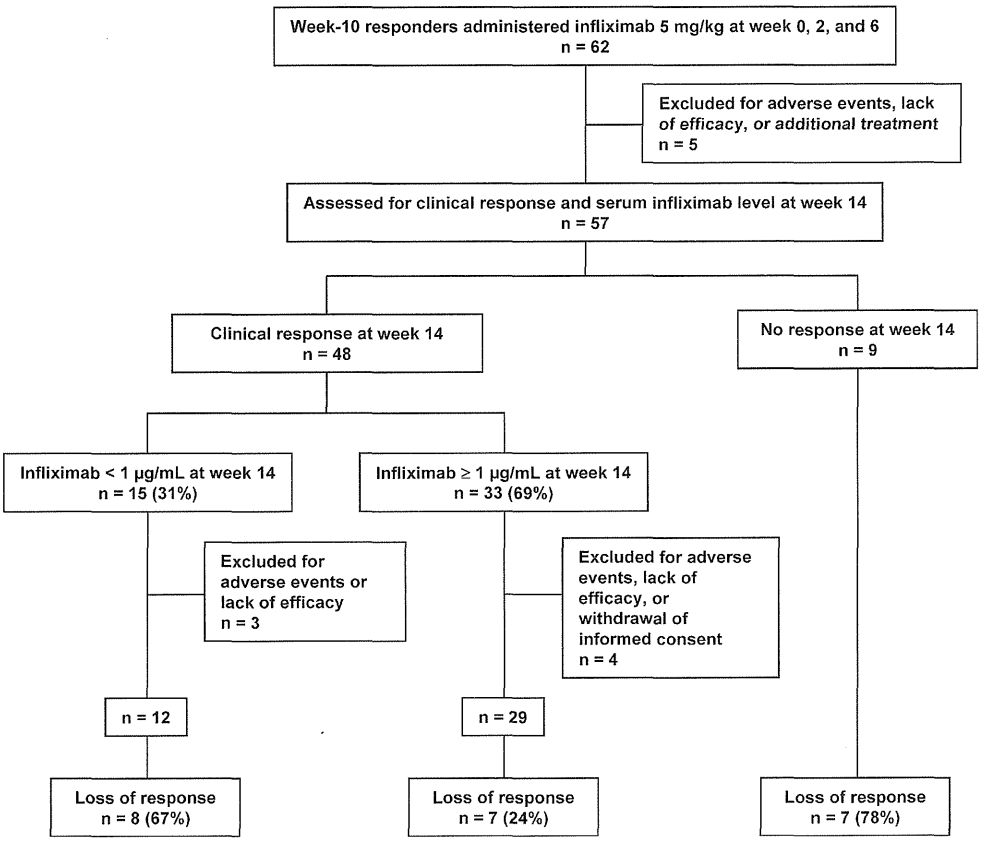

Fig. 1 Flowchart of week-10 responders administered infliximab 5 mg/kg at week 0, 2, and 6

patients whose CDAI and serum trough infliximab level were evaluated at week 14 were included in analyses (Fig. 1). Four of the five excluded patients discontinued treatment due to insufficient efficacy or adverse events up to week 14, while one patient was excluded because of additional treatment for small bowel obstruction that developed prior to week 14.

Decrease in serum infliximab level precedes loss of response

A total of 48 of 57 patients showed a clinical response at week 14, whereas 9 patients showed no response (Fig. 1). Of the 48 responders, the trough infliximab level was <1 μg/mL in 15 (31 %) at week 14. Timing of LOR was examined in 12 of these patients after exclusion of 3 patients in whom treatment was discontinued before the LOR.

At week 14, 10 of 12 patients maintained efficacy despite a trough infliximab level of <1 μg/mL, whereas the remaining 2 patients satisfied the LOR criteria (Fig. 2). The LOR was gradually observed thereafter at weeks 22, 30, 38, and 54 in 2, 1, 1, and 2 patients, respectively, giving a cumulative rate of LOR of 67 % (8/12) at week 54. Among the patients with LOR, a decrease in trough infliximab level preceded LOR in 75 % (6/8). The rate of LOR in patients whose trough infliximab level was ≥1 μg/mL at week 14 was 24 % (7/29) at week 54.

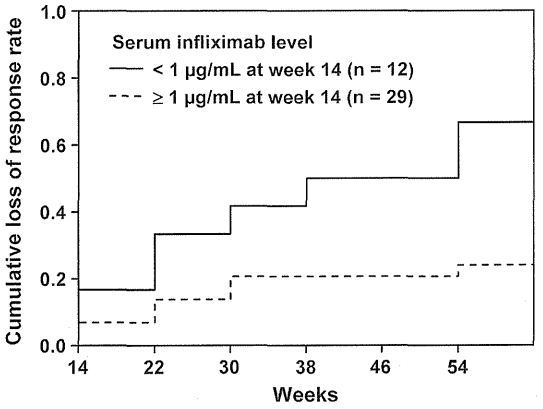

Fig. 2 Changes in the proportion of patients with loss of response in week-14 clinical responders according to serum trough infliximab level at week 14. Of the 48 patients who showed a clinical response at week 14, timing of loss of response was examined in 41 patients after excluding 7 patients in whom treatment was discontinued before the loss of response

Six of 9 non-responders at week 14 met the LOR criteria at week 14, 1 showed LOR at week 30, and the remaining 2 patients did not meet the LOR criteria.

C-reactive protein level shows better performance in detecting a decrease in serum infliximab level

Table 2 shows the AUC of each clinical parameter and laboratory parameter at week 14 by ROC curve analysis using a trough infliximab level of 1 μg/mL as cut-off. The AUC values of CRP and IL-6 were 0.928 and 0.909, respectively, and higher than those of the other parameters. From these two, we selected CRP owing to its ease of measurement in daily clinical practice. The cut-off value for CRP level was set at 0.5 mg/dL, which is the threshold value of the normal range. The accuracy, sensitivity, and specificity for detecting a trough infliximab level at week 14 of <1 μg/mL was 82, 95, and 76 %, respectively.

To investigate a correlation between CRP level and serum trough infliximab level during 8-week interval therapy, the data in patients switched to 4-week interval therapy were excluded from analysis. The CRP level showed a significantly negative correlation with trough infliximab level at weeks 14, 22, 30, 38, 46, and 54 ($p \leq 0.001$, Spearman's rank correlation coefficient) (Fig. 3a). The rho values were −0.606, −0.572, −0.639, −0.597, −0.553, and −0.626, respectively.

A trough infliximab level of ≥1 μg/mL was maintained in more than 80 % of patients whose CRP level was ≤0.5 mg/dL at weeks 14, 22, 30, 38, 46, and 54 (Fig. 3b). On the other hand, trough infliximab level was <1 μg/mL in 60–80 % of patients whose CRP level was >0.5 mg/dL. The difference in the percentage of patients with a trough infliximab level of <1 μg/mL between those with a CRP level of ≤0.5 or >0.5 mg/dL was statistically significant at all time points ($p < 0.05$, Fisher's exact test).

Table 2 Performance of clinical and laboratory data parameters in detecting an infliximab level of <1 μg/mL

	Area under the receiver operating characteristic curve at week 14 ($n = 57$)
Soft/liquid stools score	0.585
Abdominal pain score	0.584
General well-being score	0.653
Hematocrit (male, 47-Hct; female, 42-Hct)	0.644
Percent below standard body weight	0.616
C-reactive protein	0.928
Albumin	0.809
Prealbumin	0.784
Transferrin	0.614
Retinol-binding protein	0.728
Interleukin-6	0.909

No significant different in baseline CRP level was seen between patients treated with [$n = 19$, median (IQR) 1.3 (0.6–3.1) mg/dL] and without corticosteroids [$n = 38$, 1.7 (0.7–4.0) mg/dL].

C-reactive protein is an indicator of serum infliximab level in predicting loss of response

Timing of LOR in patients whose CRP was higher than the normal level

Of the 48 week-14 clinical responders, the CRP level was >0.5 mg/dL at week 14 in 18 patients. After excluding 4 patients who were discontinued before showing LOR, timing of LOR was examined in 14 patients. At week 14, 12 of these patients maintained efficacy despite an increase in CRP level, whereas the remaining 2 patients met the criteria for LOR (Fig. 4). The LOR was gradually observed thereafter at weeks 22, 30, 38, and 54 in 2, 2, 1, and 2 patients, respectively, giving a cumulative rate of LOR at week 54 of 64 % (9/14). An increased CRP level preceding LOR was observed in 78 % (7/9). The rate of LOR at week 54 was 22 % (6/27) for patients whose CRP level was ≤0.5 mg/dL at week 14.

Comparison of timing of LOR, decrease in serum infliximab level, and increase in CRP level

LOR was observed in 22 patients among the analysis population (Fig. 1). Of these 22, 10 patients were excluded from this analysis because they showed LOR when the trough infliximab level was ≥1 μg/mL. Timing of LOR, decrease in trough infliximab level to <1 μg/mL, and increase in CRP level to >0.5 mg/dL were compared in the remaining 12 patients whose trough infliximab level was decreased to <1 μg/mL at some time point during the study.

The LOR was observed at weeks 14, 22, 30, 38, and 54 in 5 (42 %), 2 (17 %), 2 (17 %), 1 (8 %), and 2 patients (17 %), respectively (Fig. 5). Trough infliximab level decreased to <1 μg/mL at week 14 in 11 patients (92 %), and at week 30 in the remaining 1 patient (8 %). The CRP level exceeded 0.5 mg/dL at week 14 in all patients showing LOR. Time to LOR [median (IQR) 22.0 (14.0–32.0) weeks] was significantly longer than both that to a decrease in trough infliximab level to <1 μg/mL [14.0 (14.0–14.0) weeks, $p < 0.05$, Wilcoxon rank test] and that to an increase in CRP level to >0.5 mg/dL [14.0 (14.0–14.0) weeks, $p < 0.01$]. The difference between the time until trough infliximab level decreased to <1 μg/mL and CRP level increased to >0.5 mg/dL was not statistically significant.

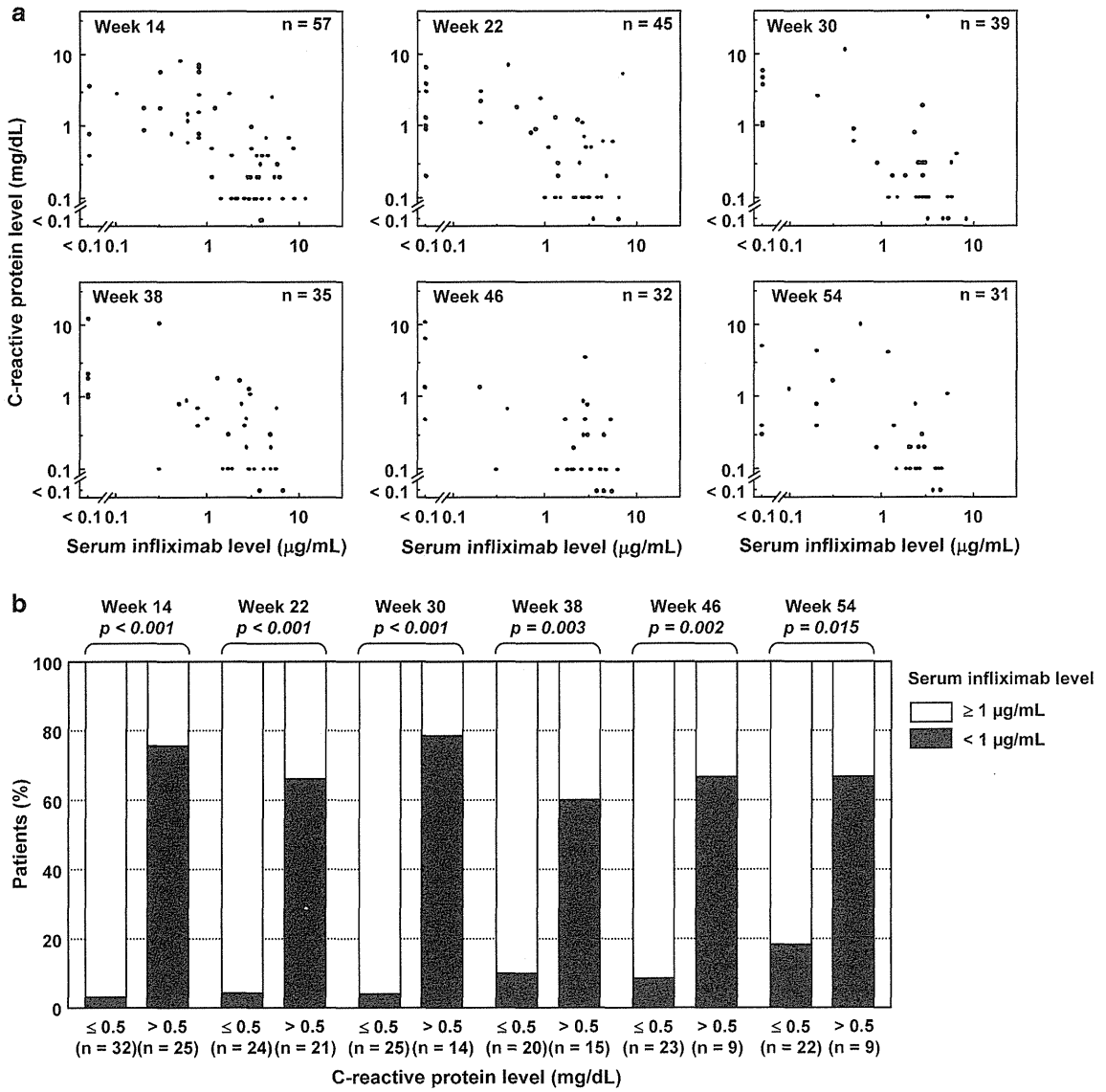

Fig. 3 Relation of C-reactive protein level to serum trough infliximab level. Data were analyzed in patients treated at 8-week intervals. **a** Correlation between C-reactive protein level and serum trough infliximab level. **b** Serum trough infliximab level according to C-reactive protein level. The cut-off value for CRP level was set at 0.5 mg/dL (threshold value of the normal range). Statistical differences in patients with a serum infliximab level <1 and ≥1 μg/mL were calculated by Fisher's exact test

C-reactive protein level is decreased after dose intensification of infliximab

Of the 22 patients who showed LOR, infusion interval was not changed in 3 patients who showed LOR at week 54. In contrast, infusion interval was shortened in 19 patients due to LOR at weeks 14, 22, 30, and 38 in 10, 4, 4, and 1 patient, respectively. Serum infliximab and CRP levels were measured in 17 of these patients after the change in interval.

Median CRP (IQR) level at baseline was 2.1 (0.7–4.1) mg/dL, and decreased to 0.4 (0.1–0.6) mg/dL at week 2. However, it was 2.4 (0.9–3.0) mg/dL at the time of LOR, and decreased again after dose intensification of infliximab to 0.6 (0.3–2.8) mg/dL at 4 weeks. It remained at around 0.5 mg/dL thereafter (Fig. 6).

Discussion

In the present study, we evaluated the hypothesis that trough infliximab level is decreased preceding LOR by analyzing the timing of LOR and a decrease in trough infliximab level to below the threshold (1 μg/mL). We demonstrated that a decrease in serum infliximab level preceded LOR, and could furthermore be easily detected by

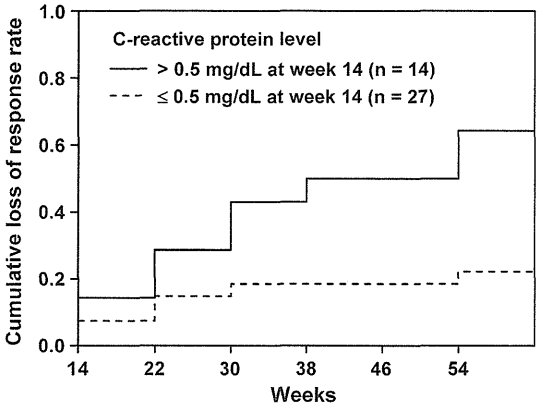

Fig. 4 Changes in the proportion of patients with loss of response in week-14 clinical responders according to C-reactive protein level at week 14. Of the 48 patients who showed a clinical response at week 14, timing of loss of response was examined in 41 patients after excluding 7 patients in whom treatment was discontinued before the loss of response

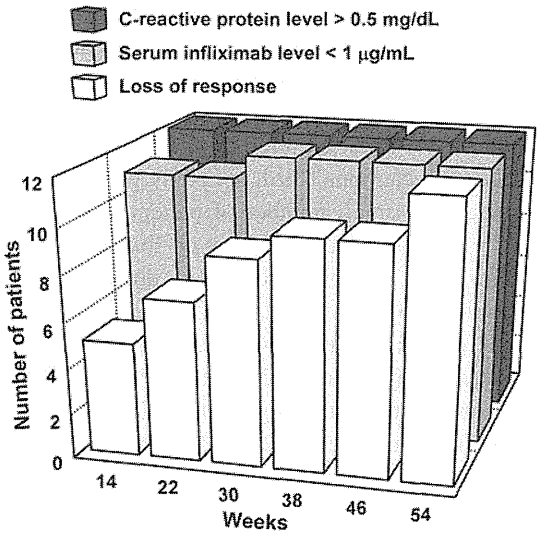

Fig. 5 Comparison of timing of loss of response, decrease in serum infliximab level, and increase in C-reactive protein level. Results are expressed as the number of patients with loss of response, with serum infliximab level <1 μg/mL, and with C-reactive protein level >0.5 mg/dL in patients showing loss of response in whom serum infliximab level decreased to <1 μg/mL at some time point ($n = 12$)

an increase in CRP, in a subset of CD patients treated with infliximab.

Various causes have been ascribed to LOR occurring during treatment with infliximab in patients with CD, many of which were reported to be associated with serum trough infliximab level [5]. Previously, we described that the threshold trough level of infliximab to obtain clinical efficacy is 1 μg/mL [7]. Maser et al., also reported a value of approximately 1 μg/mL in these patients [9]. On the other hand, some of LOR have been shown to be not relevant to trough levels of infliximab [5]. We, therefore, conducted

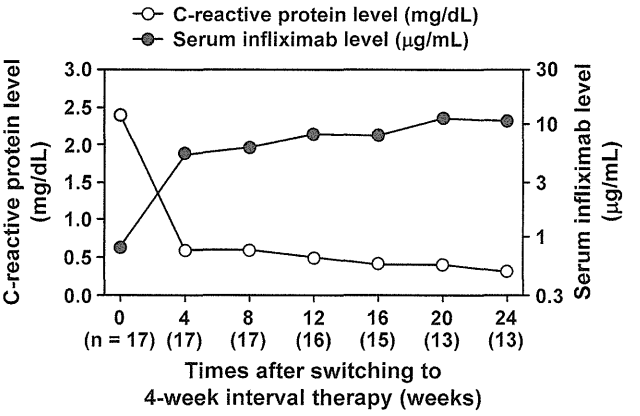

Fig. 6 Changes in C-reactive protein level after switching to 4-week interval therapy. Results are expressed as median ($n = 13$–17). The median value at each point was calculated considering the time of interval switching as baseline (week 0 on graph)

the subgroup analysis in patients whose trough infliximab level was <1 μg/mL with the aim of excluding cases of LOR presumably not associated with the reduction of infliximab level.

In our analysis, 12 patients showed a clinical response despite a trough infliximab level <1 μg/mL at week 14; of these, however, 8 (67 %) eventually experienced LOR by week 54. A decrease in trough infliximab level to <1 μg/mL preceded LOR in 6 patients (75 %). This finding suggests that a decrease in trough infliximab level precedes LOR and that monitoring infliximab level may be useful for predicting subsequent relapse.

The usefulness of monitoring infliximab levels in the treatment of inflammatory bowel disease has been described in our previous [7] and other reports [10, 11]. Afif et al. [10] reported that measurement of infliximab level was clinically useful and that a complete or partial clinical response was observed with dose escalation in 86 % of patients with subtherapeutic infliximab levels. Monitoring infliximab level in daily practice is difficult, however, and indeed not possible in some regions. We therefore used the data of our previous clinical study to identify a useful marker of a subtherapeutic serum trough level of infliximab. In ROC curve analysis using a trough infliximab level of 1 μg/mL as cut-off, AUC was highest for CRP and IL-6.

Similarly to TNF-α, IL-6 is a cytokine that is involved in the inflammatory cascade of CD [12]. The IL-6 levels correlate with the degree of inflammation [13–18], however, is not routinely measured in daily practice, and like serum infliximab may, therefore, be inappropriate as a routine monitoring marker.

The CRP is an acute-phase protein produced by hepatocytes which is induced by TNF-α, IL-6, and interleukin-1β [19]. It serves as a noninvasive marker of inflammation

in inflammatory bowel disease and is frequently measured in routine practice. A correlation between CRP and disease activity or clinical course in CD has been described [20, 21]. Early normalization of CRP levels was related to long-term response during maintenance therapy with infliximab. Consistent with our present results, Jürgens et al. [20] also stated that CRP levels were increased prior to clinical relapse in nearly 70 % of patients. However, both authors did not mention a relationship to serum infliximab level. Our present study is the first to clarify the relationship between a CRP level increase and decrease in serum infliximab level, and that both precede relapse. These results suggest that a decrease in serum trough infliximab level below the effective level, i.e., 1 μg/mL, results in insufficient suppression of TNF-α and IL-6, which subsequently results in an increase in CRP level and exacerbation of clinical symptoms. These findings highlight the critical importance of inducing a constant decrease in inflammation by maintaining serum infliximab levels above the effective level in patients receiving maintenance therapy with infliximab.

Assuming patients with a trough infliximab level below 1 μg/mL to be positive, the accuracy, sensitivity, and specificity of CRP level using the threshold value of normal levels as a cut-off were markedly high. Patients with a trough infliximab level of <1 μg/mL at weeks 14, 22, 30, 38, 46, and 54 were clearly detected by an elevated CRP level. We, therefore, suggest that CRP is a useful marker of a decrease in trough infliximab level to <1 μg/mL.

Fourteen patients showed a clinical response despite an elevated CRP level at week 14; of these, however, 9 (64 %) proceeded to LOR by week 54. A CRP level increase preceded LOR in 7 patients (78 %). This result was similar to that of patients with a decrease in serum infliximab level to <1 μg/mL prior to a LOR. In addition, the timing of LOR, a decrease in trough infliximab level, and an increase in CRP level were compared in patients showing LOR in whom the trough infliximab level decreased to <1 μg/mL. Findings confirmed that the timing of a decrease in the trough infliximab level closely coincided with that of an increase in the CRP level, which were both observed prior to LOR.

The LOR to anti-TNF-α agents is partly explained by antibody formation [5]. Because detectable antibodies to infliximab were not seen in any of our LOR patients ($n = 22$), we were unable to describe the relation between LOR and antibodies to infliximab.

Several limitations of our study warrant mention. The first concerns the threshold of the effective serum level of infliximab and CRP level. Clinical response was lost in 10 patients even when the serum trough infliximab level was ≥1 μg/mL. Of the 7 patients whose infusion interval was shortened and whose CDAI was then subsequently measured, clinical response was achieved in 5 (71 %) and no response in 2 (29 %) at week 54. The threshold of an effective serum level in these 5 responders was considered to be higher than 1 μg/mL and 2 non-responders might show LOR due to factors other than serum infliximab level, which indicates that monitoring using the normal CRP level as a threshold level is not applicable to all patients. The CRP level is normal in some patients with active CD, and was in fact ≤0.5 mg/dL before infliximab treatment in 11 of our 57 patients. Three of these patients experienced LOR, but CRP did not increase, and CRP monitoring in such patients would not be useful. It has been reported that the CRP level at diagnosis was associated with disease location in CD [22], however, in the present study, the disease location did not differ between the 11 patients with normal CRP level and the others (data not shown). Future studies are required to elucidate factors that predict LOR in patients with a higher effective infliximab serum level threshold, in patients with LOR due to factors other than infliximab level, and in patients with normal CRP levels. Second, CRP levels can also be increased by other factors, such as infection [19]. Some patients experienced a transient increase in CRP without a decrease in serum trough infliximab level. Thus, monitoring of CRP level should be performed with caution. Third, our study was a subanalysis with a relatively small number of patients, indicating the need for a larger prospective study on this topic.

In this study, we clarified that a decrease in serum trough infliximab level precedes LOR. Further, a decrease in trough infliximab level to below 1 μg/mL could be easily detected by an elevation in CRP level above the normal range. Our findings suggest that CRP is an indicator of serum infliximab level in predicting LOR. Intensive treatment, such as infliximab dose escalation, by monitoring the CRP level is expected to produce effective and persistent remission in CD. Our results also indicate the need for care in relying on the CDAI, and that this score might require the incorporation of an inflammatory marker such as CRP.

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Conflict of interest T. Hibi has received grant/research support from Ajinomoto Pharmaceuticals, JIMRO, and Mitsubishi Tanabe Pharma. M. Watanabe has received grant/research support and lecture fees from Abbott Japan, Ajinomoto Pharmaceuticals, Asahi Kasei

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Use of capsule endoscopy in patients with Crohn's disease in Japan: A multicenter survey

Motohiro Esaki,* Takayuki Matsumoto,* Kenji Watanabe,† Tetsuo Arakawa,† Yuji Naito,‡ Minoru Matsuura,§ Hiroshi Nakase,§ Toshifumi Hibi,¶ Takayuki Matsumoto,** Sadaharu Nouda,†† Kazuhide Higuchi,†† Naoki Ohmiya,‡‡ Hidemi Goto,‡‡ Sei Kurokawa,§§ Satoshi Motoya§§ and Mamoru Watanabe¶¶

*Department of Medicine and Clinical Science, Graduate School of Medical Sciences, Kyushu University, Fukuoka, †Department of Gastroenterology, Osaka City University, Graduate School of Medicine, Osaka, ‡Molecular Gastroenterology and Hepatology, Kyoto Prefectural University of Medicine, §Department of Gastroenterology and Hepatology, Graduate School of Medicine, Kyoto University, Kyoto, ¶Division of Gastroenterology and Hepatology, Department of Internal Medicine, Keio University School of Medicine, ¶¶Department of Gastroenterology, School of Medicine, Tokyo Medical and Dental University, Tokyo, **Division of Lower Gastroenterology, Department of Internal Medicine, Hyogo College of Medicine, Nishinomiya, Hyogo, ††Second Department of Internal Medicine, Osaka Medical College, Takatsuki, ‡‡Department of Gastroenterology, Nagoya University Graduate School of Medicine, Nagoya, and §§Division of Gastroenterology, Sapporo Kosei General Hospital, Sapporo, Japan

Key words
capsule endoscopy, Crohn's disease, diagnosis, retention.

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Correspondence
Dr Motohiro Esaki, Department of Medicine and Clinical Science, Graduate School of Medical Sciences, Kyushu University, Maidashi 3-1-1, Higashi-ku, Fukuoka 812-8582, Japan. Email: mesaki@intmed2.med.kyushu-u.ac.jp

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Abstract

Background and Aim: Until the approval of patency capsule, capsule endoscopy (CE) has not been routinely applied for the diagnosis of Crohn's disease (CD) in Japan. We aimed to survey current situation of CE use for patients with CD in Japan.

Methods: The nationwide survey of 40 Japanese institutions identified 94 patients with established CD (eCD) and 80 patients with suspected CD (sCD), who were examined by CE. Types and positive rates of mucosal injury under CE and capsule retention rate were investigated. In sCD, final diagnosis after CE was also analyzed.

Results: Patients with eCD comprised 82 patients of ileitis or ileocolitis type, while 12 patients had CD of colitis type. CE identified mucosal injuries in 83 of 94 patients. Eight of 12 patients with eCD of colitis type had ileal lesions under CE, thereby being reclassified as ileocolitis type. In patients with sCD, CE detected mucosal injuries in 58 patients. Linear ulceration and cobblestone appearance were depicted in 22 and 3 patients, respectively, thereby resulting in established diagnosis of CD in 23 patients. Mucosal lesion was not found in 22 patients with sCD, who were diagnosed as not having CD. Capsule retention rate was not statistically different between patients with eCD and those with sCD (7.4% *vs* 6.3%, $P = 1.0$).

Conclusions: CE is useful for the evaluation of small bowel mucosal injuries in Japanese patients with sCD and eCD. Possible intestinal stricture needs to be carefully evaluated before CE even in patients with sCD.

Introduction

Capsule endoscopy (CE) is a minimally invasive diagnostic tool that enables complete visualization of the small bowel mucosa. CE obviously allowed major implications for the diagnosis, therapeutic decision-making, and outcomes in the management of obscure gastrointestinal (GI) bleeding.[1–3] Recently, the availability of CE for other clinical conditions, including hereditary polyposis syndrome,[4,5] malabsorption syndrome,[6,7] and inflammatory bowel diseases,[8,9] have been increasingly reported. In Crohn's disease (CD), a growing body of evidence shows a favorable diagnostic yield of CE ranging between 58% and 71%,[10–13] and a recent meta-analysis confirmed significantly higher diagnostic yields of CE when compared with small bowel radiography, computed tomography (CT) enterography, or magnetic resonance enterography.[14] However, the European Crohn's and Colitis Organisation (ECCO) and the Organisation Mondiale d'Endoscopie Digestive (OMED) consensus recommended a rigorous selection for the application of CE because the risk of capsule retention is increased especially in CD patients with intestinal stenosis.[15]

Unlike in Western countries, established CD (eCD) was a contra-indication for CE in Japan until the recent approval of patency capsule. Thus, the clinical role of CE for the diagnosis of CD remains uncertain in Japan, where its original diagnostic

criteria[16] exist. Furthermore, the diagnostic process in Japan may be different from that in Western countries, especially as to the selection of small bowel examinations. We thus conducted a nationwide survey to identify the role of CE for Japanese patients with suspected or eCD.

Methods

Contents of the questionnaire sheet. As a nationwide survey by the Research Committee of Inflammatory Bowel Disease organized by Japanese Ministry of Labour and Welfare, questionnaire sheets were sent to 62 Japanese institutions majoring in inflammatory bowel diseases in October 2010. The study protocol was approved by the ethical committee at Kyushu University Hospital, and the study was conducted in accordance with the Helsinki Declaration. In addition, the study protocol was also approved by the ethical committee at each institution where CE was performed for patients with eCD, since established CE was a contra-indication for CE in Japan at the time of this survey.

The questionnaire comprised three sections. The initial section included a query regarding the total number of patients examined by CE at the time of this survey. The following two sections contained queries for eCD and for suspected CD (sCD). The information collected in both sections included the number of CE cases, positive cases of small bowel mucosal injury, and cases suffering capsule retention. In eCD, queries regarding the reason for the application of CE and possible contribution of CE in the determination of small bowel involvement were added. In sCD, the number of cases who could confirm the diagnosis of CD under CE, as well as the diagnosis and the number of cases achieving subsequent clinical diagnosis other than CD were further evaluated. Clinical information of this retrospective survey was obtained by reviewing medical records of the patients at each institution.

In order to determine the current position of CE in the diagnosis of CD, the priority of five small bowel procedures (CE, small bowel radiography, anterograde balloon endoscopy [BE], retrograde BE, and abdominal CT) was graded separately for eCD and sCD.

Definition of eCD and sCD. eCD was regarded as the condition, which fulfilled Japanese diagnostic criteria for CD.[16] The diagnostic criteria included linear ulceration, cobblestone appearance, and histologically verified granuloma within GI tract. sCD was regarded as a condition suspected of having CD based on clinical symptoms, laboratory data, or other abdominal imaging modalities, which did not fulfill the Japanese diagnostic criteria.

Types of mucosal injury under CE. The types of mucosal injury under CE were classified into four types. A whitish crater surrounded by mucosal erythema presumably measuring over 5 mm was defined as an ulcer (Fig. 1a). A superficial whitish

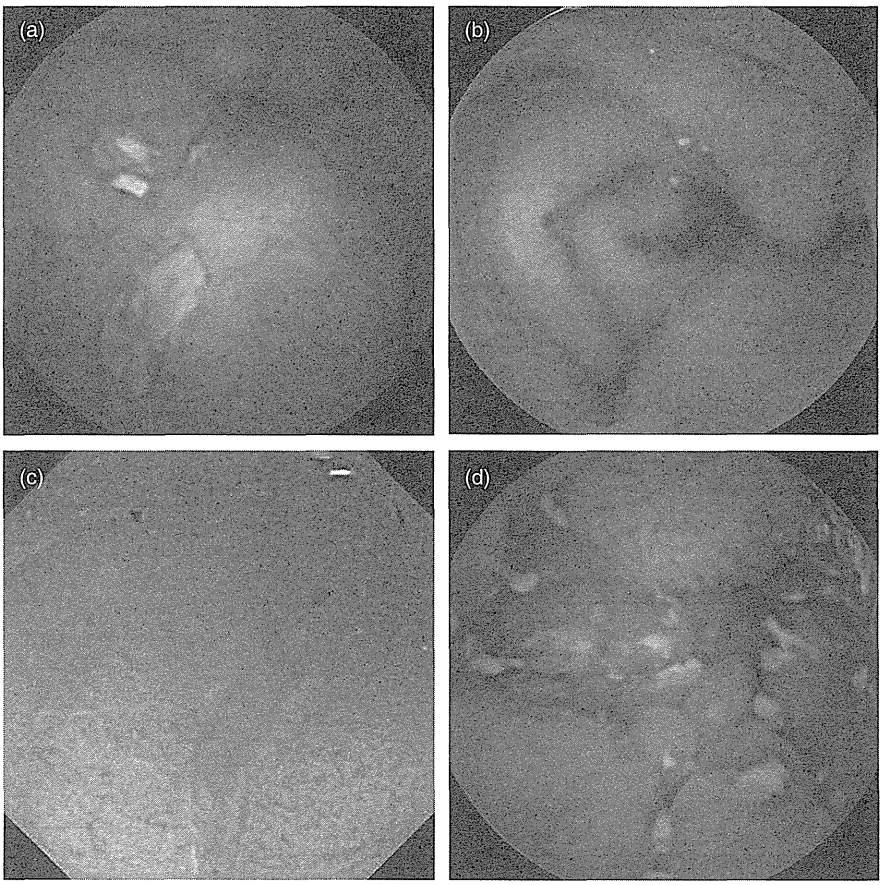

Figure 1 Typical capsule endoscopy (CE) images of mucosal injuries. Mucosal injuries were classified into four types, ulcer (a), erosion (b), linear ulceration (c), and cobblestone appearance (d).

lesion with surrounding erythema less than 5 mm in size was classified as an erosion (Fig. 1b). The diagnosis of linear ulceration (Fig. 1c) and cobblestone appearance (Fig. 1d) was based on the description in the Japanese criteria for the diagnosis of CD. Other types of mucosal injuries included luminal stenosis with or without capsule retention, and an orifice of possible intestinal fistula.

Statistical analysis. All the categorical variables were expressed as frequencies and percentages throughout the manuscript. Capsule retention rates between eCD and sCD were compared using Fisher's exact probability test. A P value of less than 0.05 was regarded as statistically significant.

Results

Of the 62 institutions, 40 institutions (65%) responded to the survey until March 31, 2011, and 5944 cases examined by CE were accumulated. CE was performed in 94 patients with eCD and 80 patients with sCD. All the patients with eCD and sCD underwent CE at the participating institutions after written informed consent for CE was obtained. These patients accounted for 2.9% of all CE examinations. The disease type of eCD was ileocolonic disease in 50 patients (53%), isolated ileal disease in 32 patients (34%), and isolated colonic disease in 12 patients (13%). In eCD, CE was applied for purposes of the possible identification of small bowel mucosal injury in 55 patients (58%), for the evaluation of therapeutic efficacy in 34 patients (36%), and for the determination of bleeding source in 5 patients (6%). In patients with sCD, ileocolonoscopy was performed in 66 patients (83%), esophagogastroduodenoscopy in 61 patients (76%), small-bowel follow-through study in 46 patients (58%), double balloon endoscopy in 6 patients (8%), abdominal CT and ultrasonography each in 5 patients (6%), barium enema examination in 2 patients (3%), and barium meal examination and Ga scintigraphy each in a patient (1%) before CE.

CE findings and capsule retention. Total enteroscopy was achieved under CE in 84 cases (89%) in eCD and 72 cases (90%) in sCD. CE identified small bowel mucosal injuries in 83 cases (88%) in eCD and 58 cases (73%) in sCD. The type and the prevalence of mucosal injury in eCD and sCD are described in Table 1. Erosions and ulcers were frequently identified in both eCD and sCD. CE also detected linear ulcerations in nearly half of the patients with eCD. Furthermore, linear ulceration was found in 22 (28%) of 80 patients with sCD.

In eCD, CE depicted intestinal strictures in 14 patients, seven of whom suffered from subsequent capsule retention. Surprisingly, intestinal strictures were found in six patients with sCD, five of whom manifested capsule retention. Among the 12 patients, the capsule retained in the jejunum in 1, the ileum in 10, and the colon in 1. The retained capsule was collected endoscopically in seven patients; however, surgery was performed in two patients. The retained capsule was naturally excreted in three patients afterwards. Consequently, the incidence of capsule retention in sCD was as high as that in eCD (6.0% *vs* 7.4%, $P = 1.0$). The luminal orifice of intestinal fistula was not found in any patients with eCD or sCD.

Clinical outcomes. CE identified small bowel mucosal injuries in 75 (91%) of 82 patients with eCD of ileitis or ileocolitis type. CD also identified small bowel mucosal injuries in 8 (67%) of 12 patients with eCD of colitis type. By means of CE, the disease type of CD was changed from colitis type to ileocolitis type in eight patients.

Table 2 shows final clinical diagnosis in sCD according to CE results. Of the 58 patients with positive CE, clinical diagnosis was established in 31 patients. The diagnoses included CD, chronic nonspecific multiple ulcers of the small intestine,[17] simple ulcers of the small intestine, intestinal Behçet's disease, and intestinal tuberculosis. However, clinical diagnosis remained obscure in the other 27 patients. The diagnosis of CD was excluded in 22 patients showing negative CE. Consequently, the diagnosis of CD was confirmed in 29% of patients with sCD.

Priority of CE in the diagnosis of CD. Corresponding Japanese gastroenterologists in 28 (70%) of 40 institutions replied that CE is considered to be useful for the evaluation of small bowel mucosal injuries in eCD. However, CE was graded to be unnecessary by 12 institutions. The reasons for such a decision were a lack of clinical advantage ($n = 7$), insufficient inspection ($n = 3$), or possible retention risk ($n = 6$). For sCD, CE was regarded to be useful in 36 (90%) of 40 institutions. However, the diagnostic yield of CE was presumed to be less than that of small bowel radiography and BE in four institutions. The result of the determination for

Table 1 Prevalence of mucosal injury in established or suspected CD

Type of injury	Established CD ($n = 94$)	Suspected CD ($n = 80$)
Erosion	66 (70%)	49 (61%)
Ulcer	53 (56%)	36 (45%)
LU	43 (46%)	22 (28%)
CA	12 (13%)	3 (4%)
Stricture	14 (15%)	6 (8%)
Fistula	0 (0%)	0 (0%)

CA, cobblestone appearance; CD, Crohn's disease; LU, linear ulceration.

Table 2 CE results and final clinical diagnoses in suspected CD

Positive CE	58 (73%)
CD	23
CNSU	3
Simple ulcer	3
Behçet's disease	1
Intestinal tuberculosis	1
Unknown	27
Negative CE	22 (27%)
Ulcerative colitis	2
Irritable bowel syndrome	2
Normal	18

CE, capsule endoscopy; CD, Crohn's disease; CNSU, chronic nonspecific multiple ulcers of the small intestine.[17]

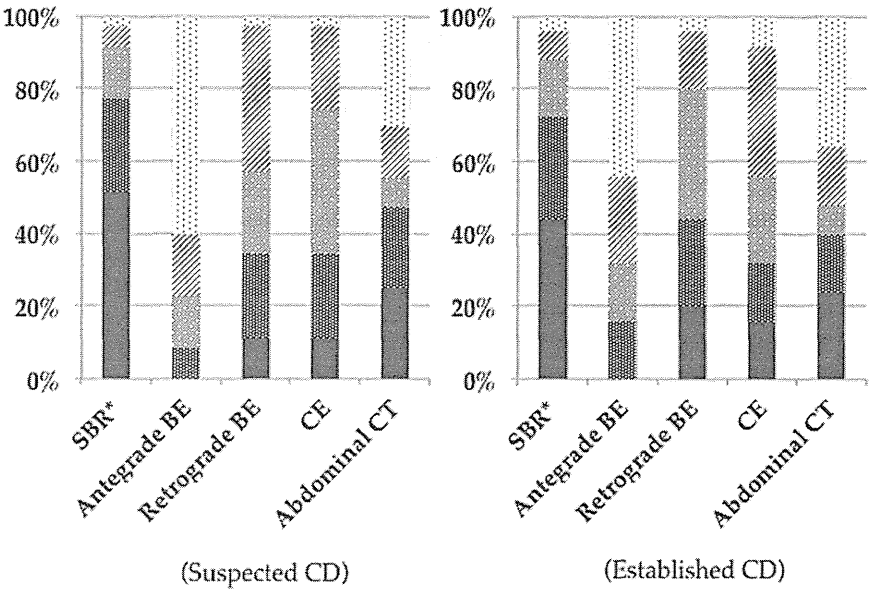

Figure 2 Priority of small bowel procedures in suspected and established Crohn's disease (CD). *SBR; small bowel radiography. †BE; balloon-assisted endoscopy. Priority grade (1 = high to 5 = low): ■, 1; ▦, 2; ▩, 3; ▨, 4; ⁝, 5.

the priority of five procedures in sCD and eCD is shown in Figure 2. The priority of CE was judged to be comparatively low in both sCD and eCD.

Discussion

CD is a chronic inflammatory disorder that may affect any segment of the gastrointestinal tract. Although any part of the gastrointestinal tract can be involved, CD most commonly affects the small bowel, with up to 30% of patients presenting isolated small bowel disease.[18] Because a diagnosis of CD is based on a combination of clinical, biochemical, radiologic, endoscopic, and histologic findings,[1] imaging procedures for the small bowel play a crucial role. With this regard, small bowel radiography (including enteroclysis and double contrast study) has been the most commonly applied in Japan, even though complicated angulation of the small bowel easily interrupts lesion detection, leading possible false negative results. Push enteroscopy and ileocolonoscopy have been also available. However, these procedures can only unveil mucosal lesions of the restricted part of the small bowel.[19,20]

CE enables direct visualization of the entire small bowel. Furthermore because CE also allows the detection of subtle mucosal changes, there has been a growing interest in the use of CE for patients with suspected and eCD.[10–13,20–25] In a recent meta-analysis by Dionisio *et al.*,[14] CE demonstrated a significantly greater diagnostic yield when compared with small bowel radiography, CT enterography, and ileocolonoscopy in patients with suspected or established diagnosis of CD. However, the meta-analysis by Dionisio *et al.*[14] included a larger amount of studies which dealt with small bowel erosions as a positive finding for CD. It thus has been criticized that the result of the meta-analysis is not necessarily representative of the diagnostic ability of CE in CD.[26] In Japan, where the proposed diagnostic criteria of CD are mainly composed of the endoscopic or macroscopic characteristics of the gastrointestinal lesions (linear ulceration and cobblestone appearance),[16] it remains uncertain whether CE is useful for the diagnosis and the evaluation of CD. We thus performed this nationwide survey to clarify the value of CE in Japanese patients with sCD or eCD.

In the present study, CE identified small bowel mucosal injuries in 88% of patients with eCD, while the detection rate of mucosal injuries characteristic of CD was not so high. This was especially the case for patients with known small bowel involvement because the detection rate of mucosal injuries by CE in those patients reached 91%. It thus seems likely that CE has a significant diagnostic yield for Japanese patients with eCD. Of interest, the procedure could identify mucosal injuries even in 67% of CD patients who had been regarded to have an isolated colonic disease. Thus, as has been suggested in an international OMED-ECCO consensus statement,[15] CE may be appropriate for CD patients having apparently isolated colonic disease under other imaging procedures. However, comparative studies with CE and other modalities are required to confirm such indication.

The major role of the imaging procedures in patients with sCD is the detection of characteristic intestinal lesions compatible with the disease. In the present survey, CE identified linear ulcerations or cobblestone appearance, which are two major items in the Japanese diagnostic criteria for CD,[16] in only 28% of the patients. It thus seems possible that the diagnostic value of CE in our patients was apparently lower than that reported previously.[1,14,22,26] However, such a discrepancy is obviously a consequence of obscure criteria for the diagnosis of CD in the previous reports.[1,14,22,26] It thus seems inevitable to evaluate a diagnostic yield of CE for CD under common diagnostic criteria. With this regard, the criteria reported by Mow *et al.*,[27] namely diffuse or multiple (> 3) small bowel ulcerations without a current consumption of nonsteroidal anti-inflammatory drugs, may be a candidate. However, the sensitivity and specificity of the criteria need to be established.

Although CE is a minimally invasive diagnostic tool for small bowel pathology, possible significant complications of the procedure should be considered. In patients with eCD prone to intestinal strictures, capsule retention seems to be a major

complication.[9,15,28,29] In the present study, capsule retention rate in patients with eCD was 7.4%, the value of which is similar to those reported from Western countries.[9,15,28,29] In addition, capsule retention rate in patients with sCD was as high as that in eCD in the present study. This observation contradicts to the OMED-ECCO consensus statement showing less incidence of capsule retention in sCD than in eCD.[15] Such a discordant result may be a consequence of the difference in the definition of sCD. Nevertheless, the present survey strongly suggests that pre-examination with the use of a patency capsule should be seriously considered when applying CE for Japanese patients with sCD.

The present survey has several limitations. First, we could not assess precise characteristics of CE findings including distribution, severity, and alignment of mucosal injuries because the aim of the study was to assess overall diagnostic yield of CE and complication rate. Second, the priority of CE in the diagnosis of CD was subjectively determined. With the introduction of patency capsule, a more objective and comprehensive role of CE for CD would be expected to be analyzed in the near future.

In summary, the present survey demonstrated favorable diagnostic yield of CE in Japanese patients with suspected and eCD, although endoscopists had been reluctant to the use of CE in consideration of the risk of capsule retention and of the lower diagnostic value of typical mucosal injuries in eCD. Therefore, cautious application of CE seems to be a useful tool for the diagnosis of CD, and this may be especially the case for sCD. Appropriate diagnostic criteria of CD by means of CE seem mandatory.

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PLOS ONE

Macrophages and Dendritic Cells Emerge in the Liver during Intestinal Inflammation and Predispose the Liver to Inflammation

Yohei Mikami[1,2], Shinta Mizuno[1], Nobuhiro Nakamoto[1], Atsushi Hayashi[1,3], Tomohisa Sujino[1], Toshiro Sato[1], Nobuhiko Kamada[4], Katsuyoshi Matsuoka[1], Tadakazu Hisamatsu[1], Hirotoshi Ebinuma[1], Toshifumi Hibi[1], Akihiko Yoshimura[2]*, Takanori Kanai[1]*

1 Division of Gastroenterology and Hepatology, Department of Internal Medicine, Keio University School of Medicine, Tokyo, Japan, **2** Department of Microbiology and Immunology, Keio University School of Medicine, Tokyo, Japan, **3** Research Laboratory, Miyarisan Pharmaceutical, Tokyo, Japan, **4** Department of Pathology and Comprehensive Cancer Center, University of Michigan Medical School, Ann Arbor, Michigan, United States of America

Abstract

The liver is a physiological site of immune tolerance, the breakdown of which induces immunity. Liver antigen-presenting cells may be involved in both immune tolerance and activation. Although inflammatory diseases of the liver are frequently associated with inflammatory bowel diseases, the underlying immunological mechanisms remain to be elucidated. Here we report two murine models of inflammatory bowel disease: RAG-2$^{-/-}$ mice adoptively transferred with CD4^{+}CD45RB^{high} T cells; and IL-10$^{-/-}$ mice, accompanied by the infiltration of mononuclear cells in the liver. Notably, CD11b^{-}CD11c^{low}PDCA-1^{+} plasmacytoid dendritic cells (DCs) abundantly residing in the liver of normal wild-type mice disappeared in colitic CD4^{+}CD45RB^{high} T cell-transferred RAG-2$^{-/-}$ mice and IL-10$^{-/-}$ mice in parallel with the emergence of macrophages (Mφs) and conventional DCs (cDCs). Furthermore, liver Mφ/cDCs emerging during intestinal inflammation not only promote the proliferation of naïve CD4^{+} T cells, but also instruct them to differentiate into IFN-γ-producing Th1 cells *in vitro*. The emergence of pathological Mφ/cDCs in the liver also occurred in a model of acute dextran sulfate sodium (DSS)-induced colitis under specific pathogen-free conditions, but was canceled in germ-free conditions. Last, the Mφ/cDCs that emerged in acute DSS colitis significantly exacerbated Fas-mediated hepatitis. Collectively, intestinal inflammation skews the composition of antigen-presenting cells in the liver through signaling from commensal bacteria and predisposes the liver to inflammation.

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* E-mail: takagast@z2.keio.jp (TK); yoshimura@a6.keio.jp (AY)

Introduction

Patients with inflammatory bowel diseases (IBD) are susceptible to developing extraintestinal disorders in the joints, eyes, skin, or liver [1]. For example, primary sclerosing cholangitis (PSC) has been diagnosed in 3.7% of patients with ulcerative colitis [2] and in 3.4% of those with Crohn's disease [3]. The liver and the biliary system are the usual sites for extraintestinal lesions, despite being located between systemic and portal circulations. The portal vein contains a large amount of gut-derived products, such as short-chain fatty acids and microbe-associated molecular patterns (MAMPs) [4]. Although MAMPs, such as LPS from gram-negative commensal bacteria, act as a strong stimulants for antigen-presenting cells (APCs) [5], the liver has been shown to be an immunologically tolerant organ [6,7]. The portal venous tolerance system is regulated by various immune compartments which contain natural killer (NK) cell, natural killer T (NKT) cell, and regulatory T cells, macrophages (Mφ) such as Kupffer cells, and dendritic cells (DCs) [8]. Recent studies have shown that plasmacytoid DCs (pDCs), a subgroup of resident DCs, induce anergy or rapid depletion of antigen-specific T cells in the liver via a CD4^{+} T cell-independent mechanism [9,10]. These findings suggest that regulation and dysregulation of APCs in the liver contribute to liver tolerance and inflammation, respectively. However, the mechanisms of immune regulation and dysregulation in human IBD and experimental colitis models are not yet fully understood. A few studies have focused on the role of gut microbiota and MAMPs in promoting high-fat induced steatohepatitis [11], however, mechanism of immunological dysregulation in the liver during colitis still remains to be elucidated.

Our group has previously reported that increased numbers of Mφs and conventional DCs (cDCs) in experimental colitis models [12] and human IBD [13] have pro-inflammatory characteristics through excess production of IL-12 and IL-23 in response to

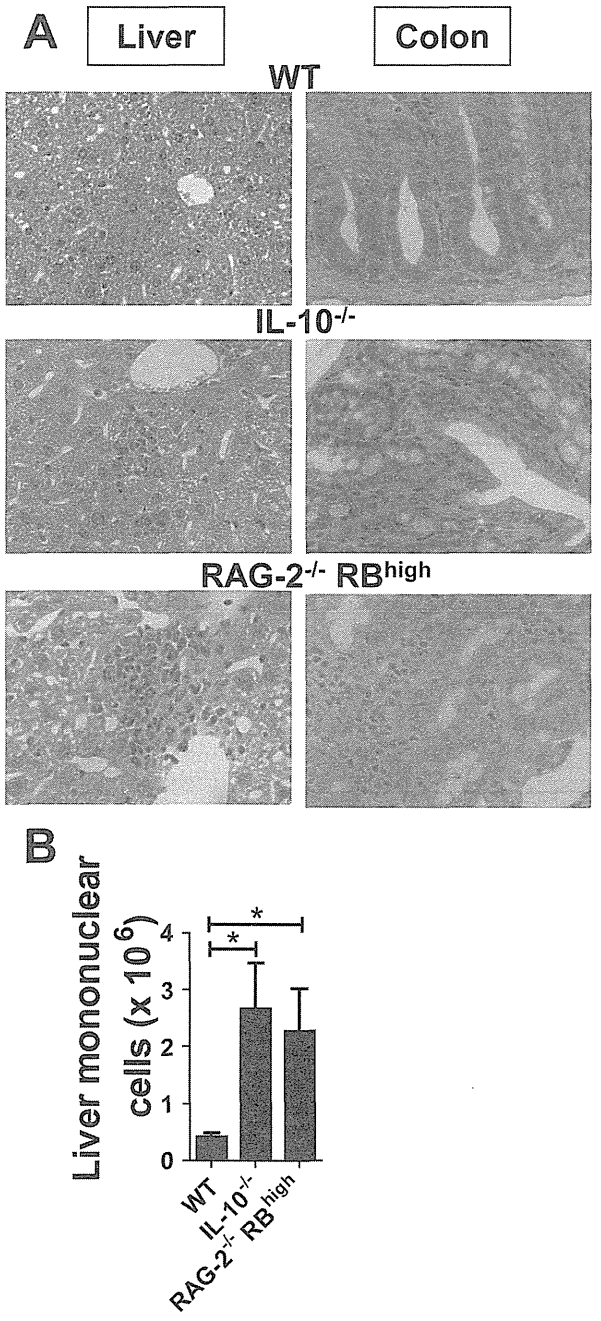

Figure 1. Accumulation of mononuclear cells in the liver develops in chronic colitis models. (**A**) H&E specimens of the liver (left) and colon (right) derived from WT, IL-$10^{-/-}$ mice, and RAG-$2^{-/-}$ RB^{high} mice. Magnification: ×100 (left) and ×400 (right). (**B**) Absolute number of hepatic mononuclear cells. FACS data are representative of three independent experiments. Values are expressed as means ± SEM for each group. WT ($n=5$), IL-$10^{-/-}$ mice ($n=7$) and RAG-$2^{-/-}$ RB^{high} mice ($n=4$). *$P<0.05$.
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bacteria. This leads to the development of Th1 immunity in inflamed intestinal mucosa. More recently, we demonstrated that migrating macrophages contribute to the induction of acute liver inflammation in murine hepatitis models [14].

To clarify hepatic immunological regulation under colitic conditions, we used three murine IBD models: (1) RAG-$2^{-/-}$ mice adoptively transferred with splenic $CD4^+CD45RB^{high}$ T cells from wild-type (WT) mice [15]; (2) an acute dextran sulfate sodium (DSS)-induced colitis model [16]; and (3) IL-$10^{-/-}$ mice [17] that spontaneously develop chronic IBD-like colitis.

Materials and Methods

Mice

WT C57BL/6J mice (8–12 weeks old) were purchased from Japan Clea (Tokyo, Japan). C57BL/6-Ly5.1 mice and RAG-$2^{-/-}$ mice were obtained from Taconic Laboratory (Hudson, NY, USA) and the Central Laboratories for Experimental Animals (Kawasaki, Japan), respectively. IL-$10^{-/-}$ mice were purchased from Jackson Laboratories (Bar Harbor, Maine, USA). Recipient RAG-$2^{-/-}$ mice were used when they were 6 or 14 weeks old. Colitic IL-$10^{-/-}$ mice were used when they were 20 weeks old. Germ-free (GF) C57BL/6-Ly5.2 mice (8 weeks old) were purchased from Sankyo Laboratories (Tokyo, Japan). GF mice were maintained in vinyl isolators within the gnotobiotic facility of the Miyarisan pharmaceutical company (Tokyo, Japan).

All experiments were approved by the Committee on the Ethics of Animal Experiments of Keio University School of Medicine, and conducted in accordance with institutional guidelines and Home Office regulations. [No. 24-026-1].

Adoptive Transfer Studies

For adoptive transfer, $CD4^+$ T cells were isolated from spleen cells of C57BL/6-Ly5.1 mice using the anti-CD4 (L3T4)-MACS system (Miltenyi Biotec, Auburn, CA, USA) according to the manufacturer's instructions. Enriched $CD4^+$ T cells (96–97% pure) were labeled with PE-conjugated anti-mouse CD4 (RM4-5; BD bioscience, San Diego, CA, USA) and FITC-conjugated anti-CD45RB (16A; BD bioscience). $CD4^+CD45RB^{high}$ cells were purified (>98.0%) using a FACS Aria (Becton Dickinson Co.). RAG-$2^{-/-}$ mice (6 weeks old) were injected i.p. with 3×10^5 $CD4^+CD45RB^{high}$ T cells. At 6 weeks post-transfer, these mice developed a wasting disease and colitis as previously reported [15].

For adoptive retransfer, lamina propria (LP) $CD4^+$ T cells were isolated from colon LP mononuclear cells of RAG-$2^{-/-}$ RB^{high} mice using the anti-CD4 (L3T4)-MACS system. Isolated LP $CD4^+$ T cells were injected i.p. into RAG-$2^{-/-}$ mice (RAG-$2^{-/-}$ LP $CD4^+$ mice). Mice were maintained under specific pathogen-free (SPF) conditions in the Animal Care Facility of Keio University.

DSS-induced Colitis Model

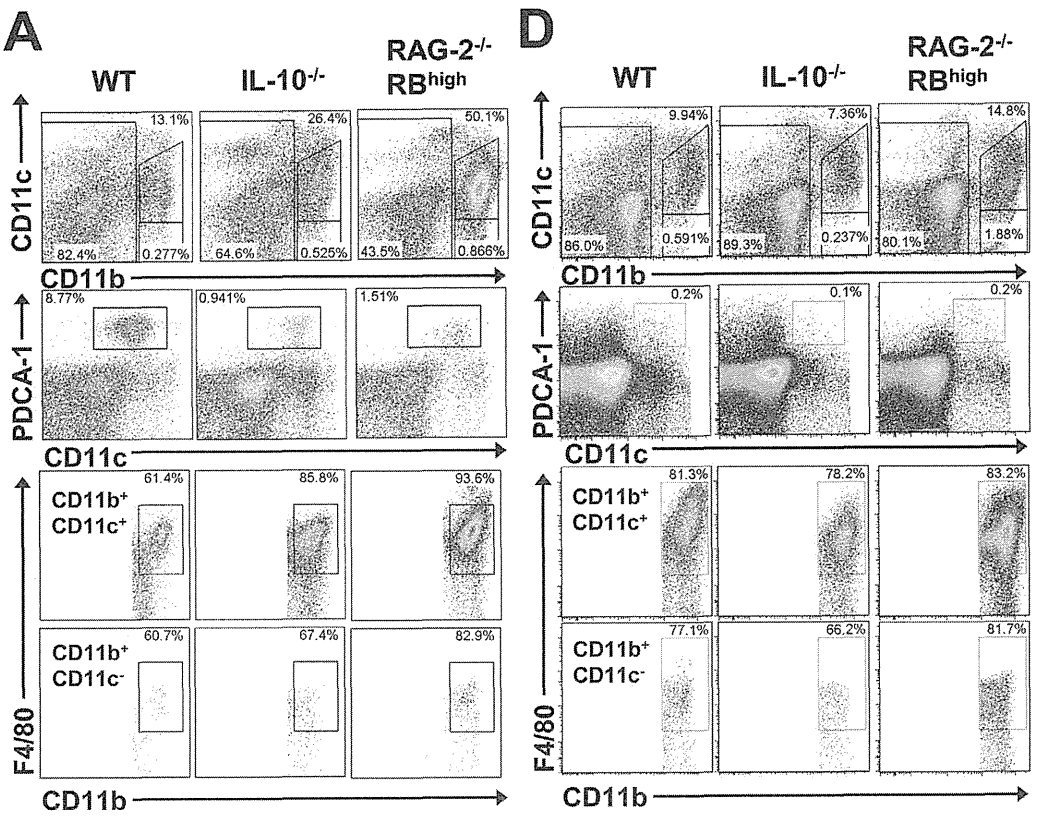
Mice were treated under SPF conditions with 2% DSS (MW 50 kDa; Ensuiko Sugar Refining Co., Yokohama, Japan) in drinking water for 7 days (>4 mice per group). Mice were treated under GF conditions with 1% DSS in drinking water for 7 days followed with regular drinking water for 3 days (>4 mice per group).

Animal Models of Liver Injury

Concanavalin A (Con A, type IV) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Intravenous injections of Con A (20 mg/kg) were administered into the tail vein of animals 10 h before examination. The Fas-activating antibody Jo2 (0.3 mg/kg of body weight; BD bioscience) was injected i.p. and mice were sacrificed 6 h later [18,19].

Preparation of Liver Mononuclear Cells

Liver mononuclear cells were separated from the liver as previously described [20]. Livers were perfused through the portal vein with PBS, then minced and passed through a 100 μm nylon mesh. The filtrate was centrifuged at 50×*g* for 1 min, and the


A
WT
IL-10-/-
RAG-2-/- RBhigh
CD11c
CD11b
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82.4%
0.277%
26.4%
64.6%
0.525%
50.1%
43.5%
0.866%
PDCA-1
CD11c
8.77%
0.941%
1.51%
CD11b+ CD11c+
61.4%
85.8%
93.6%
CD11b+ CD11c-
60.7%
67.4%
82.9%
F4/80
CD11b
D
WT
IL-10-/-
RAG-2-/- RBhigh
CD11c
CD11b
9.94%
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14.8%
80.1%
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PDCA-1
CD11c
0.2%
0.1%
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CD11b+ CD11c+
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CD11b+ CD11c-
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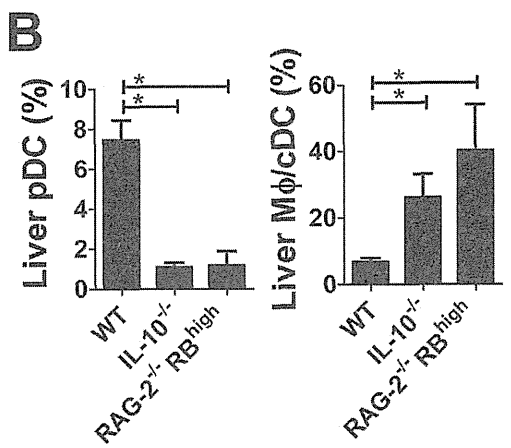
66.2%
81.7%
F4/80
CD11b


B
Liver pDC (%)
10
8
6
4
2
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WT
IL-10-/-
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Liver Mϕ/cDC (%)
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WT
IL-10-/-
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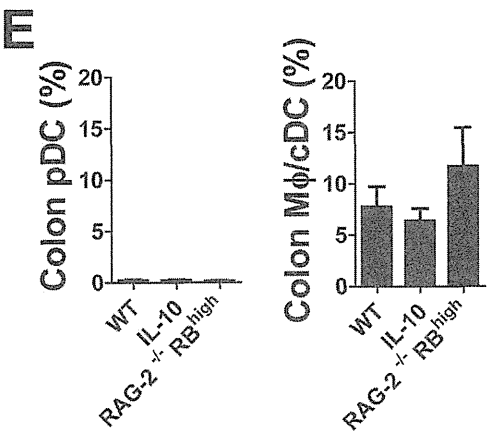
E
Colon pDC (%)
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IL-10
RAG-2-/- RBhigh
Colon Mϕ/cDC (%)
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IL-10
RAG-2-/- RBhigh

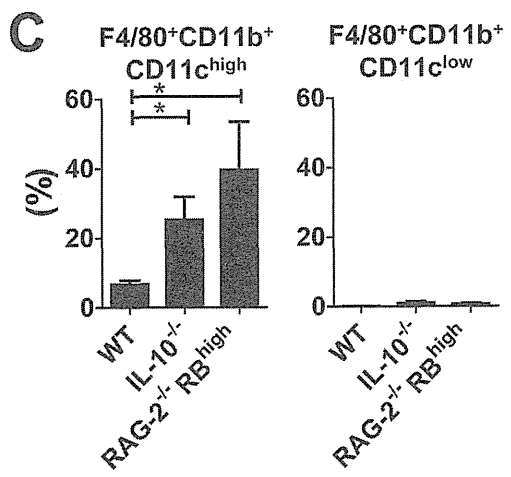
C
F4/80+CD11b+ CD11chigh
F4/80+CD11b+ CD11clow
(%)
60
40
20
0
WT
IL-10-/-
RAG-2-/- RBhigh

Figure 2. Chronic intestinal inflammation was associated with reciprocal changes in the balance of APCs. (**A**) Flow cytometry results related to mononuclear cells isolated from the livers of WT (left column), IL-10$^{-/-}$ (middle), and RAG-2$^{-/-}$ RB^{high} (right) mice. Dead cells were excluded with 7AAD staining, followed by proper use of a FSC/SSC gate. CD11b^{-}, CD11b^{+}CD11c^{high}, and CD11b^{+}CD11c^{low} cells were gated from the cells shown in the first row. CD11b^{-} cells are shown in the second row and PDCA-1^{+}CD11b^{-}CD11c^{int} cells were analyzed. The expression of F4/80 in CD11b^{+}CD11c^{high} cells and CD11b^{+}CD11c^{low} cells are analyzed in the third row and the fourth row. (**B**) Proportion of PDCA-1^{+}CD11b^{-}CD11c^{int} pDCs and F4/80^{+}CD11b^{+} Mφ/cDCs among whole mononuclear cells. (**C**) Proportion of F4/80^{+} CD11b^{+}CD11c^{high} Mφ/cDCs and F4/80^{+} CD11b^{+}CD11c^{low} Mφ/cDCs among whole mononuclear cells. (**D**) Flow cytometry analysis of mononuclear cells isolated from the colons of WT (left column), IL-10$^{-/-}$ (middle), and RAG-2$^{-/-}$ RB^{high} (right) mice. (**E**) Proportion of PDCA-1^{+}CD11b^{-}CD11c^{+} pDCs and F4/80^{+}CD11b^{+}CD11c^{-} Mφ/cDCs among whole mononuclear cells. FACS data are representative of three independent experiments expressed as means ± SEM for each group. WT ($n=4$), IL-10$^{-/-}$ ($n=4$) and RAG-2$^{-/-}$ RB^{high} ($n=3$) mice. *$P<0.05$.
doi:10.1371/journal.pone.0084619.g002

supernatant washed once. Cells were suspended in Histopaque solution (Sigma-Aldrich) and overlaid on HBSS. After centrifugation (780×*g* for 20 min), cells were collected from the upper phase.

Preparation of LP Mononuclear Cells

Cell isolation was performed as previously described [21]. Dissected colon mucosa was incubated with Ca^{2+}, Mg^{2+}-free HBSS containing 1 mM DTT (Sigma-Aldrich) and 5 μM EDTA (Gibco) for 30 min, then treated with 3 mg/ml collagenase (Roche Diagnostics GmbH, Germany) and 0.01% DNase (Worthington Biomedical Co., Freehold, NJ, USA) for 1 h. Cells were pelleted twice through a 40% isotonic Percoll solution and then subjected to Ficoll-Hypaque density gradient centrifugation (40%/75%).

Histological Examination

Liver and colon were fixed in 10% formalin and embedded in paraffin. Sections were stained with H&E and then examined. Histological examination of acute colitis was performed as described previously [22]. Briefly, histological activity score was assessed as the sum of three parameters as follows: extent, 0–3 (0, none; 1, mucosa; 2, mucosa and submucosa; 3, transmural); inflammation, 0–3 (0, none; 1, slight; 2, moderate; 3, severe); crypt damage, 0–4 (0, none; 1, basal 1/3 lost; 2, basal 2/3 lost; 3, only surface epithelium intake; 4, entire crypt and epithelium lost). The score of each parameter was multiplied by a factor of 1–4 (1, 0–25%; 2, 26–50%; 3, 51–75%; 4, 76–100%) according to the percentage of epithelial involvement.

Flow Cytometry

After blocking with anti-FcR (CD16/32, BD bioscience) for 20 min, cells were incubated with specific mAbs at 4°C for 30 min. The following mAbs were used: anti-mouse CD3e-APC-Cy7; anti-CD4-PE-Cy7; anti-NK1.1-APC; anti-CD11b-PE-Cy7; anti-CD11cFITC; 7-AAD; anti-PDCA-1-APC; anti-CCR9-PE; anti-IFN-γ-FITC; and anti-IL-17-APC (eBioscience, BD bioscience). Background fluorescence was assessed by staining with irrelevant anti-rat isotypes (BD bioscience). Stained cells were analyzed by flow cytometry (FACS Canto II, Becton Dickinson Co.) and data analyzed using FlowJo software (Tree Star Inc.) [12].

Quantitative RT-PCR (qPCR)

All qPCR assays were performed as described previously [14]. RNA was extracted from LP mononuclear cells using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and cDNA was synthesized from 100 ng of total RNA using TaqMan® Reverse Transcription Reagents (Applied Biosystems, Foster City, CA, USA). Reverse transcription was performed at 25°C for 10 min, 48°C for 30 min, and then 95°C for 5 min. cDNA was analyzed by qPCR using TaqMan® Universal PCR Master Mix (Applied Biosystems) in an Applied Biosystems StepOne™/StepOnePlus™ Real-Time PCR System. Cycling conditions for PCR amplification were 50°C for 2 min and 95°C for 10 min, followed by 40 cycles of 95°C for 10 s, then 60°C for 1 min. Relative quantification was achieved by normalizing to the β-actin gene (Applied Biosystems). The following probes were purchased from Applied Biosystems: *Ifng* (99999071_m1), *Tnf* (99999068_m1) and *Actb* (01205647_g1).

In vitro Proliferation Assays

APCs, PDCA-1^{+} pDCs from the livers of C57BL/6 mice, CD11b^{+} Mφs from the inflamed livers of Con A-injected C57BL/6 mice (Con A Mφs), IL-10$^{-/-}$ mouse Mφs, and DSS-treated C57BL/6 mouse Mφs (DSS Mφs) were isolated using a FACS Aria (Becton Dickinson Co.). Enriched naïve CD4^{+} splenocytes obtained from OT-II mice were sorted using a CD4^{+} CD62L^{+} T Cell Isolation Kit II (Miltenyi Biotech, Auburn, CA, USA) and labeled with 1 mM CFSE (Molecular Probes, Eugene, OR, USA) for 10 min at 37°C, followed by the addition of 1.0 ml of FCS for 2 min and washed three times in PBS. CFSE-labeled CD4+ naïve cells (1×10^5 cells/well) were co-cultured with pDCs or Mφs (2×10^4 cells/well) in 96-well round-bottom plates for 72 h in the presence of OVA peptides (1 μM). After incubation, cells were collected, incubated with anti-CD4-PE-Cy7 and anti-CD3e-APC-Cy7 and analyzed by FACS; 7-AAD was added to exclude dead cells. Proliferation analysis is based on division times of CFSE^{+}CD4^{+} T cells.

Unlabeled CD4^{+} naïve T cells (1×10^5 cells/well) were also co-cultured with pDCs or Mφs (2×10^4 cells/well) for 120 h in the presence of OVA peptides followed by incubation with anti-IFN-γ and/or anti-IL-17 mAbs, and then treated with a Cytofix/Cytoperm kit (BD bioscience). Culture supernatant was collected and analyzed with the BD™ Cytometric Beads Array Mouse Th1/Th2/Th17 Cytokine Kit (Becton Dickinson Co.).

Statistical Analysis

Results are expressed as mean ± SEM. Data groups were analyzed with GraphPad Prism using Tukey-Kramer test and Student's *t*-tests. A *P*-value less than 0.05 was considered statistically significant.

Results

Accumulation of Mononuclear Cells was Induced in the Liver of Mice with Chronic Colitis

To investigate hepatic immunological regulation in the colitic condition, we first used two murine IBD models, RAG-2$^{-/-}$ mice adoptively transferred with splenic CD4^{+}CD45RB^{high} T cells from WT mice (RAG-2$^{-/-}$ RB^{high} mice) and IL-10$^{-/-}$ mice. Consistent with previous reports [15], RAG-2$^{-/-}$ RB^{high} mice showed severe colitis, and infiltration of mononuclear cells in the portal vein area of the liver (Fig. 1A). This was not observed in WT mice. IL-10$^{-/-}$ mice spontaneously developed colitis, characterized by prominent epithelial hyperplasia with leukocyte infiltration into the liver (Fig. 1A). Consistently, the absolute number of liver

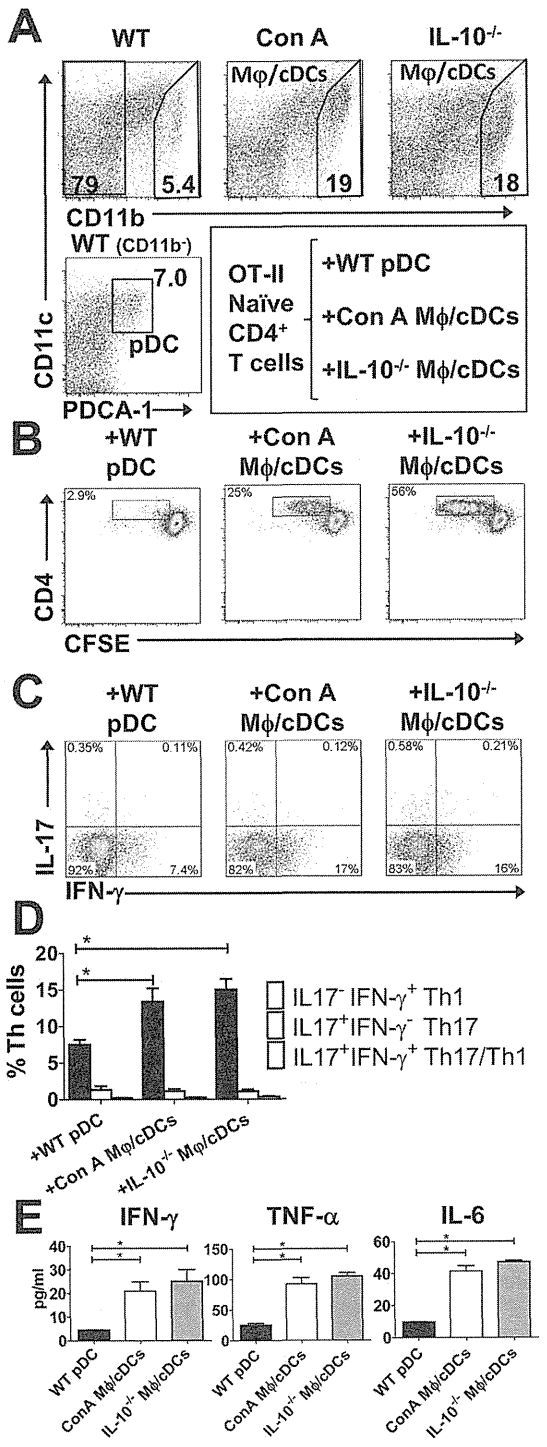

Figure 3. Hepatic Mφ/cDCs cells under colitic conditions induce a Th1 inflammatory response. (**A**) FACS analysis of PDCA-1$^+$CD11b$^-$CD11c^{int} pDCs from the livers of WT (left column) mice. We also analyzed CD11b$^+$CD11c$^-$ Mφs from the livers of ConA-treated (middle) and IL-10$^{-/-}$ (right) mice, respectively. Dead cells were excluded with 7AAD staining. (**B**) Proliferation of naïve CFSE-labeled splenic CD4$^+$ T cells from OT-II mice, and co-cultured WT pDCs, ConA Mφs, or IL-10$^{-/-}$ Mφs in the presence of OVA. Dead cells were excluded with 7AAD staining and CD4$^+$ T cells gated on CD3$^+$ CD4$^+$ cells are shown (B and C). Data are representative of three independent experiments. (**C**) Intracellular IFN-γ and IL-17A expression in CD4$^+$ T cells co-cultured with WT pDCs, ConA Mφs, or IL-10$^{-/-}$ Mφs in the presence of OVA. Data are representative of three independent experiments. (**D**) Proportion of IFN-γ$^+$IL-17A$^-$, IFN-γ$^-$IL-17A$^+$, and IFN-γ$^+$IL-17A$^+$ cells among the Th cell population. (**E**) Cytokine concentrations in the culture supernatant of OT-II CD4$^+$ T cells that were co-cultured with WT pDCs or ConA Mφs. Data are representative of three independent experiments. Each experiment was performed using duplicate samples. *P<0.05.

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mononuclear cells in both colitis models was significantly increased when compared with age-matched C57BL/6 mice (Fig. 1B). Liver enzymes (aspartate aminotransferase and alanine aminotransferase) demonstrated no significant changes between WT mice and the two colitis groups (data not shown).

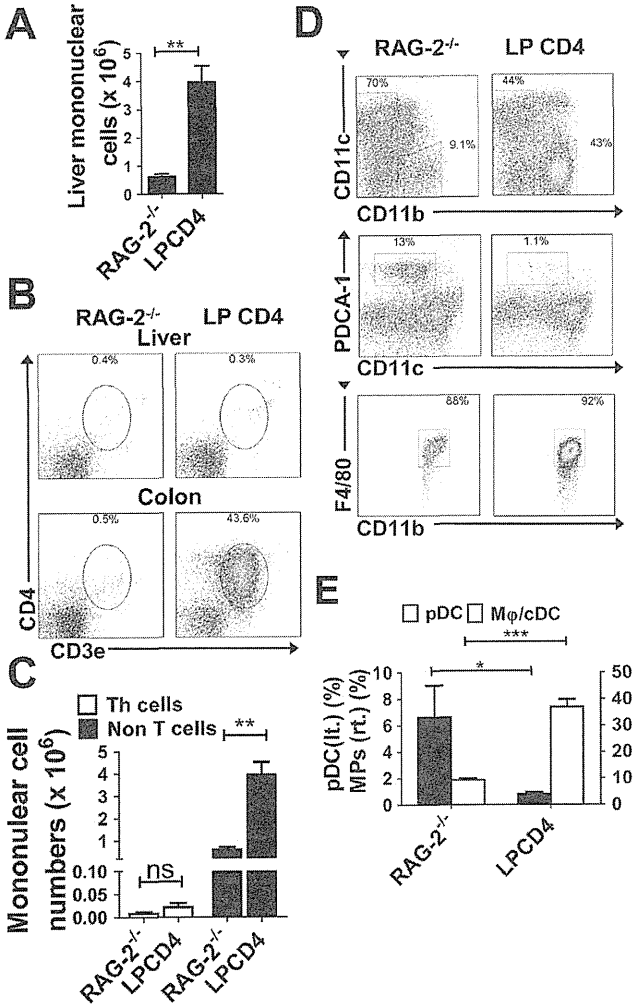

Figure 4. Immune dysregulation in the liver independent of T cell accumulation in the liver. (**A**) Numbers of hepatic mononuclear cells. Data are presented as the mean ± SEM for each group. RAG-2$^{-/-}$ mice (n=4) and RAG-2$^{-/-}$ LP CD4$^+$ mice (n=4). (**B**) Representative data from flow cytometry analysis of Th cells in each organ. Dead cells were excluded by 7AAD staining. (**C**) Numbers of hepatic CD3$^+$ CD4$^+$ Th cells and non-T cells. (**D**) Representative data from flow cytometry analysis of pDCs and Mφs in the liver of each experimental group. Dead cells were excluded using 7AAD staining. Scatter plots for CD11b$^-$CD11c^{int} and CD11b$^+$CD11c$^-$ cells are shown in the middle and bottom rows, respectively. (**E**) Proportion of PDCA-1$^+$CD11b$^-$CD11c^{int} pDCs and F4/80$^+$CD11b$^+$CD11c$^-$ Mφs among whole mononuclear cells. Data are representative of three independent experiments. Values are presented as the mean ± SEM from seven mice in each group. *P<0.05, **P<0.01, ***p<0.005.

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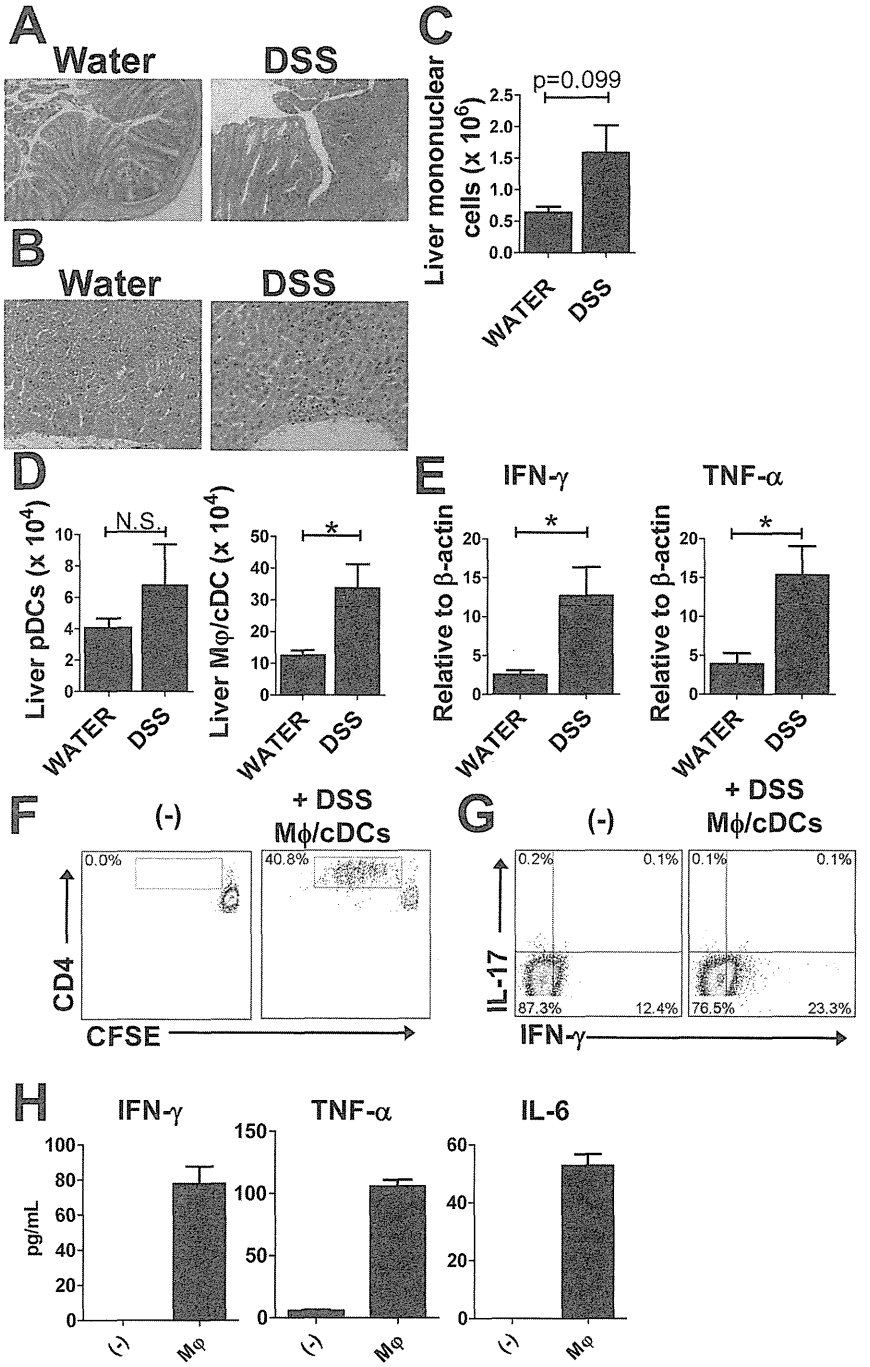

Figure 5. Accumulation of liver macrophages in acute colitis models. (**A**) H&E specimens of the colon taken from mice treated with water (left) or 2% DSS (right). Magnification, ×40 (**B**) H&E specimens of livers from mice treated with water (left) and DSS (right). Magnification, ×100 (**C**) The number of liver mononuclear cells from water- and DSS-treated mice. (**D**) The absolute number of PDCA-1^{+}CD11b^{-}CD11c^{int} pDCs and CD11b^{+}CD11c^{-} Mφs among whole mononuclear cells. Data are representative of three independent experiments. (**E**) Levels of mRNA transcripts for IFN-γ, TNF, and IL-6 in the liver. Values are presented as the mean ± SEM for each group ($n=4$, water-treated group; $n=5$, DSS-treated group). *$P<0.05$. N.S., no significant difference. (**F–H**) Hepatic DSS Mφs induce a Th1 inflammatory response. (**F**) Proliferation of naïve CFSE-labeled splenic CD4^{+} T cells. (**G**) Intracellular IFN-γ and IL-17A expression in naïve CFSE-unlabeled splenic CD4^{+} T cells from OT-II mice that were co-cultured with or without hepatic Mφs from DSS-treated WT mice in the presence of OVA. Dead cells were excluded with 7AAD staining, and CD4^{+} T cells gated on CD3^{+} CD4^{+} cells are shown. Data are representative of two independent experiments. (**H**) Representative cytokine concentrations in culture supernatants from two independent experiments. Each experiment was performed using duplicate samples.
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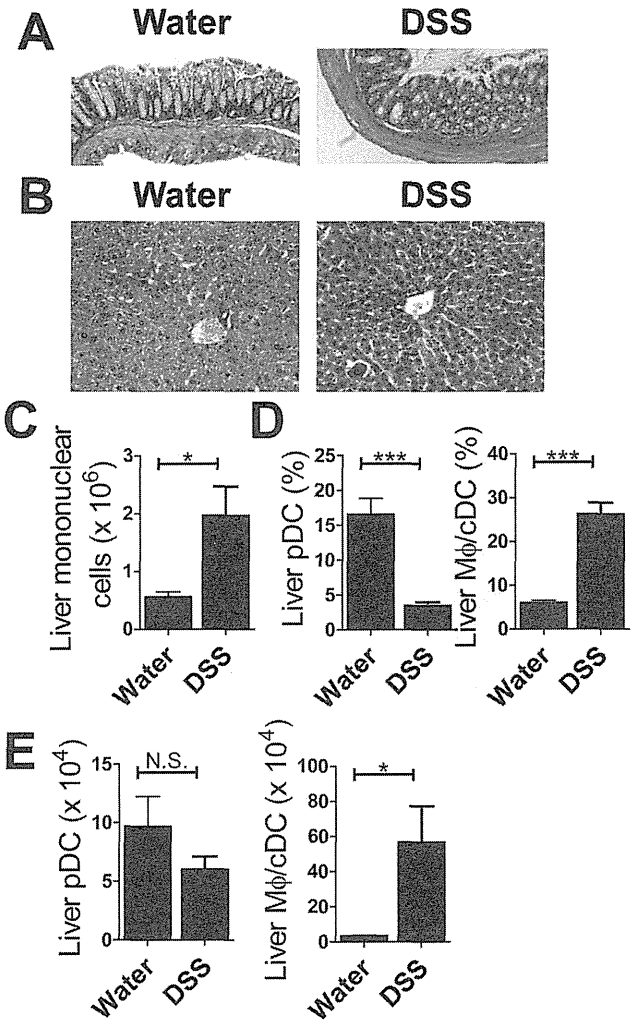

Figure 6. Hepatic infiltration of Macrophages are obserbed in DSS-treated RAG-2$^{-/-}$ mice. (**A**) H&E specimens of colon from mice treated with water (left) or 4% DSS (right). Magnification, ×100 (**B**) H&E specimens of liver from water- (left) and DSS-treated (right) mice. Magnification, ×200 (**C**) The number of liver mononuclear cells for each group of mice. (**D**) Proportion and (**E**) absolute number of PDCA-1^{+}CD11b^{-}CD11c^{int} pDCs and CD11b^{+}CD11c^{-} macrophages among whole mononuclear cells. Data are representative of two independent experiments. Values are presented as the mean ± SEM for each group ($n=4$, water-treated group; $n=4$, DSS-treated group). *$P<0.05$. N.S., no significant difference.
doi:10.1371/journal.pone.0084619.g006

Chronic Colitis is Associated with APC Balance in the Liver

Since it has been reported that Mφ/cDCs and pDCs represent subgroups of APCs differentiated from Mφ/DC precursors [23], we further investigated the composition of APCs in the liver. Analysis of flow cytometry data revealed that the proportion of CD11b^{+}CD11c$^{high/low}$ Mφ/cDCs in the livers (Fig. 2A, first row) of RAG-2$^{-/-}$ RB^{high} and IL-10$^{-/-}$ mice was significantly increased when compared with WT mice. Almost all CD11b^{+}CD11c$^{high/low}$ Mφ/cDCs expressed F4/80 (Fig. 2A, third and fourth rows), therefore we classified them as mononuclear phagocyte system cells. In contrast, the proportion of CD11b^{-}CD11c^{low}PDCA-1^{+} pDCs in the livers of WT mice was significantly higher than those in RAG-2$^{-/-}$ RB^{high} and IL-10$^{-/-}$

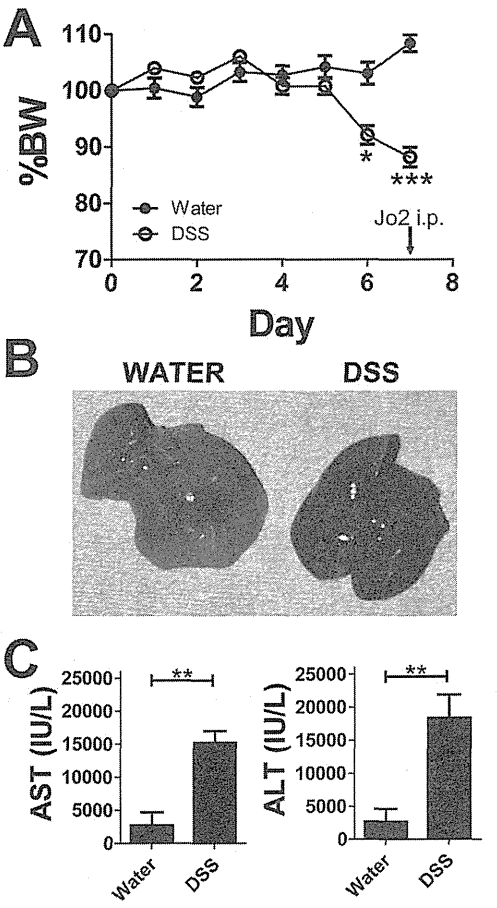

Figure 7. Newly recruited macrophages in the liver during colitis predispose it to inflammation. WT mice were treated under SPF conditions with 2% DSS for 5 days and subsequently with water for 2 days ($n=5$ mice per group). The Fas-activating antibody, Jo2, was injected i.p. into mice. (**A**) Changes in body weight are expressed as a percentage of original weight. Values are presented as the mean ± SEM for each group. Data are representative of two independent experiments. (**B**) Macroscopic view of livers from water- (left) and DSS-treated mice. (**C**) Levels of aspartate aminotransferase (left) and alanine aminotransferase (right) in water- and DSS-treated mice 6 h after Jo2 injection.
doi:10.1371/journal.pone.0084619.g007

mice (Fig. 2A, second row). Statistical analysis confirmed reciprocal changes, where a decrease in the proportion (and absolute number) of pDCs corresponded to an increase in Mφ/cDCs in the liver of colitic mice (Fig. 2B). F4/80^{+} CD11b^{+}CD11c^{high} cells, but not F4/80^{+}CD11b^{+}CD11c^{low} cells, were predominant in hepatic Mφ/cDCs (Fig. 2C). Only a small number of pDCs were found in the LP of the colon under both healthy and colitic conditions (Fig. 2D and E).

Hepatic Mφ/cDCs Under Colitic Conditions Induce a Th1 Inflammatory Response

Owing to finding drastic compositional changes of liver APCs in colitic conditions, we assessed the function of hepatic CD11b^{-}CD11c^{low}PDCA-1^{+} pDCs isolated from the livers of WT mice (WT pDCs), and CD11b^{+}CD11c$^{-/+}$ Mφ/cDCs isolated from the livers of colitic IL-10$^{-/-}$ mice (IL-10$^{-/-}$ Mφ/cDCs) (Fig. 3A). The positive controls were Mφ/cDCs isolated from ConA-treated livers (ConA Mφ/cDCs) (Fig. 3A). We co-cultured pDCs or Mφ/cDCs with naïve CFSE-labeled CD4^{+} T cells in the

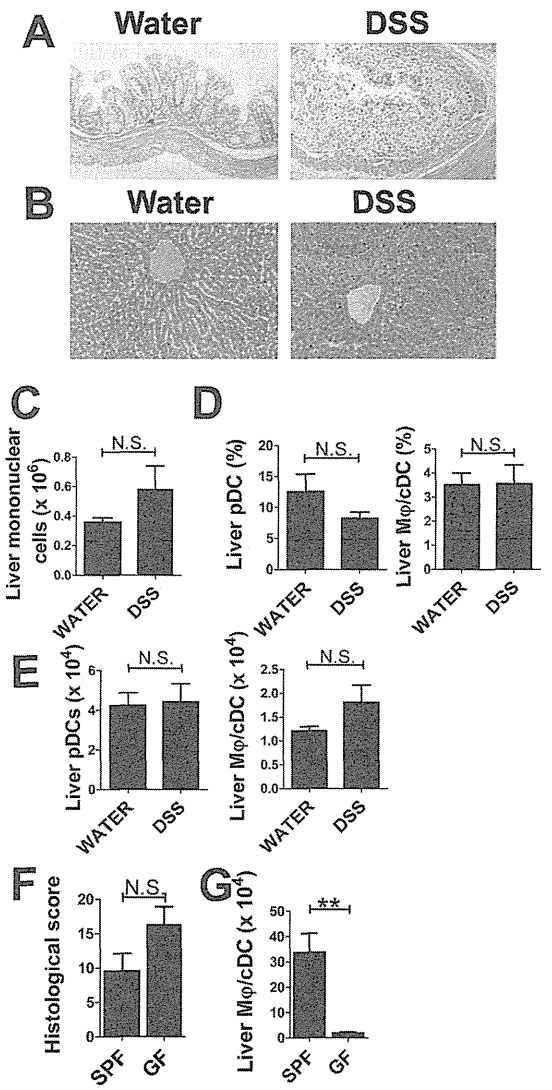

Figure 8. GF condition abrogates the compositional changes of hepatic APCs in acute colitis models. (**A**) H&E staining of colon sections taken from mice treated with water (left) or DSS (right). Magnification, ×100. (**B**) H&E staining of liver sections from water- (left) and DSS-treated (right) mice. Magnification, ×100. (**C**) Number of liver mononuclear cells. (**D**) Proportion and (**E**) absolute number of PDCA-1$^+$CD11b$^-$CD11c^{int} pDCs and CD11b$^+$CD11c$^-$ Mφs among whole mononuclear cells. (**F, G**) Comparisons between SPF and GF in the histology (F) and the numbers of Mφs (G) in DSS-treated mice. Data are representative of two independent experiments. Values are presented as the mean ± SEM for each group ($n = 5$, water-treated GF group; $n = 4$, DSS-treated GF group; $n = 5$, DSS-treated SPF group). N.S., no significant difference.
doi:10.1371/journal.pone.0084619.g008

presence of OVA peptides. After 72 h in culture, CD4$^+$ T cells had extensively divided in the presence of Mφ/cDCs from not only Con A-treated mice but also colitic IL-10$^{-/-}$ mice, but divided little in the presence of WT pDCs (Fig. 3B). To further assess pro-inflammatory responses of Mφ/cDCs, we examined cytokine production from cultured CD4$^+$ T cells. Flow cytometry showed a significant increase in the proportion of IFN-γ-expressing CD4$^+$ T cells following co-culture with IL-10$^{-/-}$ Mφ/cDCs. A similar result was seen with ConA Mφ/cDCs; however, there was no significant increase in IL-17-producing CD4$^+$ T cells (Fig. 3C and D). Consistent with these data, culture supernatants from CD4$^+$ T cells following co-culture with IL-10$^{-/-}$ Mφ/cDCs or ConA Mφ/cDCs exhibited a significant increase in IFN-γ and other pro-inflammatory cytokines such as IL-6 and TNF-α (Fig. 3E).

Immune Dysregulation in the Liver

Given the evidence that activated Mφ/cDCs in the liver instruct naïve CD4$^+$ T cells to differentiate into Th1 cells (Fig. 3C), we then examined whether the primary recruitment of colitogenic Th1 cells to the liver or other mechanisms induced a dysregulation in the balance of Mφ/cDCs and pDCs in the liver under colitic conditions, as mononuclear cells expanded in the liver in colitic RAG-2$^{-/-}$ RB^{high} mice and IL-10$^{-/-}$ mice (Fig. 1). As an alternative mechanism, the breakdown of the colonic barrier and sequential uptake of MAMPs or other gut-derived antigens during the colitis state may play an important role in drastic changes of APCs in the liver. To minimize the effects of liver-infiltrating T cells, we used an adoptive retransfer system: colitogenic LP CD4$^+$ T cells obtained from established RAG-2$^{-/-}$ RB^{high} mice were transferred into RAG-2$^{-/-}$ mice to generate RAG-2$^{-/-}$ LP CD4$^+$ mice, as colitogenic CD4$^+$ T cells residing in the intestine express gut-specific homing receptors and have an ability to preferentially migrate to the intestine but not to liver [24,25]. These mice developed severe colitis (data not shown) and also showed significant increases in the number of liver-infiltrating mononuclear cells (Fig. 4A). The RAG-2$^{-/-}$ LP CD4$^+$ mice showed almost no CD3$^+$CD4$^+$ T cell infiltration in the liver, but did exhibit severe colitis with marked infiltrations of CD3$^+$CD4$^+$ T cells in the colon (Fig. 4B). We confirmed statistically that the significant increases in liver mononuclear cells in RAG-2$^{-/-}$ LP CD4$^+$ mice was due to the emergence of non-T cells (possibly APCs) (Fig. 4C). We further investigated compartments of APCs in the liver of RAG-2$^{-/-}$ LP CD4$^+$ mice. Consistent with the data from colitic RAG-2$^{-/-}$ RB^{high} or IL-10$^{-/-}$ mice (Fig. 2), RAG-2$^{-/-}$ LP CD4$^+$ mice also showed reciprocal changes; a significant decrease in pDCs corresponded with an increase in Mφ/cDCs (Fig. 4D and E). RAG-2$^{-/-}$ LP CD4$^+$ mice exhibited severe colitis without infiltration of T cells in the liver (Fig. 4B and 4C). These data suggest that intestinal inflammation induce changes in the compartments of APCs.

Accumulation of Mφ/cDCs in the Livers of Mice with DSS-induced Colitis

To further determine whether hepatic immune dysregulation is caused by barrier disruption of the intestinal wall, we looked at livers from immune-sufficient WT mice subjected to DSS-induced colitis under SPF conditions. Seven days after the start of DSS administration, mice exhibited severe colitis and infiltration of mononuclear cells in the liver (Fig. 5A and B). Consistent with histological data, liver mononuclear cells were upregulated in DSS-treated mice when compared with water-treated mice (Fig. 5C). Flow cytometry revealed that the number of Mφ/cDCs was significantly increased in the livers of DSS-treated mice; however, there were no significant changes in the numbers of pDCs (Fig. 5D). Expression levels of IFN-γ and TNF-α in the liver were significantly increased in DSS-treated mice (Fig. 5E). Furthermore, hepatic Mφ/cDCs in the DSS-treated mice promoted proliferation of CD4$^+$ T cells (Fig. 5F), and increased the proportion of IFN-γ-expressing CD4$^+$ T cells (Fig. 5G). We detected a significant increase in pro-inflammtory cytokines in co-culture supernatants (Fig. 5H).

We also confirmed the reciprocal changes for pDCs and Mφ/cDCs in DSS-treated RAG-2$^{-/-}$ mice, which had severe colitis and infiltration of mononuclear cells in the liver (Fig. 6A, B and C). The proportion of pDCs was decreased in DSS-treated RAG-2$^{-/}$