Title:
“Acute colitis induced by dextran sulphate sodium progresses into chronicity in C57BL/6 but not in BALB/c mice – correlation between symptoms and inflammation”

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Running title: Acute and chronic colitis in C57BL/6 mice

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ABSTRACT

Exposure to dextran sulphate sodium (DSS) induces acute colitis, which is normally resolved after DSS removal. To study chronicity, mice are typically subjected to 3-5 cycles of weekly DSS-exposures, each followed by resting period of 1-2 weeks. Herein, we describe a novel and convenient way of inducing chronic, progressive colitis by a single exposure to DSS. C57BL/6 mice exposed to DSS for 5 days, developed acute colitis that progressed into severe chronic inflammation. The plasma haptoglobin levels remained high during the chronic phase, showing that the inflammation was active. Surprisingly, the mice regained their original weight along with the progression of colitis and the only apparent symptom was loose feces. Histopathological changes 4 weeks after DSS removal were dense infiltrates of mononuclear cells, irregular epithelial structure and persistent deposits of collagen. A progressive production of the cytokines IL-1β, IL-12p70 and IL-17 correlated to the extensive cellular infiltration, whereas high IFN-γ production was mainly found late in the chronic phase. Similar to C57BL/6, BALB/c mice exposed to five days of DSS developed acute colitis as previously described. The acute colitis was accompanied by elevated plasma levels of haptoglobin and increased colonic levels of IL-1α/β, IL-6, IL-18 and G-CSF. However, soon after DSS-removal BALB/c mice recovered and were symptom-free within two weeks and completely recovered 4 weeks after DSS removal in terms of histopathology, haptoglobin levels and local cytokine production. In summary, these data stress the effect of genetic background on the outcome of DSS-provocation. We believe that the current protocol to induce chronic colitis in C57BL/6 offers a robust model for validating future therapies for treatment of inflammatory bowel disease.

Key words: fibrosis, haptoglobin, recovery, IL-1, IL-12
INTRODUCTION

Ulcerative colitis (UC) and Crohn’s disease (CD) are the major chronic inflammatory bowel diseases (IBD) affecting the gastrointestinal tract in humans. IBD patients show flares of remission and relapses with symptoms of bloody diarrhea, abdominal pain and rectal bleeding. The etiology is still unknown, but the pathogenesis is likely dependent on the interaction between local immune reactions and environmental factors in genetically susceptible individuals (44).

Several animal models have been developed to understand the pathology of IBD, but no specific model for either of the diseases exists. Gut inflammation can be induced using chemical compounds e.g. dextran sulphate sodium (DSS) or by genetical targeting of specific genes, e.g. regulatory cytokines. The majority of the knockout (KO) mice develop colitis with histopathology resembling UC (e.g. IL-2KO mice) or enterocolitis (e.g. IL-10 KO mice) (4). In 1990, Okayasu and colleagues described a model in which mice receiving DSS orally developed acute and chronic colitis resembling UC (39). During the acute phase, the mice developed colonic mucosal inflammation with ulcerations, body weight loss and bloody diarrhea (39). Administration of 3-5 cycles of DSS to the mice induced chronic inflammation, characterized by severe infiltration of mononuclear cells (MNC), and regenerative changes in the epithelium (39). The mechanism by which DSS induces colitis is still not clear. To this end, proposed mechanisms have been toxic effects on the epithelium; increased exposure to luminal antigens by destruction of mucin content or altered macrophage function due to ingestion of DSS (26, 37, 39). The resulting inflammation has been shown to include polymorphonuclear cells, macrophages as well as B and T cells. Interestingly, Dieleman and colleagues demonstrated that Severed Combined Immunodeficient mice, without T and B cells, were able to
develop DSS-induced colitis based on clinical symptoms and histology, suggesting that adaptive immunity is not involved in acute colitis (12). In contrast, T cells producing both IFN-γ and IL-4 were observed in Swiss Webster mice with chronic inflammation induced by DSS (11).

Differences in susceptibility to DSS induced colitis due to genetic background are well established. In similarity to IBD in humans, there are multiple loci affecting the disease (31, 32).

In the present study, we used two different mouse strains, BALB/c and C57BL/6, to analyze the consequences of DSS exposure in a careful kinetic study of symptoms, histology and local and systemic levels of inflammatory mediators for up to 4 weeks after DSS-removal.
MATERIAL AND METHODS

Mice

Specific pathogen free female BALB/c and C57BL/6 mice, 7-12 weeks old, weighting 18-22g, were obtained from Harlan, the Netherlands. The mice were kept at the animal house facilities at AstraZeneca R&D Mölndal, Sweden, with 50% humidity; light/dark cycles of 12h and fed with a standard pellet diet (R3 pellets, Lactamin, Sweden) and tap water *ad libitum*. Mice were acclimatized for at least 2 weeks before entering the study. The Local Animal Research Board Committee in Gothenburg approved these studies.

Induction of colitis

Dextran sulphate sodium (45 kD; TdB Consultancy AB, Uppsala, Sweden) was added to tap water at a concentration of 3% for C57BL/6 and 5% for BALB/c mice. Fresh DSS solution was prepared daily. As initial studies revealed that C57BL/6 mice exposed to 5% DSS became too diseased (data not shown), a lower concentration (3%) was used. When C57BL/6 mice were exposed to DSS for 7 instead of 5 days, and then studied for recovery, the symptoms and histopathological changes were aggravated, leading to high mortality (data not shown). Therefore, we chose to study the 5-day exposure in the current experiments. Mice were divided into groups of 3-9 per time point and received DSS as scheduled in fig 1. Briefly, mice receiving DSS for 1, 3, or 5 days were used to analyze the response during the acute phase. Mice were exposed to DSS for 5 days and sacrificed after 1, 2, 3, or 4 weeks after DSS removal to study the progression from acute to chronic inflammation or to recovery. These time points are referred to as d12, d19, d26 and d33. Healthy control animals received tap water only. The clinical symptoms recorded in DSS-treated and control mice were body weight, stool consistency, fecal bleeding, and diarrhea.
Tissue and plasma sampling

Colonic tissue and plasma samples were collected at the different time points as scheduled in fig 1. At the end of each time point, mice were anesthetized by inhalation of isofluran (Abbot Scandinavia AB, Solna, Sweden), blood was drawn by retroorbital puncture, after which the mice were sacrificed by cervical dislocation. Blood was collected in tubes containing EDTA and plasma frozen and kept at -80°C. The intestines were excised and carefully rinsed with saline. The colon was cut in proximity to the ileocecal valve and the rectum, and the length was measured. One cm of the distal and proximal colon was cut out and opened longitudinally, fixed in Zinc-formalin solution (pH 7.4, Histolab Products AB, Göteborg, Sweden) and embedded in paraffin for histology analysis. The remaining part of the colon was weighed, and frozen in liquid nitrogen.

Assessment of inflammation – symptoms and inflammatory score

Clinical assessment of inflammation included daily monitoring of body weight and general health condition. At necropsy, the macroscopical appearance of colon (inflammatory score), based on the degree of inflammation and presence of edema and/or ulcerations, the stool consistency (diarrhea score) and visible fecal blood was scored separately on a scale of 0 to 3 as described in table 1.

Histology

Five µm tissue sections of distal and proximal colon were stained with hematoxylin/eosin (H&E) to address the degree of inflammation and with Masson trichrome (MT) staining to visualize the connective tissue. The stained tissue was analyzed independently by S.M. and in a blinded fashion by a pathologist (G.B.) in a standard microscope (Zeiss, Germany). The pathophysiology of the tissue was characterized by the presence of ulcerations, inflammatory cells (neutrophils,
macrophages, lymphocytes, and plasma cells), signs of edema, crypt loss, surface epithelial cell hyperplasia, goblet cell reduction and signs of epithelial regeneration.

**Analysis of local and systemic inflammatory markers**

The levels of the acute phase proteins haptoglobin (55) and serum amyloid A (SAA) (17) were determined using a Cobas Bio centrifugal analyzer with a commercial reagent kit for haptoglobin (Tridelta TP 801) and using a commercial solid phase sandwich ELISA for SAA (Tridelta Development, Ltd, Ireland).

Analyses on the production of IL-1α, IL-β, IL-6, IL-12p40, IL-12p70, IL-17, IL-18, IFN-γ and G-CSF was performed on whole colonic tissue homogenates. Dissected tissues were homogenized in a Homogenizer (Ultra Turrax T8, Tamro, Sweden) in homogenization buffer containing phosphate sodium saline (PBS, Gibco, Invitrogen Corp, UK), supplemented with complete mini proteinase inhibitor cocktail (Roche Molecular Biochemicals, Mannheim, Germany) and 10% fetal calf serum (Gibco). The homogenized solution was centrifuged for 10 minutes at 18000rpm; the supernatant was aliquoted and frozen at –80°C until analysis. The production of IL-1α, IL-6, IL-12p40, IL-12p70, IL-17 and G-CSF was analyzed using the xMAP technology developed by Luminex Corporation (Austin, Texas) (8). The concentration was determined using Bioplex Multiplex Suspension Array System kits, following the manufacture’s instructions (Bio-Rad Laboratories, Hercules, CA), and subsequently analyzed with a five-parametric curve fitting using the Bioplex Manager 3.0 software (Bio-Rad). The homogenization buffer was used to generate the standard curve, as a background to be subtracted from the standards as well as the samples, and finally to dilute the samples 1:2 before analysis. Analyses on IL-18, IFN-γ and IL-1β were made by ELISA according to the manufacture’s instructions (R&D Systems, UK). The analyses for IL-1β were made both with ELISA and xMAP technology to
compare the two techniques, obtaining similar results (data not shown). The protein values are expressed as pg/100 mg colonic tissue.

**Statistics**

Data are presented as mean ± S.E.M. Mann-Whitney’s U-test was used for statistical analysis; a value of p <0.05 was considered significant. Correlation analysis was made using Spearman r test (Graph Pad Software, Inc).
RESULTS

BALB/c and C57BL/6 mice display similar clinical symptoms and histopathological changes during acute colitis

Clinical symptoms upon exposure to DSS for five days were loss of body weight, loose feces/watery diarrhea, and fecal blood, consistent with the previous report (39). The symptoms were aggravated in both strains as disease progressed in the acute phase (fig 2a-c). Body weight loss was observed from day 4 (d4) in C57BL/6 and on d6 in BALB/c mice (fig 2a). Peak levels of diarrhea and visible fecal blood were found at d5 in both strains (fig 2b-c). Macroscopically, the inflammation was generally located to the distal colon in both strains. The inflammatory score increased as the inflammation progressed and was somewhat higher in C57BL/6 compared to BALB/c mice at d5 (fig 2d). Furthermore, the length of colon decreased as the disease developed and was significantly shorter in both strains at d5 (approx 20% in C57BL/6 and 30% in BALB/c mice, table 2). At this time point, the colonic wet weight had increased by approximately 50% in both strains (fig 3a). The plasma levels of haptoglobin were gradually elevated as the acute inflammation progressed (fig 3b) and correlated well with macroscopic inflammatory score (r = 0.82 and r = 0.85; p < 0.0001; for C57BL/6 and BALB/c mice respectively), and with colonic wet weight (r = 0.78 and r = 0.86; p < 0.0001; C57BL/6 and BALB/c mice respectively). Similar results were obtained with SAA (data not shown). Other features found at d5 in C57BL/6 mice were an enlargement of the mesenteric lymph nodes, a decreased size of cecum, and the occasional presence of blood in the small intestinal lumen, stomach and/or cecum (data not shown). Extraintestinal features found in BALB/c mice at d5 were significantly enlarged spleen and mesenteric lymph nodes (table 2).
Histopathological analysis was carried out using H&E stained sections of the proximal and distal colon (fig 4). At d3, both strains showed minimal changes in the surface epithelium, although a slight infiltration of inflammatory cells to the mucosa was observed (data not shown). The changes were more pronounced in both strains at d5, with loss of crypts and reduction of goblet cells, signs of surface epithelial regeneration, focal ulcerations, moderate infiltration of inflammatory cells to the mucosa and edema in the submucosa (fig 4b and d). The number of neutrophils was only slightly increased and remained so during the acute inflammation in C57BL/6 mice, whereas there was a marked increased in neutrophils in BALB/c mice (data not shown). In general, similar histopathological changes were observed in the proximal colon compared to distal colon of DSS-treated C57BL/6 and BALB/c mice.

Overall, the clinical symptoms and histopathological changes found in both strains are in agreement with previously described features in murine DSS-induced acute colitis (39).

**Acute colitis completely resolves in BALB/c mice while C57BL/6 mice develop severe chronic inflammation with mild clinical symptoms**

To determine the fate of inflammation after 5 days of DSS exposure, mice were monitored for an additional 4 weeks.

BALB/c mice regained their bodyweight already 2-3 days after DSS removal (fig 2a), and had no remaining signs of diarrhea/loose stools at d26 (fig 2b). No fecal blood could be found subsequent to DSS-exposure (fig 2c). Further, the inflammatory score was significantly decreased at d19, and remained low at the later time points (fig 2d). In addition, the colonic wet weight, plasma haptoglobin and SAA levels returned to normal levels at d19 (fig 3a-b and data not shown).
The symptomatic resolution of colitis in BALB/c mice correlated well to the histological changes (fig 4h-j). One week after DSS removal (at d12), we observed a reduced number of inflammatory cells, signs of regeneration of crypts and goblet cells and epithelial restitution (fig 4h). Further, 3-4 weeks after DSS removal, the epithelial architecture was fully recovered showing crypt and goblet cell regeneration and few inflammatory cells in the mucosa (fig 4j and 6f).

C57BL/6 mice continued to lose bodyweight after DSS removal (fig 2a). Not until one-week post-DSS, at d12, the mice started to gain weight, reaching their original weight after an additional two weeks (d26). At d12-d19, visible fecal blood could not longer be detected, whereas the diarrhea score remained high (fig 2b-c). At later time points, the only apparent sign of disease in C57BL/6 mice was loose stools (fig 2b), which was still present 5 weeks after DSS removal (data not shown).

Based on the symptomatic parameters, it appeared that the two mouse strains behaved similarly on a qualitative level, although C57BL/6 mice displayed a more severe disease. Upon necropsy, however, major qualitative differences were evident. The inflammatory score in C57BL/6 mice remained at the same high level as observed at d5, until termination of the experiment (d33) (fig 2d). The colon was significantly shortened at d12 and d26 (table 2) and the colonic wet weight increased progressively until d26, after which it remained high (fig 3a). The plasma levels of haptoglobin were still high during the chronic phase (d26 and d33) indicating ongoing systemic inflammation (fig 3b). As was seen during DSS-exposure, there was also a good correlation between haptoglobin and inflammatory score (r = 0.93; p < 0.0001) and the colonic wet weight (r = 0.90; p < 0.0001) during the subsequent 4 weeks. SAA levels also remained high (>190µg/ml) during these late time points (data not shown).
As the inflammation progressed in C57BL/6 mice, the colon became stiff and the middle part was often tightly adhered to the mesentery (data not shown). Other features found in C57BL/6 mice after d26 were increased size of the spleen and enlarged mesenteric lymph nodes (table 2).

In contrast to the symptoms, the microscopical progression of colitis in C57BL/6 mice involved a gradual infiltration of macrophages, lymphocytes and plasma cells into the mucosa and submucosa as the inflammation progressed (fig 4e-g). The number of neutrophils was constant during the entire period, although aggregates of neutrophils were found in conjunction to ulcerations (data not shown). Further, small formations of tightly packed mononuclear cells were observed in the lamina propria at d12, which seemed to be enlarged as the inflammation progressed (fig 4e-g). The highest number of inflammatory cells was found at d26 (fig 4g). Transmural inflammation was occasionally detected, whereas no transmural ulcerations were found at any time-point (data not shown). Marked loss of crypts, goblet cells and large ulcerations were mainly denoted at d12 (fig 4e), followed by regeneration of crypts and re-epithelisation at d19 (fig 4f). Three to four weeks after DSS removal, the regenerated epithelial structure was irregular, and contained areas with ulcerations (fig 4g and 6c). Overall, similar histopathological changes were observed in the proximal colon as in the distal colon, although with a milder degree of focal inflammation (data not shown).

Thus, the acute disease in C57BL/6 mice does not resolve after DSS removal. Instead, it progresses into a severe, chronic colitis, including systemic manifestations such as high plasma levels of acute phase proteins. Paradoxically, symptoms of diarrhea and weight loss do not correlate to the severity of the inflammation. Rather, these symptoms correlate better to the condition of the epithelium, as illustrated in
figure 5. In BALB/c mice, on the other hand, the acute inflammation as well as the acute symptoms are self-limited and start to resolve directly after DSS removal. Interestingly, we have found that the time of resolution in BALB/c is the same, despite a prolonged (9 days) DSS exposure (data not shown).

**Masson Trichrome staining demonstrates increased deposits of collagen in the mucosa and submucosa of acute and chronic inflamed colonic tissue**

One common feature of the inflamed colons, either during the acute or chronic phase, was a marked rigidity. To investigate whether this was related to infiltration/inflammation or to fibrosis, we did MT staining on distal colonic tissue from both strains during acute, recovery and chronic phase to analyze production of collagen as a marker of fibrosis (fig 6). An increase in collagen deposits was found in the mucosa and submucosa of both strains as the disease developed into the acute phase (d5) (fig 6b and e). Further, the increased deposits of collagen were still found in the mucosa and submucosa of C57BL/6 mice at d26 and d33, most likely resembling the stiffness seen in the chronic inflamed colons (fig 6c). Moreover, areas containing several bundles of collagen were found in focal regions of the serosa in the chronic C57BL/6 mice. In contrast, MT staining appeared normal in BALB/c mice at d33 (fig 6f), further supporting the total recovery of the colonic tissue in BALB/c mice.

**Progressive up-regulation of IL-1β, IL-12p70 and IL-17 follows chronic development in C57BL/6 mice.**

In order to investigate the degree of activation of the infiltrating cells during colitis development, we analyzed the production of selected cytokines in colonic
tissue homogenates. The chosen cytokines have all been implicated in the pathogenesis of IBD i.e. IL-1α/β, IL-6, IL-12, IL-17, IL-18, IFN-γ and G-CSF (14, 16, 21, 24, 35, 43). We chose to measure these mediators at the protein level rather than mRNA and without ex vivo stimulation of the cells/tissue as we consider this the most relevant condition for mimicking what is occurring in vivo.

As the symptoms were aggravated into the acute phase (at d5), the production of IL-1α, IL-1β, IL-6, IL-18 and G-CSF was elevated in BALB/c mice (fig 7a and table 3). The IL-1β production correlated well to plasma haptoglobin levels (r = 0.75; p < 0.0001), to inflammatory score (r = 0.80; p < 0.0001) and to colonic weight (r = 0.66; p = 0.0007). Further, the IFN-γ levels were very low, resembling healthy mice (fig 7e), whereas no IL-12p70 or IL-17 production was detected either in healthy or sick mice (fig 7b and d). Thus, the highly elevated levels of IL-1, IL-6, IL-18 and G-CSF coincided with the peak of infiltrating cells at d5. Interestingly, an increased production of IL-12p40 was observed in the acute phase, but the highest peak was assayed one week after DSS removal, at d12 (fig 7c). In contrast, the other cytokines were rapidly down regulated at d12 and normalized 3-4 weeks after DSS removal (fig 7a-e, table 3). The decreased cytokine production coincided with the resolution of disease in terms of clinical symptoms, haptoglobin levels and histopathology (fig 2-4 and 7).

In contrast, in C57BL/6 mice, the cytokine response of IL-1α, IL-1β, IL-12p40, IL-12p70 and IL-17 was progressively increased after DSS removal (fig 7a-d, table 3). This production coincided with the increasing number of infiltrating cells (fig 4e-g). The IL-1β production in the chronic phase (d26-33) was well correlated to haptoglobin levels (r = 0.92; p < 0.0001), to inflammatory score (r = 0.88; p < 0.0001) and to colonic weight (r = 0.86; p < 0.0001). IL-6 and G-CSF were highly elevated at
d5 and d12, followed by intermediate levels at the later time-points (table 3). IFN-γ production was heterogeneous with relatively increased levels at d5, and higher levels late in the chronic phase, at d33 (fig 7e). IL-18 production was also heterogeneous with high inter-individual values displaying no significant differences between healthy and diseased mice (table 3).

In summary, the cytokine production assayed in the acute phase of BALB/c and the progressive chronic inflammation in C57BL/6 mice, indicate that the infiltrating cells are actively involved in disease development in both models.

DISCUSSION

In the present study, we have described a new approach, where a short 5-day exposure of DSS is sufficient to induce progressive chronic colitis in C57BL/6 mice. In contrast, BALB/c mice developed acute colitis during DSS exposure, which was completely resolved after DSS removal. The clinical symptoms correlated well to inflammation in the acute phase in both strains, whereas only mild symptoms were observed in the chronic phase of C57BL/6 mice.

The data presented herein describes different phases of colitis development which we have classified into acute, chronic and recovery phases. The classification is based on clinical symptoms and histopathological changes (epithelial architecture, type of inflammatory infiltrate, and fibrosis). The acute phase (d5) was characterized by the presence of clinical symptoms i.e. body weight loss, visible fecal blood, and diarrhea, high inflammatory score, high levels of acute phase proteins, and histopathological changes e.g. ulcerations, polymorphonuclear cells and MNC infiltration, and loss of epithelial structures. The chronic phase (d26 and forward) was characterized by recovered body weight, no/few clinical symptoms, high
inflammatory score, high levels of acute phase proteins and histopathological changes e.g. irregular epithelium, dense infiltrates of MNC and plasma cells and persistent fibrosis. Finally, recovery was accomplished when no clinical symptoms or systemic markers were found, and the histological structure was recovered i.e. healed epithelial structure with crypts and goblet cells and few inflammatory cells in the mucosa.

DSS is commonly administrated in a dose range of 3-10% for 7-10 days to induce an acute inflammation depending on the susceptibility of the mouse strain, or the molecular weight of DSS (25, 31, 39). To induce “chronicity”, DSS is normally administered in 3-5 cycles with a resting period of one to two weeks in between cycles (39). Our data show that one cycle of DSS is sufficient to induce chronicity in C57BL/6 mice. Possibly, this is also true in Swiss-Webster mice (7, 11). In contrast, BALB/c mice recovered quickly after DSS removal, in terms of clinical symptoms, histopathology and inflammatory markers (present study and (12)). We do not think that the concentration of DSS is the critical factor for development of chronicity since C57BL/6 mice, exposed to 2% DSS for 5 days, still developed chronic colitis, albeit with milder symptoms during the acute stage (S Melgar, unpublished observation). Moreover, BALB/c mice exposed to 5% DSS for up to 9 days was partly recovered one week after DSS removal (S Melgar, unpublished observation), implying that neither 5 nor 9 days of DSS exposure is a critical factor for the rapid recovery in BALB/c mice. Thus, this is the first time that DSS has been used as a priming agent to induce a progressive inflammatory response in C57BL/6 mice.

In the clinical settings, one way to diagnose and monitor IBD is by recording the patient’s clinical symptoms. Symptoms often related to UC are rectal bleeding or bloody diarrhea, whereas symptoms often related to CD are abdominal pain and weight loss (44). Correlation between clinical symptoms and endoscopy score in
patients is controversial (6, 18, 19, 48). In the present experimental study, we found good correlation between clinical symptoms and inflammatory score and local cytokine response during the acute but not during chronic phase, where loose feces were the only clinical sign found. This is in contrast to the good correlations reported between the clinical symptom occult blood and inflammation in BALB/c mice treated with cycles of DSS or in chronic inflamed Swiss Webster mice (7, 39). The discrepancies may depend on the type of analysis used, i.e. hemoccult vs. gross bleeding or in strain differences.

Several sub-clinical markers are used to monitor disease activity in humans. One of these markers is serum C reactive protein (CRP), which is generally elevated in 95% of patients with active CD. However, weak correlation was reported between serum CRP levels and symptom values (51). We measured the acute phase protein haptoglobin as a marker of systemic inflammation and found good correlation to inflammatory score, colonic weight and IL-1β production in the acute phase of both strains and in the chronic phase in C57BL/6 mice. Moreover, haptoglobin levels decreased in parallel as clinical symptoms and inflammation resolved in BALB/c mice after DSS removal. In addition, SAA, another acute phase protein proven a good marker for disease activity in IL-2KO mice (9), was analyzed, yielding results similar to those of haptoglobin (data not shown). Thus, we are of the opinion that haptoglobin can be used as a systemic marker to monitor disease activity in murine experimental colitis.

Histological features typical for chronic IBD compared to other mucosal inflammations are epithelial distortion such as crypt branching and shortening, and decreased crypt density (increased spacing between crypts), and severe infiltration of inflammatory cells to the intestinal wall. Furthermore, reduction in goblet cell number
is typical for UC but not for CD (18, 52). At the late phase after DSS removal in C57BL/6 mice, we observed irregular epithelial formation with crypt distortion and globet cell depletion, equivalent to changes found in UC patients with at least 2 months of inflammation (2, 53). Similar histopathological changes were reported in chronically inflamed Swiss Webster mice, in BALB/c mice subjected to cycles of DSS and in rats exposed to peptidoglycan-polysaccharide (PG-PS) or to trinitrobenzene sulfonic acid (TNBS) (7, 11, 39, 54, 57). Another typical histological feature found in the inflamed intestinal wall of IBD patients is the severe infiltration of MNC and plasma cells to the basal lamina propria (18, 52). An increased number of inflammatory cells e.g. MNC and plasma cells was already observed at d12 in the lamina propria of C57BL/6 mice, which was even more pronounced at d26, thus reflecting the progression to chronicity rather than resolution of disease in these mice. Notably, the number of neutrophils was constant during the chronic phase, indicating that the inflammation was active in these areas, further supporting the remaining high levels of haptoglobin and SAA at this stage. Other histological features such as granuloma and transmural ulcerations, typical for CD, or crypt abscesses and cryptiditis, found in UC (18), were not observed in the current model. In general, endoscopy and histology results are not well correlated in IBD (15, 18). We observed good relationship between inflammatory score and histology at the different phases, though the histological changes were generally patchy, and the inflammatory score was based on the appearance of the entire colon. One reason for the discrepancy observed in IBD patients may be related to the size and site of the biopsies, which may not reflect the whole inflammatory profile.

Fibrosis is a non-specific feature of chronic inflammation, and is observed both in UC and CD (22, 34, 45). Fibrosis in CD is characterized by increased deposits
of collagen due to altered collagen metabolism in all layers of the intestinal wall but
specially found around and within smooth muscle (22). Moreover, excessive fibrosis
due to severe collagen deposition may result in strictures in CD patients (22, 45). In
contrast, fibrosis in UC is characterized by increased deposits of collagen in the
submucosa and lamina propria (34). In the current models, we detected extensive
deposition of collagen in the mucosa and submucosa of acute and chronic inflamed
colons and even occasionally in the muscle layers or in the serosa of the chronic
inflamed colon, features resembling fibrosis in UC rather than CD. A similar finding
was recently reported in CD1 or BALB/c mice receiving weakly doses of TNBS (29),
whereas certain rat strains injected with PG-PS develop fibrosis and granulomatous
enterocolitis resembling CD (57). One important factor for the observed differences in
fibrosis induction between the different IBD models may again be related to the
 genetic background, as shown in experimental models of lung pathology (28).

It is well established that differences in genetic background is a major
determinant for an inflammatory response, e.g. as observed after *Leishmania major*
infections (46). BALB/c mice are more susceptible to *Leishmania major* infections,
due to a defective Th1 response. In contrast, infected C57BL/6 mice effectively
mount a Th1 response leading to clearance of the infection and eventually healing
(46). Analysis of cytokine production in the current models reveals differences in the
inflammatory response between the two strains. A high production of IL-1α, IL-β, IL-
6, IL-18 and G-CSF was found in the acute phase of BALB/c mice, which coincided
with the peak infiltration of inflammatory cells. Macrophages and epithelial cells are
the main producers of these cytokines, suggesting that macrophages may be one the
drivers of the acute response in BALB/c mice as has been proposed earlier (this study
and (12, 39)). However, other studies suggest that also T cells play an important role
in DSS-induced acute colitis (20, 49). Further, IFN-\(\gamma\) production during the acute phase was low in BALB/c, but higher in C57BL/6 mice, as compared to an earlier study, where a high IFN-\(\gamma\) mRNA expression was found in BALB/c mice (13). Taken together, the data suggest that the acute response in BALB/c mice is most likely macrophage-driven, although T cells may be observed as a secondary response.

The factor(s) that regulates the resolution of disease in BALB/c mice is not known. However, an increased production of IL-12p40, without any concomitant IL-12p70 elevation was observed in the acute phase (d5) and early in the recovery phase (d12), suggesting that active IL-12 is not supporting the acute inflammation in BALB/c mice. IL-12 is an immunoregulatory cytokine involved in cell-mediated immune responses by inducing differentiation of Th1 cells and IFN-\(\gamma\) production (5). However, in murine systems, homodimers of p40 have been shown to bind to the IL-12 receptor and work as a natural inhibitor of IL-12 activity (23). Our data suggest that the increased IL-12p40 levels may form homodimers, which antagonize IL-12 activity thus inhibiting pro-inflammatory responses in BALB/c mice (23). In addition, we have also seen that an inverse correlation exists between IL-1\(\beta\) and PGE\(_{2}\), suggesting that PGE\(_{2}\) is also involved in disease resolution in BALB/c (S. Melgar, manuscript in preparation).

In contrast to BALB/c, C57BL/6 mice progressively develop a chronic inflammation subsequently to the acute response. An important question is what factor(s) are the major drivers for this response. To this end, we observed differences in the cytokine response, especially in the progressive production of IL-1, active IL-12 and IL-17, which correlated to the dense infiltration of lymphocytes, plasma cells and macrophages into the tissue.
IL-12p70 heterodimers were produced in a progressive manner during chronic development in C57BL/6 in contrast to BALB/c mice, suggesting that bioactive IL-12 favors chronic development in these mice. In other IBD-models, e.g. TNBS-induced colitis and IL-10KO mice, disease development was ameliorated by administration of anti-IL-12 antibodies (36, 47). In humans, the data on IL-12 expression is somewhat controversial. An increased expression and secretion of IL-12 was reported in CD but not in UC patients (35, 42), whereas in other studies, no significant differences in IL-12 expression were found between UC and CD patients (38, 40). A recent publication on anti-IL12 treatment to CD patients provided some evidence on the role of IL-12 in IBD. However, more studies need to be conducted (33). In addition, the p40 subunit can also bind to p19 and form the recently described cytokine IL-23, whose function is to promote proliferation of memory CD4+ T cells as well as induce production of IFN-γ and IL-17 (1, 41). IL-17, whose expression was recently reported in active UC and CD patients (16), is a T-cell derived cytokine that stimulates epithelial, endothelial and fibroblastic cells to produce e.g. IL-6 and IL-8. Notably, IL-17 is regulated by IL-23 but not by IL-12 (1). In our models, IL-17 was progressively up-regulated in C57BL/6 but not in BALB/c mice, suggesting that activated T cells may be involved in chronic development. Taken together, the data favors for a role of IL-12, but also for IL-23 in development of chronicity in C57BL/6 mice.

IL-1 have also been suggested to play an important role in initiation and perpetuation of colitis, since increased levels of IL-1β are found in IBD patients with active disease as well as in experimental IBD models (10, 21, 30). Administration of anti-mouse IL-1β antibody suppressed clinical symptoms in murine acute DSS colitis (3). In addition, IL-1β converting enzyme (ICE) knockout mice were protected from cycle induced chronic colitis in terms of clinical symptoms, histological changes, and
spontaneous secretion of IL-1β and T cell activation (50). On the other hand, Kojouharoff et al. showed that the addition of anti-IL-1 reagents aggravated acute colitis and had no effect on cycle-induced chronic colitis in BALB/c mice (27). This discrepancy could be due to genetic background, but also to the readout that was used.

The cytokines IL-4, IL-5 and IL-10 were analyzed by ELISA and multiplex technology revealing low levels (< 30 pg /100mg tissue) at all time points and in both mouse strains (data not shown). It is most likely that to detect this cytokine the analysis needs to be carried out on isolated cells or on tissue sections. Dieleman and colleagues reported an elevation of both IFN-γ and IL-4 in chronic induced colitis in Swiss Webster mice (11). Similarly, we measured an increased IFN-γ production in the chronic inflamed intestine. In humans, IFN-γ levels have been preferentially reported in CD patients, whereas no IL-4 expression has been detected in either CD or UC (14, 56). Thus, the observed differences in resolution of disease and progression to chronicity between the two strains most likely reflect the genetic ability to mount different inflammatory responses and/or healing mechanisms as observed by the different cytokine profiles. Future investigations are aimed for an in-depth analysis on what cell subsets are involved in disease processes(s), as well as to investigate what mediators regulate cellular infiltration in these two distinct models of colitis.

In summary, we believe that the acute model of colitis in BALB/c mice may be useful to study normal mucosal healing, and that the progression into chronicity observed in C57BL/6 offers a promising animal model for the pathological inflammatory changes observed in UC. Moreover, we are of the opinion that the chronic model is a powerful and convenient tool that can be used to validate future candidate treatments for IBD.
ACKNOWLEDGMENTS

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REFERENCES


FIGURE LEGENDS

Fig 1. Overview of induction of acute, chronic and recovery phase in BALB/c and C57BL/6 mice.
Mice receiving DSS for 1, 3, or 5 days respectively, were used to examine the response during the acute phase. Mice receiving DSS for 5 days followed by a water period of 1, 2, 3, or 4 weeks respectively, were used to study the progression to chronic inflammation or recovery. These time points are referred to as d12, d19, d26 and d33.

Fig 2. Clinical and inflammatory markers analyzed during acute and chronic colitis.
   a) Changes in body weight were recorded daily in C57BL/6 and BALB/c mice during the study period. Body weight change was calculated by dividing the body weight on the specified day to the starting weight at d0 and expressed in percentage.
   b) Diarrhea scoring was done at time of necropsy and scored as described in Material and methods.
   c) Visible fecal blood was scored at time of necropsy and recorded as described in Material and methods.
   d) The extent of inflammation was quantified and expressed as an inflammatory score as described in Material and methods

Fig 3. a) Colonic wet weight was obtained by dividing the weight by the length of colonic tissue and is expressed as mg/cm
   b) The expression of the acute phase protein haptoglobin was analyzed in plasma as a systemic marker of inflammation

Fig 4. Histology sections of inflamed colons of C57BL/6 and BALB/c mice.
Representative tissue sections of C57BL/6 (a-b; e-g) and BALB/c mice (c-d; h-
j) at different time points. Sections were stained with hematoxylin/eosin from mice that received DSS for 5 days (b, d); 5 days of DSS followed by one week of water (d12) (e, h); two weeks of water (d19) (f, i); three weeks of water (d26) (g, j); and healthy control mice (a, c) as described in Material and methods. Original magnification, 5x

**Fig 5.** Schematic drawing representing the histopathological changes in the epithelium and the degree of inflammation during progression of colitis. The epithelium (Ep) in healthy control mice was intact, followed by progressive degeneration during the acute phase (d5) in both strains (○ C57BL/6 Ep; □ BALB/c Ep). A marked loss of epithelium was observed at d12 in C57BL/6 mice, with a concomitant slow regeneration until the end of the study at d33. The epithelium of BALB/c mice started to regenerate at d12 and was generally fully regenerated at d33. Infiltration of immune cells (Inf) progressively increased in C57BL/6 mice during disease progression into chronicity, whereas BALB/c mice displayed a large infiltration of cells during the acute phase (d5), which was resolved at the end of the study at d33 (● C57BL/6 Inf; ■ BALB/c Inf)

**Fig 6.** Increased deposits of collagen in acute and chronic inflamed colon. Representative tissue sections of C57BL/6 (a-c) and BALB/c mice (d-f) stained with MT as described in Material and methods. Blue staining demonstrates collagen deposits depicting fibrotic thickening of tissue. C57BL/6 and BALB/c mice exposed to DSS for 5 days (acute phase) (b, e); 5 days of DSS followed by 4 weeks of water (d33, chronic phase) (e, f); and healthy control mice (a, d). Original magnification, 5x
Fig 7. Cytokine production in the acute, chronic and recovery phase in C57BL/6 and BALB/c mice. Colonic homogenates were prepared and the cytokine concentration was analyzed as described in Material and methods. Levels of IL-1β (a), IL-12p70 (b), IL-12p40 (c), IL-17 (d) and IFN-γ (e) were analyzed for each strain at the different time points. Levels are expressed as mean ± SEM and as pg/100 mg colonic tissue. Three to nine mice are analyzed per time point as outlined in fig 1.
**Table 1.** Assessment of inflammation by means of clinical and macroscopical score

<table>
<thead>
<tr>
<th>Score†</th>
<th>Diarrhea Score</th>
<th>Visible Fecal Blood</th>
<th>Inflammatory Score ‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Normal pellets</td>
<td>Normal</td>
<td>Normal</td>
</tr>
<tr>
<td>1</td>
<td>Slightly loose feces</td>
<td>Slightly bloody</td>
<td>Slight inflammation</td>
</tr>
<tr>
<td>2</td>
<td>Loose feces</td>
<td>Bloody</td>
<td>Moderate inflammation and/or edema</td>
</tr>
<tr>
<td>3</td>
<td>Watery diarrhea</td>
<td>Blood in whole colon</td>
<td>Heavy inflammation and/or ulcerations and/or edema</td>
</tr>
</tbody>
</table>

† The degree of diarrhea, visible fecal blood and inflammatory score was assessed on the day of termination and characterized on a scale of 0-3

‡ The macroscopical appearance of the colon (inflammatory score) was judged by the degree of inflammation and presence of ulcerations and/or edema in the tissue
Table 2. Colon length, and spleen weight in C57BL/6 and BALB/c during acute, chronic and recovery phase of colitis

<table>
<thead>
<tr>
<th>Days †</th>
<th>C57BL/6</th>
<th>BALB/c</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Colon length (cm)</td>
<td>Spleen weight (mg)</td>
</tr>
<tr>
<td>0 ‡</td>
<td>5.5 ± 0.4 §</td>
<td>78.0 ± 12.3</td>
</tr>
<tr>
<td>1</td>
<td>6.0 ± 0.4</td>
<td>63.1 ± 12.5</td>
</tr>
<tr>
<td>3</td>
<td>6.0 ± 0.7</td>
<td>67.0 ± 8.2</td>
</tr>
<tr>
<td>5</td>
<td>4.7 ± 0.3 ***</td>
<td>63.8 ± 17.2</td>
</tr>
<tr>
<td>d12</td>
<td>4.3 ± 0.4 ***</td>
<td>52.0 ± 30.3</td>
</tr>
<tr>
<td>d19</td>
<td>5.6 ± 0.6</td>
<td>175.3 ± 85.0 *</td>
</tr>
<tr>
<td>d26</td>
<td>5.0 ± 0.2 **</td>
<td>288.0 ± 74.5 ***</td>
</tr>
<tr>
<td>d33</td>
<td>5.1 ± 0.1</td>
<td>200.2 ± 100.8 *</td>
</tr>
</tbody>
</table>

† C57BL/6 and BALB/c mice received DSS for 1, 3, or 5 days in the acute phase, and 5 days of DSS followed by 7 (d12), 14 (d19), 21 (d26), and 28 (d33) days of water in the chronic and recovery phase

‡ Mean ± SD, values are based on records of 3–9 mice per time point as delineated in fig 1

§ d0 = control/healthy mice = mice receiving only tap water throughout the study

Statistical values using Mann-Whitney U-test comparing mice at different time points to healthy mice (d0); * p < 0.05; ** p < 0.001; *** p < 0.0001
Table 3. Fold difference in levels of cytokines IL-1α, IL-6, IL-18 and G-CSF during progression of colitis in C57BL/6 and BALB/c mice

<table>
<thead>
<tr>
<th>Days #</th>
<th>C57BL/6</th>
<th>BALB/c</th>
<th>C57BL/6</th>
<th>BALB/c</th>
<th>C57BL/6</th>
<th>BALB/c</th>
<th>C57BL/6</th>
<th>BALB/c</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.7</td>
<td>1.0</td>
<td>3.0</td>
<td>1.0</td>
<td>0.7</td>
<td>1.4</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>3</td>
<td>3.0</td>
<td>8.3</td>
<td>4.0</td>
<td>14.1</td>
<td>1.5</td>
<td>2.2</td>
<td>25.0</td>
<td>ND</td>
</tr>
<tr>
<td>5</td>
<td>15.0</td>
<td>56.3</td>
<td>513.0</td>
<td>46.3</td>
<td>1.2</td>
<td>2.7</td>
<td>283.7</td>
<td>29.3</td>
</tr>
<tr>
<td>d12</td>
<td>94.7</td>
<td>7.5</td>
<td>423.7</td>
<td>1.0</td>
<td>0.9</td>
<td>1.4</td>
<td>637.5</td>
<td>1.0</td>
</tr>
<tr>
<td>d19</td>
<td>130.1</td>
<td>11.5</td>
<td>133.9</td>
<td>1.0</td>
<td>2.0</td>
<td>1.0</td>
<td>310.1</td>
<td>1.0</td>
</tr>
<tr>
<td>d26</td>
<td>150.2</td>
<td>6.5</td>
<td>131.7</td>
<td>1.0</td>
<td>1.4</td>
<td>0.9</td>
<td>317.6</td>
<td>ND</td>
</tr>
<tr>
<td>d33</td>
<td>162.2</td>
<td>3.8</td>
<td>438.4</td>
<td>1.0</td>
<td>0.7</td>
<td>0.9</td>
<td>413.0</td>
<td>ND</td>
</tr>
</tbody>
</table>

# Number of days on DSS exposure. Homogenates from C57BL/6 and BALB/c mice exposed to DSS for 1, 3, 5 days as well as mice exposed to DSS for 5 days followed by 7, 14, 21 and 28 days of water were assayed by ELISA and multiplex analysis as described in Material and methods

¤ Fold difference compared to healthy normal mice. The analysis is based on 3-9 mice per time point as outlined in fig 1.

ND – not determined
Fig 1

1) ACUTE PHASE

Healthy  DSS
\[ \downarrow \quad \downarrow \quad \downarrow \quad \downarrow \]
\[ d_0 \quad d_1 \quad d_3 \quad d_5 \]
\[ (n = 7-9) \quad (n = 4) \quad (n = 4) \quad (n = 4-8) \]

2) RECOVERY / CHRONIC PHASE

5d DSS  WATER
\[ \downarrow \quad \downarrow \quad \downarrow \quad \downarrow \]
\[ d_{12} \quad d_{19} \quad d_{26} \quad d_{33} \]
\[ (n = 5-9) \quad (n = 5-7) \quad (n = 4-7) \quad (n = 4) \]
Fig 2

a) 

![Graph showing body weight percentage over days for different groups.]

- C57BL/6 + DSS
- C57BL/6
- BALB/c + DSS
- BALB/c

b) 

![Graph showing diarrhea score over days for different groups.]

- C57BL/6
- BALB/c
Fig 2

c) [Graph showing Fecal Blood levels over Days for C57BL/6 and BALB/c mice under DSS and Water conditions]

d) [Graph showing Inflammatory score over Days for C57BL/6 and BALB/c mice under DSS and Water conditions]
Fig 3

a) 

[Graph showing Colon wet weight/length (mg/cm) over days, with two lines representing C57BL/6 and BALB/c mice.]

b) 

[Graph showing Haptoglobin (g/l) over days, with two lines representing C57BL/6 and BALB/c mice.]
Fig 4

C57BL/6

a) Control
b) d5

c)
d)

BALB/c

e) d12
f) d19
g) d26

C57BL/6

h)
i) j)

BALB/c
Fig 5
Fig 7

a) IL-1β

![Graph showing IL-1β levels over time for C57BL/6 and BALB/c mice with different treatments (DSS vs Water)].

b) IL-12p70

![Graph showing IL-12p70 levels over time for C57BL/6 and BALB/c mice with different treatments (DSS vs Water)].
Fig 7

c) IL-12p40

- C57BL/6
- BALB/c

pg / 100 mg colonic tissue

DSS Water

Days

0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34

pg / 100 mg colonic tissue

DSS Water

Days

0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34

d) IL-17

- C57BL/6
- BALB/c
Fig 7

e) IFN-γ

- C57BL/6
- BALB/c

pg / 100 mg colonic tissue

Days

DSS  Water