assays, particularly for the insertion element 900 (IS900; highly specific for MAP), allowed the identification and/or isolation of MAP from tissues in patients with CD.[9–13] IS900 is one of the insertion elements in MAP,[14] was identified in the clinically isolated bacterial DNAs from human CD. IS900 consists of 1451 bp nucleotides and has a single open reading frame (ORF1197) encoding, predictive of 399 amino acids.

Even if the existence of MAP in intestinal tissue were proven, it remains unclear whether this organism is involved in the development of CD. Although antibodies against MAP in the serum of patients with CD have also been investigated, the results have been controversial. This is also true of the bacteria themselves, probably because of their poor specificity. Therefore, it is important in the investigation of specific immune response to use and find the specific antigen.

The aim of this study is to investigate whether MAP is involved in the pathogenesis of CD using the recombinant glutathione S-transferase (GST) fusion recombinant protein encoding portion of IS 900. We investigated the correlation between antibodies against this GST fusion protein (anti-IS900) and clinical characteristics including anti-*Saccharomyces cerevisiae* (ASCAs; Baker's yeast) antibodies, which are serologic markers associated with CD.

MATERIALS AND METHODS

Included in the study were 154 participants. Serum samples were obtained from 20 patients with CD (n = 50), ulcerative colitis (UC; n = 40), colonic tuberculosis (TB; n = 20), and non-IBD control (n = 20), in the Department of Gastroenterology, Kyoto University, and its related hospitals. The entire experimental design of this study was approved by the Kyoto University Hospital Ethics Committee. The patients' profile is shown in Table 1.

TABLE 1. Characteristics of 154 Patients According to Study Group

Characteristics	CD	UC	TB	Control
N	50	40	20	44
Mean age	35 ± 7	30 ± 8	55 ± 10	40 ± 7
Sex (M/F)	27/23	23/17	15/5	24/20
CDAI				
Mean range	212			
Range	22–320			
Involvement site				
Small bowel	13			
Small and large bowel	22		3	
Large bowel	15	20	17	
Corticosteroids	10	10		
Immunosuppressants	14	10		

Data expressed as mean ± SE.

Bacterial Strains

DNA extraction

MAP was purchased from Kaketsuken Institute (Kumamoto, Japan) with the permission of the Japanese Association of Animal Bacteriology. The method by which DNA was isolated from MAP was as follows[15]: an aliquot of freeze-dried culture was resuspended in 1 mL Tris-EDTA-sodium dodecyl sulfate buffer (containing 10 mmol/L Tris-HCl, 1 mmol/L EDTA, 0.5% SDS). The suspension was boiled for 20 minutes. After centrifugation at 17,000*g* for 5 minutes, the supernatant was subjected to 2 cycles of phenol-chloroform extraction. DNA was then recovered by ethanol precipitation with 3 mol/L sodium acetate for 1 hour at −80°C, pelleted by centrifugation at 14,500*g* for 30 min, washed in 70% ethanol, dried, and resuspended in 40 μL of TE-RNase buffer (10 mmol/L Tris-Tris-HCl, 1 mmol/L EDTA, and 2.5 μg/mL RNase).

Polymerase chain reaction

Oligonucleotide polymerase chain reaction (PCR) primers A (5′-CGCGGATCCGAGTTGATTGCGGCGGTGACGAC) and B (5′-CGGAATCCGCGAGGACGGCTTGGGTGTGG) region of MAP were selected. This amplified DNA contains the sequence of IS900 derived from the MAP genome, which yielded a 990-bp product.

Construction of plasmids

A 990-bp PCR amplified DNA sample was inserted into the pGEX-4T-2 vector, which can maintain the GST reading frame. Then, competent *Escherichia coli* cells (JM109) were transformed by the ligation reaction and a fusion protein was expressed by adding 0.1 mmol/L isopropyl β-D-thiogalactoside. A fusion protein was purified from the bacterial lysates by affinity chromatography using glutathione-Sepharose 4B, and eluted in glutathione elution buffer containing 10 mmol/L reduced glutathione in 50 mmol/L Tris/HCl (pH 8.0). Expression of the fusion protein was screened by both sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Enzyme-linked immunosorbent assay

Microtiter plates (Nunc-Immuno™ Module, F8/MAXI SORP Nunc A/S, Roskilde, Denmark) were coated with IS900-GST or control GST (1 μg/well in 50 μL of phosphate-buffered saline [PBS]) for 24 hours at 4°C and were rinsed 3 times with a washing solution (0.05% Tween 20 in PBS). Plates were then incubated for 1 hour at room temperature with 5% dried skim milk in PBS to block nonspecific sites. Human sera were tested in duplicates at varying dilutions for 2 hours at 37°C and unbound serum components were removed by a washing step. The bound antibodies then specifically reacted for 1 hour with 1:1000 dilution of peroxidase-conjugated goat antibody reactive with human immunoglobulin G (IgG) or IgA (Caltag, Burlingame, Calif.) was added and the mixture

was incubated for 1 hour at 37°C. After washing 3 times with PBS containing 0.02% Tween 80, bound reactants were developed with 100 μL of 0.06 mg/mL tetramethylbenzidine in 0.05 mmol/L citrate buffer with 0.03% hydrogen peroxide at room temperature for 20 minutes. The reaction was then stopped by the addition of 100 μL of 10% H_2SO_4. The absorbance was determined at 450 nm (A450). Optical density (OD) values of nonspecific binding sera to GST alone were subtracted from raw values of IS900-GST binding to obtain specific absorbance.

ASCAs

Serum levels of ASCA were quantitated using a commercial ELISA kit (Medipan Diagnostica, Selchow, Germany). Diluted patient and control samples were reacted with mannan immobilized on the solid phase of a microtiter plate. Serum was diluted in a ratio of 1:50 and added to the microtiter plates. After an incubation period of 60 minutes at 37°C, unbound serum components were removed by a washing step. The bound antibodies then specifically reacted with anti-human IgG or IgA antibodies conjugated to horseradish peroxidase. An incubation period of 30 minutes at 37°C was followed by a washing step. The enzyme reaction was stopped by dispensing an acidic solution (H_2SO_4) into the wells after 10 minutes at room temperature, turning the solution from blue to yellow. Plates were read at 450 nm. To each microtiter plate, 1 ASCA-positive and 1 ASCA-negative control and 1 cutoff control (20 U/mL) were added, calculated from ROC curves. Qualitative evaluation of the results was assessed by calculating the binding index = OD (sample)/OD (cutoff control). ASCA IgG and IgA were considered positive at binding index >1.0.

Statistical Analysis

The data obtained from the ELISA were expressed as mean ± SE. Analysis of variance was used for comparison of antibodies against IS900-GST between groups. Pearson's correlation coefficient analysis was used to examine the relationship between ASCA and antibodies against IS900-GST, a Crohn's Disease Activity Index (CDAI) score, and antibodies against IS900-GST. χ^2 tests were used to compare categorical variables between groups. A 2-tailed *P* value of <0.05 was used to indicate statistical significance.

RESULTS

Expression of a Fusion Protein of the Positive Clone With the GST Gene Fusion System

A recombinant GST fusion protein, IS900 peptide, containing 330 amino acids was constructed. SDS-PAGE showed a single purified band of 60 kDa consisting of a fusion IS900 protein of GST (Fig. 1). Before performing ELISA, amino acid sequence of fusion IS900-GST was analyzed and confirmed to be the same as the predicted sequence (data not shown).

Antibodies to the IS900 Recombinant Protein

A recombinant fusion IS900-GST was incorporated into an ELISA analysis of patient sera. The mean OD values of both IgG and IgA antibodies against IS900 (anti-IS900) in patients with CD (0.697 ± 0.355 and 0.354 ± 0.170, respectively) were significantly higher than those with UC (0.342 ± 0.116 and 0.200 ± 0.078, respectively), TB (0.395 ± 0.171 and 0.239 ± 0.120, respectively), or controls (0.371± 0.086 and 0.249 ± 0.065, respectively). These values were subtracted from values to control GST for obtaining the graphed values. In the present study, positive results of IgG and IgA anti-S900 were arbitrarily defined as absorbance values higher than 0.543 and 0.379, respectively, which correspond to a value that is the mean + 2 SD of normal control values. Positive results of IgG and IgA anti-IS900 were obtained in 24 of 50 (48%) and 22 of 50 (44%) patients with CD. Also, positive results of IgG (4/20, 20%) and IgA (4/20, 20%) anti-IS900 were obtained in patients with TB. No positive results were obtained in patients with UC and normal controls (Fig. 2, A and B).

Correlation Between Serum Levels of Anti-IS900 and Disease Activity

We determined whether the serum levels of anti -IS900 were involved with disease activity. As shown in Figure 3A

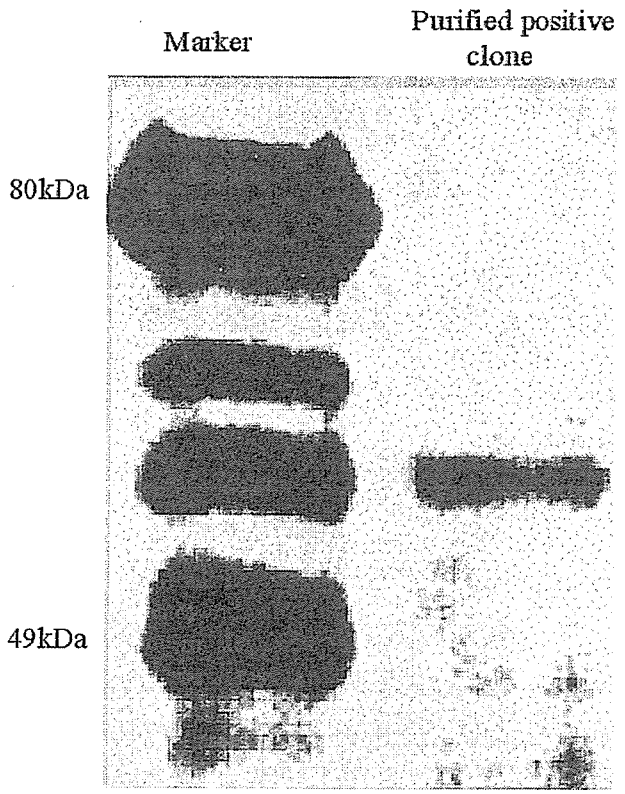


FIGURE 1. SDS/PAGE of the positive clone expressed as a fusion protein with GST.

and B, no significant correlation was observed between CDAI and the IgG and IgA anti-IS900s in patients with CD.

Relationship Between Serum Levels of Anti-IS900 and Clinical Features in CD Patients

We investigated whether a clinical phenotype was associated with the serum levels of anti-IS900. As shown in Figure 4, the serum levels of IgG and IgA anti-IS900 in CD patients with small intestinal involvement (0.75 ± 0.381 and 0.383 ± 0.175, respectively) tended to be higher than in patients with CD limited to the colon (0.574 ± 0.2560, 286 ± 0.140, respectively), although there was no significant difference among these groups. On disease behavior, serum levels of anti-IS900 in patients with penetrating- and stricture-type CD are significantly higher than those with the inflammatory type (Fig. 5). Also, a negative correlation was found between serum levels of IgG anti-IS900 and disease duration (Fig. 6). Furthermore, serum levels of IgG anti-IS900 were not associated with the use of immunosuppressants, nor were they associated with surgical treatment (Fig. 7).

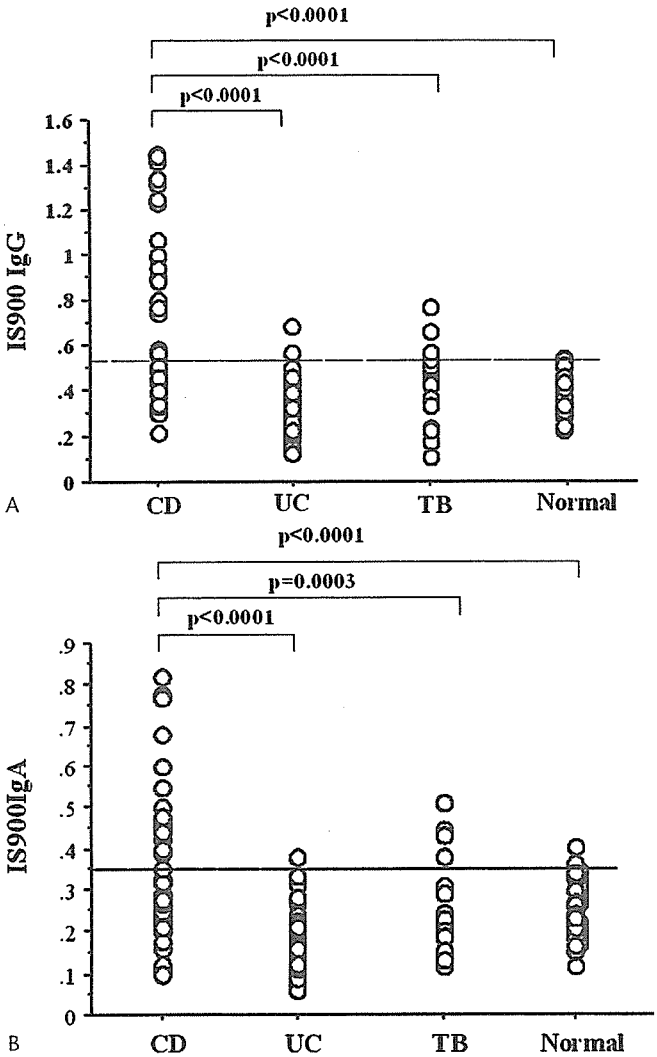


FIGURE 2. Scatterplots showing IgG (A) and IgA (B) against IS900-GST values as determined by ELISA in patients with CD vs those with UC, colonic TB, and normal controls. IS900-GST ELISA wells were reacted with human sera at 1:50 dilution and detected with antihuman IgG and IgA antibodies. Dotted line indicates a cutoff value (cutoff value = mean ± 2 SD for the normal group).

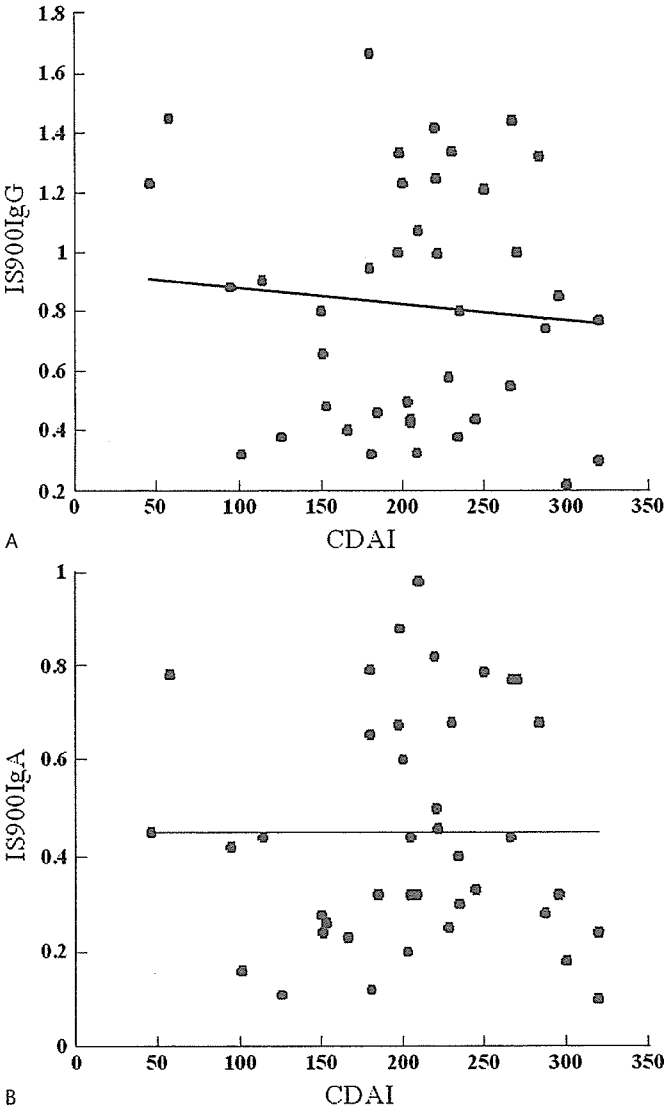


FIGURE 3. Correlation between antibody responses against IS900-GST protein and CDAI in the (A) CD IgG against IS900-GST protein and (B) CDAI (R^2 = 0.001, P = 0.8342) IgA against IS900-GST protein and CDAI (R^2 = 0.003, P = 0.7893). There was no significant correlation between the seroactivity against GST-IS 900 and against CDAI.

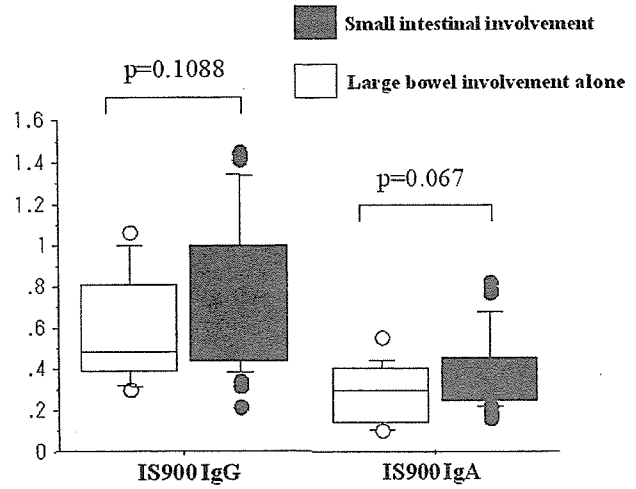


FIGURE 4. Association between IgG or IgA against IS900 and disease sites. Both IgG and IgA against IS900 tended to be higher in CD patients involved with small bowel than those with large bowel involvement.

Correlation Between ASCA and Antibodies to IS900-GST

Figure 8 shows that no significant correlation was observed between the serum levels of IgG anti-IS900 and ASCA in CD patients.

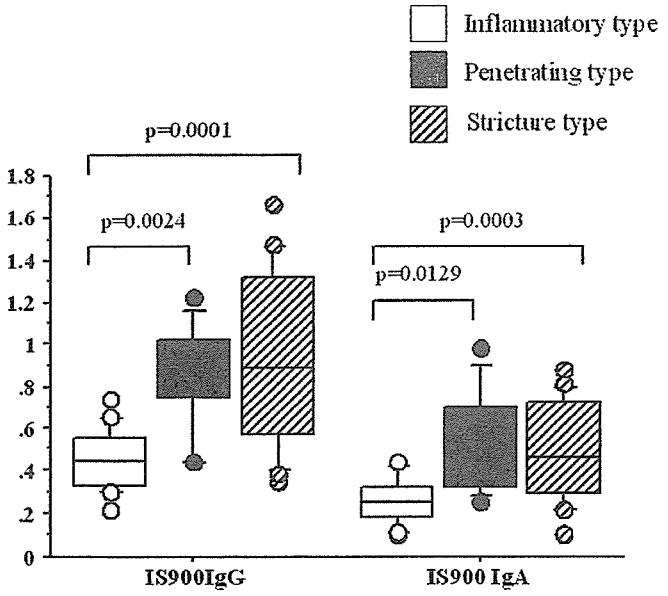


FIGURE 5. Phenotypic association with IgG or IgA against IS900. Both IgG and IgA against IS900 were significantly higher in patients with penetrating- or stricture-type CD than those with inflammatory-type CD.

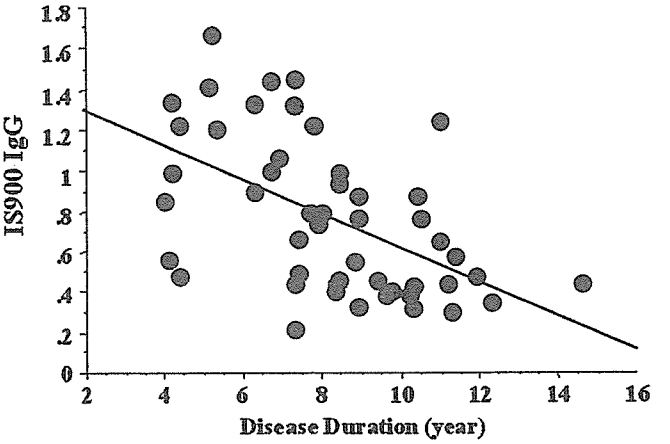


FIGURE 6. Negative correlation was observed between IgG against IS900-GST and disease duration ($Y = 1.459–0.084X$, $R^2 = 0.285$, $P < 0.0001$).

DISCUSSION

CD is a chronic IBD characterized by transmural inflammation and granuloma formation. Several bacteria have been proposed in the etiology of CD. Among them, infection with MAP, which causes a similar disease in animals and is present in the human food chain, has been focused on because of more frequent identification of MAP by both PCR and in situ hybridization in the intestinal tissue of patients with CD compared with that of healthy people.[16–19] In particular, the discovery of IS900, which is a specific site for MAP, has enabled the differentiation of MAP from other mycobacteria. However, the identification of IS900 PCR products in tissues from healthy controls, even with a small prevalence rate, suggests that infectivity of this organism in human is extremely rare. Therefore, the pathogenic roles of MAP infection in CD have been controversial.[20,21]

Serologic tests in patients with CD are essential for detecting the presence of abnormal antibodies against self or nonself proteins, because these are not present in healthy people and their presence reflects subclinical immune dysregulation. Mycobacteria usually cross-react with one another or other acid-fast organisms. Moreover, patients with CD have been reported to demonstrate various antibodies against intestinal bacterial and food antigens.[22–24] Therefore, demonstration of more specific immune responses against MAP could help clarify the involvement of this organism in the pathophysiology of CD. Recently, by analysis with a genomic library constructed using MAP chromosomal DNA in an expression vector, 2 putative clones encoding MAP-specific antigens 35K (p35) and 36K (p36) were identified and their seroactivity in patients with CD was evaluated by immunoblotting. The results showed that 77% of 61 sera in patients with CD reacted with both antigens compared with 8% of 12 sera in those with UC; 0 of 35 sera

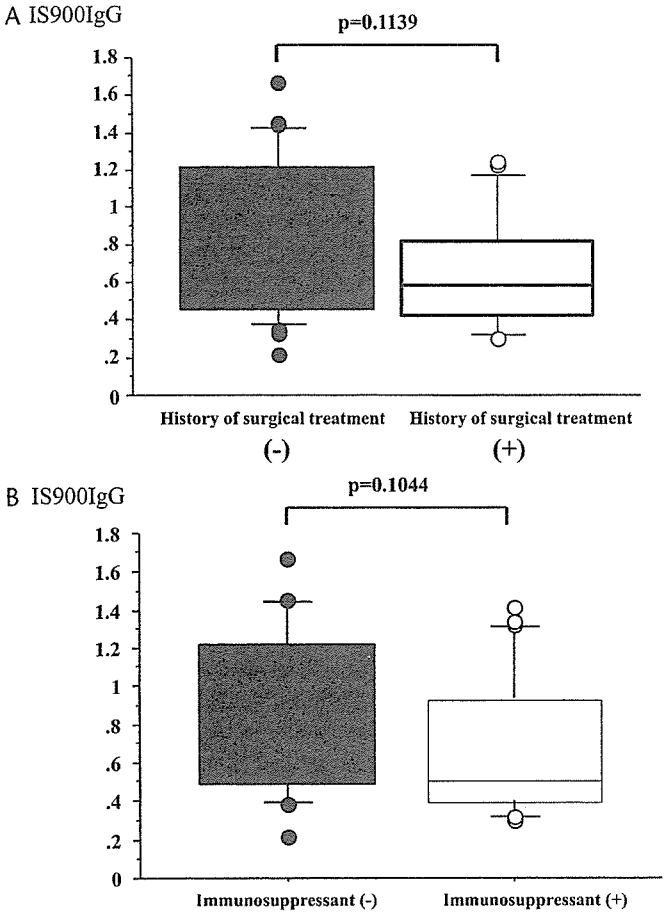


FIGURE 7. The association between clinical features and IgG against IS900. A, History of surgical treatment. B, Use of immunosuppressant. The serum level of IgG against IS900 was not associated with history of surgery or immunosuppressant use.

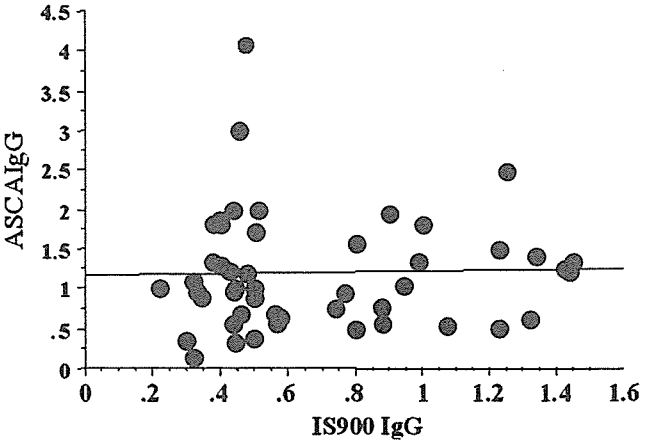


FIGURE 8. Levels of IgG against IS900-GST is independent of ASCA ($Y = 1.161 + 0.054X$; $R^2 = 0.001$).

from normal controls reacted.[25] Sunenaga et al reported that patients with CD have a higher titer of antibodies against the protoplasmic antigen of MAP than that in patients with UC and control patients using a commercial ELISA kit for the serological diagnosis of Johne's disease in cattle (Johnelisa Kit, Kyoritsu Seiyaku Co., Tokyo, Japan).[26] There are several reports showing the negative correlation between MAP and CD in the serum levels of antibodies against MAP antigen. Brunello et al reported no significant difference of positive values of IgG antibodies against MAP protoplasmic antigen between patients with CD (3.7%) and those with UC (5%).[27] Walmsley et al reported no statistical elevation of IgG or IgA antibodies against 18 kDa protease-resistant antigen corresponding to MAP bacterioferritin of patients with CD over normal controls.[28] Bernstein et al also reported that there was no difference in MAP seropositivity rate by ELISA among CD patients (37.8%), UC patients (34.7%), and healthy control (33.6%).[29] Thus, regarding seropositivity against MAP, the possibility of an association between MAP and CD remains inconclusive. In the present study, we investigated anti-MAP antibodies using more specific antigens encoding IS900. Our results revealed that the serum level of IgG and IgA anti-IS900 in patients with CD was significantly higher than those in patients with UC, colonic TB, and normal controls. These results suggest that this organism could be involved in the pathophysiology of CD as well as previous reports. Moreover, the positive results of serum levels of anti-IS900 in patients with colonic TB tend to be higher than normal controls. It was reported that the serum IgG level in CD patients was raised in response not only to MAP but also to other mycobacteria strains. This may mean a high degree of mimicry between MAP and other mycobacteria strains in their antigenic determinants. However, in this study, there should be little possibility that higher positive results of anti-IS900 in patients with colonic TB is caused by antigenic mimicry because IS900 sequence is considered the gold standard in differentiating other types of mycobacteria. Therefore, the reason for this finding is unclear at present. In this regard, we should examine greater number of patients with colonic TB to confirm these results and investigate the reason for the higher tendency of serum levels of anti-IS900 in patients with colonic TB.

We investigated the relationship between the disease site and the serum levels of anti-IS900. CD patients with small intestinal involvement have higher levels of IgG and IgA anti-IS900 compared with those limited to the colon. In the previous serological study with ASCA, CD patients with small intestinal involvement (including both small and large bowel involvement) tended to have higher IgG and IgA ASCA compared with those limited to the colon.[30] In general, CD patients have more enhanced mucosal permeability and abnormal epithelial barrier function.[1,31] In addition, when CD patients have small intestinal lesions, including short bowel syndrome, stenosis, intestinal permeability, and barrier function worsen compared with patients who have colonic lesions alone because of a loss of protein and an inability to

absorb, following malnutrition. This means that in CD patients with small intestinal involvement, various bacteria easily both invade the alimentary tract and encounter immune-regulating cells such as dendritic cells, macrophages, and lymphocytes. These conditions may elicit unusual immune responses, which result in the induction of higher levels of antibodies against specific pathogens. In this study, no significant association between the serum level of anti-IS900 and disease activity was observed, whereas the serum level of anti-IS900 in patients with inflammatory-type CD is significantly lower than that in patients with penetrating- and stricture-type CD. These findings suggest that MAP may not always elicit strong immune responses in the human intestine even if it is infected, but chronic MAP infection interferes with the intestinal immune system, which affect the development of disease behavior such as penetrating or stricture type.

Furthermore, negative correlation was found between anti-IS900 and disease duration. Similar findings have been already reported. Kreuzpainterner et al speculated on such a decline in antimycobacterial antibodies after surgery concurrent with a reduced activity of disease.[32] Olsen et al also discussed lower antibody levels in CD patients with long-term disease caused by lower disease activity[33]; however, our present results reveal that there is no correlation between serum levels of anti-IS900 and CDAI, and serum levels of IgG anti-IS900 were not associated with a history of surgical treatment and the current use of immunosuppressants. Therefore, the reason for the negative correlation between anti-IS900 and disease duration is still unclear. One possible explanation is that MAP may also be eliminated naturally along with other intestinal bacteria after it infected an already-diseased intestinal tract, although it is still unclear how long elimination may take because of its slow growth.

The results of this study also revealed no correlation between ASCA and antibodies against IS900-GST, which suggests that the antigenic property of MAP is different from *S cerevisiae* and may be involved in the pathophysiology of CD.

Recently, it was reported that the insertion mutation in the *NOD2* gene has been associated with CD.[34,35]*NOD2*, a gene that encodes a protein with homology to plant disease–resistance gene product, is located in the peak region of linkage on chromosome 16, which is restricted to monocytes and activates NF-κB. The leucine-rich repeats (LRRs) domain of *NOD2* has binding activity for bacterial lipopolysaccharides and its deletion stimulates the NF-κB pathway.[36] The reason why a mutation of LRRs in *NOD2* may develop CD is considered to be altering intracellular recognition of microbial components, although it remains controversial whether its mutation activates NF-κB. *Mycobacterium* species bind to both Toll-like receptors 2 and 4.[37] Particularly, Toll-like receptor 4, a member of a family of NF-κB activators, is known to bind lipopolysaccharides through its LRR domain.[38] If MAP is involved in the development of CD, mutation of LRRs in *NOD2* may induce susceptibility to MAP with changes in the intracellular recognition of mycobacterial components, followed by noncaseous granuloma, which was thought to be involved in IS900 PCR products in macrophages in patients with CD.[16] Moreover, several reports of antimycobacterial therapy showed the effect on patients with CD, which may support the involvement of MAP in the pathophysiology of CD.[39–41] Considering that common variants in *NOD2* were not found in Japanese CD patients,[42] alternative signal transduction may be involved in the pathophysiology of Japanese CD patients.

It was reported that patients with CD express antibody responses to several bacterial species, which represent mucosal disruption and intensified microbial exposure, leading to a diversely targeted antimicrobial immune response.[20–24] Therefore, the seroactivity against IS900 may represent the results of secondary microbial exposure. Judging from the pathogenesis of CD associated with abnormal responsiveness to microbial components,[34,35] however, we consider that chronic intracellular infection by bacteria including MAP may trigger a T_H1-driven immune response that results in the clinical, histological manifestation of CD. In addition, Naser et al reported viable MAP in peripheral blood in a higher population of patients with CD than in controls.[43] These data also support the notion that MAP could invade intestinal mucosa with a generalized defect of barrier function in patients with CD.

In conclusion, our study demonstrated high titer against IS900 in patients with CD compared with those with UC, colonic TB, and normal controls. These results may support the theory that mycobacteria are the cause of CD.

REFERENCES

1. Sartor RB. Current concept of the etiology and pathogenesis of ulcerative colitis and Crohn's disease. *Gastroenterol Clin North Am.* 1995;24:475–507.
2. Dalziel TK. Chronic intestinal enteritis. *BMJ.* 1913;ii:1068–1070.
3. Crohn BB, Ginzburg L, Oppenheim GD. Regional ileitis, a pathologic and clinical entity. *JAMA.* 1932;99:1323–1329.
4. Burnham WR, Leunard-Jones JE, Stanford JL, et al. Mycobacteria as a possible cause of inflammatory bowel disease. *Lancet.* 1978;2:693–696.
5. Chiodini RJ, Vankruiningen HJ, Merkal RS. Ruminant paratuberculosis (Johne's disease): the current status and future prospects. *Cornell Vet.* 1984;74:218–262.
6. Chiodini RJ. Crohn's disease and the mycobacterioses. A review and comparison of two disease entities. *Clin Microbiol Rev.* 1989;2:90–117.
7. Chiodini RJ, Van Kruiningen HJ, Merkal RS, et al. Characteristics of an unclassified mycobacterial species isolated from Crohn's disease. *J Clin Microbiol.* 1984;20:966–971.
8. Mc Fadden JJ, Butcher PD, Chiodini R, et al. Crohn's disease-isolated mycobacteria are identical to *Mycobacterium paratuberculosis*, as determined by DNA probes that distinguish between mycobacterial species. *J Clin Microbiol.* 1987;25:796–801.
9. Haagama J, Mulder CJJ, Eger A, et al. Mycobacterium paratuberculosis isolated from patients with Crohn's disease in the Netherlands. *Scand J Gastroenterol.* 1989;24:S158–S168.
10. Gitnick G, Collins J, Beaman B, et al. Preliminary report on isolation of mycobacteria from patients with Crohn's disease. *Dig Dis Sci.* 1989;34:925–932.
11. Graham DY, Markesich DC, Yoshimura HH. Mycobacteria and inflammatory bowel disease. *Gastroenterology.* 1987;92:436–442.

12. Naser SA, Schwartz D, Shafran I. Isolation of *Mycobacterium avium* subsp. *paratuberculosis* from breast milk from Crohn's disease patients. *Am J Gastroenterol.* 2000;95:1094–1095.
13. Dell'Isola B, Poyart C, Goulet O, et al. Detective of mycobacterium paratuberculosis by polymerase chain reaction in children with Crohn's disease. *J Infect Dis.* 1994;169:449–451.
14. Green EP, Tizard MLV, Moss MT, et al. Sequence and characteristics of IS 900, an insertion element identified in human CD. *Nucleic Acids Res.* 1989;17:9063–9073.
15. Moss MT, Sanderson JD, Tizard ML, et al. Polymerase chain reaction detection of *M. paratuberculosis* and *M. avium* subsp *silvaticum* in long term cultures from Crohn's disease and control tissues. *Gut.* 1992;33:1209–1213.
16. Hulten K, El-Zimaity HM, Karttunen TJ, et al. Detection of *Mycobacterium avium* subspecies *paratuberculosis* in Crohn's diseased tissues by in situ hybridization. *Am J Gastroenterol.* 2001;96:1529–1535.
17. Chamberlin W, Graham DY, Hulten K, et al. Review article: *Mycobacterium avium* subsp. *paratuberculosis* as one cause of Crohn's disease. *Aliment Pharmacol Ther.* 2001;15:337–346.
18. Sanderson JD, Moss MT, Tizard ML, et al. *Mycobacterium paratuberculosis* DNA in Crohn's disease tissue. *Gut.* 1992;33:890–896.
19. Mishina D, Katsel P, Brown ST, et al. On the etiology of Crohn's disease. *Proc Natl Acad Sci USA.* 1996;93:9816–9820.
20. Stainsky KJ, Lowes JR, Allan RN, et al. Antibodies to *Mycobacterium paratuberculosis* and nine species of environmental mycobacteria in CD and control subjects. *Gut.* 1993;34:371–374.
21. Cho SN, Brennan PJ, Yoshimura HH, et al. Mycobacterial aetiology of Crohn's disease: serological study using common mycobacterial antigens and a species-specific glycolipid antigen from *Mycobacterium paratuberculosis*. *Gut.* 1986;27:1353–1356.
22. Davidson IW, Lloyd RS, Whorwell PJ, et al. Antibodies to maize in patients with Crohn's disease, ulcerative colitis, and coeliac disease. *Clin Exp Immunol.* 1976;35:147–148.
23. Koninckx CR, Giliams JP, Polanio J, et al. IgA antigliadin antibodies in celiac and inflammatory bowel disease. *J Pediar Gastroenterol Nutr.* 1984;3:676–680.
24. Danielssen D, Kjellander J, Persson S, et al. Investigation of the immune response to aerobic and anaerobic intestinal bacteria in a patient with Crohn's disease. *Scand J Infect Dis.* 1979;19:52–60.
25. Naser S, Shafran I, El-Zaatari FAK. *Mycobacterium avium* subsp. *paratuberculosis* in Crohn's disease is serologically positive. *Clin Diagn Lab Immunol.* 1999;6:282.
26. Sunenaga K, Yokoyama Y, Nishikawa I, et al. Serum antibodies to *Mycobacterium paratuberculosis* in patients with Crohn's disease. *Dig Dis Sci.* 1999;44:1202–1207.
27. Brunello F, Pera A, Martini S, et al. Antibodies of *Mycobacterium paratuberculosis* in patients with Crohn's disease. *Dig Dis Sci.* 1991;36:1741–1745.
28. Walmsley RS, Ibbotson JP, Chahal H, et al. Antibodies to *Mycobacterium paratuberculosis* in Crohn's disease. *QJM.* 1996;89:217–221.
29. Bernstein CN, Blanchard JF, Rawsthorne P, et al. Population-based case control study of seroprevalence of *Mycobacterium paratuberculosis* in patients with Crohn's disease and ulcerative colitis. *J Clin Microbiol.* 2004;42:1129–1135.
30. Ruemmele FM, Targan SR, Levy G, et al. Diagnostic accuracy of serological assays in pediatric inflammatory bowel disease. *Gastroenterology.* 1998;115:822–829.
31. Sartor RB. Pathogenesis and immune mechanisms of chronic inflammatory bowel disease. *Am J Gastroenterol.* 1997;92:5s–11s.
32. Kreuzpaintner G, Das PK, Stronkhorst A, et al. Effect of intestinal resection on serum antibodies to the mycobacterial 45/48 kilodalton doublet antigen in Crohn's disease. *Gut.* 1995;37:361–366.
33. Olsen I, Wiker HG, Johnson E, et al. Elevated antibody response in patients with Crohn's disease against a 14-kDa secreted protein purified from *Mycobacterium* avium subsp. *paratuberculosis*. *Scand J Immunol.* 2001;53:198–203.
34. Ogura Y, Bonen DK, Inohara N, et al. A frameshift mutation in *NOD2* associated with susceptibility to Crohn's disease. *Nature.* 2001;411:603–606.
35. Hugot JP, Chamaillard M, Zouali H, et al. Association of *NOD2* leucine-rich repeat variants with susceptibility to Crohn's disease. *Nature.* 2001;411:599–603.
36. Ogura Y, Inohara N, Benito A, et al. *NOD2*, a *NOD1*/Apaf-1 family member that is restricted to monocytes and activates NF-kappa B. *J Biol Chem.* 2001;276:4812–4818.
37. Means TK, Jones BW, Schromm AB, et al. Differential effects of a Toll like receptor antagonist on *Mycobacterium tuberculosis*-induced macrophage response. *J Immunol.* 2001;166:4074–4082.
38. Poltrak A, He X, Smirnova I, et al. Defective LPS signaling in C3H/HeJ and C57 BL/10 ScCr mice: mutations in Tlr4 gene. *Science.* 1998;282:2085–2088.
39. Hermon-Taylor J, Barnes N, Clarke C, et al. *Mycobacterium paratuberculosis* cervical lymphadenitis, followed five years later by terminal ileitis similar to Crohn's disease. *BMJ.* 1998;516:449–453.
40. Gui GP, Thomas PR, Tizard ML, et al. Two-year-outcomes analysis of Crohn's disease treated with rifabutin and macrolide antibiotic. *J Antimicrob Chemother.* 1997;39:393–400.
41. Graham DY, Al-Assi MT. Prolonged remission in Crohn's disease following therapy for *Mycobacterium paratuberculosis* infection. *Gastroenterology.* 1995;108:A826.
42. Inoue N, Tamura K, Kinouchi Y, et al. T. Lack of common *NOD2* variants in Japanese patients with Crohn's disease. *Gastroenterology.* 2002;123:86–91.
43. Naser SA, Ghobrial G, Romero C, et al. Culture of *Mycobacterium avium* subspecies *paratuberculosis* from the blood of patients with Crohn's disease. *Lancet.* 2004;364:1039–1044.

GASTROENTEROLOGY 2007;132:176–189

Bone Marrow Retaining Colitogenic CD4+ T Cells May Be a Pathogenic Reservoir for Chronic Colitis

YASUHIRO NEMOTO,* TAKANORI KANAI,* SHIN MAKITA,* RYUICHI OKAMOTO,* TERUJI TOTSUKA,* KIYOSHI TAKEDA,‡ and MAMORU WATANABE*

*Department of Gastroenterology and Hepatology, Graduate School, Tokyo Medical and Dental University, Tokyo, Japan; and ‡Department of Molecular Genetics, Medical Institute for Bioregulation, Kyushu University, Fukuoka, Japan

Background & Aims: **Although bone marrow (BM) is known as a primary lymphoid organ, it also is known to harbor memory T cells, suggesting that this compartment is a preferential site for migration and/or selective retention of memory T cells. We here report the existence and the potential ability to induce colitis of the colitogenic BM CD4+ memory T cells in murine colitis models.** ***Methods:*** **We isolated BM CD4+ T cells obtained from colitic severe combined immunodeficient mice induced by the adoptive transfer of $CD4^+CD45RB^{high}$ T cells and colitic interleukin (IL)-$10^{-/-}$ mice that develop colitis spontaneously, and analyzed the surface phenotype, cytokine production, and potential activity to induce colitis. Furthermore, we assessed the role of IL-7 to maintain the colitogenic BM CD4+ T cells.** ***Results:*** **A high number of CD4+ T cells reside in the BM of colitic severe combined immunodeficient mice and diseased IL-$10^{-/-}$ mice, and they retain significant potential to induce type-1 T helper-mediated colitis in an IL-7–dependent manner. These resident BM CD4+ T cells have an effector memory (T_{EM}; $CD44^{high}CD62L^{-}IL\text{-}7R^{high}$) phenotype and preferentially are attached to IL-7–producing BM cells. Furthermore, the accumulation of BM CD4+ T_{EM} cells was decreased significantly in IL-7–deficient recipients reconstituted with the colitogenic lamina propria CD4+ T_{EM} cells.** ***Conclusions:*** **Collectively, these findings suggest that BM-retaining colitogenic CD4+ memory T cells in colitic mice play a critical role as a reservoir for persisting lifelong colitis.**

It has long been known that T-cell precursors generated in the bone marrow (BM) migrate to the thymus, where T-cell development occurs. However, a fact often neglected is that under physiologic conditions, mature CD4+ and CD8+ T cells undergo extensive migration from the blood to the BM and vice versa. In both human beings and mice, T-cell receptor $\alpha\beta^+$ cells constitute approximately 3%–8% of nucleated BM cells.[1,2] BM CD4+ and CD8+ T-cell populations contain a high proportion of cells displaying a memory phenotype, that is, expressing low levels of CD45RA in human beings[3] and high levels of CD44 in mice.[4,5]

As early as 1974 it was documented that mouse CD4+ T cells migrate to the BM after priming, and it was proposed that BM CD4+ T cells contributed to the development of a memory antibody response in this organ.[6] Recently, T cells persisting in extralymphoid organs such as the liver, lung, and skin have attracted increasing interest because it has been recognized that these T cells contribute considerably to the long-lived memory T-cell pool.[7,8] In this context, BM has been shown to harbor a high number of antigen-specific CD8+ T cells for several months after resolution of acute infection.[9] For instance, adoptive transfer of BM cells from lymphochoriomeningitis virus–immune mice (>90 days after acute infection) to immunodeficient recipients provides antiviral protection, and thus CD8+ memory T cells from the BM are able to mount an effective secondary response.[10]

Primary T-cell responses to blood-borne antigens also can be initiated in the BM. This was shown initially in conditions of altered lymphocyte trafficking in splenectomized mice and then in individuals with normal lymphoid organs, for both CD4+ and CD8+ T-cell responses.[11] Thus, the BM resembles a secondary lymphoid organ, although it lacks the organized T- and B-cell areas found in the spleen, lymph nodes, and Peyer's patches. Although accumulating evidence suggests that BM plays an important role in the communication with mature naive/memory T cells, there is no evidence for the role of BM memory CD4+ cells in chronic immune diseases, such as inflammatory bowel diseases (ulcerative colitis and Crohn's disease) and autoimmune diseases. Crohn's disease is characterized by chronic inflammation of the small and large intestine and structures apart from the

Abbreviations used in this paper: Ag, antigen; APC, antigen-presenting cell; BM, bone marrow; BrdU, bromodeoxyuridine; CBA, cecal bacterial antigen; CSFE, carboxyfluoroscein succinimidyl ester; ELISA, enzyme-linked immunosorbent assay; FITC, fluorescein isothiocyanate; IFN, interferon; IL, interleukin; LP, lamina propria; mAb, monoclonal antibody; MLN, mesenteric lymph node; PE, phycoerythrin; SCID, severe combined immunodeficient; Th1, type-1 T helper.

0016-5085/07/$32.00
doi:10.1053/j.gastro.2006.10.035

bowel. Surgery does not cure Crohn's disease, and recurrence after surgery is the rule rather than the exception.[12] There is also no correlation between recurrence of the disease and the dissection of regional lymph nodes and spleen.[13] The evidence suggests that other sites might play a critical role in the recurrence of diseases as reservoirs of colitogenic memory $CD4^+$ T cells.

Furthermore, it is well known that interleukin (IL)-7 is important as a critical factor for the survival and homeostatic proliferation of memory $CD4^+$ T cells, and that BM is a major site of IL-7 production.[14] We have shown previously that mucosal $CD4^+$ T cells in colitic mice express IL-7Rα highly, and they are pathogenic cells responsible for chronic colitis.[15] In vitro stimulation of these colitic lamina propria (LP) $CD4^+$IL-$7R^{high}$ T cells by IL-7, but not IL-15 and thymic stromal lymphopoietin, enhanced significant proliferative responses and survival of colitic $CD4^+$ T cells.[16] These backgrounds prompted us to investigate the role of the resident BM memory $CD4^+$ T cells in persisting lifelong colitis using a murine model of chronic colitis induced by the adoptive transfer of $CD4^+CD45RB^{high}$ T cells.

Materials and Methods

Mice

Female BALB/c, CB-17 severe combined immunodeficient (SCID), and C57BL/6 mice were purchased from Japan Clea (Tokyo, Japan). Female C57BL/6 Rag-$2^{-/-}$ mice were provided by Central Laboratories for Experimental Animals (Kawasaki, Japan). C57BL/6 Rag-$1^{-/-}$ mice and IL-$7^{-/-}$ mice were kindly provided by Dr. Zamoyska (National Institute for Medical Research, London, UK).[17] IL-$7^{-/-}$ × Rag-$1^{-/-}$ mice and littermate IL-$7^{+/+}$ × Rag-$1^{-/-}$ mice were generated in our laboratory. All mice were maintained under specific-pathogen-free conditions in the Animal Care Facility of the Tokyo Medical and Dental University. The Institutional Committee on Animal Research approved the experiments.

Antibodies and Flow Cytometry

The following monoclonal antibodies (mAbs) other than biotin-conjugated anti-mouse IL-7Rα (A7R34; Immuno-Biological Laboratories, Takasaki, Japan) were obtained from BD PharMingen (San Diego, CA) and used for purification of cell populations and flow-cytometric analysis: Fcγ (CD16/CD32)-blocking mAb (2.4G2), phycoerythrin (PE)-, peridinin chlorophyll protein, and phycoerythrin-phycoerythrin- 5'- disulfonatoindodicarbocyanine conjugated anti-mouse CD4 (RM4-5); fluorescein isothiocyanate (FITC)-conjugated anti-mouse CD3 (145-2C11); PE- and allophycocyanin-conjugated anti-mouse CD44 (IM7); FITC- and PE-conjugated anti-mouse CD62L (MEL-14); FITC-conjugated anti-mouse CD69 (H1.2F3); PE-conjugated anti-mouse integrin $\alpha_4\beta_7$ (DATK32); FITC-conjugated anti-mouse CD45RB (16A); FITC-conjugated hamster anti-mouse Bcl-2 (3F11); PE-conjugated streptavidin; biotin-conjugated rat IgG2; PE-conjugated mouse IgG; and PE-conjugated rat IgG. Flow cytometric 3-color analysis was performed as described.[18]

Induction of Colitis

Colitis was induced in SCID/Rag-$2^{-/-}$ mice by the adoptive transfer of $CD4^+CD45RB^{high}$ T cells as described.[18] Colitic mice were killed at 6–8 weeks after transfer, and $CD4^+$ T cells were isolated from BM, mesenteric lymph nodes (MLNs), and colonic LP.

Cytokine Enzyme-Linked Immunosorbent Assay

To measure cytokine production, 3×10^4 $CD4^+$ T cells from MLN, LP, and BM were cultured in 200 μL of culture medium at 37°C in a humidified atmosphere containing 5% CO_2 in 96-well plates (Costar, Cambridge, MA) precoated with 5 μg/mL hamster anti-mouse CD3ϵ mAb (145-2C11; BD PharMingen) and 2 μg/mL hamster anti-mouse CD28 mAb (37.51; BD PharMingen) in phosphate-buffered saline (PBS) overnight at 4°C. Culture supernatants were removed after 48 hours and assayed for cytokine production. Cytokine concentrations were determined by specific enzyme-linked immunosorbent assay (ELISA) as per the manufacturer's recommendation (R&D, Minneapolis, MN).

Interferon-γ Production by $CD4^+$ T Cells Stimulated With APCs Pulsed With Cecal Extracts

Colitic SCID mice were killed and their cecums were removed. The cecums were opened and placed in 1 mL of PBS, and the cecal bacteria were expelled by mixing with a vortex, and residual cecal tissue was removed. After the addition of DNase (10 μg/mL), 1 mL of this bacterial suspension was added to 1 mL of glass beads.[19] The cells were disrupted at 5000 revolutions per minute in a Mini-Bead Beater (BioSpec Products, Bartlesville, OK) for 3 minutes and then iced. The glass beads and unlysed cells were removed by centrifuging at 5000 × g for 5 minutes. The lysates were filter-processed in a similar manner. For antigen-presenting cells (APCs), spleen cells from normal BALB/c mice were prepared and treated with the appropriate concentration of cecal bacterial antigens (CBAs) as indicated at 2×10^7 cells/5 mL in a 15-mL tube overnight at 37°C. After washing twice, these APCs were treated with mitomycin-c before being added to T-cell cultures. BM, MLN, and LP $CD4^+$ T cells obtained from normal mice and colitic $CD4^+CD45RB^{high}$ T-cell–transferred SCID mice were cultured in the presence of APCs pretreated with cecal extract antigens in complete media. The culture supernatants were collected on day 3 of culture for interferon (IFN)-γ assay by ELISA.

Bromodeoxyuridine Incorporation

Colitic mice and age-matched normal BALB/c mice were given 1 mg of bromodeoxyuridine (BrdU) in PBS by intraperitoneal injection. Twenty-four hours later, mice were killed and the lymphocytes were prepared from BM, MLN, and colonic LP. Cells were first stained with PE-conjugated anti-CD4 mAbs for 2-color flow-cytometric analysis, or peridinin chlorophyll protein-conjugated anti-CD4 mAbs, APC-conjugated anti-CD44 mAbs, and PE-conjugated anti-CD62L mAbs for 4-color flow-cytometric analysis, and fixed and permeabilized with Cytofix-Cytoperm (BD PharMingen) solution according to the manufacturer's instructions. Cells were stained with FITC-conjugated anti-mouse BrdU (BD PharMingen) diluted in perm/wash buffer.

Cell-Cycle Analysis

A total of 1×10^6 cells from colitic mice induced by the adoptive transfer of $CD4^+CD45RB^{high}$ T cells were stained for PE-conjugated anti-CD4 mAbs, and fixed and permeabilized with Cytofix-Cytoperm (BD PharMingen) solution according to the manufacturer's instructions. 7-AAD (10 μg/mL) and RNase (200 μg/mL) were added, and cells were incubated for 20 minutes at room temperature. Cells were acquired on a FACSCalibur (BD PharMingen) in their staining solution. Cell-cycle analysis of DNA histograms was performed with Cell Quest Software (BD PharMingen).

Immunohistochemistry

Consecutive cryostat bone marrow sections (6 μm) were fixed and stained with the following rat antibodies: biotinylated CD4 (RM4-5) and polyclonal anti-IL-7 antibodies (R&D Laboratories). Alexa 594 goat anti-rat IgG, Alexa 488 goat anti-hamster IgG, and Alexa 488 rabbit anti-goat IgG (Molecular Probes, Eugene, OR) were used as second antibodies. All confocal microscopy was performed on a BioZERO BZ8000 (Keyence, Tokyo, Japan).

Adoptive Transfer Experiments

To assess the in vivo potential of the residual BM $CD4^+$ T cells in colitic SCID mice induced by the adoptive transfer of $CD4^+CD45RB^{high}$ T cells to induce colitis, $CD4^+$ T cells (1×10^5 cells/mouse) isolated from the BM, MLN, and LP of colitic mice or BM of age-matched normal BALB/c mice were injected into new SCID mice. In another set of experiments, BM $CD4^+$ T cells (1×10^5 cells/mouse) isolated from colitic IL-$10^{-/-}$ mice (age, 20 wk) or age-matched normal C57BL/6 mice (1×10^5 cells/mouse) were injected into C57BL/6 RAG2$^{-/-}$ mice. To assess the role of commensal bacteria in the development of colitis and the retention of colitogenic BM $CD4^+$ effector-memory T (T_{EM}) cells, we used broad-spectrum antibiotics in another adoptive transfer experiment. CB-17 SCID mice were treated with or without ampicillin (1 g/L; Sigma, St. Louis, MO), vancomycin (500 mg/L; Abbott Labs, Abbott Park, Illinois), neomycin sulfate (1 g/L; Pharmacia/Upjohn, New York, NY), and metronidazole (1 g/L; Sidmak, Gujarat, India) in drinking water 4 weeks before beginning the adoptive transfer and during the course of the development of colitis based on a variation of the commensal depletion protocol of Fagarasan et al.[20] All recipient mice were weighed initially, then 3 times/wk after the transfer. They then were observed for clinical signs of illness as previously described.[18]

Adoptive Transfer Experiments Into IL-7$^{-/-}$ × Rag-1$^{-/-}$ Mice

To assess the role of IL-7 in the maintenance of BM $CD4^+$ T cells, we further transferred LP $CD4^+$ T cells (2×10^6 cells/mouse) isolated from colitic $CD4^+CD45RB^{high}$ T-cell–transferred mice into IL-$7^{-/-}$ × Rag-$1^{-/-}$ and IL-$7^{+/+}$ × Rag-$1^{-/-}$ mice. Mice were killed 5 days after transfer, and the spleen and BM cells were isolated and stained with PE-conjugated rat anti-CD3ε mAbs and FITC-conjugated rat anti-CD69 mAbs or isotype FITC-conjugated control antibody. Before staining for intracellular Bcl-2, cells (2×10^6) were stained with PE-conjugated rat anti-CD3 mAbs as described earlier. After washing, cells were fixed and permeabilized with Cytofix-Cytoperm (BD PharMingen) solution according to the manufacturer's instructions. Cells were stained with either FITC-conjugated hamster anti-mouse Bcl-2 or a control antibody diluted in perm/wash buffer. To further assess the proliferative responses of $CD4^+$ T cells in IL-$7^{+/+}$ × Rag-$1^{-/-}$ and IL-$7^{-/-}$ × Rag-$1^{-/-}$ recipients, LP $CD4^+$ T cells from SCID mice with colitis induced by the adoptive transfer of $CD4^+CD45RB^{high}$ T cells were labeled with carboxyfluoroscein succinimidyl ester (CFSE) (Molecular Probes) by incubating at 5 μmol/L in PBS, quenching with fetal calf serum, and washing with PBS 3 times. Cells were resuspended in PBS, and 3×10^6 total cells were transferred by intravenous injection into IL-$7^{+/+}$ × Rag-$1^{-/-}$ and IL-$7^{-/-}$ × Rag-$1^{-/-}$ mice. In another set of experiments, we transferred with colitogenic BM $CD4^+$ T cells from colitic $CD4^+CD45RB^{high}$ T-cell–transferred Rag-$2^{-/-}$ mice into IL-$7^{+/+}$ × Rag-$1^{-/-}$ and IL-$7^{-/-}$ × Rag-$1^{-/-}$ recipients to clarify whether these mice develop colitis. Mice were killed at 10 weeks after transfer.

Statistical Analysis

The results were expressed as the mean ± SD. Groups of data were compared by the Mann–Whitney *U* test. Differences were considered statistically significant when the *P* value was less than .05.

Results

Effector Memory T Cells Reside in the BM of Colitic Mice

To investigate the role of BM in consecutive immunopathology in immune-mediated diseases, we first compared the composition and phenotype of $CD4^+$ T

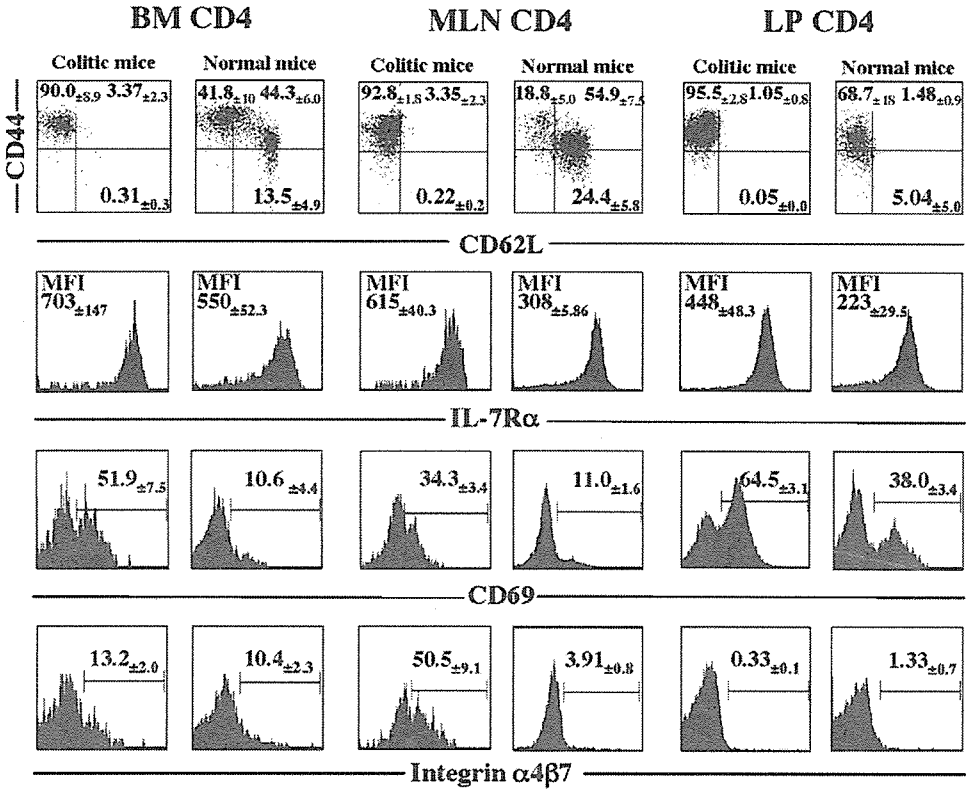


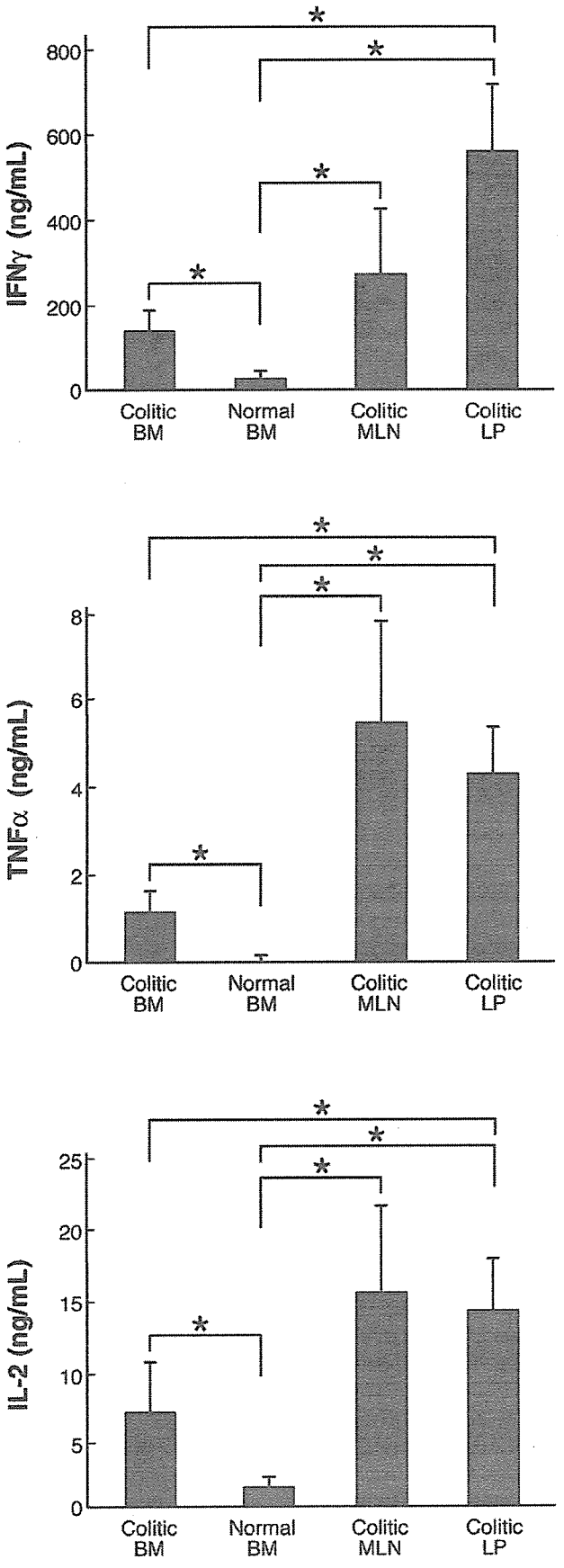
Figure 1. Colitic BM CD4+ T cells are CD44^{high}CD62L^{-}IL-7Rα^{high}. Expression of CD44, CD62L, IL-7Rα (CD127), CD69, and integrin $\alpha4\beta7$ on CD4+ T cells obtained from spleen, MLN, LP, and BM in colitic mice induced by adoptive transfer of CD4+CD45RB^{high} T cells into CB-17 SCID mice (6 weeks after transfer) and normal BALB/c mice (age, 8 wk). Freshly isolated cells from colitic mice and normal BALB/c mice were stained with FITC-labeled anti-CD4, and PE-labeled anti-CD44, anti-CD62L, anti-IL-7Rα, anti-CD69, or anti-integrin $\alpha4\beta7$ mAbs. Samples were analyzed by flow cytometry. Lymphocytes were identified by characteristic forward angle and side-scatter profiles. Data are displayed as a dotted plot (4-decade log scale) and quadrant markers were positioned to include more than 98% of control Ig-stained cells in the lower left. Percentages in each quadrant are indicated. Representative of 3 mice in each group.

cells in BM, MLN, and colonic LP of colitic mice induced by the adoptive transfer of CD4+CD45RB^{high} T cells into recipient CB-17 SCID mice and with those of age-matched normal BALB/c mice. CD3+CD4+ mature T cells were found to reside in BM, MLN, and LP (colitic mice: BM, 12.7 ± 4.4 × 10^5 per mouse; MLN, 7.01 ± 4.2 × 10^5; and LP, 187 ± 99 × 10^5; normal mice: BM, 16.6 ± 3.8 × 10^5; MLN, 99.6 ± 18 × 10^5; and LP, 4.17 ± 1.2 × 10^5). As shown in Figure 1, the BM CD4+ T cells, as well as MLN and LP CD4+ T cells, from the colitic mice, exclusively have a phenotype of CD44^{high}CD62L^{-} cells. Furthermore, these colitic BM CD4+ T cells expressed IL-7Rα highly, indicating that the colitic BM CD4+ T cells have a characteristic of T_{EM} cells. In contrast, the BM CD4+ T cells from normal mice are composed of 3 subpopulations: CD44^{low}CD62L+ naive cells, CD44^{high}CD62L+ central-memory T cells, and CD44^{high}CD62L^{-} T_{EM} cells (Figure 1). CD69, which is associated with cell activation, was expressed by a significantly higher proportion of CD4+ T cells from colitic mice than from normal mice. Interestingly, BM CD4+ T cells from colitic mice expressed relatively, but not significantly, high levels of integrin $\alpha4\beta7$, a homing receptor to the gut, as compared with BM CD4+ T cells from normal mice, but lower levels than did MLN CD4+ T cells from colitic mice. These data indicate that the integrin $\alpha4\beta7$-expressing CD4+ memory T cells, which are instructed to express the molecule in MLN or Peyer's patches,[21,22] migrate to the BM.

Colitic BM CD4+ Memory T Cells Produce a Large Amount of Th1 Cytokines

We next examined whether the colitic BM CD4+ T cells retained the ability to produce type-1 T helper (Th1) cytokines as well as the colitic CD4+ T cells in other sites. The production of IFN-γ, tumor necrosis factor-α, and IL-2 by anti-CD3/CD28 mAb-stimulated BM CD4+ T cells from colitic mice was significantly higher than that by normal BM CD4+ T cells, but lower than those by anti-CD3/CD28 mAb-stimulated LP CD4+ T cells (Figure 2), indicating that the colitic BM CD4+ T cells could be primed to Th1-type cells, and sustained in the BM.

To determine whether the BM CD4+ T cells from colitic mice express their pathogenic potential on stim-



ulation with antigens derived from resident enteric bacteria, we examined in vitro IFN-γ secretion by normal and colitic BM, MLN, and LP $CD4^+$ T cells stimulated with various concentrations of CBA. The results show that significantly higher levels of IFN-γ were produced by colitic BM $CD4^+$ T cells in response to a high dose (1000 μg/mL) of CBA as compared with normal BM $CD4^+$ T cells, but significantly lower than those by colitic LP $CD4^+$ T cells, which responded to much lower concentrations (10, 100, 1000 μg/mL) of CBA (Figure 3). The similar result was obtained by paired samples of MLN (Figure 3) and splenic (data not shown) $CD4^+$ T cells. These results indicated that the colitic BM $CD4^+$ T cells have the potential to respond against bacterial antigens and thus have the possibility to be colitogenic similar to the colitic LP $CD4^+$ T cells as we have shown previously.[18]

IL-7–Expressing Cells are Scattered Throughout BM and Colocalized in Close Proximity to $CD4^+$ T Cells

We next examined the distribution of IL-7–producing cells[23] and their interaction with $CD4^+$ T cells in the colitic BM. The IL-7–expressing cells were scattered throughout the BM as has been reported previously[24] and most $CD4^+$ T cells were in close contact with the bodies of IL-7–expressing cells (Figure 4). In contrast, IL-7 was not expressed, and $CD4^+$ T cells did not reside in the BM of IL-$7^{-/-}$ × Rag-$1^{-/-}$ mice used as a negative control (Figure 4).

BM Contains the Most Actively Dividing Pool of $CD4^+$ T Cells

To examine the homeostatic proliferation of the colitic BM $CD4^+$ T cells, 2 experimental approaches were used. First, we examined memory $CD4^+$ T cells from each tissue for evidence of active cell division by DNA staining using 7AAD (Figure 5*A*). Cells actively synthesizing DNA could be identified by their increased DNA content, allowing us to identify tissues where active cell division was occurring. A larger percentage of $CD4^+$ T cells was actively synthesizing DNA in both the colitic and normal BM than in any other tissues (Figure 5*A*). Although the difference was slight, it was reproducible over 3 independent experiments.

Second, colitic mice were injected with BrdU to provide evidence of recent DNA synthesis. To accurately examine the differences in cell proliferation in different tissues, it was necessary to give a short pulse of BrdU because

Figure 2. Colitic BM $CD4^+$ T cells produce Th1 cytokines. Cytokine production by $CD4^+$ T cells. Isolated $CD4^+$ T cells were stimulated with anti-CD3 and anti-CD28 mAbs for 48 hours. The indicated cytokines in these supernatants were measured by ELISA. Data are indicated as the mean ± SD of 7 mice in each group.

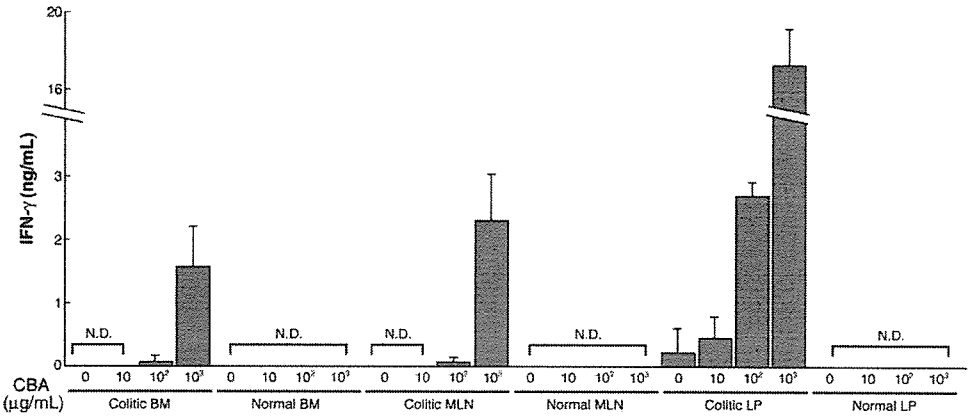


Figure 3. IFN-γ production by CD4+ T cells stimulated with APCs pulsed with CBA from colitic mice induced by adoptive transfer of CD4+CD45RB^{high} T cells. Supernatants collected on day 3 of culture were assayed for IFN-γ by ELISA. Data are indicated as the mean ± SD of 5 mice in each group. *$P < .05$. ND, not detected.

longer treatment with BrdU might obscure the differences among the various tissues, probably because of the migration of dividing cells among the tissues. Mice thus were killed 24 hours after the injection of BrdU, and BrdU incorporation was measured in the CD4+ T cells obtained from BM, MLN, and LP (Figure 5*B*). Significantly higher percentages of memory T cells were synthesizing DNA in the colitic BM, MLN, and LP as compared

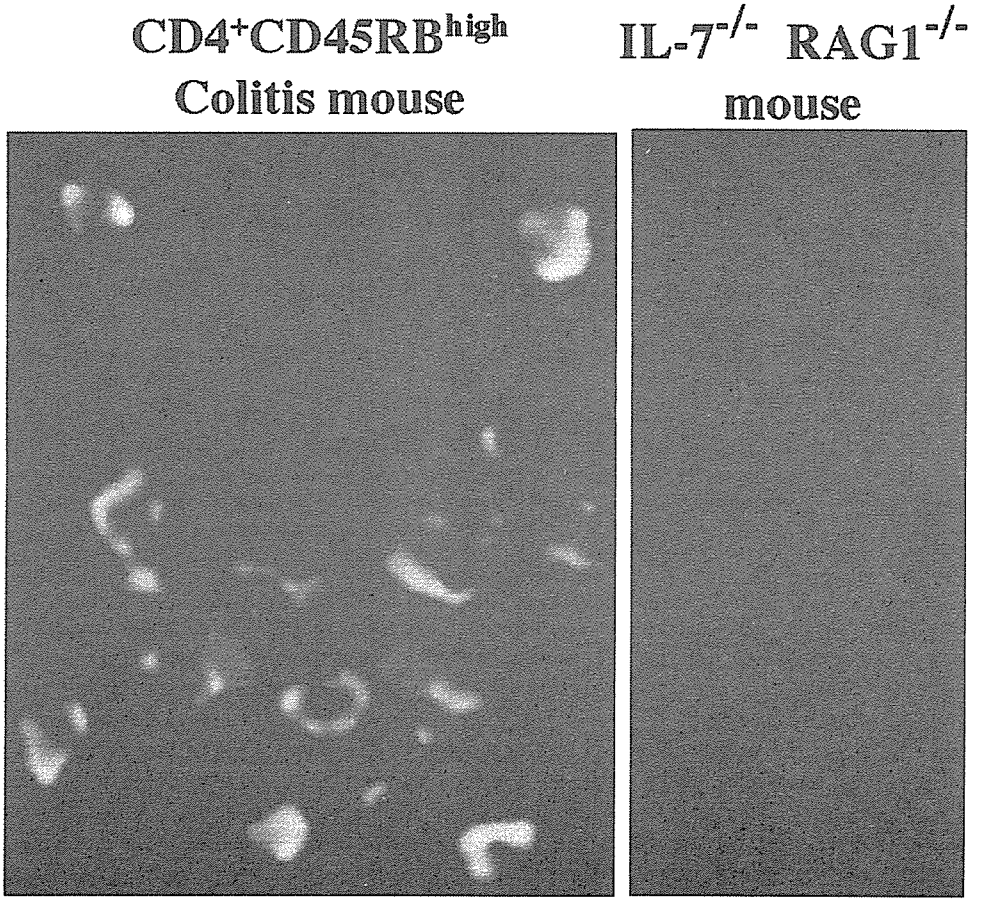


Figure 4. Cluster formation between CD4+ T cells and IL-7–expressing stromal cells within BM. Frozen sections of BM from colitic mice induced by adoptive transfer of CD4+CD45RB^{high} T cells (*left*) and untreated IL-7$^{-/-}$ × Rag-1$^{-/-}$ control mice (*right*) were stained with corresponding monoclonal antibodies. The IL-7–expressing cells (*green*) are scattered uniformly throughout the BM CD4+ T cells (*red*). CD4+ T cells lie close to IL-7–expressing stromal cells.

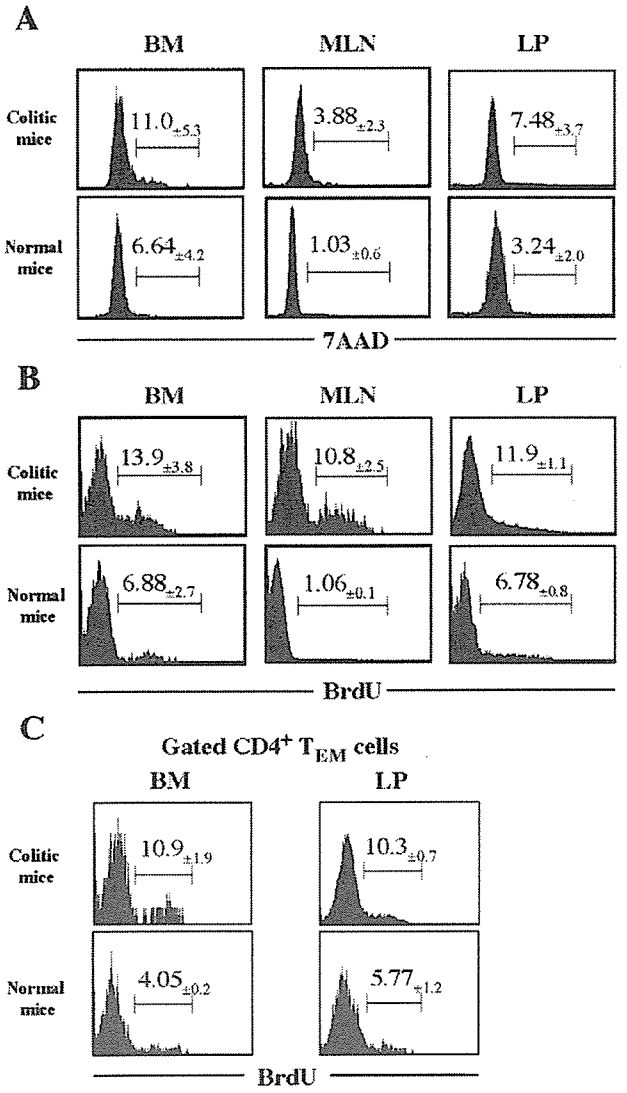


Figure 5. Colitic BM contains the actively dividing pool of memory CD4+ T cells. (*A*) BM, MLN, and LP CD4+ T cells from colitic mice or age-matched normal BALB/c mice were stained for DNA content using 7AAD. One representative mouse is shown of 5 mice analyzed. (*B*) Colitic mice and normal control mice were injected with BrdU for pulse-chase studies of BrdU incorporation. One representative mouse of 4 is shown. (*C*) Colitic mice and normal control mice were injected with BrdU as described in the Materials and Methods section. CD4+ T cells were stained with CD4, CD44, and CD62L before intracellular staining for BrdU, and then the gated $CD4^+CD44^{high}CD62L^-$ T_{EM} cells in the BM and LP from colitic and normal mice were assessed by the BrdU incorporation. One representative mouse of 3 is shown.

with those in the paired normal BM, MLN, and LP. Because we compared dissimilar subsets in this setting because normal BM contains all subsets, such as naive, central memory, and T_{EM} $CD4^+$ T cells, yet in contrast colitic BM $CD4^+$ T cells are constituted of T_{EM} cells exclusively (Figure 1), we next compared colitic BM and LP $CD4^+CD44^{high}CD62L^-$ T_{EM} cells with the paired normal T_{EM} cells. As shown in Figure 5*C*, DNA synthesis in colitic BM and LP $CD4^+CD44^{high}CD62L^-$ T_{EM} cells was increased significantly as compared with that in the paired normal gated T_{EM} cells (Figure 5*C*).

Transfer of the BM Memory CD4+ T Cells From Colitic Mice Into SCID Mice Reproduce Th1-Mediated Colitis

Based on the earlier-described results, we hypothesized that the colitic BM retaining $CD4^+$ T_{EM} cells is a pathogenic reservoir for persisting lifelong colitis. To prove this, we performed an adoptive transfer experiment by transferring colitic BM, MLN, and LP $CD4^+$ T_{EM} cells obtained from $CD4^+CD45RB^{high}$-transferred SCID mice and normal BM $CD4^+$ T cells into new SCID mice (Figure 6*A*). As shown in Figure 6*B*, mice transferred with the colitic BM, MLN, and LP $CD4^+$ T cells manifested progressive weight loss at 8 weeks after transfer. These mice had diarrhea with increased mucus in the stool, anorectal prolapse, and hunched posture by 4–6 weeks. In contrast, mice transferred with normal BM $CD4^+$ T cells appeared healthy, showing a gradual increase of body weight and no diarrhea during the period of observation (Figure 6*B* and *C*). At 8 weeks after transfer, colitic BM $CD4^+$ T-cell–transferred mice, but not mice transferred with normal BM $CD4^+$ T cells, had enlarged colons with greatly thickened walls (Figure 6*D*). The assessment of colitis by clinical scores showed a clear difference between mice transferred with colitic BM $CD4^+$ T cells and mice transferred with normal BM $CD4^+$ T cells (Figure 6*C*). In addition, the clinical scores of mice transferred with colitic BM $CD4^+$ T cells were comparable with those of mice transferred with colitic MLN or LP $CD4^+$ T cells. Histologic examination showed prominent epithelial hyperplasia with glandular elongation and massive infiltration of mononuclear cells in LP of the colon from colitic BM $CD4^+$ T-cell–transferred mice as well as colons from the colitic MLN or LP $CD4^+$ T-cell–transferred mice (Figure 6*E*). In contrast, pathologic findings were not observed in the LP of the colon from mice transferred with normal BM $CD4^+$ T cells (Figure 6*E*). This difference also was confirmed by histologic scoring of multiple colon sections (Figure 6*F*).

A further quantitative evaluation of $CD4^+$ T-cell accumulation was made by isolating $CD3^+CD4^+$ T cells. Few $CD3^+CD4^+$ T cells were recovered from the colonic LP in the normal BM $CD4^+$ T-cell–transferred mice as compared with the mice transferred with the colitic BM, MLN, or LP $CD4^+$ T cells (Figure 6*G*). Somewhat unexpectedly, the number of $CD4^+$ T cells recovered from the BM of normal BM $CD4^+$ T-cell–transferred mice was comparable with that from mice transferred with the colitic BM, MLN, or LP $CD4^+$ T cells (Figure 6*G*). Importantly, the number of $CD4^+$ cells recovered from the colitic BM $CD4^+$ T-cell–transferred mice far exceeded the number of cells originally injected (1×10^5), indicating extensive T-cell migration and/or proliferation in each

tissue. We also examined the cytokine production by isolated LP CD4+ T cells. As shown in Figure 6*H*, LP CD4+ T cells from colitic BM CD4+ T-cell–transferred mice produced significantly higher levels of IFN-γ and tumor necrosis factor-α than those from normal BM CD4+ T-cell–transferred mice on in vitro anti-CD3/anti-CD28 mAbs stimulation. In contrast, the production of IL-4 or IL-10 was not affected significantly (data not shown).

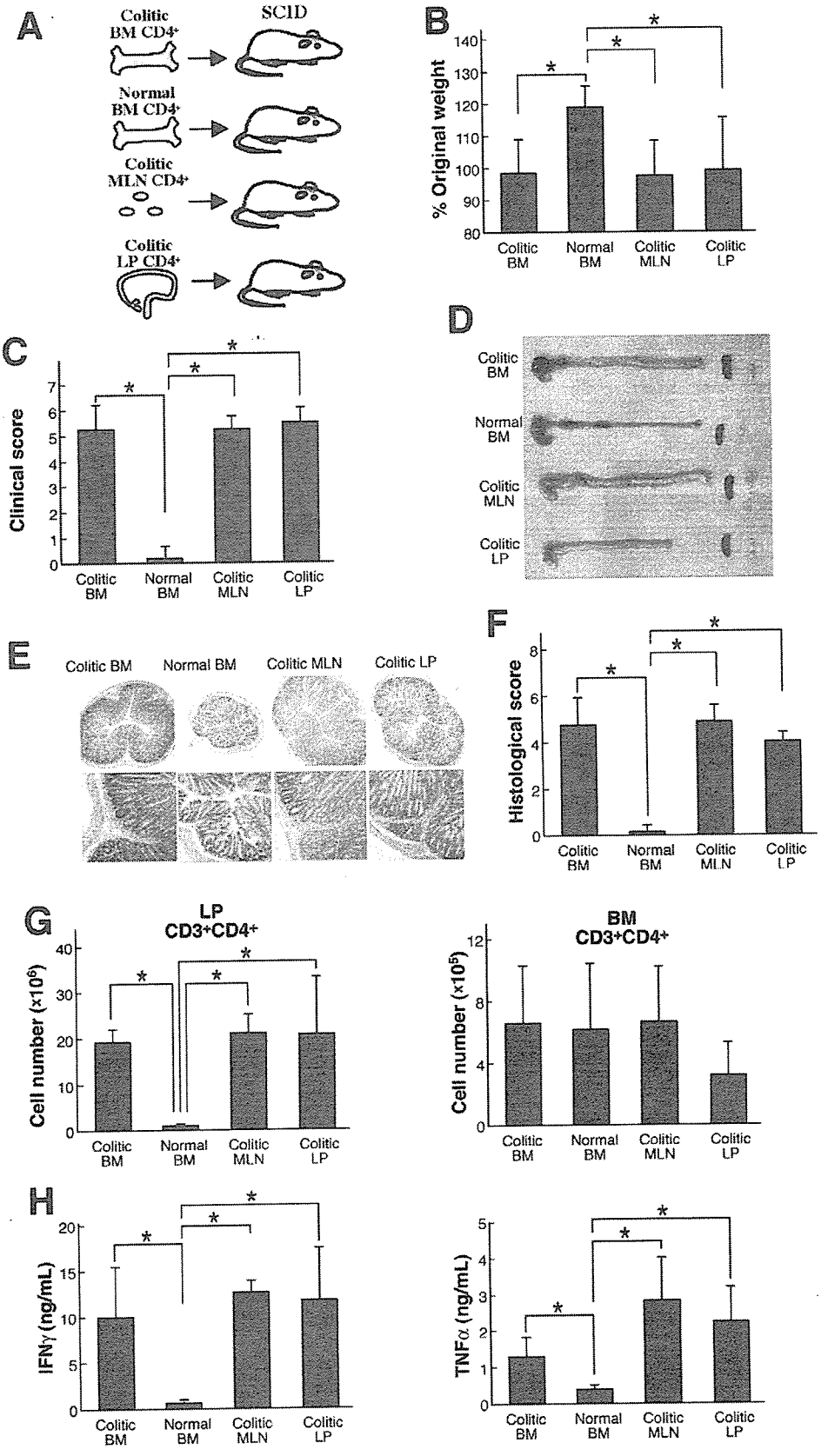
IL-7 Is Essential for the Survival and Homeostatic Proliferation of Colitogenic BM CD4+ Memory T Cells

To further analyze the role of IL-7 in the survival and homeostatic proliferation of the colitogenic BM CD4+ T cells, we retransferred CFSE-labeled LP CD4+ T cells obtained from CD4+CD45RB^{high} T-cell–transferred colitic mice into IL-7$^{+/+}$ × Rag-1$^{-/-}$ and IL-7$^{-/-}$ × Rag-1$^{-/-}$ mice (Figure 7*A*). Rapid proliferation of donor colitic LP CD4+ T cells was observed in the BM from IL-7$^{-/-}$ × Rag-1$^{-/-}$ mice 5 days after the transfer, although the relative size of the expanded T-cell populations in IL-7$^{-/-}$ × Rag-1$^{-/-}$ BM CD4+ T cells was approximately 80% of that observed in the control IL-7$^{+/+}$ × Rag-1$^{-/-}$ BM CD4+ T cells (Figure 7*B*). Somewhat unexpectedly, however, the recovered cell numbers of the BM and spleen CD4+ T cells from IL-7$^{-/-}$ × Rag-1$^{-/-}$ mice were strikingly lower than those from IL-7$^{+/+}$ × Rag-1$^{-/-}$ mice (BM: IL-7$^{-/-}$ × Rag-1$^{-/-}$ 2.3 ± 1.9 × 10^5; IL-7$^{+/+}$ × Rag-1$^{-/-}$ mice, 45 ± 19 × 10^5; spleen: IL-7$^{-/-}$ × Rag-1$^{-/-}$ 3.8 ± .1 × 10^5; IL-7$^{+/+}$ × Rag-1$^{-/-}$ mice, 32 ± 13 × 10^5) (Figure 7*C*), indicating that the IL-7 was essential for the survival rather than the homeostatic proliferation of the colitogenic CD4+ T cells in the BM. Consistent with this notion, we next assessed if regulation of Bcl-2 requires IL-7 at day 5 after the transfer, because induction of the anti-apoptotic protein, Bcl-2, is a hallmark of responses to IL-7.[14] As expected, the BM CD4+ T cells in IL-7$^{-/-}$ × Rag-1$^{-/-}$ mice expressed lower levels of Bcl-2 than those in IL-7$^{+/+}$ × Rag-1$^{-/-}$ mice (Figure 7*D*). Furthermore, the cell activation marker CD69 also was down-modulated significantly on the BM CD4+ T cells in IL-7$^{-/-}$ × Rag-1$^{-/-}$ mice as compared with those in IL-7$^{+/+}$ × Rag-1$^{-/-}$ mice (Figure 7*E*).

Finally, we asked whether adoptive transfer of colitogenic BM CD4+ T cells into IL-7$^{-/-}$ × Rag-1$^{-/-}$ or IL-7$^{+/+}$ × Rag-1$^{-/-}$ mice induces colitis and results in the retention of BM CD4+ T cells (Figure 8*A*). Expectedly, transfer of colitogenic BM CD4+ T cells into the control IL-7$^{+/+}$ × Rag-1$^{-/-}$ mice led to a severe wasting disease 4–6 weeks after transfer, but IL-7$^{-/-}$ × Rag-1$^{-/-}$ mice transferred with colitogenic BM CD4+ T cells appeared healthy and continued to gain weight during 10 weeks of observation (data not shown). The clinical score of IL-7$^{-/-}$ × Rag-1$^{-/-}$ recipients was almost zero, and significantly lower than that of IL-7$^{+/+}$ × Rag-1$^{-/-}$ recipients at 10 weeks after transfer (Figure 8*B*). The colon, the spleen, and the MLN from IL-7$^{+/+}$ × Rag-1$^{-/-}$ recipients, but not those from IL-7$^{-/-}$ × Rag-1$^{-/-}$ recipients, were enlarged and had a greatly thickened wall of colon (Figure 8*C*). Consistent with the lack of clinical signs in IL-7$^{-/-}$ × Rag-1$^{-/-}$ recipients, they displayed no histologic evidence of intestinal inflammation in contrast to IL-7$^{+/+}$ × Rag-1$^{-/-}$ recipients with severe inflammation (Figure 8*D*). Histologic analysis of colonic mucosa showed development of severe colitis in IL-7$^{+/+}$ × Rag-1$^{-/-}$, but not in IL-7$^{-/-}$ × Rag-1$^{-/-}$, recipients (Figure 8*E*). The total cell numbers of isolated BM, MLN, and LP CD3+CD4+ T cells from IL-7$^{-/-}$ × Rag-1$^{-/-}$ recipients were significantly lower than those from IL-7$^{+/+}$ × Rag-1$^{-/-}$ recipients (Figure 8*F*). Collectively, these results indicated that IL-7 is essential to develop colitis for colitogenic BM CD4+ T cells and to sustain these cells in the BM and in the LP and the MLN.

SCID Mice Transferred With CD4+CD45RB^{high} and Administered With Broad-Spectrum Antibiotics Did Not Develop Colitis, but Retained CD4+ T_{EM} in BM

It generally is accepted that colitis-inducing CD4+CD45RB^{high} T cells recognize bacterial and/or self-antigens that are induced by the presence of intestinal bacteria, and germ-free conditions prevent the development of intestinal inflammation in many animal models of colitis including the CD4+CD45RB^{high} T-cell–transfer model.[25] We therefore assessed whether SCID mice transferred with CD4+CD45RB^{high} T cells and treated with or without oral administration of a mixture of antibiotics (vancomycin, neomycin, metronidazole, and ampicillin) develop colitis and the persistence of BM CD4+ T cells (supplemental Figure 1*A*; supplementary material online at www.gastrojournal.org). As expected, we found that SCID mice transferred with CD4+CD45RB^{high} T cells without oral administration of antibiotics developed wasting disease (supplemental Figure 1*B*) and severe colitis (supplemental Figure 1*C*), whereas those with administration of antibiotics did not develop wasting disease and colitis 4 weeks after transfer (supplemental Figures 1*B* and *C*). The blinded histologic score of mice treated with antibiotics was almost zero in contrast to control recipient mice without administration of antibiotics (6.2 ± 1.3) (supplemental Figure 1*D*). The average number of CD3+CD4+ T cells recovered from recipient mice that transferred with CD4+CD45RB^{high} T cells and given drinking water without antibiotics was 11.0 ± 0.7 × 10^5 per mouse in BM, 52 ± 20 × 10^5 in MLN, and 240 ± 40 × 10^5 in LP (supplemental Figure 1*E*). In contrast, the cell number in mice transferred with CD4+CD45RB^{high} T cells and treated with antibiotics was decreased significantly compared with mice transferred with CD4+CD45RB^{high} T cells and given the antibiotics (BM, 2.2 ± 1.8 × 10^5 per mouse; spleen, 11 ± 11 × 10^5;


A
Colitic BM CD4+
SCID
Normal BM CD4+
Colitic MLN CD4+
Colitic LP CD4+
B
% Original weight
Colitic BM
Normal BM
Colitic MLN
Colitic LP
C
Clinical score
D
E
Colitic BM
Normal BM
Colitic MLN
Colitic LP
F
Histological score
G
LP CD3+CD4+
Cell number (x10^6)
BM CD3+CD4+
Cell number (x10^5)
H
IFNγ (ng/mL)
TNFα (ng/mL)

and LP, 28 ± 24 × 10^5) (supplemental Figure 1*E*). Therefore, the administration of antibiotics significantly suppressed colitis and resulted in the reduced expansion of BM $CD3^+CD4^+$ T cells and MLN and LP.

Transfer of BM CD4+ T Cells From Colitic IL-10–Deficient Mice, but not Normal Mice, Into Rag-2−/− Mice Reproduces Th1-Mediated Colitis

We finally addressed whether latent colitogenic $CD4^+$ T cells reside in the BM in a colitis model that develops colitis spontaneously, rather than the adoptive transfer model, in this case, IL-$10^{-/-}$ mice[26] (supplemental Figure 2*A*; supplementary material online at www.gastrojournal.org). We first isolated the BM $CD4^+$ T cells from diseased IL-$10^{-/-}$ mice and age-matched normal C57BL/6 mice, and analyzed the expression of CD44 and CD62L on $CD4^+$ T cells by flow cytometry. Similar to the BM $CD4^+$ T cells in colitic mice induced by the adoptive transfer of $CD4^+CD45RB^{high}$, $CD4^+CD44^{high}CD62L^-$ T_{EM} cells preferentially resided in the BM of colitic IL-$10^{-/-}$ mice as compared with age-matched normal C57BL/6 mice (supplemental Figure 2*B*, upper). We next transferred the BM $CD4^+$ T cells from diseased IL-$10^{-/-}$ mice and age-matched normal C57BL/6 mice into recipient C57BL/6 Rag-$2^{-/-}$ mice (supplemental Figure 2*A*). Mice transferred with the colitic IL-$10^{-/-}$ BM $CD4^+$ T cells manifested progressive weight loss (wasting disease) at 10 weeks after transfer as compared with the mice transferred with normal C57BL/6 BM $CD4^+$ T cells (data not shown). These mice had significant clinical symptoms by 4–6 weeks after transfer, but mice transferred with normal BM $CD4^+$ T cells appeared healthy without diarrhea during the whole period of observation. The assessment of colitis by clinical scores showed a clear difference between the mice transferred with colitic IL-$10^{-/-}$ BM $CD4^+$ T cells and the mice transferred with normal BM $CD4^+$ T cells (supplemental Figure 2*C*). At 10 weeks after transfer, the colitic IL-$10^{-/-}$ BM $CD4^+$ T-cell–transferred mice, but not those transferred with normal BM $CD4^+$ T cells, had enlarged colons with greatly thickened walls (supplemental Figure 2*D*). Histologic examination showed severe signs of colitis, including epithelial hyperplasia and massive infiltration of mononuclear cells, in LP from the colitic IL-$10^{-/-}$ BM $CD4^+$ T-cell–transferred mice as compared with the colons from the normal BM $CD4^+$ T-cell–transferred mice (supplemental Figure 2*E*). This difference also was confirmed by histologic scoring of multiple colon sections (supplemental Figure 2*F*). Furthermore, few $CD4^+$ T cells were recovered from the colonic LP in the normal BM $CD4^+$ T-cell–transferred mice as compared with the mice transferred with the colitic IL-$10^{-/-}$ BM $CD4^+$ T cells (supplemental Figure 2*G*). As in the model of $CD4^+CD45RB^{high}$ T-cell–transferred colitis, the number of recovered BM $CD4^+$ T cells from the normal BM $CD4^+$ T-cell–transferred mice was comparable with that from mice transferred with the colitic IL-$10^{-/-}$ BM (supplemental Figure 2*G*). We finally examined the cytokine production by isolated LP $CD4^+$ T cells. LP $CD4^+$ T cells from the normal BM $CD4^+$ T-cell–transferred mice produced significantly less IFN-γ and tumor necrosis factor-α than those from the colitic IL-$10^{-/-}$ $CD4^+$ T-cell–transferred mice on in vitro stimulation (supplemental Figure 2*H*). These results suggested that the colitic IL-$10^{-/-}$ BM $CD4^+$ T cells have potent colitogenic $CD4^+$ T cells to reproduce Th1-mediated colitis in normal recipient SCID mice.

Discussion

In the present study, we showed that $CD4^+CD44^{high}CD62L^-$IL-$7R\alpha^{high}$ T_{EM} cells, but not central-memory T cells and naive T cells, preferentially reside in the BM obtained from Th1-mediated colitic SCID/Rag-$2^{-/-}$ mice induced by the adoptive transfer of $CD4^+CD45RB^{high}$ T cells. Importantly, these resident BM $CD4^+$ T_{EM} cells are attached closely to IL-7–producing stromal cells in the BM, and retain significant potential to induce colitis by the adoptive retransfer into new SCID/Rag-$2^{-/-}$ mice. Of particular importance, we showed here that IL-7 is essential for the development of colitis induced by the adoptive transfer of colitogenic BM $CD4^+$ T_{EM} cells using IL-$7^{-/-}$ × Rag-$1^{-/-}$ and the control IL-$7^{+/+}$ × Rag-$1^{-/-}$ mice. Furthermore, the accumulation

Figure 6. SCID mice transferred with the BM $CD4^+$ T cells obtained from $CD4^+CD45RB^{high}$ T-cell–transferred colitis develop chronic colitis. (*A*) CB-17 SCID mice were injected intraperitoneally with normal splenic $CD4^+CD45RB^{high}$ T cells. Six weeks after transfer mice developed chronic colitis, and $CD4^+$ T cells were isolated from each organ. Doses of 2 × 10^5 BM, MLN, or LP $CD4^+$ T cells were injected into new CB-17 SCID mice. As a negative control, 2 × 10^5 BM $CD4^+$ T cells obtained from normal BALB/c mice also were injected into SCID mice. (*B*) Mice transferred with the colitic BM $CD4^+$ T cells did not gain weight. *$P < .05$. (*C*) Mice transferred with the colitic BM $CD4^+$ T cells showed severe clinical signs of colitis. Data are indicated as the mean ± SEM of 7 mice in each group. *$P < .05$. (*D*) Gross appearance of the colon, spleen, and MLN from mice transferred with the colitic BM $CD4^+$ T cells (*first row*), the normal BM $CD4^+$ T cells (*second row*), the colitic MLN $CD4^+$ T cells (*third row*), or LP $CD4^+$ T cells (*fourth row*). (*E*) Histopathologic comparison of distal colon from mice injected with the colitic BM, the normal BM, the colitic MLN, or the colitic LP $CD4^+$ T cells. Original magnification: *upper*, 40×; *lower*, 100×. (*F*) Histologic scores were determined at 8 weeks after transfer as described in the Materials and Methods section. Data are indicated as the mean ± SEM of 7 mice in each group. *$P < .05$. (*G*) LP and BM $CD4^+$ T cells were isolated from mice injected with colitic BM, normal BM, colitic MLN, or colitic LP $CD4^+$ T cells 8 weeks after transfer, and the number of $CD3^+CD4^+$ cells was determined by flow cytometry. Data are indicated as the mean ± SEM of 7 mice in each group. *$P < .05$. (*H*) Cytokine production by LP $CD4^+$ T cells. IFN-γ and tumor necrosis factor-α concentrations in culture supernatants were measured by ELISA. Data are indicated as the mean ± SD of 6 mice in each group. *$P < .05$.

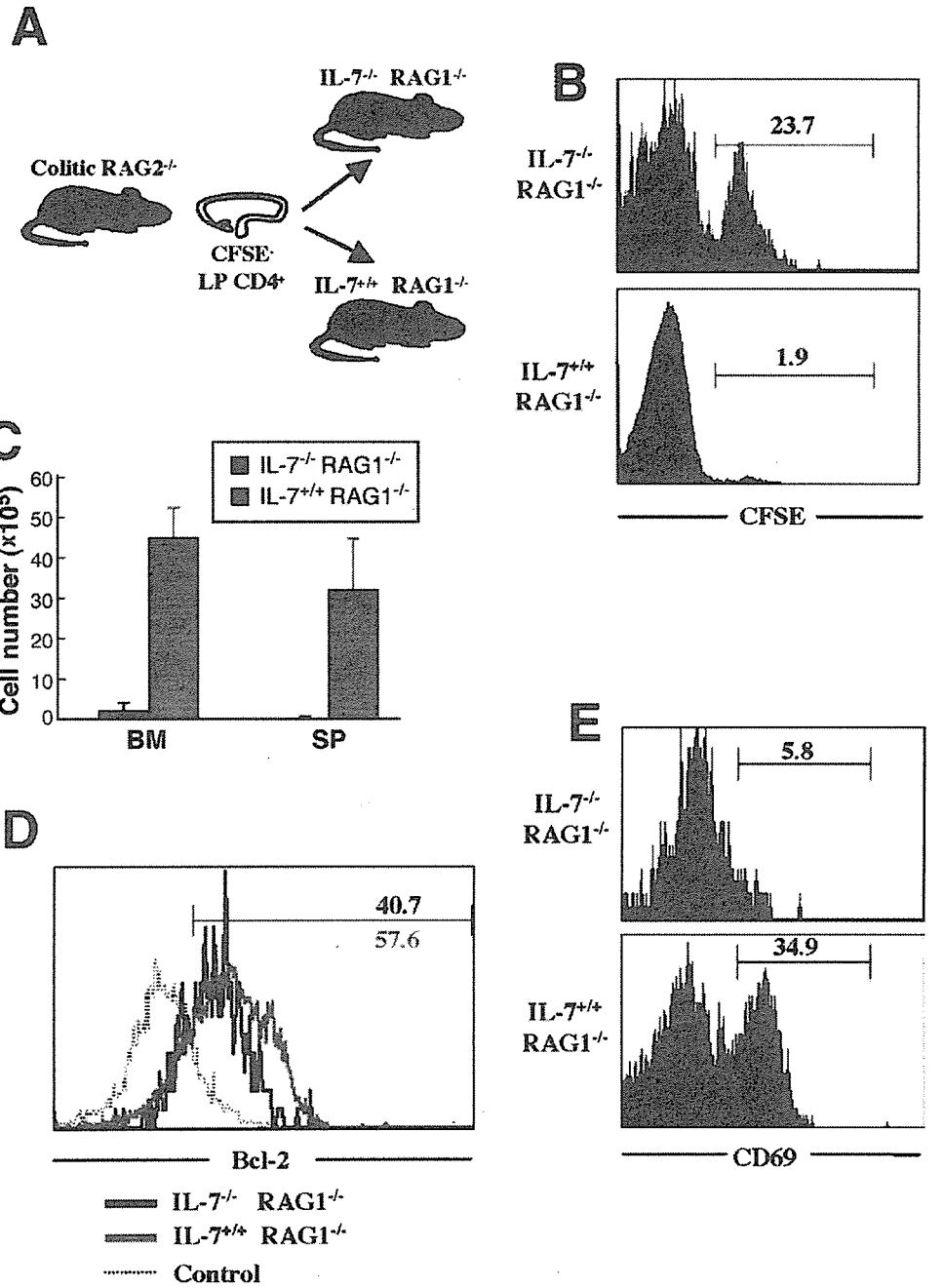


Figure 7. IL-7 is essential for the survival and in part for the cell turnover of colitogenic BM CD4+ T cells. (*A*) C57BL/6 Rag-2$^{-/-}$ mice were injected intraperitoneally with normal splenic CD4+CD45RB^{high} T cells. Six weeks after transfer, the LP CD4+ T cells were isolated. Colitogenic LP CD4+ T cells were labeled with CFSE and adoptively transferred into new IL-7$^{+/+}$ × Rag1$^{-/-}$ or IL-7$^{-/-}$ × Rag1$^{-/-}$ mice. Five days after transfer, CFSE incorporation was determined by flow cytometry. Histograms are gated on CD3+ T cells. (*C*) The BM and spleen (SP) CD4+ T cells were isolated from IL-7$^{+/+}$ × Rag1$^{-/-}$ or IL-7$^{-/-}$ × Rag1$^{-/-}$ mice injected with the colitic LP CD4+ T cells 5 days after transfer, and the number of CD4+ cells was determined by flow cytometry. Data are indicated as the mean ± SEM of 7 mice in each group. *$P < .05$. (*D*) Representative flow-cytometric histograms showing the expression of Bcl-2 in BM CD4+ T cells from IL-7$^{+/+}$ × Rag1$^{-/-}$ or IL-7$^{-/-}$ × Rag1$^{-/-}$ mice injected with the colitogenic LP CD4+ T cells 5 days after transfer from 3 independent similar experiments. (*E*) Representative flow-cytometric histograms showing the expression of CD69 on BM CD4+ T cells from IL-7$^{+/+}$ × Rag1$^{-/-}$ or IL-7$^{-/-}$ × Rag1$^{-/-}$ mice injected with the colitogenic LP CD4+ T cells 5 days after transfer from 3 independent similar experiments.

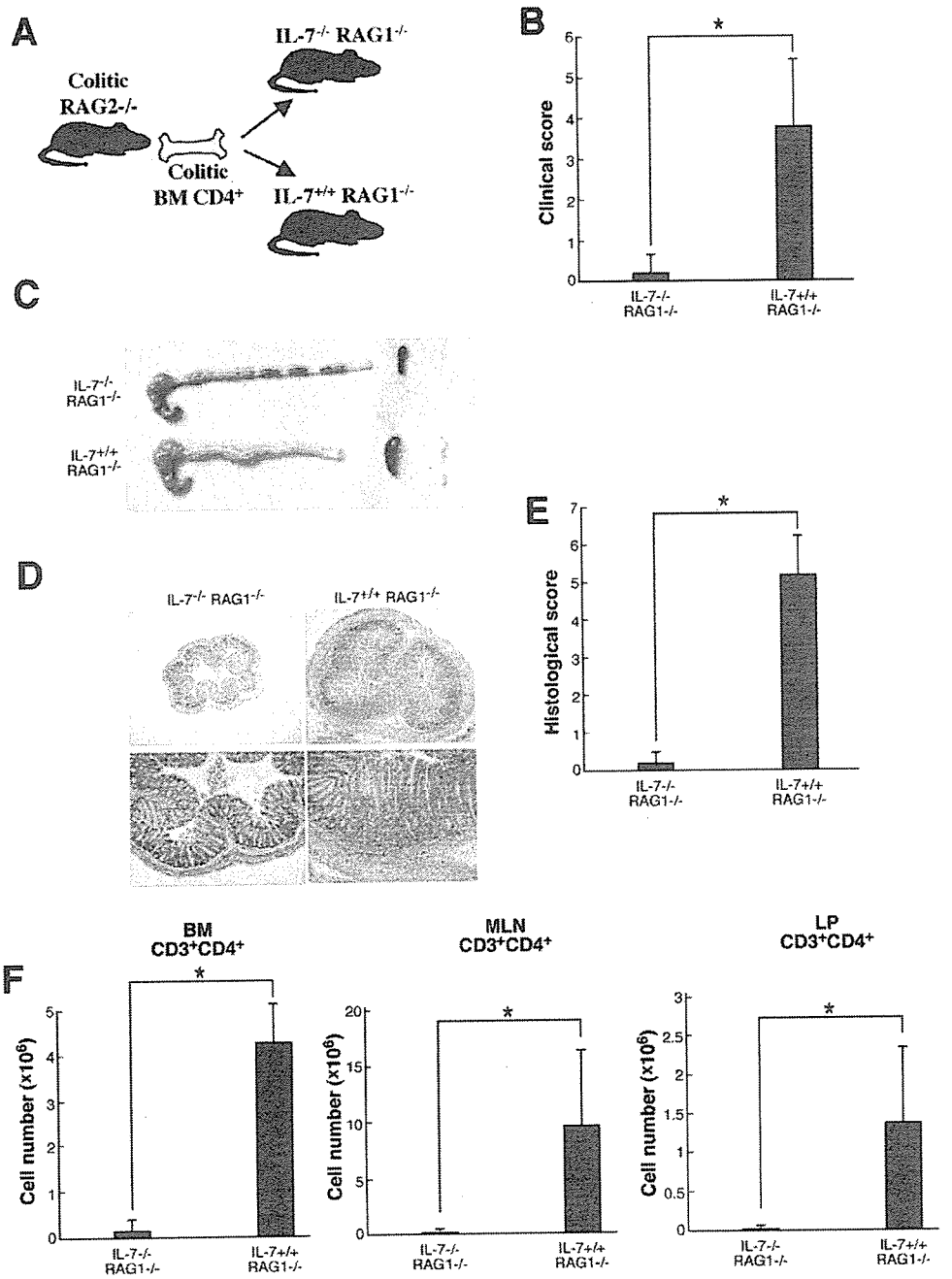


Figure 8. IL-7$^{-/-}$ × Rag-1$^{-/-}$ mice transferred with colitogenic BM CD4$^+$CD44^{high}CD62L$^-$ T_{EM} cells did not develop colitis. (*A*) IL-7$^{+/+}$ × Rag-1$^{-/-}$ (n = 5) and IL-7$^{-/-}$ × Rag-1$^{-/-}$ (n = 5) mice were transferred with colitic BM CD4$^+$ T cells. (*B*) Clinical scores were determined 10 weeks after transfer. Data are indicated as the mean ± SEM of 7 mice in each group. *$P < .005$. (*C*) Gross appearance of the colon, spleen, and MLN from IL-7$^{-/-}$ × Rag-1$^{-/-}$ (*top*) and IL-7$^{+/+}$ × Rag-1$^{-/-}$ (*bottom*) recipients 10 weeks after transfer. (*D*) Histologic examination of the colon from IL-7$^{-/-}$ × RAG-1$^{-/-}$ and IL-7$^{+/+}$ × RAG-1$^{-/-}$ mice transferred with colitogenic BM CD4$^+$ T cells 10 weeks after transfer. Original magnification: *upper*, 40×; lower, 100×. (*E*) Histologic scoring of IL-7$^{+/+}$ × Rag-1$^{-/-}$ and IL-7$^{-/-}$ × Rag-1$^{-/-}$ recipients 10 weeks after transfer. Data are indicated as the mean ± SEM of 7 mice in each group. *$P < .005$. (*F*) BM, LP, and spleen cells were isolated from IL-7$^{+/+}$ × Rag-1$^{-/-}$ and IL-7$^{-/-}$ × Rag-1$^{-/-}$ recipients 10 weeks after transfer, and the number of CD3$^+$CD4$^+$ cells was determined by flow cytometry. Data are indicated as the mean ± SEM of 7 mice in each group. *$P < .0005$.

of BM CD4$^+$ T cells was decreased significantly in IL-7-deficient recipients reconstituted with the colitogenic LP CD4$^+$ T_{EM} cells. Collectively, these findings suggest that the BM CD4$^+$ T_{EM} cells residing in mice with chronic colitis play a critical role as a reservoir for persisting lifelong colitis in an IL-7-dependent manner.

The present data raise the most important question of whether the colitogenic BM CD4$^+$CD44^{high}CD62L$^-$ T

cells can be defined as T_{EM} cells rather than effector T cells in the presence of antigens (Ags), in this case, probably intestinal bacteria. First, we found that these colitogenic BM $CD4^+$ T cells highly expressed IL-7Rα in accordance with the evidence that IL-7Rα is one of memory, but not effector, T-cell markers. Second, it is well known that memory, but not effector, $CD4^+$ T cells are critically controlled by the homeostatic proliferation and the survival by IL-7.[14] Consistent with this, we found that the BM $CD4^+$ T cells were decreased markedly in IL-$7^{-/-}$ × Rag-$1^{-/-}$ mice transferred with the colitogenic LP or BM $CD4^+$ T cells as compared with IL-$7^{+/+}$ × Rag-$1^{-/-}$ recipients. Further, we showed that IL-$7^{-/-}$ × Rag-$1^{-/-}$ mice transferred with the colitogenic BM $CD4^+$ T cells did not develop colitis in contrast to IL-$7^{+/+}$ × Rag-$1^{-/-}$ recipients with colitis. Collectively, these data indicate that the colitogenic BM $CD4^+$ T cells in our colitis model are T_{EM} cells rather than effector T cells.

IL-7 originally was discovered in the BM stromal cells.[23] However, the role for $CD4^+$ T cells in the BM is largely unknown, especially in pathologic conditions, although it has been recognized recently that a high number of antigen-specific $CD8^+$ memory T cells persist in the BM for several months after resolution of acute viral infection.[7,8] Furthermore, recent accumulating evidence suggests that IL-7 is a critical factor for the survival and homeostatic proliferation of memory $CD4^+$ T cells.[14] Thus, we hypothesized that IL-7–producing BM harbors the colitogenic memory $CD4^+$ T cells as a reservoir, causing persistent lifelong colitis. Consistent with this hypothesis, we found that IL-7–expressing cells were scattered throughout the BM and most $CD4^+$ T cells were in close contact with the bodies of IL-7–expressing BM cells in colitic SCID mice induced by the adoptive transfer of $CD4^+CD45RB^{high}$ T cells (Figure 5). However, the possibility cannot be excluded of a recently described novel pathway for dendritic cell migration that allows dendritic cells to collect Ags in peripheral sites and traffic them to the BM to elicit recall responses by the resident BM T cells.[27] This, however, is unlikely in this case because the production of IFN-γ by anti-CD3/CD28- or CBA-stimulated colitic BM $CD4^+$ T cells was significantly lower than that of anti-CD3/CD28- or CBA-stimulated colitic LP $CD4^+$ T cells (Figures 2 and 3), indicating that the BM colitogenic T cells in colitic mice might be indicative of a recent encounter with Ags in the LP, and may migrate into the BM, which is abundant in IL-7, but not in Ags.

In this article we asked how $CD4^+$ memory T cells accumulate in the BM in mice with chronic colitis. Indeed, BM stromal cells can support lymphoid precursor cell differentiation into mature T cells in vitro[28] and in athymic mice in vivo.[29] Mature T cells in the BM are probably immigrants from the blood because T cells normally are produced in the thymus. However, the mechanisms by which in vivo–generated memory cell subsets are recruited to tissues have been difficult to study in the case of polyclonal and physiologic systems rather than the monoclonal T-cell receptor transgenic system because such studies require unattainable numbers of purified cells for in vivo assay. In this study, however, we were able to circumvent this obstacle by using the SCID/Rag-$2^{-/-}$-colitis model induced by the adoptive transfer of $CD4^+CD45RB^{high}$ T cells because a large number of $CD4^+$ T cells infiltrated the colonic LP in this model, and they technically could be isolated in the order of approximately 1×10^7 cells per mouse. By using the present adoptive transfer system, we found that $CD4^+$ T cells resided in the BM from Rag-$1^{-/-}$ mice transferred with colitogenic LP $CD4^+$ T cells at the early time point of 5 days after transfer (Figure 7). We also found that the recovered cell number of BM $CD4^+$ T cells was parallel to that of LP $CD4^+$ T cells in mice given antibiotics without colitis and the control mice with colitis. These results indicate that colitogenic LP $CD4^+$ T cells exit from the gut, and directly migrate into the BM, (Supplemental Figure 1, see supplemental material online at www.gastrojournal.org although further studies will be needed to show direct evidence for this issue.

Although the Ags driving the T-cell immune response in the experimental system of T-cell–induced IBD have not yet been identified with certainty, and thus it is impossible to chase the biological behavior of antigen-specific T cells, overwhelming evidence supports the idea that the triggering factor in this experimental system is of bacterial origin. Furthermore, the present study significantly complements recent reports that BM harbors Ag-specific memory $CD8^+$ T cells.[2,30-31] A recent report has shown very efficient interactions between T cells and dendritic cells in the BM microenvironment.[11] It may be that the similar environment that promotes T-cell priming also triggers homeostatic proliferation and survival of the colitogenic BM T_{EM} cells by IL-7. Perhaps, as has been suggested for plasma cells and Ag-specific $CD8^+$ memory T cells, a unique combination of the cytokine milieu including IL-7 and contact-dependent interactions in the BM supports the colitogenic BM T_{EM} cells. Furthermore, the possibility that other sites, such as MLN and spleen, also might play a role as other reservoirs for colitogenic $CD4^+$ T_{EM} cells, as well as the BM in colitic mice, cannot be excluded. Further studies will be needed to address this issue.

In conclusion, our findings show that a proportion of colitogenic $CD4^+$ T cells in colitic mice may leave peripheral tissues, such as LP and MLN, and gain access to the IL-7–abundant BM via the bloodstream. By using adoptive transfer protocols, we have shown that these BM $CD4^+$ T_{EM} cells possess the ability to induce colitis, suggesting that the colitogenic BM $CD4^+$ T cells residing in colitic mice play a critical role as a reservoir for persisting lifelong colitis and participate in relapses after remissions in IBDs.[17]