at 1.5 x 10^5 cells/well in medium with or without human recombinant IL-7 (100 ng/mL), with fresh medium added once per week. Four weeks later, the CD4^+ T cells were collected and analyzed by flow cytometry.
2-12, Cytokine production by cultured and primary LP CD4⁺ T cells in response to intestinal bacterial antigens. Cecal bacterial antigens (CBA) were prepared from the cecums from colitic SCID mice injected with CD4⁺CD45RB<sup>high</sup> T cells as previously reported (9, 10). Each cecum was opened, placed in 1 ml PBS and vortexed. After addition of DNase (10 μg/ml), 1 ml of this bacterial suspension was added to 1 ml of glass beads. The cells were disrupted at 5,000 rpm in a Mini-Bead Beater (BioSpec Products, Bartlesville, OK) for 3 min and placed on ice. The glass beads and unlysed cells were removed by centrifugation at 8,000 rpm for 5 min. The lysates were filter-processed in a similar manner.

To prepare APCs, spleen cells (2 x 10⁷ cells/5 ml in a 15-ml tube) from normal BALB/c mice were incubated with appropriate concentrations of lysates overnight at 37°C, washed twice, treated with mitomycin-C, and added to T cell cultures. SP CD4⁺ T cells obtained from normal mice (Normal SP), LP CD4⁺ T cells freshly isolated from colitic CD4⁺CD45RB<sup>high</sup> T cell-transferred SCID mice (Primary-LP) and colitic LP CD4⁺ T cells cultured with IL-7 for 8 weeks (Cultured-LP) were cultured in the presence of APCs pretreated with CBA in complete media. The supernatants were collected 3 days later, and concentrations of IFN-γ, IL-17 and IL-2 were measured by ELISA.

2-13, Re-transfer of Cultured- and Primary-LP CD4⁺ T cells into new SCID mice. LP CD4⁺ T cells isolated from colitic SCID mice previously injected with CD4⁺CD45RB<sup>high</sup> T cells were cultured with 100 ng/ml IL-7 for 4 weeks, as above (Cultured-LP CD4⁺). 1 x 10⁵ of these Cultured-LP CD4⁺ T cells or the same number of LP CD4⁺ T cells freshly isolated from colitic SCID mice previously injected with CD4⁺CD45RB<sup>high</sup> T cells (Primary-LP CD4⁺) were injected intraperitonially into new...
SCID mice (n=5 each). The mice were monitored for up to 15 weeks, sacrificed and analyzed.

2-14, Co-transfer of Ly5.2\(^+\) cultured LP CD4\(^+\) T cells and Ly5.1\(^+\) primary LP CD4\(^+\) T cells into RAG-2\(^-\)/- mice. LP CD4\(^+\) T cells isolated from colitic RAG-2\(^-\)/- mice previously injected with Ly5.2\(^+\)CD4\(^+\)CD45RB\(^{\text{high}}\) T cells were cultured with 100 ng/ml IL-7 for 8 weeks (Ly5.2\(^+\)Cultured-LP CD4\(^+\)). 3 x 10\(^5\) Ly5.2\(^+\)Cultured-LP CD4\(^+\) T cells and the same number of LP CD4\(^+\) T cells freshly isolated from colitic RAG-2\(^-\)/- mice previously injected with Ly5.1\(^+\)CD4\(^+\)CD45RB\(^{\text{high}}\) T cells (Ly5.1\(^+\)Primary-LP CD4\(^+\)) were co-injected intraperitoneally into 8 RAG-2\(^-\)/- mice. The mice were monitored for up to 9 weeks, sacrificed and analyzed.

2-15, Re-transfer of 4 and 8 week-cultured LP CD4\(^+\) T cells into SCID mice. 3 x 10\(^5\) LP CD4\(^+\) T cells from colitic SCID mice cultured with 100 ng/ml IL-7 for 4 (cLP 4W; n=5) or 8 (cLP 8W; n=5) weeks were injected intraperitoneally into new SCID mice. The mice were monitored for up to 7 weeks, sacrificed and analyzed.

2-16, Statistical analysis. We examined the normality of the distribution of results in each group. If results from either group were not normally distributed, differences between two groups were analyzed using the Mann–Whitney \(U\)-test. If results from both groups were normally distributed, the variance of the population to which each group belonged was assessed using the \(F\)-test. When the populations were homoscedastic, differences between two groups were assessed using Student’s \(t\)-tests; otherwise, differences were assessed using Welch’s \(t\)-test. Statcel software was utilized for all statistical analyses. Results are expressed as mean ± SEM. Differences were considered significant when \(P<0.05\).
3. Results

3-1. Most CD4+ T cells in mouse models of chronic colitis are effector memory cells.

We first assessed the diversity of memory CD4+ T cells in several mouse IBD models. We observed large numbers of T_{EM} cells and small numbers of T_{CM} cells in the SP and MLN of SCID mice previously injected with CD4+CD45RB^{high} T cells (Supplemental Fig.1A). In contrast, large numbers of T_{CM}, T_{Naive} and T_{SCM} cells and small numbers of T_{EM} cells were observed in normal Balb/c mice. Surprisingly, there were no T_{SCM} in colitic SCID mice, although continuous colitis occurs in this model. Consistent with previous reports, only T_{EM} cells were present in the colonic LP of both colitic SCID and normal mice.

We also compared the expression of Bcl-2, an anti-apoptotic molecule and T_{SCM} marker, in T_{CM} and T_{EM}. SP T_{CM} expressed higher level of Bcl-2 than SP T_{EM}. LP T_{EM} also expressed a high level of Bcl-2, similar to that of SP T_{CM} (Supplemental Figure 1B).

3-2. T_{CM} and T_{EM} from colitic SCID mice injected with CD4+CD45RB^{high} T cells induce colitis to the same extent.

There were no T_{SCM} in colitic mice, but T_{CM} expressed higher level of Bcl-2 than T_{EM}, suggesting that T_{CM} have higher survival ability and are more important in chronic colitis than T_{EM}. We therefore compared the ability of T_{CM} and T_{EM} to induce colitis in SCID mice, by transferring T_{CM} and T_{EM} from SP and MLN of colitic into new SCID mice and comparing the severity of colitis (Figure 1A). Both T_{CM} and T_{EM} from colitic SCID mice could induce colitis, with no difference in severity, including clinical symptoms (Figure 1B, C), histological findings (Figure 1D, E), the number of CD3+CD4+ T cells infiltrating the colonic LP (Figure 1F) and the production of IFN-γ, TNF-α and IL-17 by LP CD4+ T cells (Figure 1G). Interestingly, following the
development of colitis, both transferred T\textsubscript{CM} and T\textsubscript{EM} cells differentiated to T\textsubscript{EM} cells in recipient mice (Figure 1H). Therefore, we concluded that hierarchy of memory T cells previously reported cannot apply to IBD model mice, and that T\textsubscript{EM} in colitic mice are not short-lived cells but include long-lived cells.

3-3, IL-7, but not IL-2 or IL-15, promotion of the \textit{in vitro} survival of LP CD4\textsuperscript{+} T cells from colitic SCID mice in a quiescent state.

We next attempted to purify long-lived colitic CD4\textsuperscript{+} T cells by culturing with IL-7, the most important factor for the maintenance of memory CD4\textsuperscript{+} T cells (11, 12). LP CD4\textsuperscript{+} T cells of colitic SCID mice previously injected with CD4\textsuperscript{+}CD45RB\textsuperscript{high} T cells were isolated and cultured in medium that included anti-CD3e antibody, IL-2, IL-15 or IL-7. IL-7 dose-dependently maintained colitic LP CD4\textsuperscript{+} T cells in culture for 4 weeks (Figure 2A). In contrast, these cells were unable to survive in medium containing anti-CD3e antibody, IL-2 or IL-15. Next, CD4\textsuperscript{+} T cells in the SP, BM and LP were isolated from these colitic SCID mice and from normal Balb/c mice and were cultured with IL-7. Interestingly, CD4\textsuperscript{+} T cells of normal Balb/c mice, which must include a substantial number of memory T cells, could not survive, even in the presence of IL-7, whereas CD4\textsuperscript{+} T cells of colitic mice survived for 4 weeks (Figure 2B), indicating that these cells were more sensitive to or dependent on IL-7 than normal CD4\textsuperscript{+} T cells. CFSE assays showed that colitic CD4\textsuperscript{+} T cells divided a maximum of five times in 4 weeks (Figure 2C), indicating that these cells were in a quiescent state and divided intermittently. Thus, colitic T\textsubscript{EM} are not solely short-lived, but may include long-lived cells.
3-4, LP CD4+ T cells express higher levels of Bcl-2, integrin-α4β7, CXCR3 and CD25 after than before culture.

We next examined the difference between freshly isolated colitic LP CD4+ T cells (Primary-LP CD4+) and those cultured with IL-7 for 4 weeks (Cultured-LP CD4+). Cultured-LP CD4+ expressed higher levels of Bcl-2 than Primary-LP CD4+, indicating that the former contains higher numbers of long-lived cells than the latter (Figure 3). The proportion of T_CM was higher in Cultured- than in Primary-LP CD4+ cells, with the former expressing higher levels of the T_SC_M marker CXCR3; integrin-α4β7, a homing receptor for intestines. CD25, the α-chain of the IL-2 receptor, was expressed by most Cultured- but by fewer Primary-LP CD4+ cells. In contrast, the levels of expression of the activation marker CD69 and of IL-7Rα were lower on Cultured- than on Primary-LP CD4+ cells. There was no difference in the expression of the stem cell markers c-kit and sca-1 (Figure 3). IL-7 dose dependently increased the proportion of dividing Cultured LP CD4+ cells and dose-dependently increased the expression by these cells of CD25 and Bcl-2, but had no effect on the proportion of T_C_M (Supplemental Figure 2).

3-5, Production of IL-2, IFN-γ and IL-17 by Cultured-LP CD4+ T cells by intestinal bacterial antigens.

We also assessed whether LP CD4+ T cells could produce cytokines in response to intestinal bacterial antigens. Cultured- and Primary-LP CD4+ T cells were co-cultured for 72 hours with antigen presenting cells previously pulsed with intestinal bacterial antigens, and the concentrations of IL-2, IFN-γ and IL-17 in the supernatant were analyzed by ELISA. Cultured-LP CD4+ T cells produced high concentrations of IL-2 and low concentrations of IFN-γ and IL-17, whereas Primary-LP CD4+ T cells produced low concentrations of IL-2 and high concentrations of IFN-γ and IL-17. This
result suggests that, during culture, LP CD4+ T cells did not lose their effector function against intestinal bacterial antigens. Second, their pattern of cytokine production was similar to that of TCM, although the pattern of their surface markers was similar to that of TEM (Figure 4).

3-6, Induction of chronic Th1/Th17-mediated colitis by Cultured- and Primary-LP CD4+ T cells in vivo.

To assess whether Cultured-LP CD4+ T cells could induce chronic colitis, 1 x 10^5 of Cultured- or Primary-LP CD4+ T cells were transferred into new SCID mice. SCID mice injected with Cultured-LP CD4+ T cells (Cultured-LP) showed apparent on-going clinical signs of colitis (Figure 5A), but their end-point clinical score was the same as that of mice injected with Primary-LP CD4+ T cells (Primary-LP) (Figure 5B). Both Cultured-LP and Primary-LP developed colitis in histological findings (Figure 5C). The histological score of the Cultured-LP was higher than that of the Primary-LP group (Figure 5D). The numbers of LP CD4+ T cells were similar in both groups (Figure 5E), and there were no differences between both groups in the concentrations of IFN-γ, IL-17 and TNF-α produced by LP CD4+ T cells (Figure 5F). Cultured-LP CD4+ T cells could induce chronic colitis to the same extent as primary LP CD4+ T cells. The SP, MLN and LP CD4+ T cells in each group were mostly CD44+CD62L+IL-7RαhighCD69+ TEM type cells (Supplemental Figure 3A), and the TCR Vβ repertoire of SP CD4+ T cells from both groups were similar (Supplemental Figure 3B).

3-7, Distribution of Ly5.1+ Primary-LP CD4+ T cells and Ly5.2+ Cultured-LP CD4+ T cells co-transferred into new RAG-2-/- mice.
Although the effector function of Cultured-LP CD4$^+$ T cells was less than that of Primary-LP CD4$^+$ T cells in vitro, they had similar colitogenic ability when transferred into new SCID mice (Figure 6). We hypothesized that, although Cultured-LP CD4$^+$ T cells are quiescent, they can differentiate into effector cells in vivo. We therefore co-transferred Ly5.1$^+$ Primary-LP CD4$^+$ T cells and Ly5.2$^+$ Cultured LP CD4$^+$ T cells into new RAG-2$^{-/-}$ mice and assessed the distribution of Ly5.1$^+/Ly5.2^+$ cells in each organ after the recipients developed colitis (Figure 6A). Fewer Ly5.2$^+$ Cultured- than Ly5.1$^+$ Primary-LP CD4$^+$ T cells were observed in the MLN and LP, whereas more Ly5.2$^+$ Cultured- than Ly5.1$^+$ Primary-LP CD4$^+$ T cells were present in the SP (Figure 6B). In addition, the proportion of IFN-γ$^+$ IL-17Th1 cells was lower in Ly5.2$^+$ Cultured- than in Ly5.1$^+$ Primary-LP CD4$^+$ T cells (Figure 6C). These results suggest that Cultured-LP CD4$^+$ T cells are more quiescent than Primary-LP CD4$^+$ T cells and tend to be preserved in the SP.

**3-8, LP CD4$^+$ T cells cultured for 8 weeks induced more severe colitis than LP CD4$^+$ T cells cultured for 4 weeks.**

Since Cultured-LP CD4$^+$ T cells include larger numbers of quiescent, long-lived memory cells than Primary-LP CD4$^+$ T cells, we hypothesized that, during culture, long-lived cells can be selected from primary T cells. We therefore compared the ability of LP CD4$^+$ T cells cultured for 4 and 8 weeks to induce colitis. LP CD4$^+$ T cells cultured with IL-7 for 4 (cLP 4W) and 8 (cLP 8W) weeks were transferred into new SCID mice. Four weeks after transfer, mice in the cLP 8W group lost weight, whereas mice in the cLP 4W group gained weight (Figure 7A). In addition, the clinical score of the cLP 8W group increased gradually 4 weeks after transfer (Figure 7B). The clinical and histological scores, as well as the numbers of LP CD4$^+$ T cells, were higher in the
cLP 8W than in the cLP 4W group (Figure 7C-E), with cells in the former group secreting higher concentrations of IFN-γ, IL-17 and TNF-α (Figure 7F). Most of the SP, MLN and LP CD4+ T cells in each group were CD44+CD62L-IL-7RαhighCD69+ T_EM cells (Supplement Figure 4A), with no between group differences in TCR Vβ repertoire of SP CD4+ T cells (Supplemental Figure 4B).

_3-9, LP CD4+CXCR3+CD25+ T cells in colitic mice showed similar feature to Cultured-LP CD4+

To exclude the potential artifact of _in vitro_ system, we checked whether there is a population similar to cultured-LP CD4+ T cells in vivo or not. As shown in figure 8, in the colitic mice, LP CD4+CXCR3+CD25+ cells expressed higher level of Bcl-2, integrin-α4β7 and IL-2 than the other subsets, which means that this subset is similar to cultured-LP CD4+ T cells (Figure 8). These results suggest that this subset will be novel and effective target of IBD.

_4, Discussion_

This study was performed to define a population of long-lived, highly proliferative memory T cells, similar to memory stem cells. In assessing the presence of T_SCM in colitic mice, we observed no CD4+CD44+CD62L+ T cells and no difference between T_CM and T_EM in the ability to induce colitis, indicating that the previously described hierarchy of memory T cells cannot be applied to this colitis model.

In colitic mice, most CD4+ T cells were T_EM. Moreover, most T_CM transferred into new SCID mice differentiated to T_EM. T_EM cells have been regarded as terminally differentiated short-lived cells. Most CD4+ T cells in the colonic LP were thought to be T_EM or effector T cells, both of which are short-lived and undergo apoptosis in the LP
without re-circulation. Furthermore, CD4\(^+\) T cells in the T cell transfer model are not regarded as memory cells, but as memory-phenotype cells (MP), a type of effector cell that differentiates during homeostatic expansion (11, 12).

We recently reported that LP CD4\(^+\) T cells in colitic SCID mice could re-circulate to the systemic blood circulation (13) and that colitic LP CD4\(^+\) T cells could induce colitis upon transfer into new SCID mice (3). We therefore hypothesized that colitic LP CD4\(^+\) T cells include long-lived memory cells. Although distinguishing memory from effector T cells based only on their surface markers is difficult, the most important difference between these cell types is their maintenance factors. Effector T cells cannot survive without cognate antigens, whereas memory T cells can survive in the presence of homeostatic cytokines, including IL-7 or IL-15, even without cognate antigens (14, 15).

We have shown here that colitic LP CD4\(^+\) T cells included a long-lived population, which could survive for more than 8 weeks \textit{in vitro} when cultured with IL-7. Although these cells responded to intestinal antigens, both \textit{in vitro} and \textit{in vivo}, they could survive in the absence of these antigens. During culture, they were quiescent, dividing a maximum of five times in 4 weeks, very similar to the homeostatic proliferation of memory T cells \textit{in vivo} (16). To our knowledge, this is the first evidence that colitic LP CD4\(^+\) T cells include long-lived “memory” T cells.

Although Primary-LP CD4\(^+\) T cells did not include any T\(_{CM}\), Cultured-LP CD4\(^+\) T cells included a small but substantial number of T\(_{CM}\), suggesting that T\(_{EM}\) can differentiate into T\(_{CM}\) and that the differentiation pathway from T\(_{CM}\) to T\(_{EM}\) is not always one way, but may be plastic. Although T\(_{CM}\) were shown to differentiate from T\(_{EM}\) or effector T cells (17, 18), we found that IL-7 may participate in this pathway.

Cultured-LP CD4\(^+\) cells expressed higher levels of Bcl-2 than Primary-LP CD4\(^+\) T cells, suggesting that the former are more long-lived. On the other hand, they didn’t express
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$T_{SCM}$ marker Sca-1 and c-kit, suggesting that this subset is different from memory stem cells previously reported (Figure 3).

Surface markers expressed on Cultured-LP CD4$^+$ cells were unique. They had features of both memory and effector. They expressed high levels of CD25, while they expressed low levels of CD69, both of which are effector marker. The expression of memory marker IL-7R $\alpha$ on Cultured-LP CD4$^+$ was lower than that on Primary-LP CD4$^+$ cells. This result is not inconsistent with our finding that Cultured-LP CD4$^+$ are long-lived memory cells, inasmuch as the expression of IL-7R$\alpha$ is reduced after interaction with a high concentration of IL-7. The reason why CD25 on Cultured-LP CD4$^+$ were dramatically higher than those on Primary-LP CD4$^+$ is not clear. CD25 is the alpha chain of IL-2 receptor, a molecule with multiple functions for both effector and regulatory T cells (19). Furthermore, IL-2/IL-2R signaling was shown to play an important role in the expression of IL-7R$\alpha$ on memory CD4$^+$ T cells (20, 21, 22). Additional investigations are needed to determine the function of CD25 on Cultured-LP CD4$^+$ T cells.

Cultured-LP CD4$^+$ expressed homing receptors for the intestines, such as integrin-$\alpha$4$\beta$7 and CD103, but expressed high levels of CXCR3, which is also expressed on $T_{SCM}$, suggesting that they have a potential to migrate both to intestine like $T_{EM}$ and another site (Figure 3). Actually, as shown in figure 6, a larger number of Cultured-LP CD4$^+$ were maintained in SP than Primary-LP CD4$^+$. Since Cultured-LP CD4$^+$ also expressed higher level of gut homing receptors, in the early time point, larger number of Cultured-LP CD4$^+$ might accumulate to LP than Primary-LP CD4$^+$. However, after the development of colitis, in the effector site, LP, Primary-LP CD4$^+$ have already expanded dramatically, while Cultured-LP CD4$^+$ are quiescent and they have not
expanded as same as Primary-LP CD4+, which can explain the smaller number of Cultured-LP CD4+ in LP.

In addition, they secreted a large amount of IL-2, a small amount of TNF-α and IFN-γ in response to intestinal antigens, which was very similar of T_{CM} cells (Figure 4).

In summary, Cultured-LP CD4+ T cells were Bcl-2^{high} long-lived cells, which can be defined as CD44^{+}CD62L^{−}CD25^{−} integrin-α 4 β 7^{high}CXCR3^{high}. They had potential to migrate to both intestine and spleen. Their production of cytokines was similar to T_{CM}. Thus, Cultured-LP CD4+ T cells were different from any memory subsets previously reported, so we concluded that they were novel and unique subset.

Upon co-transfer with Primary LP CD4+ T cells, their proliferation activity was lower. When each was transferred alone, however, their effector function and induction of colitis were similar. These findings suggested that Cultured-LP CD4+ cells include a larger number of long-lived memory cells, which were selected during the culture period, than Primary-LP CD4+ T cells, and that fewer Cultured-LP CD4+ T cells were required to induce colitis. Indeed, we found that LP CD4+ T cells cultured for 8 weeks induced more severe colitis than cells cultured for 4 weeks.

Although several papers showed that primary CD4+ T cells could be cultured in the absence of TCR stimulation up to 2 weeks (23, 24), we here for the first time showed that IL-7 could promote more survival of memory CD4+ T cells more than 8 weeks.

Tumor-protective memory CD8+ T cells could be maintained, in vitro without TCR stimulation, but in the presence of IL-2 and IL-7, for more than 6 weeks (25). Thus, the maintenance systems for CD4+ and CD8+ memory T cells differ. Indeed, we found that CD4+ memory T cells divided a maximum of five times in 4 weeks, consistent with results showing that memory CD8+ T cells divided about once a week after the
disappearance of cognate antigens \textit{in vivo} (16). These findings emphasize the novelty and uniqueness of our CD4$^{+}$ memory T cell culture system.

Our finding, that CD4$^{+}$ T cells isolated from normal Balb/c mice could not be cultured by this method, suggests that memory CD4$^{+}$ T cells from colitic mice previously injected with CD4$^{+}$CD45RB$^{\text{high}}$ T cells may be more sensitive or dependent on IL-7. Importantly, similar subset, CD4$^{+}$CD25$^{\text{high}}$CXCR3$^{\text{high}}$integrin-\(\alpha_4\beta_7\)high Bel-2$^{\text{high}}$IL-2$^{\text{high}}$ cells existed in LP of colitic mice, which suggests that this subset is not an artificial one induced by in vitro system, but a physiological subset.

In summary, we developed a novel culture system to purify a long-lived, highly pathogenic memory subset from activated LP CD4$^{+}$ T cells. IL-7 promoted long-term in vitro survival of this subset in a quiescent state. This subset will be a novel, effective target for the treatment of IBD.

**Competing Interests**

There are no conflicts of interest.

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References


Figure Legends

Figure 1

CD4+ T_{CM} and CD4+ T_{EM} from colitic SCID mice previously injected with CD4+CD45RB^{high}T cells induced colitis. (A) Experimental design. SP CD3+CD4+CD44+CD62L+ (SP T_{CM}), SP CD3+CD4+CD44+CD62L- (SP T_{EM}), MLN...
CD3⁺CD4⁺CD44⁺CD62L⁺ (MLN T_CM) and MLN CD3⁺CD4⁺CD44⁺CD62L⁻ (MLN T_EM) cells were sorted from colitic SCID mice previously injected with CD4⁺CD45RB^{high} T cells and injected into new SCID mice (n=4 per group). The mice were monitored for up to 16 weeks, sacrificed and analyzed. (B) Gross appearance of the colon, SP and MLN 16 weeks after transfer. (C) Clinical scores. (D) Histopathology of the distal colon. (E) Histological scores. (F) Numbers of CD4⁺CD3⁺ cells infiltrating the colonic LP. (G) Cytokine production by LP CD4⁺ T cells. (H) Expression of CD44 and CD62L on CD3⁻CD4⁺ T cells in the SP, MLN and LP. The graph on the right shows the percentages of T_EM and T_CM cells in SP, MLN and LP CD3⁺CD4⁺ cells. Pictures and dot plots of flow cytometry show representative mice in each group. All data are reported as the mean ± SEM. N.S., not significant. *p<0.01.

Figure 2

IL-7, but not IL-2 or IL-15, promotes the in vitro survival of LP CD4⁺ T cells from colitic SCID mice previously injected with CD4⁺CD45RB^{high} T cells. (A) CFSE-labeled LP CD4⁺ T cells obtained from colitic SCID mice previously injected with CD4⁺CD45RB^{high} T cells were cultured in medium containing anti-CD3e antibody, IL-2, IL-15 or IL-7. Four weeks later, LP CD4⁺ T cells were analyzed by flow cytometry and the numbers of CD3⁺CD4⁺ T cells and percentages of CFSE^{Low} dividing cells were calculated. (B) SP, BM and LP CD4⁺ T cells of colitic and/or normal mice were cultured in the presence or absence of IL-7. Four weeks later, LP CD4⁺ T cells were analyzed by flow cytometry and the numbers of CD3⁺CD4⁺ T cells and percentages of CFSE^{Low} dividing cells were calculated. (C) CFSE-intensity on colitic SP, BM and LP CD4⁺ T cells cultured with IL-7 for 4 weeks. Histogram of flow cytometry shows
representative samples from each group. All data are shown as the mean ± SEM for 3 samples per group. N.S., not significant. *p<0.01.

Figure 3

Cultured-LP CD4+ T cells express higher levels of Bcl-2, integrin-α4β7, CXCR3 and CD25 than Primary-LP CD4+ T cells. Expression of various surface markers on Cultured- and Primary-LP CD4+ T cells. Histograms or dot plots of flow cytometry show a representative sample from each group. Numerical values are shown as the mean for 3 samples per group.

Figure 4

Cultured-LP CD4+ T cells secrete high concentrations of IL-2 and low concentrations of IFN-γ and TNF-α in response to intestinal bacterial antigens in vitro. Cultured- and Primary-LP CD4+ T cells were each co-cultured for 3 days with antigen presenting cells previously pulsed with CBA, and the concentrations of IFN-γ, IL-17 and IL-2 in the supernatant were analyzed by ELISA. The negative control consisted of SP CD4+ T cells from normal Balb/c mice. Data are expressed as the mean ± SEM of 3 samples per group.

Figure 5

Cultured- and Primary-LP CD4+ T cells induce chronic Th1/Th17-mediated colitis to the same extent. 1 x 10^5 of Cultured- or Primary-LP CD4+ T cells were transferred into new SCID mice (n=5 per group). The mice were monitored for up to 11 weeks, sacrificed and analyzed. (A) Change in on-going colitis scores. (B) End point-clinical
scores. (C) Histopathology of the distal colon. Original magnification: ×100. (D) Histological scores. (E) Numbers of LP CD4⁺CD3⁺ cells. (F) Intracellular staining of IFN-γ and IL-17 in LP CD4⁺ T cells. Numerical values are shown as the mean for 5 samples per group. Pictures and dot plots of flow cytometry of representative samples from each group. All data are shown as the mean ± SEM for 5 mice per group. Data are representative of three independent experiments. N.S., not significant. *p<0.01.

Figure 6

Distribution of Ly5.1⁺Primary- and Ly5.2⁺Cultured-LP CD4⁺ T cells in the SP, MLN and LP after co-transfer into new RAG-2⁻ mice. (A) Experimental design. The same numbers of Ly5.1⁺ Primary- and Ly5.2⁺ Cultured-LP CD4⁺ T cells were co-injected intraperitoneally into new RAG-2⁻ mice (n=8 per group). The mice were monitored for up to 9 weeks, sacrificed and analyzed. (B) Ratio of Ly5.1⁺ to Ly5.2⁺ CD3⁺CD4⁺ T cells in the SP, MLN and LP of RAG-2⁻ recipients 9 weeks after transfer. (C) Intracellular staining of IFN-γ and IL-17 in Ly5.1⁺ and Ly5.2⁺ LP CD4⁺ T cells in RAG-2⁻ recipients. Numerical values are shown as the mean for 8 mice per group. Graphs on the right show the percentages of Ly5.1⁺ and Ly5.2⁺ LP CD4⁺ T cells that were IFN-γ⁺IL-17⁻ and IFN-γ⁺IL-17⁺. All data are shown as the mean ± SEM for 8 mice per group. N.S., not significant. *p < 0.01.

Figure 7

LP CD4⁺ T cells cultured for 8 weeks induced more severe colitis than LP CD4⁺ T cells cultured for 4 weeks. LP CD4⁺ T cells obtained from colitic SCID mice and cultured with IL-7 for 4 (cLP 4W) or 8 (cLP 8W) weeks were transferred into new
SCID mice (n=5 per group). Seven weeks later, the mice were sacrificed and analyzed. (A) Changes in body weight over time, expressed as the percentage of initial body weight. (B) Changes in on-going colitis score. (C) Gross appearance of the colon, spleen and mesenteric lymph nodes. (D) Clinical scores. (E) Histopathology of the distal colon. Original magnification: ×100. (F) Histological scores. (G) Numbers of SP, MLN and LP CD4⁺CD3⁺ cells. (H) Cytokine production by LP CD4⁺ T cells. Pictures show a representative mouse from each group. All data are shown as the mean ± SEM for 5 mice per group. N.S., not significant. *p<0.01.

**Figure 8**

LP CD4⁺CXCR3⁺CD25⁺ T cells in colitic mice showed similar feature to Cultured-LP CD4⁺

LP cells from colitic SCID mice previously transferred with CD4⁺CD45RB³ T cells were obtained and analyzed. All samples are gated as CD3⁺CD4⁺. Histograms or dot plots of flow cytometry show a representative sample from each group. Numerical values are shown as the mean for 3 samples per group.
Figure 1

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Figure 2

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Revised Figure 3
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Fig. 3
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Figure 4
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Figure 5

A. On-going Colitis Score

B. Clinical Score

C. Cultured-LP vs Primary-LP

D. Histological Score

E. Number of LP CD4+CD8− (×10^4)

F. IL-17 and IFN-γ

N.S. indicates no significant difference.

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Figure 6
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Figure 7
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