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Germ-free and Antibiotic-treated Mice are Highly Susceptible to Epithelial Injury in DSS Colitis

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Abstract

Background and Aims: Intestinal microbiota is required to maintain immune homeostasis and intestinal barrier function. At the same time, intraluminal bacteria are considered to be involved in inflammatory bowel disease and are required for colitis induction in animal models, with the possible exception of dextran sulphate sodium [DSS] colitis. This study was carried out to ascertain the mechanism underlying the induction of colitis by DSS in the absence of bacteria.
Methods: Conventional and germ-free [GF] Naval Medical Research Institute [NMRI] mice were used, plus conventional mice treated with an antibiotic cocktail to deplete the intestinal microbiota ['pseudo-GF' or PGF mice]. The differential response to DSS was assessed.
Results: Conventional mice developed DSS-induced colitis normally, whereas GF mice showed only minimal inflammation [no colonic thickening, lower myeloperoxidase activity, IL-6, IL-17, TNF-α, and IFN-γ secretion by splenocytes and mesenteric cell cultures, etc.]. However, these mice suffered enhanced haemorrhage, epithelial injury and mortality as a consequence of a weakened intestinal barrier, as shown by lower occludin, claudin 4, TFF3, MUC3, and IL-22. In contrast, PGF mice had a relatively normal, albeit attenuated, inflammatory response, but were less prone to haemorrhage and epithelial injury than GF mice. This was correlated with an increased expression of IL-10 and Foxp3 and preservation barrier-related markers.
Conclusions: We conclude that enteric bacteria are essential for the development of normal DSS-induced colitis. The absence of microbiota reduces DSS colonic inflammation dramatically but it also impairs barrier function, whereas subtotal microbiota depletion has intermediate effects at both levels.

Key Words: Germ-free; mucosal; barrier function; microbiota

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1. Introduction

The gastrointestinal tract is the home of the largest bacterial population in the body, which is maximal in the caecum, followed by the colon and then the ileum, jejunum and finally duodenum.[1] Despite the enormous bacterial load carried by the gastrointestinal tract and the sheer variety of species present, an exquisite balance is maintained at almost all times. The combination of an efficient, self-repairing barrier, abundant mucus secretion, continuous luminal flow of contents and a vigorous yet finely regulated immune system is capable of keeping a massive foreign population contained within the limits of the mucosa. This delicate equilibrium represents a well-balanced opposition of considerable forces. Alteration of this equilibrium is pivotal in the development of inflammatory bowel disease [IBD], which is considered to be strongly dependent on the presence of luminal bacteria. Although both Crohn's disease and ulcerative colitis are the result of the combined effects of genetic variants and environmental factors, a dysregulated immune response toward the normal microbiota is a pivotal factor in IBD.[2]

Far from being simply a source of pathogenic germs, the intestinal microbiota has a complex relationship with the host, in which both benefit from the association. Thus the microbiota contributes to the maturation and activation of the immune system and to the maintenance of the intestinal mucosal barrier, which requires the input of microbiota-derived signals such as short chain fatty acids or toll receptor ligands.[3] Germ-free [GF] animals have been an excellent research tool to study the role of bacteria in the maturation of the mucosal immune system. The study of laboratory animals in GF conditions, available now for ~ 50 years, soon revealed that mice and rats survive fairly well without bacteria. Reproduction and overall appearance and physiology are essentially normal. In fact, it was shown early on that GF mice survive much longer than the conventionally reared mice,[4] and this seemed to be the case also for rats.[5] Nevertheless, GF animals exhibit an underdeveloped mucosal immune system in the gut, whereas conventional rodents have what has been described as 'physiological inflammation', ie. a controlled status of sustained, low-grade immune stimulation or surveillance. Another relevant change observed in GF animals is the occurrence of a defect in hydroelectrolytic transport, albeit this is compensated and results only in softening of the faeces.[6] Little attention has been paid to study the effects on the mucosal barrier function of the immature immune system in GF animals.

One of the main arguments that supports the importance of the intestinal microbiota in this context is the fact that intestinal inflammation is very difficult to induce in experimental animals in GF conditions. Nevertheless, some studies have indicated that dextran sulphate sodium [DSS] colitis can be induced in GF conditions with similar or enhanced severity compared with that in regular mice.[7,8] Hence, the role of luminal bacteria in this model is unclear. DSS is a high molecular weight glucose polymer which incorporates straight and branched chains of variable length, with approximately three molecules of sulfate per monomer. It is structurally related to carrageenan, one of the first compounds used to induce experimental colitis, and it was first applied as a colitogenic agent in the 1980s.[9] In this model of IBD, DSS is included in the drinking water of mice and eventually reaches the colon, where it modulates tight junction proteins and probably exerts cytotoxic effects on epithelial cells, leading to augmented permeability and subsequent immune stimulation. This mode of action would be predicted to be dependent on the presence of luminal microbiota. In this regard, the antibiotics metronidazole and ciprofloxacin ameliorate acute, although not chronic, DSS colitis in mice, and oral administration of bacterial sonicates dampens DSS colitis, possibly via tolerogenic mechanisms.[9,10]

Taking in consideration these facts and the relationship between the intestinal microbiota and the intestinal immune system and barrier function, we formulated the hypothesis that these two elements may be differentially regulated in GF conditions. In order to test this hypothesis, we assessed the response to DSS in conventional mice, GF mice, and conventional animals with acquired depletion of the microbiota [which we will refer to as 'pseudo-GF' or PGF here].

2. Materials and Methods

2.1. Reagents

Except where indicated, all reagents and primers were obtained from Sigma [Barcelona, Spain]. RNeasy Mini Kit was obtained from QIAGEN [Hilden, Germany]. Reverse transcription was achieved with the iScript™ cDNA Synthesis Kit, and iQ™ Sybr® Green Supermix was used for amplification [Biorad, Alcobendas, Madrid, Spain]. Mouse enzyme-linked immunosorbent assay [ELISA] kits were obtained from eBioscience [San Diego, CA, USA]. DSS was obtained from ICN Biomedicals [Costa Mesa, CA, USA]; average molecular weight: 36–50 kDa.

2.2. Animals

A total of 24 GF Naval Medical Research Institute [NMRI], half females and half males, were obtained from the Karolinska Institutet Core Facility for Germ-free Research [Comparative Medicine, Karolinska Institutet, Stockholm, Sweden] and maintained there. Newborn litters of GF NMRI mice were placed and raised in special plastic isolators until they reached 16 to 18 weeks [wk] of age. Animals were maintained on autoclaved R36 Lactamin Chow and kept in 12-h light-dark cycles. Males and females were kept separately. The GF status was checked weekly as routine quality control by culturing faecal samples, both aerobically and anaerobically. A total of 48 conventional NMRI mice were purchased from Janvier-Labs [Rennes, France] and maintained at the University of Granada in air-conditioned animal quarters with a 12-h light-dark cycle. Animals were housed in makrolon cages and were given free access to autoclaved tap water and food [Harlan-Teklad 2014, Harlan Ibérica, Barcelona, Spain]. Males and females were kept separately. Acquired depletion of colonic microbiota was achieved in 24 of these NMRI mice [half females and half males] by the administration of an antibiotic cocktail: ampicillin 1 g/l [Applichem, Darmstadt, Germany], neomycin 1 g/l, metronidazole 0.25 g/l, and vancomycin 0.5 g/l. Antibiotic treatment was applied for 4 wk before DSS was started and was maintained until the end of the experiment. Subtotal bacterial depletion was confirmed by conventional culture as above and, additionally, DNA from faeces was extracted with QIAamp DNA stool Minikit [QIAGEN], quantified, and analysed for total 16S by quantitative polymerase chain reaction [qPCR] with a Biorad CFX connect real-time PCR device [Alcobendas, Madrid, Spain], using a nonvariant amplicon [16SDPO-forward 5′-AGAGTTTGATCMTGGCTCA-I-I-I-I-I-AACGCT-3′; 16SDPO-reverse 5′-CGCGGCTGCTGGCA-I-I-I-A-I-TTRGC-3′][11] [see Supplementary Figure. 1, available as Supplementary data at *ECCO-JCC* online].

All animal procedures in this study were carried out in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the U.S. National Institutes of Health, and were approved by the Animal Experimentation Ethics Committee of the University of Granada [registry number 710].

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2.3. Induction of colitis and experimental design

Colitis was induced by adding 2% or 4% DSS to drinking water for 7 days. The status of the animals was monitored by general examination and body weight evolution. Mice were randomly assigned as follows. Conventional, GF and PGF mice were divided into three groups, namely control, 2% DSS, and 4% DSS [n = 8]. The control groups did not receive DSS. The same batch of DSS was used in conventional, GF, and PGF mice. Food intake, water intake and body weight were measured generally every day. Animals were sacrificed by cervical dislocation under isoflurane anesthaesia, and different organs were obtained: colon, ileum, liver, spleen, and mesenteric lymph nodes, plus a blood sample. The entire colon was removed, gently flushed with saline and blotted on filter paper, placed on an ice-cold plate, cleaned of fat and mesentery, and longitudinally opened so as to exhaustively eliminate faecal remains. Each specimen was weighed and its length was measured under a constant load [2 g]. A small segment was dissected from the intestine and used for RNA isolation. The colon was subsequently divided longitudinally into several pieces for biochemical determinations. The fragments were immediately frozen in liquid nitrogen and kept at -80ºC until used.

2.4. Myeloperoxidase [MPO] and alkaline phosphatase [AP] activities

Colonic tissue homogenisation was carried out with the protocol for intestinal tissue homogenisation in a Bullet Blender® [Next Advance, Inc., NY, USA] in 50 mM Tris base buffer with 0.5% hexadecyl trimethyl ammonium bromide, pH 6.0. MPO activity was measured spectrophotometrically as the peroxidase enzymatic activity extracted from colonic tissue after homogenisation [1:20 w/v]. The homogenate was sonicated and subjected to three freeze-thaw cycles before measurement. The enzymatic reaction was performed in presence of 0.0005% hydrogen peroxide and 0.168 mg/ml o-dianisidine as substrate. AP activity was measured spectrophotometrically, using 5.5 mM disodium nitrophenyl phosphate as substrate in 50 mM glycine buffer with 0.5 mM $MgCl_2$, pH 10.5. The sensitivity to the AP inhibitor levamisole was also tested *in vitro*. AP and MPO enzymatic activities are expressed as mU/mg protein.[12–14]

2.5. RNA isolation and quantitative reverse-transcription polymerase chain reaction [RT-qPCR] analysis

Total RNA was isolated using RNeasy Mini Kit [QIAGEN] according to the manufacturer's instructions. Quantification was determined by the 260/280 nm absorbance ratio; 1 µg RNA was subjected to reverse transcription; iQ™ Sybr® Green Supermix was used for amplification, and specific DNA sequences were amplified with a Biorad CFX connect real-time PCR device [Alcobendas, Madrid, Spain]. Primers used are shown in Table 1. Results are expressed as 2^{-ddCt} using 18S as reference gene.

2.6. Western blot analysis

Tissue samples were homogenised in lysis buffer [0.1% w/v SDS, 0.1% w/v sodium deoxycholate, 1% v/v Triton X-100 in PBS] with protease inhibitor cocktail 1:200 [v/v] and phosphatase inhibitor cocktail 1:100 [v/v] [Santa Cruz]. Then homogenates were sonicated and centrifuged at 10000 rpm for 5 min at 4 °C. Protein concentration was determined by the BCA assay. Samples were boiled for 5 min in Laemmli buffer [Bio-Rad], separated by SDS–PAGE, electroblotted to nitrocellulose membranes [Millipore], and probed with the corresponding antibody. The bands were detected by enhanced chemiluminescence [PerkinElmer] and quantitated with the software Image J. The primary antibody anti-claudin 2 [1:1000], anti-claudin 4 [1:1000], and anti-occludin [1:1000] were purchased from Life Technologies [Grand Island, NY, USA]; anti-PCNA [1:1000] and anti-cytokeratin 5/8 from BD Biosciences [1:1000, Bedford, MA, USA]; anti-phospho STAT3 [1:2000] and anti-STAT3 [1:1000] from Cell Signaling Techonlogies [Danvers, MA, USA]; and anti-ZO-1 [1:1000] and anti-beta actin [1:1500] from Abcam [Cambridge, UK]. The bands were quantified with the National Institute of Health software Scion Image.

2.7. Mesenteric lymph node cells [MLNC]

MLNC were extracted from the mice in the study using sterile technique and dissected mechanically. Cells were washed once with fresh medium and were filtered using a 70-µM filter [cell strainer BD Falcon™, Ref. 352350] to obtain a mononuclear suspension. The

Table 1. Mouse primers [sequence 5′-3′].

	Forward	Reverse
18S	ACA CGG ACA GGA TTG ACA GAT TG	GCC AGA GTC TCG TTC GTT ATC G
IL-1β	AAG GGC TGC TTC CAA ACC TTT GAC	TGC CTG AAG CTC TTG TTG ATG TGC
IL-10	CAG GAC TTT AAG GGT TAC TTG	ATT TTC ACA GGG GAG AAA TC
IL-17A	ACG TTT CTC AGC AAA CTT AC	CCC CTT TAC ACC TTC TTT TC
IL-22	ATC AGT GCT ACC TGA TGA AG	CAT TCT TCT GGA TGT TCT GG
IL-27	AAT CTC GAT TGC CAG GAG	CTC AGA GTC AGA GAG GTG
IFN-γ	GCT CTG AGA CAA TGA ACG CTA CAC	TTC TTC CAC ATC TAT GCC ACT TGA G
TNF-α	CGT GGA ACT GGC AGA AGA GG	CAG GAA TGA GAA GAG GCT GAG AC
ZO-1	GGG GCC TAC ACT GAT CAA GA	TGG AGA TGA GGC TTC TGC TT
Occludin	ACG GAC CCT GAC CAC TAT GA	TCA GCA GCA GCC ATG TAC TC
TFF3	CCT GGT TGC TGG GTC CTC TG	GCC ACG GTT GTT ACA CTG CTC
MUC3	AAA GAT TAC CTC CCA TCT CC	TAA AAC TAA GCA TGC CCT TG
KGF	AAA GAA CGG CAG TAA ATA CG	CCA GCA TCC TCA AAA GTT AC
S100A8	GCC CTC TAC AAG AAT GAC TTC AAG	ATC ACC ATC GCA AGG AAC TCC
REG3γ	CAG AGG TGG ATG GGA GTG GAG	CAC AGT GAT TGC CTG AGG AAG AG
FOXP3	AAT AGT TCC TTC CCA GAG TTC	GGT AGA TTT CAT TGA GTG TCC
VEGFa	TAG AGT ACA TCT TCA AGC CG	TCT TTC TTT GGT CTG CAT TC
MYC	TTT TGT CTA TTT GGG GAC AG	CAT AGT TCC TGT TGG TGA AG
Cyclin D1	CTA AGA TGA AGG AGA CCA TTC C	GTT CTG CTT GTT CTC ATC C

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cells were incubated in RPMI-1640 medium containing fetal bovine serum [10%], 2 mM L-glutamine, 100 U/ml penicillin, 0.1 mg/ml streptomycin, 2.5 mg/ml amphotericin B, and 0.05 mM mercaptoethanol. The cells were plated at a density of 10^6 cells/ml and a final volume of 500 µl/well in 24-well plates, and stimulated with concanavalin A [ConA] at a final concentration of 5 µg/ml in a humidified 5% CO_2 atmosphere at 37°C.

2.8. Mice spleen mononuclear cells

The spleen was extracted aseptically and a cell suspension was obtained by disrupting the tissue between dissecting forceps in Dulbecco's Modified Eagle's Medium supplemented with fetal bovine serum [10%], 2 mM L-glutamine, 100 U/ml penicillin, 0.1 mg/ml streptomycin, and 2.5 mg/ml amphotericin B. After centrifuging, cells were cleared of erythrocytes by suspension on hypotonic lysis buffer [red blood cell lysis buffer, Sigma, Barcelona, Spain] for 5 min at room temperature. Cells were washed once with fresh medium and were filtered using a 70-µm filter [cell strainer BD Falcon™, Ref. 352350] to obtain a mononuclear suspension; 10^6 cells/ml cells were cultured on a final volume of 500 µl/well in 24-well plates, and stimulated with ConA at a final concentration of 5 µg/ml in a humidified 5% CO_2 atmosphere at 37°C.

2.9. Cell viability assay

Cell viability was quantified with the Trypan blue exclusion assay. Cell suspensions were diluted 1:10 for MLNC and 1:20 for splenocytes in 0.4% Trypan blue in PBS, and incubated for 2 min while shaking, and viable [unstained] and total cells were counted.

2.10. Cytokine determination

Cell culture medium was collected after 48 h, cleared by centrifugation [9300 g/10 min/4°C] and frozen at -80°C until assayed for cytokine content by commercial ELISA, following the protocols recommended by the manufacturer. The cytokines determined were IL-6, IL-17, IFN-γ, and TNF-α [eBiosciences]. Plates [Nunc™ Inmuno plate, Roskilde, Denmark] were read at 450 nm using a plate reader [Tecan, model Sunrise-basic, Austria].

2.11. Faecal occult blood determination

Stool samples were freshly collected individually from each mouse and stored at –80°C. The protocol used for the faecal occult blood determination is an adaptation of the one first developed by the Dr Donald S. Young.[15] Briefly, stool samples were homogenised in distilled ultrapure water [Spibio] [0.105 mg of wet faeces/µl]. Then, 200 µl of that solution was taken into a 15-ml tube, incubated at 100°C for 10 min, and mixed with 1.2 ml of acetic acid and distilled water [30/70]. Further, 1.8 ml of pure ethyl acetate were added on top and the whole mixture vortexed and centrifuged [2500 rpm/3 min/room temperature]. Finally, the upper phase was collected and exposed to 3,3',5,5' tetramethylbenzidine in the presence of H_2O_2 and the reaction was monitored spectrophotometically after 30 and 60 s at 660 nm. As a reference, a haemoglobin curve was generated in distilled water and subjected to the same protocol above described. Results are expressed as µg haemoglobin/mg of faeces.

2.12. Data and statistical analysis

Samples were run at least in triplicate and results are expressed as mean ± standard error of the mean [SEM]. The obtained values are the result of the average values between males and females. Differences among means were tested for statistical significance by two-way analysis of variance [ANOVA] and *a posteriori* Fisher's least significant difference [LSD] tests on preselected pairs. All analyses were carried out with GraphPad Prism [La Jolla, CA, USA]. Differences were considered significant at $p < 0.05$.

3. Results

3.1. Colitis evolution and animal status

The supplementation of drinking water with 4% DSS caused significant body weight loss in all conditions [Figure 1]. In addition, GF mice treated with 2% DSS showed a significantly lower body weight just before sacrifice. Three GF mice died in the 4% DSS group, compared with none in the conventional and PGF groups ($p < 0.05$ by log-rank [MantelCox] test).

As expected, conventional mice treated with 2% or 4% DSS showed an inflammatory reaction characterised by loss of the mucosal vascular pattern, hyperaemia and occasional haemorrhage, oedema, and fibrotic features, resulting in a dose-dependent increase in colonic weight to length ratio compared with the non colitic group [Table 2]. Conventional mice with DSS colitis also showed splenomegaly but no change in liver weight [Table 2]. GF mice appeared significantly sicker than conventional mice, especially with the 4% dose, as they showed reduced spontaneous movement, increased huddling behavior and eye and ear decolouration. GF DSS treated mice exhibited diarrhoea and haematochezia, beginning around Day 4. After sacrifice, it was interesting to note that intestinal hyperaemia was completely absent, the blood sample appeared pink rather than red and the liver had a brownish color [4% dose]. Taken together, these findings are suggestive of substantial blood loss in DSS-treated GF mice, which was confirmed by the faecal occult blood test [Figure 2]. In addition to the lack of hyperaemia, the colon of these animals was fragile and rigid but not thickened. It is also worth mentioning that the colon was very difficult to clean of faecal material, which had a dark blue colour and a very thick consistency. Ileal weight to length ratio was reduced with 4% DSS in these animals, compared with no change in the normal and PGF groups.

The appearance of PGF mice largely resembled that of conventional mice, with hyperaemia but, interestingly, no significant colonic thickening [Table 2]. As in conventional mice, DSS colitis was associated with splenomegaly but no changes in liver weight. However, it should be noted that liver weight was significantly lower in both GF and PGF mice than in the normal animals, with the sole exception of the control PGF group. Blood loss as assessed with the faecal occult blood test was elicited similarly by 4% DSS in PGF and conventional mice [Figure 2]. In addition, PGF mice showed changes in water absorption and colonic transporter expression that are comparable to those in GF mice compared with conventional controls [data not shown]. Besides, PGF mice also developed the characteristic caecum enlargement observed in GF mice. GF mice also showed splenomegaly [4% dose only] but no hepatomegaly.

These data indicate that GF conditions result in lower intestinal inflammation but, despite the occurrence of only minimal inflammatory reaction, they are more prone to blood loss, general deterioration and death. PGF mice exhibited an intermediate phenotype, with hyperaemia but no thickening of the colon, and they were protected from enhanced blood loss and overall deterioration. The impact of DSS on epithelial cells was further assessed by measuring cytokeratin 5/8 in colonic tissue by western blot. Consistent with the above, the levels of the epithelial marker were severely depressed in GF animals but not in PGF conditions.

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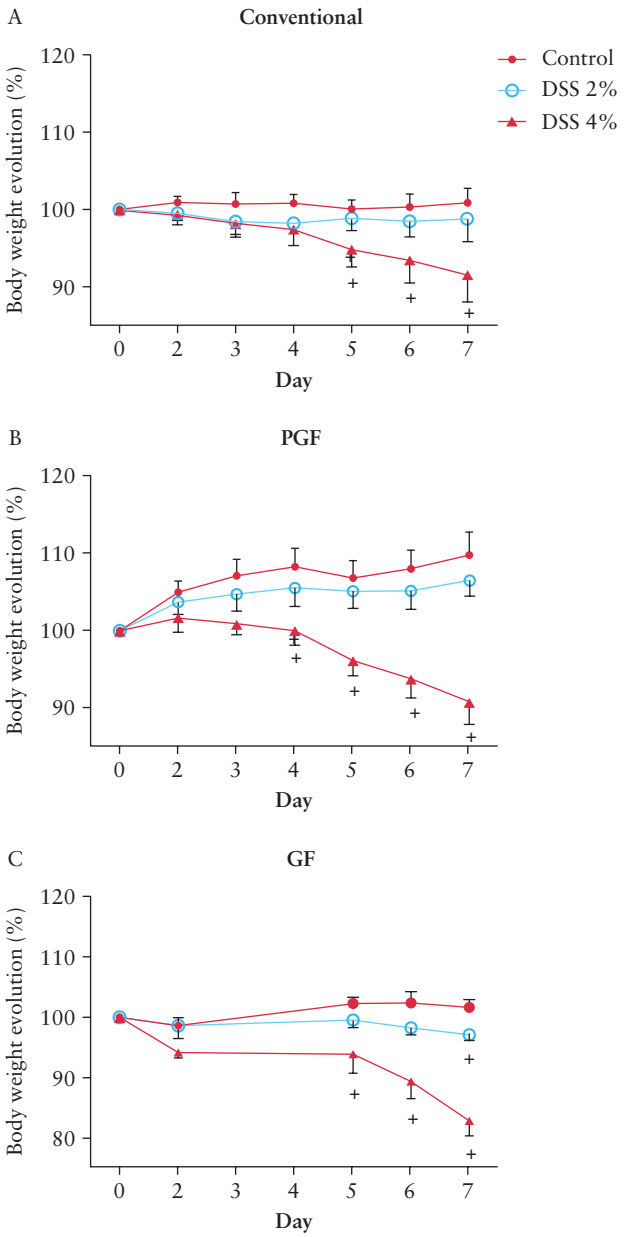

Figure 1. Body weight evolution [%] during DSS-induced colitis in conventional [A], PGF [B] and GF [C] Naval Medical Research Institute [NMRI] mice. Body weight is expressed as percentage [%] ± SEM; $^{+}p < 0.05$ vs control. DSS, dextran sodium sulphate; PGF, pseudogerm-free; GF, germ-free; SEM, standard error of the mean.

3.2. Colonic inflammatory markers

In conventional mice treated with DSS there was a dose-dependent increase in neutrophil recruitment to the colonic tissue, as measured by colonic MPO activity, compared with the non colitic group [Figure 3A]. Colonic AP activity, a marker of intestinal inflammation and epithelial stress, was also significantly greater in the colitic groups than in the control group [Figure 3B]. In addition, the sensitivity of this enzymatic activity to levamisole *in vitro* was increased in colitic tissue compared with the control, consistent with neutrophil infiltration and the isoform shift previously described in enterocytes [Figure 3C].[16] In the three parameters, the increase was significant with 2% and 4% DSS [$p < 0.05$]. Conversely, GF mice exhibited no change in MPO activity, whereas PGF mice had a diminished response to 4% DSS [Figure 3A].

Table 2. Morphological indicators of inflammation in conventional, PGF and GF NMRI mice.

	Colon weight:length ratio	Ileum weight:length ratio	Spleen weight[a]	Liver weight[a]
	[mg/cm]		[g organ/body weight mouse]·1000	
Conventional				
Control	33.1 ± 2.4	22.1 ± 1.9	3.52 ± 0.20	48.25 ± 1.35
DSS 2%	46.9 ± 5.2^{+}	25.0 ± 1.6	4.73 ± 0.48^{+}	52.64 ± 1.52
DSS 4%	52.8 ± 4.6^{+}	22.9 ± 1.4	5.14 ± 0.76^{+}	52.23 ± 1.42
PGF				
Control	30.3 ± 2.3	22.0 ± 1.3	3.33 ± 0.16	44.55 ± 1.47
DSS 2%	38.2 ± 3.6	26.3 ± 0.9	4.00 ± 0.19	43.26 ± 2.47$^{\#}$
DSS 4%	40.2 ± 2.1$^{\#}$	23.9 ± 1.5	6.00 ± 0.44^{+}	42.31 ± 2.96$^{\#}$
GF				
Control	21.0 ± 0.9$^{\#}$	21.4 ± 0.8	2.86 ± 0.09	36.35 ± 0.94$^{\#}$
DSS 2%	22.4 ± 1.5$^{\#}$	21.1 ± 0.8	3.12 ± 0.31$^{\#}$	36.73 ± 0.88$^{\#}$
DSS 4%	20.5 ± 1.0$^{\#}$	15.1 ± 2.3$^{+\#}$	5.01 ± 0.83^{+}	35.21 ± 1.88$^{\#}$

PGF, pseudo-germ-free; GF, germ-free; DSS, dextran sodium sulphate; SEM, standard error of the mean; NMRI, Naval Medical Research Institute.

[a]Refers to the animal's relative weight. Values are means ± SE; $^{+}p < 0.05$ vs corresponding control; $^{\#}p < 0.05$ vs the same condition in the conventional group.

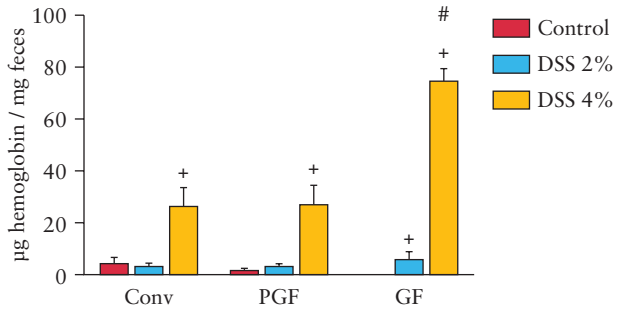

Figure 2. Faecal occult blood; faeces were obtained from the colon of individual mice straight after sacrifice and blood content measured as described in Materials and Methods; $^{+}p < 0.05$ vs control; $^{\#}p < 0.05$ vs conventional mice.

Colonic AP activity was similarly increased in the three microbiota conditions [Figure 3B]; however, enzyme inhibition by levamisole *in vitro* was lower in both PGF and specially in GF mice [Figure 3C].

Colonic mRNA levels of several inflammatory markers, including IL-17, TNF-α, IFN-γ, IL-1β, and S100A8, were measured by RT-qPCR. All five markers were upregulated by 2–4% DSS in conventional mice [Figure 4]. Interestingly, the increase was lower with 4% DSS than with 2% in some cases [TNF-α, IL-1β, S100A8]. GF mice exhibited dramatic changes in this profile, as the expression of IFN-γ, IL-1β, and S100A8 was very low and unaffected by DSS, whereas IL-17 and TNF-α levels were higher than in conventional mice. In contrast, PGF mice showed a profile that was largely similar to that of conventional animals. Thus GF mice displayed distinct changes in the inflammatory response to DSS, whereas PGF mice were more similar to conventional mice.

3.3. Cytokine secretion by MLNC and splenocytes

DSS colitis was associated with increased cytokine production by MLNC of conventional mice *ex vivo* [Figure 5]. In sharp contrast,

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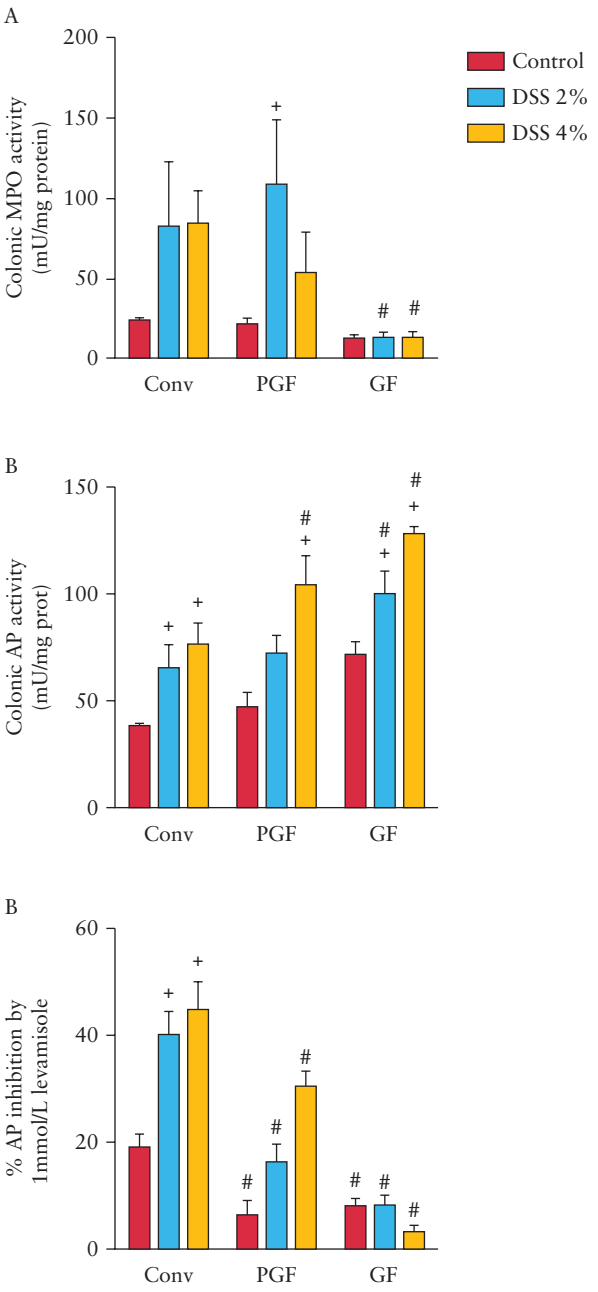

Figure 3. Colonic MPO [A] and AP [B] activities, plus AP sensitivity to the specific inhibitor levamisole [C]. Values are means ± SEM; $^{+}p < 0.05$ vs control; $^{\#}p < 0.05$ vs conventional mice. MPO, myeloperoxidase; AP, alkaline phosphatase; SEM, standard error of the mean.

cells obtained from GF mice showed unaltered secretion of IL-6 and negligible production of TNF-α, IL-17A, and IFN-γ. MLNC from PGF mice were generally comparable to those of normal mice, although higher production of IL-6 and IFN-γ with 2% DSS and a lower output of IL-17A with 4% DSS were observed. Conversely, only IL-17A production was consistently associated with DSS colitis in the case of splenocytes [Figure 6]. Again, cells isolated from GF mice displayed an almost null level of cytokine secretion. In the case of PGF animals, cytokine levels were either similar to or lower than those in conventional mice. In both MLNC and spleen cells, IFN-γ exhibited the highest overall level of production.

Therefore GF mice showed a greatly diminished cytokine production by MLNC and splenocytes, whereas cells obtained from PGF and conventional animals were generally comparable.

3.4. Mucosal barrier function

In order to explore the possible mechanism underlying the discrepant results presented so far, we measured by RT-qPCR a number of parameters related to mucosal barrier function, based on the hypothesis that this plays a central role in the overall response to DSS. As shown in Figure 7, the epithelial junctional genes occludin and ZO-1 were downregulated in GF vs conventional mice in basal conditions, but PGF mice were comparable to those in the conventional group. A similar pattern was noted in the case of MUC3 and TFF3. In contrast, REG3γ expression was negligible in basal conditions in all control groups.

Conventional mice receiving DSS showed a significantly reduced expression of occludin but not of ZO-1 at the mRNA level. REG3γ was markedly upregulated, and MUC3 and TFF3 were unchanged. In GF mice, the REG3γ upshot was enhanced but MUC3, TFF3, and occludin remained at very low levels. ZO-1 was unchanged. Again, PGF mice data were quite similar to those in conventional mice, except in that TFF3 was further upregulated.

We additionally measured occludin and ZO-1, plus claudin 2/4, by western blot [Figure 8]. Protein levels of ZO-1, but not occludin, were reduced in control GF mice compared with the conventional and PGF groups. Importantly, the expression of both tight junction components was compromised in GF mice after DSS exposure [non-significant with the 2% dose], consistent with damaged barrier function. These effects were largely absent in PGF animals. In contrast, protein levels of claudin 2 and 4 were downregulated in both PGF and GF mice. Conventional mice, however, displayed an enhanced expression of claudin 4 but not claudin 2, in response to DSS.

Keratinocyte growth factor [KGF], IL-22, and IL-27, mucosal mediators with known epithelial proliferative actions, were generally increased in normal mice in response to DSS [Figure 9]. Of these, only IL-27 was upregulated in GF mice, showing actually an enhanced response to 4% DSS. PGF mice showed a singular pattern, with a significantly higher basal IL-22 expression which was not further upregulated by exposure to DSS, negligible IL-27 levels, and a KGF response comparable to that of conventional mice, and that was even enhanced with 4% DSS.

We hypothesised that IL-10, a cytokine that enhances mucosal barrier function, may be involved in the differential effects observed in conventional, GF, and PGF mice. IL-10 levels were significantly augmented in the colon of conventional and PGF mice exposed to DSS, but not in GF animals [Figure 9]. Interestingly, IL-10 expression in PGF animals was comparable to that in conventional mice. Thus, the changes in occludin, ZO-1, MUC3, and TFF3, among others, correlate with IL-10 expression. We further hypothesised that previous normal exposure of PGF mice to bacteria and the resulting induction of Treg cells in the colonic mucosa may exert protective effects in these mice that are absent in GF. Thus, we measured the expression of the regulatory T cell [Treg] marker Foxp3 [Figure 9]. Indeed, DSS treatment resulted in a robust increase of Foxp3 in either conventional or PGF mice, but not in GF mice.

In order to assess the possible impact of DSS and germ status on the epithelial proliferative response, we measured PCNA and pSTAT3/STAT3 by western blot in colonic tissue [Figure 9]. Although PCNA levels were comparable among the control groups, conventional animals showed an increased expression after DSS challenge, that was absent in PGF and GF mice. STAT3 phosphorylation was

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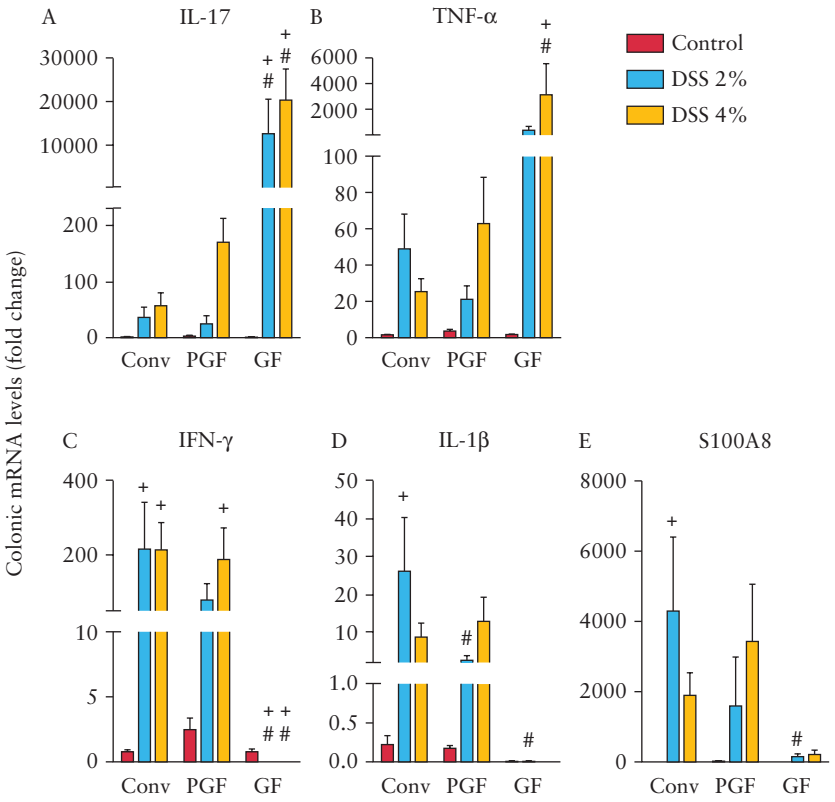

Figure 4. Colonic expression of inflammatory markers assessed by RT-qPCR; colonic mRNA levels of IL-17 [A], TNF-α [B], IFN-γ [C], IL-1β [D], and S100A8 [E] are shown. Values are means [fold change] ± SEM; $^{+}p < 0.05$ vs control; $^{\#}p < 0.05$ vs conventional mice. RT-qPCR, real-time quantitative polymerase chain reaction; SEM, standard error of the mean.

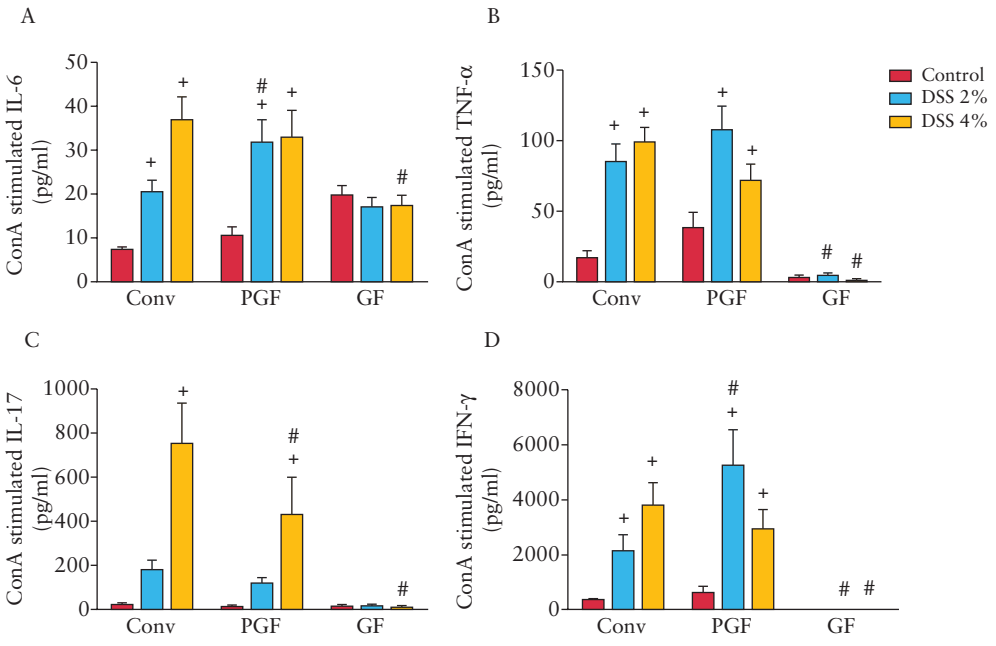

Figure 5. Cytokine secretion by MLNC. [A] IL-6; [B] TNF-α; [C] IL-17; [D] IFN-γ. MLNC were cultured *ex vivo* and stimulated with ConA 5 μg/ml and the supernatant was collected at 48 h and analysed for cytokine secretion by ELISA. Concentrations are expressed as means ± SEM [pg/ml]; $^{+}p < 0.05$ vs control; $^{\#}p < 0.05$ vs conventional mice. MLNC, mesenteric lymph node cells; ELISA, enzyme-linked immunosorbent assay; SEM, standard error of the mean.

similarly augmented in the DSS-treated groups, but this effect was dampened significantly in GF conditions. The colonic adaptive response to DSS injury was additionally evaluated by means of the mRNA levels of MYC, cyclin D1, and VEGF. Consistent with the decreased proliferative response, MYC and cyclin D1 expression was very low in GF animals compared with conventional and PGF mice. A similar pattern was noted with VEGF, which is in keeping with enhanced blood loss in the GF group.

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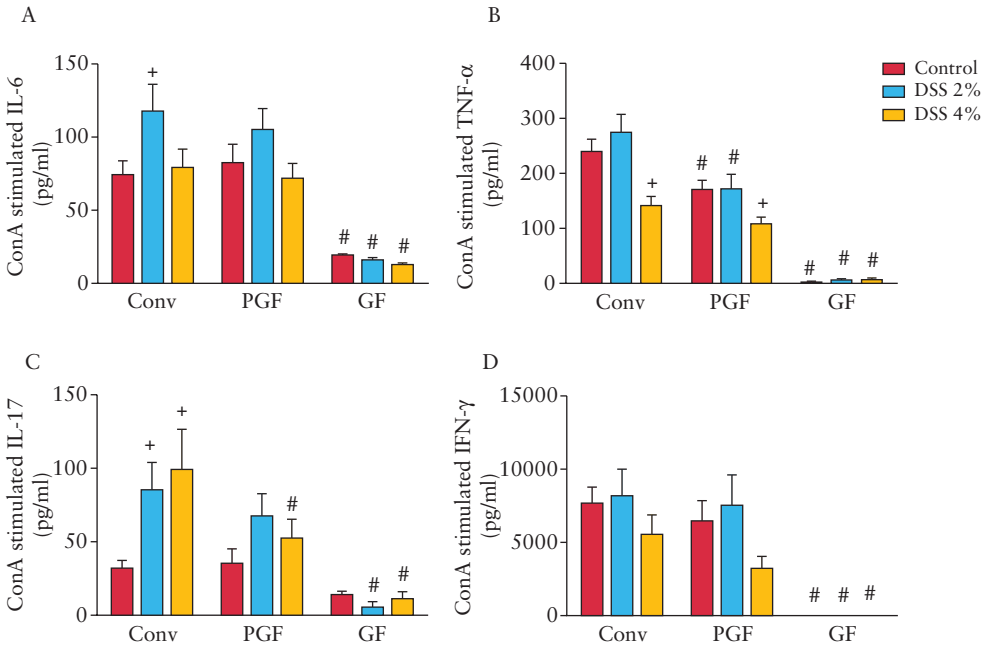

Figure 6. Cytokine secretion by splenocytes. [A] IL-6; [B] TNF-α; [C] IL-17; [D] IFN-γ. Splenocytes were cultured *ex vivo* and stimulated with ConA 5 µg/ml, and the supernatant was collected at 24 h and analysed for cytokine secretion by ELISA. Concentrations are expressed as means ± SEM [pg/ml]; $^{+}p < 0.05$ vs control; $^{\#}p < 0.05$ vs conventional mice. ELISA, enzyme-linked immunosorbent assay; SEM, standard error of the mean.

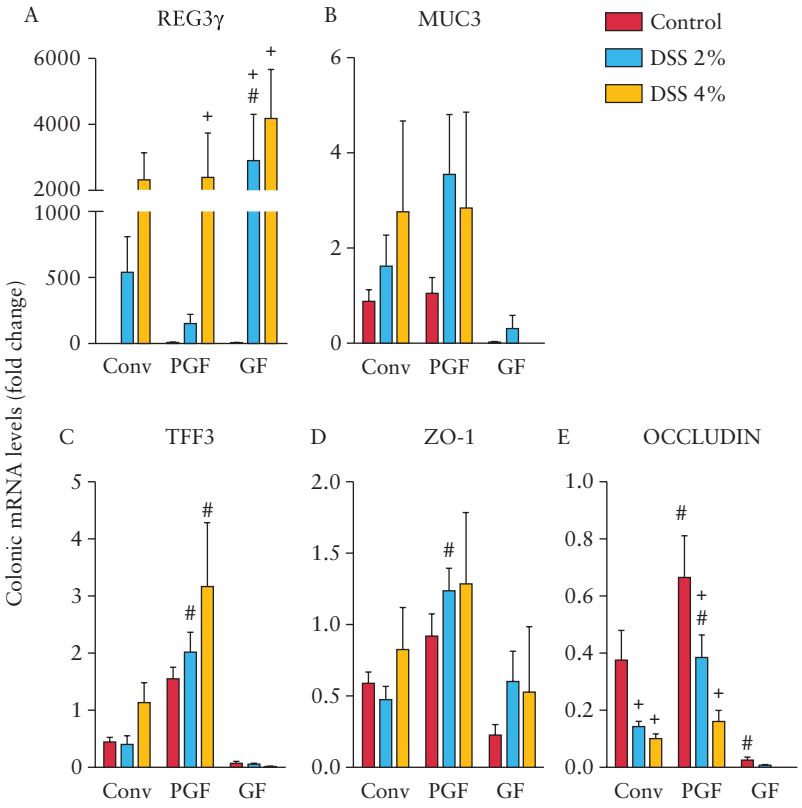

Figure 7. Colonic expression of mucosal barrier function markers assessed by RT-qPCR: colonic mRNA levels of REG3γ [A], MUC3 [B], TFF3 [C], ZO1 [D], and occludin [E] are shown. Values are means [fold change] ± SEM; $^{+}p < 0.05$ vs control; $^{\#}p < 0.05$ vs conventional mice. RT-qPCR, real-time quantitative polymerase chain reaction; SEM, standard error of the mean.

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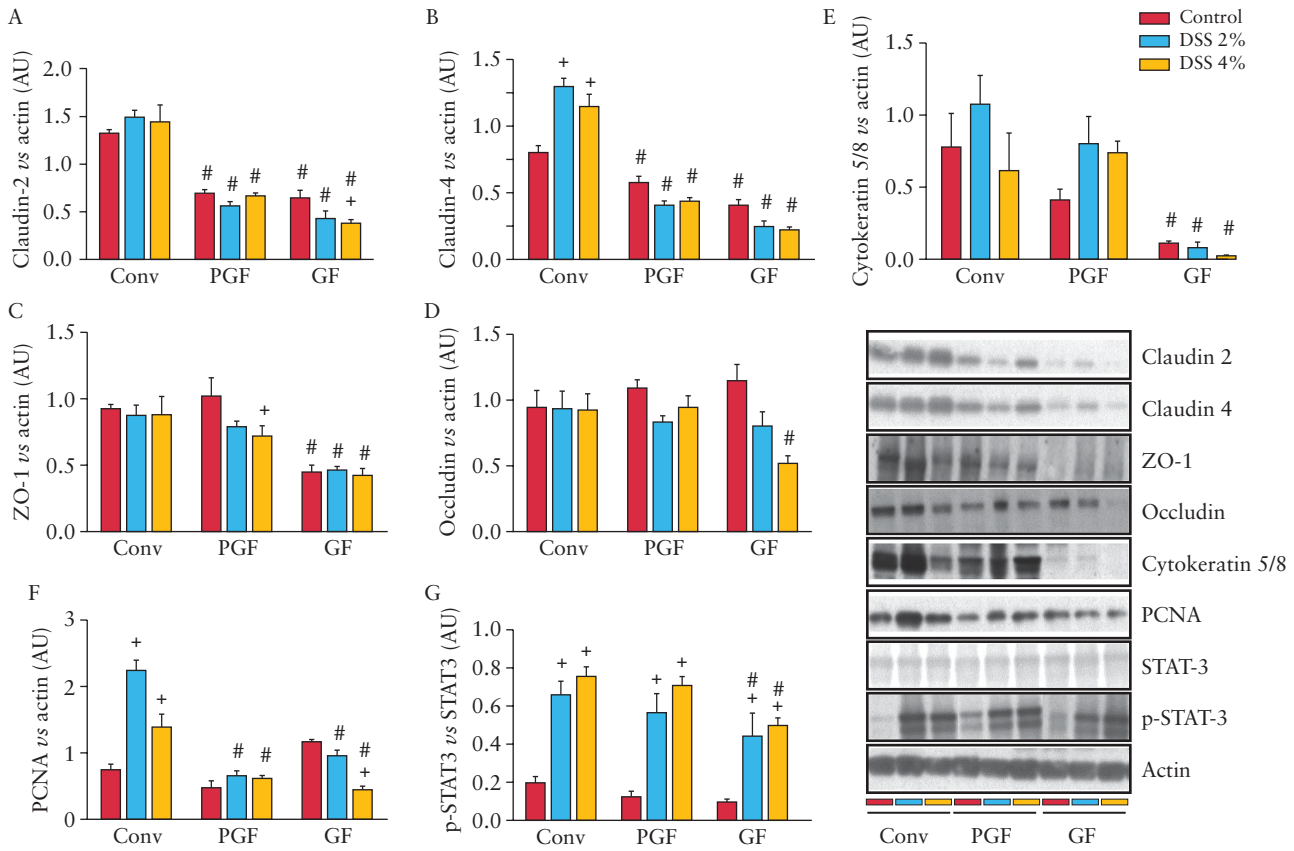

Figure 8. Colonic expression of mucosal barrier function markers assessed by western blot: colonic protein levels of: claudin 2 and 4 [A, B]; ZO-1 [C]; occludin [D]; cytokeratin [E]; PCNA [F]; and p-STAT3 [G] are shown. Representative blots and quantitation values as means [fold change] ± SEM are displayed; $^{+}p < 0.05$ vs control; $^{\#}p < 0.05$ vs conventional mice. SEM, standard error of the mean.

4. Discussion

The importance of the microbiota in IBD has been revealed to a large extent by evidence obtained in animal models. As a rule, intestinal inflammation develops weakly or not at all in GF conditions in multiple types of models, including genetic [IL-10 KO, IL-2 KO], lymphocyte transfer, and chemically induced models (trinitrobenzenesulphonic acid, (TNBS]).[16–22] There is also clinical evidence pointing to a significant involvement of the microbiota in 'human' IBD, particularly regarding Crohn's disease.[23] TNBS and DSS colitis are the two models of IBD most used for pathophysiological studies and, particularly, for the testing of pharmacological and nutritional treatments. Although TNBS is recognised as a microbiota-dependent model, in line with virtually all others,[21] there have been conflicting reports regarding DSS. Thus, DSS colitis has been claimed to develop normally in the absence of bacteria,[24] or even to be a more efficient colitogenic stimulus in these conditions,[8] whereas other authors have reported a weak inflammatory response to DSS colitis in GF conditions,[25] and DSS colitis is amenable to antibiotic treatment.[26] The reasons for these discrepancies are unknown.

Interestingly, Kitajima *et al*. showed that GF mice [IQI/Jic strain] died early after treatment with 5% DSS was started, with an enhanced decrease in haematocrit and weight loss.[8] Postmortem analysis showed signs of luminal blood loss but, notably, little sign of inflammation. In contrast, using a chronic protocol with a 14-day administration of 1% DSS, GF animals again exhibited weight loss and signs of haemorrhage compared with conventional animals, but inflammation was noted in the colon and it was higher than that observed with conventional mice, which was of low grade. The authors concluded that early death in the acute DSS protocol was due to massive blood loss, precluding the development of inflammation, whereas the chronic protocol allowed colitis to ensue in GF animals, showing that these animals are more susceptible than regular [ie conventional] animals. Enhanced blood loss in GF conditions was also observed by Maslowski *et al*. in C57BL/6 mice.[27] In another study, NMRI mice were also shown to exhibit a high mortality rate after treatment with 5% DSS. This was not due to strain specific sensitivity, because conventional NMRI mice responded normally.[24] However, the inflammatory status of the colon was not assessed in either study. IL-10 KO and wild type control GF mice were also found to be more affected clinically than conventional mice after DSS administration, in spite of lower infiltration of the mucosa and colonic damage scores that comparable to those of the control DSS group.[28]

Our results, obtained with the NMRI strain, also show that GF mice are globally more affected by DSS than conventional mice. DSS-treated GF mice looked sicker than regular mice, had increased mortality [at 4% DSS] and higher blood loss, consistent with previous observations. Conversely, the colon of GF mice showed few signs of inflammatory changes, with two major exceptions, namely colon IL-17 and TNF-α mRNA levels, which were increased beyond the response in conventional and PGF mice to DSS [see below]. Of note, colonic thickness was also much lower in GF animals, by approximately 50%. These features are consistent with the well-characterised atrophy of the intestinal mucosal immune system in GF animals, which extended to the mesenteric nodes and spleen in our study.[16] Therefore, our data indicate

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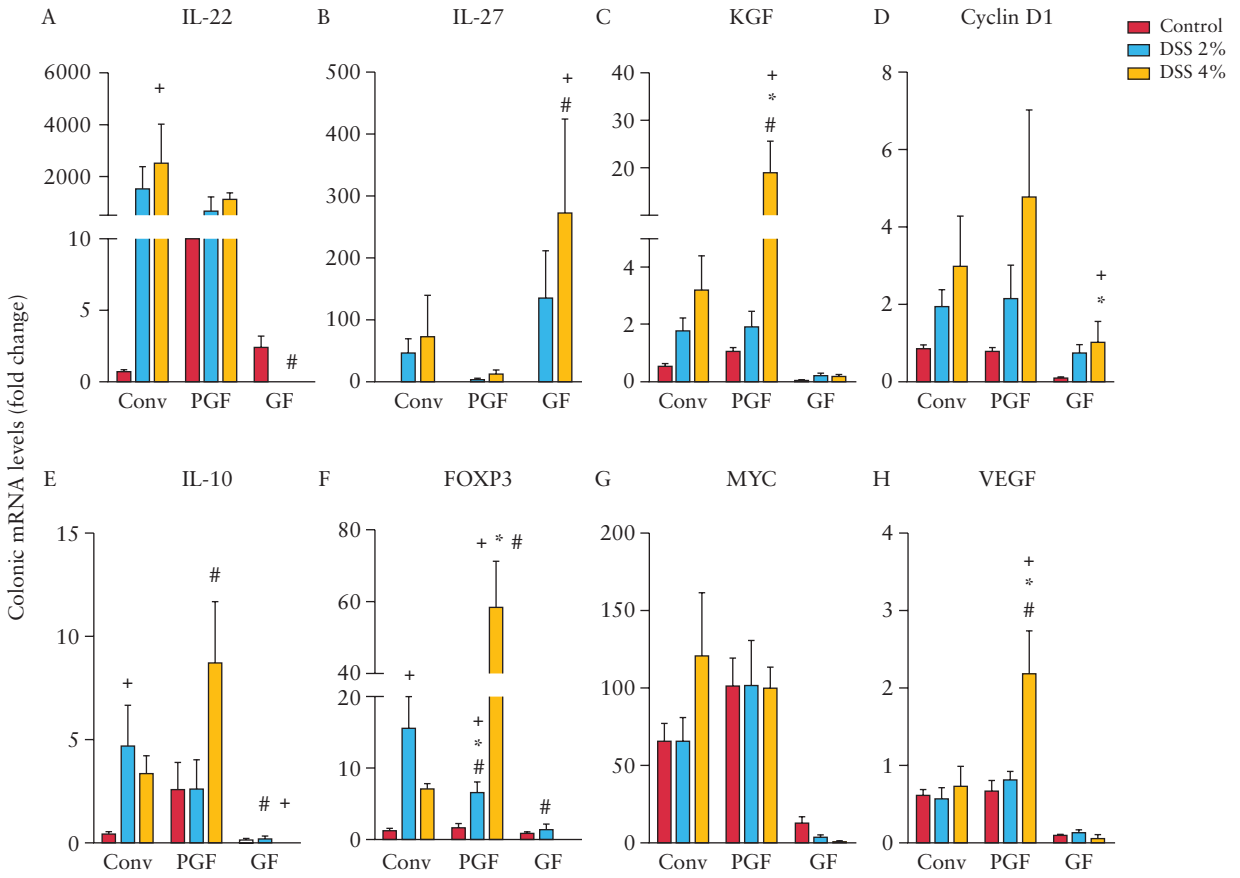

Figure 9. Colonic expression of various inflammatory/barrier function modulators assessed by RT-qPCR: colonic mRNA levels of IL-22 [A]; IL-27 [B]; KGF [C]; cyclin D1 [D]; IL-10 [E]; FOXP3 [F]; MYC [G]; and VEGF [H] are shown. Values are means [fold change] ± SEM; $^{+}p < 0.05$ vs control; $^{\#}p < 0.05$ vs conventional mice. RT-qPCR, real-time quantitative polymerase chain reaction; SEM, standard error of the mean.

unambiguously that DSS-induced colonic inflammation and overall immune response are greatly diminished in GF mice. Previous studies mentioned above have either documented global deterioration with minimal inflammation, or increased mortality and blood loss wrongly interpreted as signs of enhanced inflammation. Indeed, the absence of microbiota appears to have a dual effect on the response to DSS, ie attenuated inflammation but heightened epithelial injury and haemorrhage, leading to deterioration of animal status and even to death in some cases.

Colonic inflammation, either experimental or clinical, is frequently associated with rectal bleeding or subclinical haematochezia, and DSS colitis in particular is a model characterised by a marked visible blood loss in faeces. Such bleeding is consistent with a mechanism of epithelial disruption, especially if subepithelial cells are further affected by DSS, resulting in capillary lesions and blood loss to the lumen. Because DSS is continuously administered to animals, this process goes on indefinitely, resulting in protracted haemorrhage. In addition, DSS exerts anticoagulant effects, due to its similarity to heparin.[8,29] However, it is unclear why GF and conventional/PGF mice should respond differently to this challenge. One possibility is that anticoagulant effects are potentiated by lack of DSS degradation by bacteria, which has been claimed to occur normally in conventional mice.[24,30] However, this hypothesis has been disputed,[31] and it does not explain the effect on PGF mice. Thus, this mechanism is unlikely to be involved in the observed differences. Instead, enhanced bleeding may be the result of compromised mucosal barrier function, due to factors such as alteration of epithelial dynamics[32] or thinning of the mucus layer in GF animals.[33] Consistent with this hypothesis, ligation of TLR receptors has been associated with protective effects against inflammation.[3]

The administration of DSS results in erosion of the intestinal epithelium. In general, epithelial erosion triggers epithelial restitution, followed by a proliferative response,[34] but these responses depend on input from inflammatory mediators and the stimulation of TLRs. Epithelial restitution involves the migration of cells surrounding the wounded area to the lesion to initiate wound healing, and is induced by cytokines like IL-1β or IFN-γ through different pathways that involve TGF-β_1 or other molecules like TFF or galectin-2/4. Diminished IL-1β, IFN-γ, and TFF3 are therefore consistent with compromised restitution in GF animals. Hours or days after epithelial restitution, the proliferative response increases the pool of intestinal epithelial cells available to cover the lesion. The proliferative response is triggered again not only by cytokines like IL-22 and IL-6, but also by bacterial TLR ligands, activating transcription factors like NF-κB and STAT3 to induce cell survival and proliferation. Consistent with the lack of TLR activation and the low IL-22 response to DSS, we showed a lower colonic STAT3 activation and a decreased colonic content of PCNA in GF mice. This, plus a decreased expression of KGF, indicate an impaired proliferative response in the absence of microbiota. Cytokeratin is a marker of intestinal epithelial cells and its quantity indicates the relative abundance of intestinal epithelial cells in the colon. The decreased presence of cytokeratin in GF animals treated with DSS also supports

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the impaired proliferative response of this cell type. In addition, our data are consistent with an effect on mucosal tight junctions. Claudins-2 and -4, ZO-1, and occludin were found to be decreased in the colon of GF mice that received DSS. These findings should be viewed with caution because we could not perform any permeability studies to confirm compromised barrier function. Because TNF-α has known deleterious effects at this level, barrier defects may be partly ascribed to TNF-α upregulation.[35]

To further characterise the interaction between DSS and the microbiota, conventionally reared mice were depleted of the microbiota [PGF conditions] by treatment with an antibiotic cocktail. This approach allowed us to test whether the acquired absence of luminal microorganisms had similar effects to those of inborn GF conditions [it should be noted that bacterial depletion is not absolute in PGF conditions].[27] Interestingly, GF and PGF mice differed markedly, in that the latter were largely protected from blood loss, epithelial injury, and mortality, but at the same time they exhibited a relatively normal, albeit attenuated, inflammatory response to DSS. In this regard, GF mice displayed very low colonic expression of various genes involved in mucosal barrier function, including MUC3, TFF3, occludin [compensated partly at the protein level], KGF, VEGF, and cyclin D1, whose levels were basically normal in PGF mice and, as noted, this was also the case for cytokeratin 5/8. These findings therefore confirm a dual effect of the microbiota at this level. The differences observed between GF and PGF mice may be explained either by the presence of a remnant microbiota, or by previous exposure of PGF mice to a normal microbiota. In this regard, the fact that faecal extracts of both GF and PGF mice are devoid of immunomodulatory effects,[36] attributable to either TLR ligation or the production of short chain fatty acids, which exert anti-inflammatory effects via activation of GPR43 receptors,[27,36] points to the latter hypothesis.

Notably, the mRNA levels of IL-10 and FOXP3, a marker of Treg cells, are comparable in conventional and PGF-DSS treated mice, but not in GF mice. IL-10 has well-documented enhancing effects on mucosal barrier function,[35] and its expression correlates with that of mucosal barrier function markers. Thus our data are consistent with protection in PGF conditions arising from Treg cells generated by exposure to the normal microbiota before antibiotic treatment or, alternatively, Treg cells sustained by a minimal microbiota.[17,37] Although IL-10 was also increased in animals showing a positive DSS colitic response [ie conventional mice], the protective effects on ZO-1 and occludin are likely to be overturned by the parallel upshot in inflammatory cytokines [IL-6, IL-17, and IFN-γ], with known inhibitory effects at this level.[38,39]

On the other hand, the relatively robust inflammation observed in PGF mice suggests that the presence of a minimal microbe presence in the colonic lumen is enough to fuel the immune response to significant levels. In a seminal study by Rakoff-Nahoum *et al.*,[40] PGF mice were generated with a protocol almost identical to ours, except for a higher metronidazole dose resulting in low inflammation but increased mortality after DSS administration, ie a phenotype very similar that of GF mice in our study.[8,28] This suggests that the protection conferred by Tregs in PGF mice may depend critically on the magnitude of the remnant microbiota, although other confounding factors cannot be discarded. In support of this critical balance, in a separate experiment performed with C57/BL6 mice exposed to 3% DSS in PGF conditions, we obtained a similar degree of protection against epithelial injury and blood loss, but even greater limitation of inflammation [Supplementary Figure. 2, available as Supplementary data at *ECCO-JCC* online].

Despite the absence of a normal immune/inflammatory response to DSS, GF mice displayed an enhanced colonic expression of IL-17, TNF-α, and IL-27 [at 4% DSS]. Recently, a subset of Th17 cells named natural Th17 [nTh17] has been described.[41] nTh17 are different from the so-called induced Th17 in that they mature in the thymus, do not require symbiotic microbiota or IL-6 for their generation, and readily produce IL-17.[42] We may speculate that nTh17 cells tend to predominate in GF conditions, possibly by lack of modulation by other immune cells and/or by IL-10, an antiinflammatory cytokine with known inhibitory properties on IL-17 production.[43] However, we do not know the cell source of IL-17 in GF mice.[44] The same is true of TNF-α, although it is feasible that it can be produced by nTh17 cells as well. IL-27 is a pleiotropic cytokine with anti- and pro-inflammatory effects. It is an inhibitor of Th17 cells and an inductor of Treg cell differentiation, and has proliferative effects on IECs.[45] IL-27 has been previously shown to be upregulated in GF animals.[45] Since epithelial proliferation is impaired in these animals, it is possible that IL-27 acts as a compensatory mechanism.

In conclusion, the presence of luminal microorganisms is required for the colitic response to DSS, whereas the deleterious effects of DSS on mucosal barrier function are greatly mitigated by previous exposure to a normal microbiota and/or the presence of a minimal bacterial population. Although these data cannot be extrapolated to the clinical arena, they may help explain the limitations of antibiotic treatment of IBD patients, inasmuch as the anti-inflammatory effects may be associated with impaired epithelial/blood vessel compliance.

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Conflct of Interest

The authors have no competing interests.

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Author Contributions

JJC, MDS, AZ, FSM, and OMA contributed to the concept and design of the study. FCC, MOG, BO, CHC, CJA, FSM, and OMA performed the experiments. FSM and OMA wrote the manuscript. All the authors contributed to the analysis and interpretation of data and critically reviewed and approved the final draft.

Supplementary Data

Supplementary data are available at *ECCO-JCC* online.

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