Title: Hypoxia-inducible factor (HIF) hydroxylase inhibition enhances the protective effects of cyclosporine in colitis

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Abstract:

Inflammatory Bowel Disease (IBD) is characterized by epithelial barrier dysfunction with resultant inflammation as the mucosal immune system becomes exposed to luminal antigens. The hydroxylase inhibitor dimethyloxalylglycine (DMOG) reduces symptoms in experimental colitis through the upregulation of genes promoting barrier function and inhibition of epithelial cell apoptosis. The immunosuppressive drug cyclosporine reduces inflammation associated with IBD via suppression of immune cell activation. Given the distinct barrier protective effect of DMOG and the anti-inflammatory properties of cyclosporine, we hypothesised that combining these drugs may provide an enhanced protective effect by targeting both barrier dysfunction and inflammation simultaneously. We used the dextran sulfate sodium (DSS) model of colitis in C57BL/6 mice to determine the combinatorial efficacy of cyclosporine and DMOG. While cyclosporine and DMOG administered alone ameliorated disease progression, in combination they had an additive protective effect that surpassed the level of protection afforded by either drug alone. The ability of DMOG to augment the anti-inflammatory effects of cyclosporine was largely due to preservation of barrier function and at least in part due to ZO-1 regulation. We propose that combining the barrier protective effects of a hydroxylase inhibitor with the anti-inflammatory effects of cyclosporine provides added therapeutic benefit in colitis.

New and Noteworthy:

Inflammatory bowel disease is the result of decreased intestinal epithelial barrier function leading to exposure of the mucosal immune system to luminal antigens causing inflammation, which in turn further decreases epithelial barrier function. We demonstrate for the first time that strengthening the epithelial barrier with a hydroxylase inhibitor in combination with the administration of the immunosuppressive cyclosporine provides additive therapeutic advantage in a murine model of colitis.
Introduction:

Inflammatory Bowel Disease (IBD) which comprises Crohn’s disease and ulcerative colitis is characterised by increased epithelial permeability coupled with a dysfunctional mucosal immune response, which together result in uncontrolled inflammation of the intestinal mucosa (24). Though the precise mechanisms underpinning the pathogenesis of IBD remain unclear, a combination of immune, genetic, microbial and environmental factors are thought to be contributory (1). There is currently no pharmacological cure for IBD and most treatments are aimed at managing symptoms and promoting remission. The currently used therapies for IBD include aminosalicylates, corticosteroids, biologics (e.g. anti-TNFα antibodies) and immunosuppressives (6). Corticosteroids and aminosalicylates are the frontline therapy for new cases and although these drugs have proven clinical effectiveness, 20-40% of ulcerative colitis patients do not respond to these conventional therapies (27). Despite pharmacological advances, 25-35% of ulcerative colitis patients still ultimately require surgical intervention (16). This reflects a relative failure of currently available therapies to adequately manage the disease, and as such, there is a clear clinical need for new treatment strategies. Notably, despite loss of barrier integrity being recognized as central to the pathogenesis of IBD (40), there are currently no approved drugs which target this aspect of the disease.

Hypoxia occurs when the demand for oxygen by a cell exceeds its vascular supply. Growing appreciation of the hypoxic microenvironment that exists within inflamed sites of affected tissue in IBD has led to multiple studies investigating the impact of hypoxia on mucosal inflammation (8). Two key transcription factors regulated by hypoxia which contribute to the transcriptional response are the hypoxia-inducible factor (HIF) and Nuclear factor-kappa B (NF-κB) (5, 9). The HIF pathway plays a key role in the adaptive transcriptional response to hypoxia and upregulates a cohort of genes that increase blood supply, and thus oxygen...
delivery, as well as promote anaerobic metabolism by upregulating glycolytic genes (13). NF-κB is a master transcriptional regulator of inflammation, immunity and cell survival. Notably, NF-κB is known to regulate the transcription of a host of cytokines, chemokines and adhesion molecules, and its activation strongly regulates inflammation (20, 26). Both NF-κB and HIF are key effectors in colitis (20, 32, 37). The oxygen sensitivity of both HIF and NF-κB is conferred by a family of oxygen-dependent prolyl hydroxylases, termed PHD1-3 (5, 9). These enzymes represent a promising point of convergence for therapeutic targeting and in fact, pharmacological and genetic inhibition of these enzymes (e.g. through the use of dimethylxalylglycine, ‘DMOG’) has been shown to be protective in a host of models of inflammatory disease, including colitis (10, 14, 15, 19, 29). With regard to colitis, this protective role has been proposed to be through the upregulation of barrier protective genes including CD73 and intestinal trefoil factor, but also in part due to an anti-apoptotic effect on intestinal epithelial cells, thus ameliorating barrier dysfunction (10, 18). Formation of a more effective barrier in IBD should minimize the exposure of the mucosal immune system to luminal antigens and thereby reduce inflammation (3). As a result, manipulation of the ability to optimize barrier integrity holds significant therapeutic potential for this disease.

Another drug which has proven efficacy in IBD is the immunosuppressive cyclosporine, a calcineurin inhibitor which has potent anti-inflammatory properties. Cyclosporine inhibits T-cell signaling, reduces the expression of pro-inflammatory cytokines and inhibits the migration of leukocytes (11, 23). As a result, the drug has been used as an effective treatment in a number of inflammatory diseases, and is particularly useful in cases of severe ulcerative colitis (21). Cyclosporine has also been shown to be protective in experimental models of colitis such as the dextran sulfate sodium (DSS) model, via both suppression of immune signaling and inhibition of intestinal epithelial cell apoptosis (25, 31).
Owing to the barrier protective role of DMOG, and the anti-inflammatory properties of cyclosporine, we hypothesised that combining these drugs as a two-pronged approach to alleviating the barrier dysfunction and inflammation associated with IBD may provide additive therapeutic effects. To test this hypothesis, we used the DSS model of colitis to compare the efficacy of cyclosporine and DMOG, both alone and in combination. Here we show that the combination of cyclosporine and DMOG produces an additive protective effect on mice in DSS-colitis through concomitant barrier protection and immunosuppression, and therefore represents a potential new therapeutic approach for mucosal inflammation associated with IBD.
Materials and Methods:

DSS colitis:

Female C57BL/6 mice aged between 10 – 12 weeks were used in all experiments. All procedures were reviewed and approved by the Ulster University Animal Research Ethics Committee and UK Home Office, under Project license number PL2768. Mice were administered 2.5% (w/v) DSS (mol wt. 36,000 – 50,000. MP Biomedicals, OH) in their drinking water to induce colitis over a period of 6 days. Designated mice were given a loading dose of 50mg/kg cyclosporine via i.p injection on Day 0, with subsequent daily doses of 25mg/kg (25). 200mg/kg DMOG (Cayman Chemical, MI) was administered via i.p injection to designated animals on alternate days. 200mg/kg DMOG is a lower dose than has been shown previously to be effective (10, 38). Intermediate doses of both drugs were selected with the aim of increasing the likelihood of observing any potential combinatorial efficacy. Daily observations of weight loss, stool consistency and presence of fecal occult blood were recorded in order to determine the Disease Activity Index (DAI, see supplementary table 1). On day 6, all mice were sacrificed by cervical dislocation. Following sacrifice, the colon of each mouse was removed, rinsed with PBS and measured in length.

Histology:

1cm sections of mouse distal colon were fixed in 10% (v/v) formalin prior to embedding in paraffin wax. 4μm sections were deparaffinized and rehydrated in decreasing concentrations of alcohols prior to staining with hematoxylin and eosin using an Autostainer XL (Leica, Nussloch, Germany), and images were acquired using an Aperio ScanScope XT (Leica, Nussloch, Germany) slide scanner. Images were randomized and distributed to three experienced individual assessors who gave the tissues a composite ‘Tissue Inflammation’ score based on extent and severity of inflammation, crypt damage and the percentage
involvement of disease (see supplementary table 2). Images were also scored on the extent of edema, neutrophil infiltration and damage to the epithelial cell layer (see supplementary table 3).

**In vivo intestinal permeability assay:**

4 hours prior to sacrifice, mice were orally administered with 600mg/kg 4kDa FITC-labelled dextran. 4µm sections of distal colon were sectioned from paraffin blocks. Sections were deparaffinized and rehydrated in decreasing concentrations of alcohols prior to cover-slipping. Widefield fluorescence microscopy was used to image the tissues. Image J software (National Institute of Health, Bethesda, MD) was used to quantify the permeation of FITC-labelled dextran. Rectangles the width of the mucosa were drawn in three independent, random locations on each imaged tissue. The intensity of FITC-labelled dextran in each of the three locations was recorded and the mean was calculated. Representative images shown were acquired by confocal microscopy.

**Immunofluorescence:**

4µm sections of distal colon were sectioned from paraffin blocks. The sections were deparaffinized and rehydrated in decreasing concentrations of alcohols prior to heat-induced antigen retrieval. The tissues were blocked in 5% normal donkey serum for 1 hour and probed for ZO-1 (#61-7300, Thermo Fisher, MA) overnight at 4ºC in a humidified chamber. Secondary antibody (AlexaFluor 568, Thermo Fisher, MA) was applied for 2 hours at room temperature. DAPI (Boster Biological, CA) was used to counterstain the nuclei. Widefield fluorescence microscopy was used to image the tissues. Image J software (National Institute of Health, Bethesda, MD) was used to quantify the expression of ZO-1 using the same
method as described above for FITC-labelled dextran. Representative images shown were acquired by confocal microscopy.

Quantitative reverse transcription polymerase chain reaction (RT-qPCR):

10 mg sections of colon were homogenized using a TissueLyser (Qiagen, Venlo, Netherlands). RNA was isolated by TRIzol-based extraction and cDNA was synthesized using MMLV (Promega, WI). RT-qPCR was run for TNFα (#4331182, Thermo Fisher, MA) and 18s rRNA (#4310893E, Thermo Fisher, MA) using specific TaqMan® probes. The relative mRNA was calculated using the ΔΔCt method, normalizing the TNFα Ct of each sample to the corresponding 18s rRNA Ct.

Statistical analysis:

Each treatment group consisted of 5-6 individual mice. All statistical analysis was carried out using the GraphPad Prism 5 statistics package. Data was first tested for normality to determine whether to use a parametric or non-parametric ANOVA. In the case of ANOVA, Bonferroni post-tests were used. In the case of non-parametric ANOVA, Dunn’s post-tests were used. P-values of less than 0.05 were deemed to be statistically significant. Data are presented as mean ± standard error of the mean (S.E.M).
Results:

Combinatorial efficacy of cyclosporine and DMOG in murine colitis

We first investigated the therapeutic effects of cyclosporine and DMOG (alone and in combination) on intestinal pathophysiology in DSS colitis. After day three, mice treated with DSS began to develop a marked increase in disease activity index (DAI), a composite score based upon percentage weight loss, stool consistency and presence of fecal occult blood. Cyclosporine treated mice exhibited partial protection, with the DAI of these mice approximately half that of DSS control mice on days 5 and 6 (Figure 1A). DMOG treatment transiently attenuated the induction of disease up to day five, however DAI scoring for this group matched DSS control mice upon the final day before sacrifice (Figure 1B). The 200mg/kg dose of DMOG used in this experiment is significantly lower than what has previously been shown to be effective (10, 38). Notably, the combination of cyclosporine and DMOG afforded a strikingly additive level of protection, with mice in this group showing no significant signs of disease activity following 6 days of DSS treatment, demonstrating significantly enhanced efficacy over cyclosporine alone (Figure 1C).

Colon shortening and inflammation-associated diarrhea are common features of colitis. DSS treated mice demonstrated characteristic colon shortening when measured (Figure 1D-E), while treatment with either cyclosporine or DMOG attenuated this to varying degrees. As with the DAI, the combination of cyclosporine and DMOG provided an additive protective effect, preventing colon shortening from occurring entirely. Moreover, physical examination of the colons showed that DSS treatment leads to a loss of the ability of the mice to adequately form fecal pellets, resulting in poorly formed stool (red rectangle) with intestinal blood also typically apparent. Mice receiving the combination of cyclosporine and DMOG retained the ability to form pellets (red arrows) and displayed minimal intestinal blood, demonstrating an overall preservation of intestinal health (Figure 1D).
We next investigated the integrity of the colonic architecture using hematoxylin and eosin staining of sections of distal colon. In DSS control mice, we found severe inflammation with widespread loss of crypts and epithelium when compared to healthy mice (*Figure 2A+B*). Cyclosporine alone provided a high degree of protection with less widespread inflammation and much of the colonic architecture preserved (*Figure 2C*). Though DMOG alone appeared to preserve some of the structure, this effect was not statistically significant (*Figure 2D+F*). However, the combination of cyclosporine and DMOG had an additive ability to reduce inflammation and preserve crypt architecture when compared to either treatment alone (*Figure 2E+F*). This data provides further evidence that DMOG can augment the protective effects of cyclosporine.

Next, histological sections were scored based on the extent of edema, neutrophil infiltration and damage to the epithelial cell layer (*Figure 3A-C*). Extensive edema was a feature of DSS control mice when compared to healthy controls. Both cyclosporine and DMOG reduced edema to various degrees, with cyclosporine providing the greater protection. The combination of cyclosporine and DMOG however reduced edema to the greatest extent, with no statistical difference between this group and healthy controls (*Figure 3A*). DSS also led to an influx of neutrophils throughout the mucosa. Though DMOG treatment did not prevent this, cyclosporine was relatively potent in terms of suppressing this immune response. However, the combination of cyclosporine and DMOG was the most effective at reducing neutrophil infiltration (*Figure 3B*). Finally, DSS treated mice exhibited significant epithelial damage when compared to healthy mice. Similar to the effect on edema, both cyclosporine and DMOG provided a protective effect on the epithelium, with cyclosporine providing the more significant protection. However, the combinatorial effects of cyclosporine and DMOG again surpassed the level of protection afforded by either drug alone (*Figure 3C*). In summary, both cyclosporine and DMOG provide intermediate degrees of protection when
administered alone. However, when the two drugs are combined, there is an additive level of protection that surpasses the protection afforded by either drug in isolation.

Having observed the combinatorial efficacy of cyclosporine and DMOG, we next wanted to discern whether this additive effect was due to an anti-inflammatory effect, a barrier protective role or a combination of both. We first investigated mRNA levels of TNFα, a key pro-inflammatory cytokine involved in IBD. Treatment with DSS caused an increase in TNFα mRNA when compared to healthy controls (Figure 4). While cyclosporine inhibited the DSS induction of TNFα, DMOG had no effect. The combination of cyclosporine and DMOG was effective at preventing DSS induction of TNFα, however there was no additional protection afforded by the combination therapy when compared to cyclosporine alone. This led us to conclude that the complementary effect of DMOG to the well characterised anti-inflammatory effects of cyclosporine is not via additive suppression of TNFα production.

Owing to the importance of TNFα in IBD, we hypothesised that the ability of DMOG to augment the anti-inflammatory effects of cyclosporine may instead be related to preservation of barrier function.

In order to determine the effects of cyclosporine and DMOG on intestinal barrier function a FITC-labelled dextran permeability assay was carried out. We found that treatment with DSS led to a significant increase in the permeation of FITC-labelled dextran into the mucosa, indicating reduced epithelial barrier function. Treatment with either cyclosporine or DMOG alone did not significantly reduce this DSS-induced barrier dysfunction. However, the combination of cyclosporine and DMOG significantly reduced the permeation of FITC-labelled dextran when compared to DSS controls, indicating restored barrier function (Figure 5). This again highlights the additive protective effect when the combination therapy is administered and suggests that the mechanism by which DMOG augments cyclosporine is through preservation of barrier function.
To gain mechanistic insight into the mechanism by which the combination of cyclosporine and DMOG maintains barrier function, in particular preventing the leakage of FITC-labelled dextran via the paracellular route, we next analyzed the expression of a key regulator of paracellular permeability: ZO-1 (34). We found that DSS treated mice displayed a significant loss of ZO-1 that pervaded the entire tissue when compared to healthy mice. Treatment with either cyclosporine or DMOG ameliorated this DSS-induced loss of ZO-1, with cyclosporine proving to be the more effective. Combining cyclosporine with DMOG further enhanced this protective effect, rescuing ZO-1 to a significantly greater extent than either drug alone (Figure 6). By inhibiting loss of ZO-1, this data demonstrates the barrier protective role of both cyclosporine and DMOG but importantly, it also provides evidence for an additive protective effect on the epithelial barrier when the two drugs are administered together.
Discussion:
In this study we investigated the combinatorial efficacy of cyclosporine and DMOG in an attempt to provide a potential alternative treatment for IBD. While our group and others have previously shown a reduction in colitis-like symptoms in the DSS model when either DMOG or cyclosporine is employed alone (10, 25), in this study, we took the novel approach of attempting to simultaneously target the epithelial barrier dysfunction and uncontrolled inflammation associated with IBD. We hypothesised that these distinct mechanisms of action may facilitate an enhanced level of protection. Here we show that when cyclosporine and DMOG are combined, there is an additive protective effect. The potency of this combination was such that there was no statistical difference between mice receiving this treatment and healthy controls under multiple parameters, both clinical and molecular (DAI, colon length, tissue inflammation, TNFα mRNA, ZO-1 expression). The significance of this is further compounded by the fact that apparent additivity may allow us to use lower doses of each drug as part of a combination than what may be required for the use of each drug individually. This is particularly important owing to the potential side effects associated with both cyclosporine and DMOG.

Cyclosporine alone was a powerful treatment in terms of alleviating colitis-like symptoms throughout the study, and also reduced TNFα mRNA. DMOG alone reduced disease under a number of parameters however had no effect on TNFα transcript levels. Owing to the importance of TNFα in IBD, signified by the fact that the most commonly used biologics in the treatment of the disease are anti-TNFα antibodies, we hypothesised that DMOG is likely contributing a barrier-protective rather than an anti-inflammatory role. This hypothesis is supported by previous studies demonstrating an upregulation of barrier protective genes by DMOG as well as an inhibitory effect on epithelial cell apoptosis (10, 18). To investigate this, we carried out a FITC-labelled dextran permeability assay in order to assess barrier function. We found that while cyclosporine and DMOG alone were unable to prevent the DSS-induced
increase in permeability to FITC-labelled dextran, the combination of cyclosporine and DMOG significantly reduced the permeation of FITC-labelled dextran into the intestinal tissue, demonstrating enhanced barrier integrity. Owing to the fact that barrier dysfunction is a crucial step in the pathogenesis of IBD and that there are currently no drugs which target barrier function, this suggests that the combination of cyclosporine and DMOG could be of significant therapeutic potential for this disease.

To determine the mechanism by which the combination of cyclosporine and DMOG enhances barrier function we examined the expression of ZO-1, a key regulator of paracellular permeability. Redistribution of ZO-1 has been linked to altered tight junction permeability and reduced barrier function (33). We found that both cyclosporine and DMOG enhanced ZO-1 expression in DSS-treated tissues. Importantly, the combination of cyclosporine and DMOG produced an additive effect on ZO-1 expression. This suggests that the ability of DMOG to augment the anti-inflammatory effects of cyclosporine is at least in part due to preservation of barrier function, and in particular ZO-1. This provides further support for the use of a combination of cyclosporine and DMOG due to the ability of the combination to significantly rescue ZO-1 levels and maintain barrier integrity. A more effective barrier should reduce the leakage of luminal antigens into the lamina propria and thus break the cycle of inflammation and barrier dysfunction characteristic of IBD. This barrier enhancing effect combined with the traditional immunosuppressive properties of cyclosporine could potentially be useful in human colitis by both dampening the chronic inflammatory response and resealing the epithelial barrier.

Interestingly, IBD patients have been shown to have significantly reduced ZO-1 expression (2, 12). Whether this loss of ZO-1 and other tight junction proteins precedes or occurs as a result of inflammation remains unconfirmed however there is some clinical data to support the hypothesis that loss of barrier function comes first. IBD patients display altered intestinal
permeability during both active and quiescent disease (39). Two separate studies have also shown that increased permeability to inert tracers predicted relapse and therefore preceded active inflammation in Crohn’s disease patients (4, 41). Interestingly, an individual with a family history of IBD displayed elevated intestinal permeability despite being entirely asymptomatic, before ultimately being diagnosed with Crohn’s disease 8 years later (17). These data all suggest barrier dysfunction may precede inflammation in human disease. This suggests that a therapy which enhances barrier function such as the combination of cyclosporine and DMOG may be beneficial.

However, several issues must be resolved before a combination of cyclosporine and DMOG could be considered a realistic treatment option. Firstly, there are a number of issues associated with systemic exposure to DMOG. For example, excessive erythrocytosis due to stabilization of HIF and subsequent EPO production (36, 38). While erythrocytosis is beneficial in anemia, where clinical trials for hydroxylase inhibitors are currently progressing well (28), this could cause unwanted cardiovascular side-effects for non-anemic patients. One mechanism by which these issues may be overcome is through more sophisticated drug delivery. Our group has shown previously that by formulating DMOG as orally-ingestible polymer coated minispheres, we can colon-target a dose that is 40-fold lower than that required for systemic administration, which maintains therapeutic efficacy in the DSS model but minimizes systemic side effects (38).

Similarly, despite its powerful effects, the clinical usefulness of cyclosporine has typically been limited to severe cases of ulcerative colitis owing to several serious systemic side effects including nephrotoxicity and hypertension (7, 35). Previous attempts to overcome this issue have encountered problems of their own. For example, rectal enema reduces systemic exposure of cyclosporine but only treats distal areas of an affected colon and has issues of
patient compliance (22, 30). Therefore, colon-targeted cyclosporine which retains efficacy yet negates systemic exposure, and that is orally ingestible is an attractive option.

In summary, both cyclosporine and DMOG have therapeutic efficacy in the DSS model and when combined, there is an additive protective effect that surpasses the level of protection afforded by either drug alone. As discussed above, low dose, colon-targeted formulations of both drugs appear to be the key to retaining efficacy while minimizing systemic side effects. We propose that producing a co-formulation of colon-targeted DMOG with colon-targeted cyclosporine may be a potentially promising new therapy for IBD.
References:


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**Figure Legends:**

**Figure 1:** Additive protection of cyclosporine and DMOG in the DSS model of colitis.

C57BL/6 mice were administered 2.5% (w/v) DSS or plain drinking water for 6 days. 25 mg/kg cyclosporine (i.p) was administered to designated mice each day, with 200 mg/kg DMOG (i.p) administered every alternate day. **A-C:** A disease activity index (DAI) score was determined daily by analyzing the weight, stool consistency and presence of fecal occult blood for each mouse. ‘Healthy’ and ‘DSS’ groups are repeated in panels A-C for clarity. ns = non-statistically significant, * p < 0.05, **** p < 0.0001, Two-way ANOVA with Bonferroni post-tests (n = 5-6). **D-E:** Following sacrifice on day 6, the colon of each mouse was resected and measured in length to analyze contractility. Red arrows indicate fecal pellets, red rectangle highlights inflammation-associated diarrhea. ns = non-statistically significant. ** p < 0.01, *** p < 0.001, **** p < 0.0001, One-way ANOVA, mean of each group is compared to ‘Healthy’ group (n = 5-6).

**Figure 2:** Preservation of colonic architecture by Cyclosporine and DMOG in the DSS model of colitis. Sections of distal colon were stained with hematoxylin and eosin and imaged. These images were then randomized and distributed to three experienced individual assessors who scored the tissues based on the extent and severity of inflammation as well as crypt damage and the percentage involvement of the tissue. These scores were then aggregated into a ‘Tissue Inflammation Score’ with a maximum score of 40. Representative images are magnified 20x. ns = non-statistically significant, * p < 0.05, ** p < 0.01, One-way ANOVA with Bonferroni post-tests (n = 4-6).
Figure 3: Cyclosporine and DMOG combine to reduce edema, neutrophil infiltration and epithelial barrier damage in the colon of DSS-treated mice. Sections of distal colon were stained with hematoxylin and eosin and imaged. These images were then randomized and distributed to three experienced individual assessors who scored the tissues based on the extent of edema (A), neutrophil infiltration (B) and damage to the epithelial cell layer (C). ns = non-statistically significant, * p < 0.05, *** p < 0.001, **** p < 0.0001, One-way ANOVA with Bonferroni post-tests (n = 4-6).

Figure 4: Cyclosporine but not DMOG inhibits DSS-induction of TNFα. RNA was isolated from sections of colon tissue and the levels of TNFα mRNA was analyzed by RT-qPCR. ns = non-statistically significant, * p < 0.05, ** p < 0.01, Non-parametric one-way ANOVA (Kruskal Wallis test) with Dunn’s post-tests (n = 4-6).

Figure 5: Enhanced epithelial barrier function in mice treated with a combination of cyclosporine and DMOG. Mice were orally administered with FITC-labelled dextran four hours prior to sacrifice. The permeation of FITC-labelled dextran into the intestinal tissue was determined by widefield fluorescence microscopy. FITC was quantified using Image J software. Representative images were acquired by confocal microscopy and are magnified 20x. ns = non-statistically significant, * p < 0.05, One-way ANOVA with Bonferroni post-tests (n = 4-6)
Figure 6: Cyclosporine and DMOG prevent DSS-induced loss of ZO-1. Sections of distal colon were immunostained for ZO-1, with DAPI staining of nuclei. Images were acquired by widefield fluorescence microscopy and the expression of ZO-1 was quantified using Image J software. Representative images shown were acquired by confocal microscopy and are magnified 20x. * p < 0.05, ** p < 0.01, *** p < 0.001, One-way ANOVA with Bonferroni post-tests (n = 4-6)
DMOG enhances epithelial barrier function.

Cyclosporine suppresses immune response.