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Novel polyurethane based particulate formulations of infliximab reduce inflammation in DSS induced murine model of colitis – A Preliminary study

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Abstract

Our recent study showed that novel infliximab (INF) loaded polyesterurethane (INF-PU) and INF-PU-PEG particulate formulations reduced inflammation in an in-vitro epithelial inflammation model. In this study we investigated therapeutic potential of novel INF-PU and INF-PU-PEG particulate formulations to reduce inflammation in a dextran sodium sulfate (DSS) induced murine model of colitis. Severity of colitis was assessed by measurement of disease activity index (DAI) score, inflammatory markers (neutrophil infiltration, TNFα) and histological score. Treatment groups orally administered with INF-PU and INF-PU-PEG particulate formulations showed improvement in the clinical signs of colitis, similar to that observed with intraperitoneally administered INF, in both, moderate and severe DSS induced colitis model. This was related to a significant reduction in inflammatory cytokines, resulting in a significant reduction in histological score (ANOVA; $p < 0.05$), indicative of mucosal healing, a key goal of IBD therapy. This could be attributed to its targeted delivery to the inflamed colon and higher permeation of these particulate formulations across the inflamed colonic mucosa, as observed by confocal images, resulting in local inhibition of TNFα at its site of production. These promising preliminary results warrant further investigation of orally administered INF and its novel particulate formulations in a wider preclinical study.

Keywords: Colitis, Infliximab, Biomaterials, particulate formulation, drug delivery

Highlights

- Novel polyurethane based particulate formulations of infliximab reduced inflammation in moderate and severe colitis model
- Significant reduction in histological score was observed, indicative of mucosal healing, a key goal in IBD therapy
- Significant improvement in the clinical signs of colitis, as observed by a significant decrease in disease activity score (DAI)
- Targeted delivery to the inflamed colon and permeability across the inflamed colonic mucosa confirmed by confocal studies.

1. Introduction

Inflammatory bowel disease (IBD) comprising of ulcerative colitis (UC) and crohns disease, is a chronic progressive inflammatory condition of the gastro-intestinal (GI) mucosa, characterised by varying degrees of intestinal injury (Ungaro, Colombel, Lissoos, & Peyrin-Biroulet, 2019). It is a chronic relapsing and remitting condition with rising incidence worldwide, with 6.8 million cases globally in 2017 (Alatab et al., 2020). If not optimally treated, IBD can lead to serious complications and disability, and is also a risk factor for developing GI cancers (J. F. Colombel et al., 2011). Clinical signs and symptoms of UC include weight loss, passing blood with loose stools. This can lead of loss in education, reduced productivity at work, resulting in economic burden (Burisch, Jess, Martinato, Lakatos, & EpiCom, 2013; Ungaro et al., 2019).

In mild to moderate cases of UC, treatment is initiated with conventional small molecule comprising of immunosuppressive agents such as aminosalicylates which mainly provide symptomatic relief, and do not induce repair of the inflammatory lesions' in IBD (J.-F. Colombel, D'haens, Lee, Petersson, & Panaccione, 2020; J. F.

Colombel et al., 2011). Consequently, disease progression ultimately leads to surgical intervention in 46.9% of crohns disease and 15.6% of ulcerative colitis patients, within 10 years of diagnosis (Frolkis et al., 2013).

Consequently non-responders to conventional therapy are then switched to therapeutics for the treatment of moderate to severe colitis which include: anti-TNFα monoclonal antibody (mAbs): infliximab (Remicade®, FDA approval in 2006), adalimumab (Humira®, FDA approval in 2012), golimumab (Simponi®, FDA approval in 2013), certolizumab pegol (under Phase 2 clinical trial), anti-integrin α 4β 7 mAb: vedolizumab (Entyvio®, FDA approval in 2014), anti-IL -12 / -23 mAb: ustekinumab (Stelara®, FDA approval in 2019), small molecule targeting janus kinase (JAK) signaling: tofacitinib (Xeljanz®, FDA approval in 2018) and sphingosine-1-phosphate (S1P) receptor: ozanimod (being considered for FDA approval) (Binienda, Fichna, & Salaga, 2020; Hazel & O'Connor, 2020).

In the last decade, there has been a paradigm shift in the goal of UC treatment, which is to initiate and maintain clinical remission thus allowing mucosal healing, a key goal to reduction in hospitalisation and surgery (J.-F. Colombel et al., 2020; Sandborn et al., 2009).

Biological therapies such as anti-TNFα monoclonal antibodies mAbs, particularly infliximab (INF) have been reported to achieve this goal, and have shown good clinical benefits in IBD, improving quality of life (J. F. Colombel et al., 2011; Griffiths et al., 2020; Nurbhai et al., 2019; Singh, Fumery, Sandborn, & Murad, 2018; Troncone, Marafini, Blanco, Di Grazia, & Monteleone, 2020). However, parenteral (intravenous or subcutaneous) administration of mAbs is associated with life threatening side effects, including risk of serious infections, due to high dose related long term systemic immune suppression and poor patient compliance (Nurbhai et al., 2019). In addition,

mAbs are associated with non-drug costs including outpatient appointments and administration costs, adding to the economic burden of the healthcare system (Cronin, Moore, Lenihan, O'Shea, & Woods, 2019).

Consequently, orally administered gut-selective treatment with high intestinal exposure exhibiting local effect and minimal systemic exposure and side-effects is desirable(Bhol et al., 2013; Tambuwala et al., 2015). INF stability has been measured and been found to be reasonably stable in the colon (Yadav, Varum, Bravo, Furrer, & Basit, 2016)**.** The desire to attend to this current unmet clinical need is increasing, and is reflected in recent studies surrounding development of novel oral anti-TNFα and evaluation of its efficacy in preclinical models (Almon et al., 2021).

Various experimental models of colitis have been reported in the literature, however no single model replicates all the aspects and features of human UC. Chemically induced model of colitis is most commonly used due to advantages of rapid onset of inflammation and reproducibility (Wirtz et al., 2017).

In 2,4,6-trinitro-benzene sulfonic acid (TNBS) and oxazolone induced colitis model, intrarectal administration of TNBS and Oxazolone results in T-cell mediated immunity against haptenised luminal antigens and microbiodata derived proteins. TNBS induced colitis model have been reported to exhibit many histopathological features similar to Crohns disease (Alex et al., 2009; Wirtz et al., 2017), whereas oxazolone has been reported to resemble many disease features of human UC. It induces colitis via elevated interleukins (IL) production, therefore Oxazolone induced colitis model has been used to evaluate efficacy of anti-IL therapeutics (Kasaian et al., 2014; Wirtz et al., 2017).

On other hand in DSS model, colitis is induced by oral administration of DSS dissolved in drinking water, available *ad libitum*. DSS is believed to induce colitis by disrupting

intestinal epithelial barrier, resulting in increased permeability and allowing entry of luminal antigens to the underlying mucosal immune system, resulting in rapid and profound inflammatory response and further damage to the intestinal mucosa (Wirtz et al., 2017). DSS induced model of colitis resembles many clinical characteristics similar to human UC (Alex et al., 2009). It is easy to generate and is highly reproducible, therefore it has been used extensively to investigate the potential of therapeutics and novel drug delivery system to reduce inflammation in UC (Beloqui et al., 2014; Naeem et al., 2015; Xiao et al., 2017).

Pathophysiological changes in UC offers therapeutic potential as it allows targeted delivery of polymeric particulate formulations (NPs) by the virtue of its preferential accumulation at the inflamed site via electrostatic interactions. This potentially promotes its residence time, allowing translocation of NPs across the compromised intestinal barrier to the underlying layer of the intestinal mucosa, the site of inflammation. This also prevents its rapid elimination by diarrhoea, a common symptom in IBD (Collnot, Ali, & Lehr, 2012; Maisel, Ensign, Reddy, Cone, & Hanes, 2015; Schmidt et al., 2013).

Numerous authors have successfully explored this therapeutic potential by engineering polymeric particulate formulations as carriers for targeted delivery of conventional small molecules. Ali et al (Ali et al., 2014) and Xiao et al (Xiao, Si, Zhang, & Merlin, 2015) reported higher therapeutic efficacy of budesonide and curcumin based PLGA polymeric particles in DSS induced murine model of colitis by virtue of maximized local drug concentrations at inflamed sites, while minimizing any systemic side effects. In first in vivo study in IBD human patients, rectally administered non-functionalized polymeric PLGA particles showed accumulation and uptake in intestinal ulcerous lesions (Schmidt et al., 2013).

INF has been reported to be effective in ameliorating the severity of colitis in DSS induced murine model of colitis, administered via IV and rectal route (Lopetuso et al., 2013). Limited studies have been carried out in engineering particulate formulations designed for oral delivery of anti-TNFα mAbs to treat inflammation in IBD. Recently Kim et al (J. M. Kim et al., 2020) and Wang et al (Wang et al., 2020) reported higher therapeutic efficacy of orally administered INF loaded coated/uncoated liposomal nanocomposites particles and natural polyphenol tannic acid/PEG particles, respectively in DSS induced murine model of colitis, compared to INF suspensions.

In our recently published work, we compared novel polyesterurethane (PU) biomaterial with conventional PLGA and PCL as polymeric drug carrier platform and observed that novel INF loaded PU (INF-PU) based particulate formulations (NPs) showed a rapid rate and extent of recovery of the epithelial barrier function in an in-vitro inflamed epithelial cell culture model (Pabari et al., 2019). This was related to the higher cellular interaction, uptake and permeability of these novel PU based polymeric NPs (Clara Mattu et al., 2013; Pabari et al., 2019).

Hence in this work we investigated the potential of these novel polymeric biomaterial of PU, and its PEGylated (PU-PEG) form as carriers of INF to reduce inflammation in a moderate and severe DSS induced murine model of colitis. Differential localisation of the fluorescently labelled PU and PU-PEG NPs in healthy and colitis models was also studied.

2. Materials and Methods

2.1. Formulation and characterisation of infliximab-loaded particulate formulations

INF-loaded particles were formulated by solvent dispersion technique as outlined by us previously (Pabari et al., 2019). Polymer, PU or PU-PEG was dissolved at 10mg/ml or 20mg/ml in acetone, and added dropwise to aqueous Tween® 80 solution at 2% w/v or 0.02% w/v, while stirring at 480rpm. This mixture was stirred overnight to allow complete solvent evaporation, then washed and incubated with INF solution (1mg/ml in sterile water for injection) for 2h at 4°C, under mild stirring. This was then pre-frozen at −80°C and lyophilised at −56°C and 0.056 mbar (Labconco Freezone 6, model no. 7752030; Labconco, Kansas City, MO, USA). Particle size, polydispersity index (PDI) and zeta potential (ZP) of particulate formulations dispersed in deionised water was measured by zetasizer (Nanoseries, Nano-ZS, Malvern Instruments, Worcestershire, UK). Bioactivity of INF was measured using ELISA (Human TNF-α ELISA MAX Deluxe, BioLegend San Diego, CA).

2.2. Development of DSS induced colitis model

An in vivo preclinical study was performed in C57BL/6 Male mice (23–30 g, 10-12 weeks; Biomedical Research Facility, RCSI). Animals were housed under standard conditions, with a standard pellet diet and water available *ad libitum*. A 12-h light:dark cycle was maintained (light hours 0700–1900 h). Protocols were approved by the Research Ethics committee (REC1153b).

Colitis was induced in male mice (C57BL/6) by treatment with up to 2.5% dextran-sodium sulphate (DSS) (MW: 36-50k) (MP Biomedicals, Solon, OH) dissolved in the drinking water for 7 consecutive days, available *ad libitum* (Tambuwala et al., 2010; Tambuwala et al., 2015). The animals were grouped as follows: Group 1: Untreated

Control (Healthy), Group 2: Untreated Control (Colitis), Group 3: INF administered by IP injection, Group 4: INF solution administered by Oral Gavage (OG), Group 5: INF-PU NPs administered by OG, Group 6: INF-PU-PEG NPs administered by OG. Each control and experimental group consisted of a minimum of 4 mice.

INF solution and INF loaded PU and PU-PEG NP suspension, equivalent to 10mg/kg of INF was administered by OG (200uL), every day starting on day 3 after DSS is initiated, over the 7-day experiment. INF administered by IP injection, and untreated colitis mice were used as controls.

On day 8 mice were sacrificed, the colon was excised and placed onto a non-absorbent surface while taking care not to stretch, to measure colon length with a ruler, an indicator of the severity of inflammatory response (Han et al., 2015).

Colon was divided into three portions: proximal, transverse and distal. Proximal and distal portion were flash frozen in liquid nitrogen and stored for later assessment of neutrophil infiltration in the colonic mucosa by measuring colonic MPO activity and cytokine TNFα, analysis by ELISA, respectively. Transverse section was washed with PBS and neutral buffered formalin, and stored in neutral buffered formalin (10%) for histological analysis (J. J. Kim, Shajib, Manocha, & Khan, 2012; Whittem, Williams, & Williams, 2010).

2.3. Clinical activity scoring by Disease Activity Index (DAI)

After beginning treatment with DSS, induction and progression of colonic inflammation was assessed daily where body weight, occult blood in faeces, and stool consistency/diarrhoea was recorded daily for each mouse to determine the disease activity index (DAI). Stool consistency was visually monitored, whereas faecal blood was determined by Hemdetect kit (DiproMed, Vosendorf, Austria). DAI was calculated

as the sum of scores of weight loss, stool consistency and blood in faeces. The maximum DAI score was 12 based on assigning a scoring system of 1–4 for each parameter: weight loss, stool consistency and blood in faeces (supplementary table 1) (Cummins et al., 2008; Tambuwala et al., 2010)

2.4. Histological assessment of inflammation in colonic tissue

Formalin fixed colonic tissue was embedded in paraffin, sections of 10μm thickness were cut and stained with hematoxylin and eosin (H&E) for evaluation of changes in colonic tissue mucosal architecture, related to extent and severity of inflammation. Histologic assessment and scoring of colon tissue sections was carried out in a blind manner according to a scoring procedure for colitis as reported previously (supplementary table 2) (Beloqui et al., 2013; Tambuwala et al., 2010).

2.5. Myeloperoxidase activity (MPO)

The tissue-associated MPO was measured using Myeloperoxidase Colorimetric Activity Assay Kit (Sigma-Aldrich, Wicklow, Ireland), to quantitate degree of neutrophil/inflammatory infiltration, which corresponds to the severity of colitis (Wéra, Lancellotti, & Oury, 2016). Briefly, flash frozen colonic tissue samples were homogenized in four volumes (of the tissue weight) of MPO assay buffer. The tissue homogenates were sonicated for 10 seconds, vortexed for 15-20 seconds and centrifuged at 13,000 rpm for 15 minutes. Reaction mix consisting of MPO substrate was added to this as per the manufacturer's instructions. This was mixed using a horizontal shaker, incubated at room temperature away from light. The reaction was stopped after 60 min, and the absorbance was measured at 412 nm by the microplate

reader Varioscan microplate reader (Perkin-Elmer) (Luzardo-Ocampo, Campos-Vega, Gonzalez de Mejia, & Loarca-Piña, 2020; Sheng, Sun, Li, Wang, & Ma, 2019).

2.6. Tissue cytokine quantitation by ELISA

Levels of cytokine, TNFα, a marker of inflammation in colonic tissues were determined by ELISA (Mouse TNF-α ELISA MAX Deluxe, BioLegend, San Diego, CA, US). Briefly, flash frozen colonic tissue samples were homogenized in 0.5ml of extraction buffer; RIPA buffer containing complete protease inhibitor cocktail (Roche Diagnostics, Vilvoorde, BE, ref 04693124001). The tissue homogenates were sonicated for 0.5-1 minute, vortexed for 15-20 seconds, followed by centrifugation at 15,000rpm for 45 minutes at 4^{o}C (Beloqui et al., 2013; Beloqui et al., 2014). The supernatants were collected and used for the quantitation of TNFα by ELISA analysis following the manufacturer's instructions (Mouse TNF-α ELISA MAX Deluxe, BioLegend, San Diego, CA, US).

2.7. Localization of coumarin-6 loaded particulate formulations in the colonic tissue

In a separate study, fluorescently, coumarin-6 (C6) loaded PU and PU-PEG particulate formulations were formulated as per our previously published work (Pabari et al., 2019). These were administered by OG (200uL) every day from day 3 to heathy groups and to DSS treated groups, over the 7-day experiment. On day 8, mice were sacrificed and freshly excised colons were cut longitudinally, washed with PBS and rolled to form a "swiss roll". This was fixed in neutral buffered formalin (10%) for 1-2 hours, cryoprotected in 30% sucrose, embedded in OCT compound (Laboratory Instrument & Supplies, Meath, Ireland) and frozen at -80^{o}C for subsequent cryosectioning using

a cryostat and imaging by Zeiss confocal laser-scanning microscope (CLSM) (Carl Zeiss, Jena, Germany) (Beloqui et al., 2013; Beloqui et al., 2014).

3. Results

3.1. Influence of increasing DSS concentrations on the severity of colitis induced

In this study, DSS at increasing concentrations from 0 to 2.5 %w/v was administered in drinking water from day 0 to day 7, and severity of colitis induced was compared. Three major clinical parameters of colitis i.e. degree of weight loss, alterations in stool consistency and blood in the stools determined by Hemdetect kit was recorded daily and calculated as disease activity index (DAI) score to determine severity of colitis induced.

Clinical signs of colitis were observed in all the DSS treated groups, thus indicating that DSS effectively induced colitis at all concentrations from 1 to 2.5% w/v. Severity of colitis induced, as indicated by the DAI score, was found to be directly proportional to the concentration of DSS and are shown in Figure 1A. At day 7, significantly high DAI score was observed in group treated with DSS at 2.5%w/v, compared to rest of the DSS treated groups (1.5%w/v and 1%w/v) and control (ANOVA, post-hoc, $p < 0.001$). DSS 1.5% w/v treated group showed significantly high DAI score compared to the group treated with DSS at 1%w/v (ANOVA; post-hoc, $p < 0.05$). Similarly, group treated with DSS at 1%w/v showed significantly high DAI score compared to healthy control (ANOVA; post-hoc, $p < 0.05$).

On day 8 after induction of colitis, mice were sacrificed, colon was resected from the ileo-caecal junction till the proximal rectum, and colon length was measured.

Representative photographs of colon length from each group are illustrated in Figure 1B. Mean colon length of healthy mice was found to be longest at 7.3cm. All DSS treated groups showed a reduction in mean colon length, indicating induction of colitis(Han et al., 2015). Change in colon length was found to be inversely proportional to the concentration of DSS administered (Figure 1C). DSS 1.5%w/v and 2.5%w/v treated groups showed significantly lower colon length compared to healthy control and group treated with DSS at 1%w/v (ANOVA; post-hoc, $p < 0.05$).

Colon was then cut into 3 sections, proximal, transverse and distal section. Middle section of the colon was assessed for colitic-induced histological changes by H&E staining. Proximal portion of the colon was assessed for neutrophil infiltration in the colonic mucosa by measuring colonic MPO activity, whereas distal portion of colon was measured for the level of TNFα secreted by ELISA.

Histological assessment of colon sections from healthy groups and DSS treated groups was carried out by H&E staining, and scoring was carried out in a blinded manner, as per the literature (Beloqui et al., 2013; Tambuwala et al., 2010). Representative colon sections from healthy and colitis groups are illustrated in Figure 1D. Colon tissue section from healthy mice showed intact colonic mucosal architecture and epithelial layer including presence of goblet cells and crypts. Whereas, colon tissue section from DSS treated control groups exhibited DSS-concentration dependent signs of inflammation such as epithelial disruption, crypt distortion, ulcerated mucosa, depletion of goblet cells, recruitment of inflammatory cells in the lamina propria region and moderate-to-severe submucosal lesions and oedema.

These histological changes were confirmed by an increase in the histological score observed in colonic tissues sections of all DSS groups, indicative of disruption in mucosal architecture, compared to healthy control. Level of histological score, i.e. degree of colonic mucosal damage and injury was dependent on the concentration of DSS, as illustrated in Figure 1E. Colon tissue sections from DSS groups can be arranged in decreasing order of histological score as DSS 2.5% > DSS 1.5% > DSS 1% (Figure 1D & E). All groups were statistically different from each other and from control (ANOVA; post-hoc, $p < 0.05$).

Typical mucosal changes occurring during colitis include influx of neutrophils to the inflamed mucosa(C. Zhang et al., 2018), and activation of mucosal immune cells causing secretion of proinflammatory cytokines, including TNFα (Chassaing, Aitken, Malleshappa, & Vijay-Kumar, 2014). MPO, a lysosomal protein, is released by activated neutrophils, therefore colonic MPO activity was measured to quantify neutrophil infiltration into the colon (Ali et al., 2014; Xiao et al., 2017). A significant increase in neutrophil infiltration indicated by the higher degree of colonic MPO activity, was observed in all DSS groups, compared to control (ANOVA, post-hoc, $p < 0.05$). An increasing trend in colonic MPO activity hence neutrophil infiltration was observed with increasing DSS concentration, related to corresponding increase in the severity of colitis induced(Wéra et al., 2016) (Figure 1F).

Level of proinflammatory cytokine TNFα secreted in colon tissues was determined using ELISA. Colon from healthy mice did not show any secretion of TNFα, as expected. Colon from all DSS groups showed TNFα secretion, indicating induction of colitis. Increasing levels of TNFα, was observed potentially reflecting increasing severity of inflammation induced with increasing DSS concentrations, which were

statistically different from each other and from control (ANOVA; post-hoc, $p < 0.05$) (Figure 1G).

Increase in the severity of colitis induced with increasing concentrations of DSS was expected (da Costa Gonçalves et al., 2013; Eichele & Kharbanda, 2017). DSS groups treated at 1.5%w/v and 2.5%w/v were chosen moderate severe and colitis model, respectively, to assess anti-inflammatory properties of orally administered INF and its polymeric particulate formulations (NPs).

3.4. Therapeutic Efficacy of Infliximab loaded particles in DSS induced colitis model

INF loaded particulate formulations: INF-PU and INF-PU-PEG, formulated by solvent dispersion technique and characterised for size and zeta potential by Zetasizer during our previously published work (Pabari et al., 2019) was used in this work. Physicochemical characteristics of particulate formulations used in this study such as particle size, zeta potential, INF loading and INF bioactivity were similar to that observed in our previously published work (Pabari et al., 2019).

INF-loaded particulate formulations showed particle size of 1550.67 ± 473.53 for INF-PU and 1667.60 ± 423.57 for INF-PU-PEG particulate formulations, and zeta potential of -5.43 ± 2.98 and -2.16 ± 0.17 respectively. Bioactivity of INF in INF-loaded particulate formulations was found to be at 99.19 ± 1.07 for INF-PU and 100.10 ± 1.11 for INF-PU-PEG. Therapeutic efficacy was studied by oral administration of INF solution and its particulate formulations of INF-PU and INF-PU-PEG suspension, at the INF dose of 10mg/kg, daily from day 3 to day 7 to DSS induced moderate and severe murine model of colitis (Figure 2a).

3.4.1. Disease activity index (DAI) score

Change in clinical features of colitis such as weight loss, alteration in stool consistency and blood in stool detected by Hemdetect kit, was calculated as a DAI score. Groups treated with INF or INF-NPs administered by OG or IP showed a decrease in mean DAI score, compared to colitis control in moderate colitis model (DSS 1.5%w/v), indicating a decrease in the severity of inflammation (Figure 2A). Groups can be arranged in decreasing level of DAI score as: DSS colitis control (1.5%w/v) > INF (OG) > INF-PU (OG) > INF-PU-PEG (OG) > INF (IP) (Figure 2A). Groups treated with INF-PU (OG), INF-PU-PEG (OG) and INF (IP) showed a statistically significant decrease in DAI score, compared to untreated control group (DSS 1.5%w/v) at day 7 (ANOVA; post-hoc, $p < 0.05$).

A similar trend of decreasing DAI score was observed after oral administration of INF or INF-NPs in 2.5%w/v DSS induced severe colitis groups (Figure 3A). Groups treated with INF-PU (OG), INF-PU-PEG (OG) and INF (IP) showed a statistically significant decrease in DAI score compared to DSS 2.5% untreated control group at day 7 (ANOVA; post-hoc, $p < 0.05$).

DSS induced colitis groups treated with INF (OG) showed lower mean DAI score in both, moderate and severe colitis model, however this decrease was not statistically significant compared to corresponding untreated controls (ANOVA; post-hoc, $p > 0.05$).

3.4.2. Colon length

On day 8, all mice were euthanized and colon was dissected out between ileo-caecal junction till the proximal rectum, and measured for colon length using a ruler. Representative photographs of colon from each group are shown in Figure 2B and 3B for moderate (DSS 1.5%w/v) and severe (DSS 2.5%w/v) colitis model, respectively.

A significant colon length shortening was observed in both DSS-treated groups of 1.5%w/v at 5.6cm (Figure 2C) and 2.5%w/v at 4.9cm (Figure 3C), compared to healthy group at 7.3cm (ANOVA, post-hoc, $p < 0.05$), owing to the induction of inflammation (Biton et al., 2018). All the treatment groups showed less shortening of mean colon length. In moderate colitis model (DSS 1.5%w/v), treatment with INF or INF-NPs showed colon length in the range of 6.2 to 6.4cm, whereas in severe colitis model (DSS 2.5%w/v), treatments with INF or INF-NPs showed colon length in the range 5.7 to 6.2cm.

Groups treated with PU-INF and PU-PEG-INF showed significantly lower colitis-related decrease in colon length in both, moderate (DSS 1.5% w/v) and severe (DSS 2.5%w/w) colitis model (ANOVA, post-hoc, $p < 0.05$).

3.4.3. Histological assessment and scoring of colon tissue inflammation

Alterations in colon tissue histological morphology in DSS control and groups treated with INF and INF-NPs was evaluated by haematoxylin-eosin (H&E) staining of colon tissue sections. Representative images from control and various INF or INF-NPs treatment groups from moderate colitis model (DSS 1.5%w/v) and severe colitis model (2.5%w/v), is illustrated in Figure 2D and 3D, respectively.

Colon tissue sections from treatment groups showed a decrease in signs of inflammation, with some degree recovery in epithelial tissue architecture in all groups treated with INF or its INF-NPs administered by IP or OG. These observations were

confirmed by the histological scoring of the tissue sections illustrated in Figure 2F and 3F.

Untreated control group showed significantly high histological score compared to healthy control for both, moderate (DSS 1%w/v) and severe (DSS 2.5%w/v) colitis model (ANOVA, post-hoc, $p < 0.05$), indicative of inflammation related mucosal damage. Colonic tissue from all treatment groups i.e. INF-NPs administered orally, or INF administered by IP or OG, showed significantly lower histological score compared to DSS control in moderate (DSS 1.5%w/v) colitis model (ANOVA, post-hoc, $p < 0.05$). Furthermore, groups treated with INF administered by OG showed significantly higher histological score compared to groups treated with INF-NPs administered by OG or INF administered by IP (ANOVA, post-hoc, $p < 0.05$).

However, in severe colitis model (DSS 2.5%w/v), groups treated with INF administered by OG showed statistically similar histological score compared to corresponding control (ANOVA, post-hoc, $p > 0.05$). Whereas, groups treated with INF-NPs administered by OG or INF administered by IP showed significantly lower histological score compared to corresponding DSS control in severe (DSS 2.5%w/v) colitis model (ANOVA, post-hoc, $p < 0.05$).

Tissue colon from groups treated with INF-PU and INF-PU-PEG particulate formulations showed a lot of resemblance to the colon from healthy control, illustrating major regeneration of mucosal architecture, presence of crypts, goblet cells, lower submucosal oedema. This was confirmed by the significantly lowest mean histological score observed in groups treated with INF-PU and INF-PU-PEG in both, moderate (DSS 1.5%w/v) and severe (DSS 2.5%w/v) colitis model (ANOVA, post-hoc, $p < 0.05$).

3.4.4. **Myeloperoxidase activity (MPO) in colonic tissue**

Colonic MPO activity, a measure of neutrophil infiltration, was quantified in healthy, DSS untreated control group and in treatment groups. Colon tissues from untreated DSS control showed high neutrophil infiltration, indicated by significantly high MPO activity in both, moderate (DSS 1.5%w/v) (Figure 2F) and severe (DSS 2.5%w/v) colitis model (Figure 3F), compared to control (ANOVA, post-hoc, $p < 0.05$). This was expected for an inflamed colonic tissue.

Colonic MPO activity was found to significantly lower in colon from all treatment groups of INF or INF-NPs administered by IP or OG, compared to both, moderate (DSS 1.5%w/v) and severe (DSS 2.5%w/v) colitis model (ANOVA; post-hoc, $p < 0.05$) (Figure 2F & 3F). Colon from INF-PU and INF-PU-PEG treatment showed significantly lowest colonic MPO activity, and reduction in inflammation in both, moderate (DSS 1.5%w/v) and severe (DSS 2.5%w/v) colitis model (ANOVA; post-hoc, $p < 0.05$). Anti-TNFα therapy has been reported to significantly downregulate infiltration of neutrophils in inflamed intestinal mucosa (C. Zhang et al., 2018).

3.4.5. Inflammatory cytokine TNFα levels in the colonic tissue

Colon from untreated DSS control groups from both concentrations, moderate (1.5%w/v) (Figure 2G) and severe (2.5%w/v) (Figure 3G) colitis model showed significantly high secretion of pro-inflammatory cytokine, TNFα, compared to healthy control, indicating induction of inflammation (ANOVA, post-hoc, $p < 0.001$). Colon from all treatment groups showed a significant reduction in secretion of TNFα level in both, moderate (DSS 1.5%w/v) and severe colitis model (DSS 2.5%w/v) (ANOVA, post-hoc, $p < 0.05$). Colon from group treated with INF-PU and INF-PU-PEG-INF showed significantly lowest mean TNFα secretion (ANOVA, post-hoc, $p < 0.05$).

3.4.6. Localisation of Infliximab loaded particles in severe and moderate colitis model

Fluorescent label, coumarin-6 (C6) loaded particulate formulations (NPs) of PU and PU-PEG were formulated and orally administered to healthy and DSS induced moderate (1.5%w/v) and severe (2.5%w/v) colitis model from day 3 to day 7.

Healthy control treated with C6-PU and C6-PU-PEG particulate formulations showed no change in body weight or DAI score, thus indicating biocompatibility of novel PU and PU-PEG polymeric biomaterials (Supplementary Figure 1). Biodegradability and biocompatibility of PU and PU-PEG polymers have been reported in the literature (Clara Mattu et al., 2012; C. Mattu et al., 2018). Similarly, DSS moderate (1.5%w/v) and severe (2.5%w/v) colitis model treated with C6-PU or C6-PU-PEG particulate formulations showed no change in body weight or DAI score compared to corresponding controls, indicating that these polymeric biomaterials do not have any inherent anti-inflammatory properties and also it does not worsen inflammation in DSS-induced colitis models (Supplementary Figures 2 and 3).

On day 8 mice colon was dissected and cryoprotected for subsequent cryosectioning and visualisation under confocal microscope (CLSM). Representative images of colon sections from healthy and DSS treated groups are presented in Figure 4A. Fluorescence pixel intensity of C6 in each image was calculated by image analysis using R statistical computing (Barthelmé & Tschumperlé, 2019; Villanueva & Chen, 2019), and is illustrated in Figure 4B.

CLSM showed higher accumulation and penetration of C6-NPs in the colon from healthy tissues as well as inflamed colon from DSS treated groups, compared to C6

solution. Thus, PU and PU-PEG polymeric biomaterials are acting as a cargo allowing transport of C6 across the colonic mucosa.

Overall higher translocation of C6 and its particulate formulations, C6-PU and C6-PU-PEG was observed across inflamed mucosa from DSS treated groups, compared to the healthy mucosa. PEGylated version showed comparatively highest permeation across both, healthy and inflamed mucosa, which is consistent with the literature(Lautenschläger, Schmidt, Lehr, Fischer, & Stallmach, 2013).

4. Discussion

In IBD, a complex interaction between genetic factors and environmental stress is believed to cause impairment in epithelial barrier integrity, allowing entry of luminal toxins and microbes to the underlying intestinal mucosa. This results in abnormal over activation of intestinal mucosal immune responses, and secretion of pro-inflammatory cytokines causing mucosal damage. Excessive secretion of pro-inflammatory cytokine, particularly TNFα, both soluble and membrane-bound forms are reported to play a central role in the pathogenesis of IBD and at the top of the inflammatory cascading network (Bhol et al., 2013; Schreiner et al., 2019; Y.-Z. Zhang & Li, 2014). Consequently, anti-TNFα mAb, INF has been reported to be effective in inducing and maintaining long term clinical remission, resulting in mucosal healing, hence fewer IBD-related hospitalisations or surgery (J. F. Colombel et al., 2011; Papamichael et al., 2019). Oral anti-TNFα which will reduce its systemic exposure and associated serious side-effects, including long-term systemic immunosuppression, commonly encountered with parenteral administration of anti-TNFα mAbs, is highly desirable.

Consequently a recent surge in research has been seen surrounding development of oral anti-TNFα mAb (Bhol et al., 2013; Crowe et al., 2018) or formulation INF particles (J. M. Kim et al., 2020; Wang et al., 2020).

A novel oral polyclonal anti-TNFα antibody, AVX-470, functionally comparable to infliximab, orally administered twice a day in dextran sodium sulfate (DSS) colitis model showed concentration dependent decrease in endoscopy score (Bhol et al., 2013). Recently, AVX-470 showed clinical response in first human clinical trial (Phase 1 clinical trial, NCT1759056) in 25.9% of UC patients, with greatest response at 3.5g/day (Harris et al., 2016). This indicates that orally administered anti-TNFα neutralises proinflammatory cytokine TNFα locally, present in the inflamed GI mucosa of IBD, resulting in a decrease in disease severity and potentially facilitating mucosal healing (Hartman et al., 2016).

In another study, V565 a novel anti-TNFα single domain antibody designed for oral administration in IBD patients was reported to show similar potency as adalimumab. In DSS colitis model, V565 was shown to penetrate to lamina propria region of the colon, i.e site of TNFα production, attributed to the compromised barrier function of the inflamed colon(Crowe et al., 2018). V565 has been reported to inhibit of mucosal inflammatory processes after 6–7 days oral dosing in UC patients (Parkes et al., 2018). Recently, Nurbhai et al (Nurbhai et al., 2019) reported that oral V565 showed reduction in markers of inflammation in UC patients. V565 is currently being studied in Crohns disease under phase 2 clinical trial (NCT02976129). Despite several successful research in oral anti-TNFα therapy, none are in general clinical use yet (Griffiths et al., 2020).

Particulate formulations have been reported to be preferentially up taken by the inflamed colonic mucosa of mice model of colitis and human patients by virtue of

electrostatic interactions (Ali et al., 2014; Beloqui et al., 2014; Schmidt et al., 2013; Xiao et al., 2017). In our previously published work, we successfully developed INF loaded particulate formulations using various polymeric biomaterials and observed that INF-NPs of novel PU polymer and its PEGylated version showed high uptake and permeability across in-vitro inflamed epithelial cell culture model. This resulted in rapid recovery of the epithelial barrier integrity, a pathophysiological hallmark of reduction in inflammation (Pabari et al., 2019). This was related to higher cellular interactions of PU based polymeric NPs compared to PLGA and PCL based NPs, which was also observed in an another study by Mattu et al (Clara Mattu et al., 2013). In this study we evaluated the therapeutic efficacy of INF loaded PU and PU-PEG particulate formulations, in an in-vivo DSS induced colitis model with moderate and severe inflammation.

Initially we assessed the effect of increasing DSS concentrations from 0 to 2.5%w/v on the severity of colitis induced. DSS i.e. dextran sodium sulfate is a sulphated polysaccharide, dissolved in drinking water to induce colitis in murine model (McCarthy et al., 2013; Tambuwala et al., 2010). DSS induced model of colitis has been reported to closely resemble human UC. It disrupts intestinal epithelial cell barrier, allowing entry of luminal antigens to underlying mucosal immune cells, resulting in a rapid and profound inflammatory immune response and mucosal damage. DSS model of colitis is widely used because of its simplicity and reproducibility (Chassaing et al., 2014; Wirtz et al., 2017).

DSS treated groups showed dose dependent increase in clinical signs of colitis indicated by the DAI score. At day 7, DAI score between all the DSS groups were significantly different from each other, and from control (ANOVA, post-hoc, $p < 0.05$). Interestingly, a 2-fold increase in the DAI score was observed between day 6 and 7 in

this study. This was consistent with the literature. Beloqui et al (Beloqui et al., 2013) observed a nearly 4-fold increase in the clinical activity score between day 6 and 7 of the study in 3% DSS induced colitis model. Similarly, Kim et al (J. M. Kim et al., 2020) and Wang et al (Wang et al., 2020) reported a colitis-related decrease in body weight from day 6 onwards in 1.5% and 2% DSS induced colitis model, respectively. It has been reported that physiologically signs of induction of inflammation can be seen from day 1 of DSS administration. This include alteration in the expression of epithelial tight junction proteins, followed by apoptosis of intestinal epithelial cells, contributing to the damage of protective epithelial barrier function (Eichele & Kharbanda, 2017). This may indicate that physiologically inflammation should be well progressed before the clinical signs of inflammation is apparent.

During active intestinal inflammation, infiltration of leukocytes such as neutrophils in the intestinal mucosa is one of the early steps in the immune response (Holma et al., 2001). In addition, activation of intestinal mucosal immune cells, macrophages and dendritic cells in submucosa and lamina propria causes secretion proinflammatory cytokines, including TNFα, resulting in further mucosal damage to the intestinal epithelium, and progression of colitis resulting in clinical signs of colitis (Ali et al., 2014; Collnot et al., 2012; Lautenschläger et al., 2013; Neurath, 2014).

A DSS dose-dependent decrease in colon length was expected because inflammation has been reported to cause shortening of colon length (Ali et al., 2014; Naeem et al., 2015; Tambuwala et al., 2010). Biton et al(Biton et al., 2018) studied the dose dependence of DSS induced colitis at DSS concentrations of 0.5%, 1%, 1.5% and 2% and reported a decrease in colon length in groups treated with ≥ 1% DSS, and no change in colon length in 0.5% DSS-treated group, compared to healthy control.

Authors further report no significant difference in colon length among groups treated with 1 to 2% DSS.

DSS induced histological changes in tissue structure typically includes depletion of goblet cell, erosion of epithelium, infiltration of granulocytes and neutrophil into the intestinal submucosa including lamina propria region. These were scored as per the literature (Beloqui et al., 2013; Eichele & Kharbanda, 2017; Tambuwala et al., 2010). Typical DSS-colitis induced histological changes such as increasing erosion of epithelium and changes in the crypt architecture was observed with increasing severity of inflammation. This was confirmed by significantly higher histological scoring with higher DSS concentration from 1 to 2.5%w/v. This trend of increasing histological score and mucosal injury with increasing DSS concentrations is consistent with the literature(Biton et al., 2018; Egger et al., 2000). Progressive disruption of colonic mucosal architecture with increasing DSS concentrations from 1 to 2.5%w/v can be related to increasing severity of inflammation. This was confirmed by increasing neutrophil infiltration and level of pro-inflammatory cytokine, TNFα, secreted observed with increasing DSS concentrations from 1 to 2.5%w/v. Colonic MPO activity was used to measure level of neutrophil infiltration because MPO is the most abundant protein present in neutrophils and is released when neutrophils is activated during active inflammation in colitis (Beloqui et al., 2014).

DSS dose-dependent increase in the severity of colitis is in line with the literature (Eichele & Kharbanda, 2017). Colitis model developed at DSS concentrations of 1.5%w/v and 2.5%w/v were designated as moderate and severe model, respectively, to evaluate therapeutic efficacy of orally administered INF and its particulate formulations, INF-PU and INF-PU-PEG.

In this study, groups orally administered INF-PU and INF-PU-PEG particulate formulations, similar to groups treated with INF (IP), showed a significant improvement in the clinical signs of colitis, reflected by a significantly lower DAI score, compared to corresponding controls, in both moderate and severe colitis model.

Improvement in clinical signs of colitis in INF-NP treated groups could be related to a significant physiological decrease in colonic inflammation. This was further supported by results from colonic tissue histology studies, and level of inflammatory markers such as neutrophil infiltration and TNFα levels.

Colon tissues from groups treated with INF-PU and INF-PU-PEG particulate formulations showed significantly lower decrease in colitis related colon length shortening and a significant restoration in the tissue architecture, confirmed by statistically lowest histological score, thus implying mucosal healing. Regeneration in tissue histological morphology of the colon was related to a significant decrease in mucosal inflammation in groups treated with INF-NPs, as indicated by a significant decrease in neutrophil infiltration, measured by colonic tissue MPO activity as well as a significant decrease in pro-inflammatory cytokine, TNFα.

In some studies, authors report no change in clinical signs of colitis however further report significant physiological reduction in colitis (Beloqui et al., 2013; Beloqui et al., 2014; McCarthy et al., 2013), demonstrating that physiological reduction in colitis precedes improvement in clinical signs of colitis.

Interestingly in this study, groups treated with oral INF solution also showed a decrease in inflammatory markers (neutrophil infiltration, TNFα) in both, moderate and severe colitis model. Mean decrease in inflammatory cytokines such as IL-6 and IL-1β after oral administration of INF to 2% DSS induced colitis mice has been observed by Wang et al (Wang et al., 2020).

Treatment with oral INF solution showed lower histological score compared to DSS control in moderate colitis model (DSS 1.5%w/v). This can be probably related to the lower severity of inflammation induced, and highest possible dose of INF used in this study of 10mg/kg, compared to 5mg/kg as recommended by the summary product characteristics (SPC) of INF (Remicade®). No significant decrease in histological score compared to control was observed after administration of INF oral solution to in severe colitis model (DSS 2.5%w/v).

No significant decrease in clinical signs of colitis (i.e. DAI score) or changes in colitis-induced decrease in colon length was observed in moderate or severe DSS colitis groups treated with oral INF. This was consistent with the literature. Recently Kim et al (J. M. Kim et al., 2020) studied orally administered INF and its liposomal-aminoclay nanocomposites over 9 days (from day 0), in a 7-day 1.5% DSS induced colitis model and a lower mean DAI was observed in groups treated with orally administered INF suspension. Similarly, Wang et al (Wang et al., 2020) studied orally administered INF infusions and its self-assembled particles based on natural polyphenol tannic acid and PEG in a 7-day study in 2% DSS induced colitis mice and observed a decrease in mean DAI score in groups orally administered INF suspension.

Particulate formulations comprising of microparticles (Lautenschläger et al., 2013; Naeem et al., 2015; Schmidt et al., 2013; Xiao et al., 2015) or nanoparticles (Ali et al., 2014; Beloqui et al., 2014; Xiao et al., 2017) have been reported to show targeted delivery due to its preferential adherence at the inflamed mucosa by virtue of its electrostatic interactions (Collnot et al., 2012; Li, Lu, Yang, Yu, & Rao, 2020).

Lautenschlager et al (Lautenschläger et al., 2013) reported that PEG functionalised PLGA particles showed higher translocation through inflamed mucosa, compared to non-functionalized particles, related to the hydrophilic PEG. A similar trend was

observed in this study during confocal studies whereby PEGylated PU particles showed higher permeation across the inflamed mucosa compared to PU particles. Consequently INF-PU-PEG showed lower mean DAI score compared to INF-PU, however these were not statistically significant.

PU and PU-PEG particulate formulations used in this study showed a negative surface charge (Pabari et al., 2019), allowing electrostatic interactions with the positively charged proteins at the damaged epithelium of the inflamed mucosa. Consequently, this increased residence time of INF-PU NPs and INF-PU-PEG NPs at the inflamed mucosa could have facilitated its translocation across the compromised epithelial barrier, to the submucosal layers where the active mucosal immune system can be targeted to treat inflammation at its site of origin, resulting in local effect thus enhancing its therapeutic efficacy (Beloqui et al., 2014; Maisel et al., 2015; Wang et al., 2020; Xiao et al., 2017). This concept was confirmed by the NP localisation studies which showed translocation of fluorescently labelled, C6-PU NPs and C6-PU-PEG NPs across the colonic mucosa, and corresponding higher fluorescence pixel intensity.

5. Conclusion

In this study INF loaded PU and PU-PEG particulate formulations adhered to the inflamed mucosa, allowing targeted delivery of INF, anti-TNFα mAb to the site of inflammation in DSS induced murine model of colitis. This enabled local inhibition of TNFα driven pathways of inflammation at its source, reducing inflammation in both moderate and severe colitis model, and increasing therapeutic potential of INF in bringing mucosal healing, a key goal of IBD therapy.

These promising preliminary results warrant further investigation of orally administered INF and its novel formulations in a wider preclinical study. This will potentially pave the

way for oral administration of biological therapeutics such as INF loaded particulate formulations, which will result in greater patient compliance, reduced systemic side effects and lower non-drug costs associated with the administration of intravenous infusions, thus lowering the economic burden on the healthcare budget.

Author contributions

Ritesh M. Pabari: Conceptualisation, Methodology, Investigation, Visualisation, Formal analysis, Original draft preparation, Funding acquisition. Murtaza M Tambuwala: Conceptualisation, Methodology, Formal analysis, Supervision, Writing: reviewing and editing, Natalia Lajczak-McGinley: Methodology, Alaa Aljabali: Formal analysis, Writing: reviewing and editing, Brian Kirby: Supervision, Stephen Keely: Supervision, Zebunissa Ramtoola: Supervision

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Figures

Figure 1 Effect of increasing DSS concentrations from 0 to 2.5%w/v on **A C**linical signs of colitis measured as disease activity index (DAI) assessed in healthy control (light green line) and DSS treated control groups: 1%w/v DSS treated control (blue line), 1.5%w/v DSS treated control (pink line), and 2.5%w/v DSS treated control (red line), **B & C** Colon length in cm from healthy control group and DSS treated control groups, **D** Changes in Histological features via H&E staining observed in images of colon sections from healthy control groups and DSS control groups, **E** Histological scores from healthy control groups and DSS control groups, **F** Changes in colonic MPO activity indicating neutrophil infiltration, and **G** Levels of TNFα secreted in the colon. Data (n > 3) are expressed as mean ± SD. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$. Statistical significance was assessed using Student's t-test and one-way ANOVA followed by Tukey multiple comparisons to compare the results

Figure 1

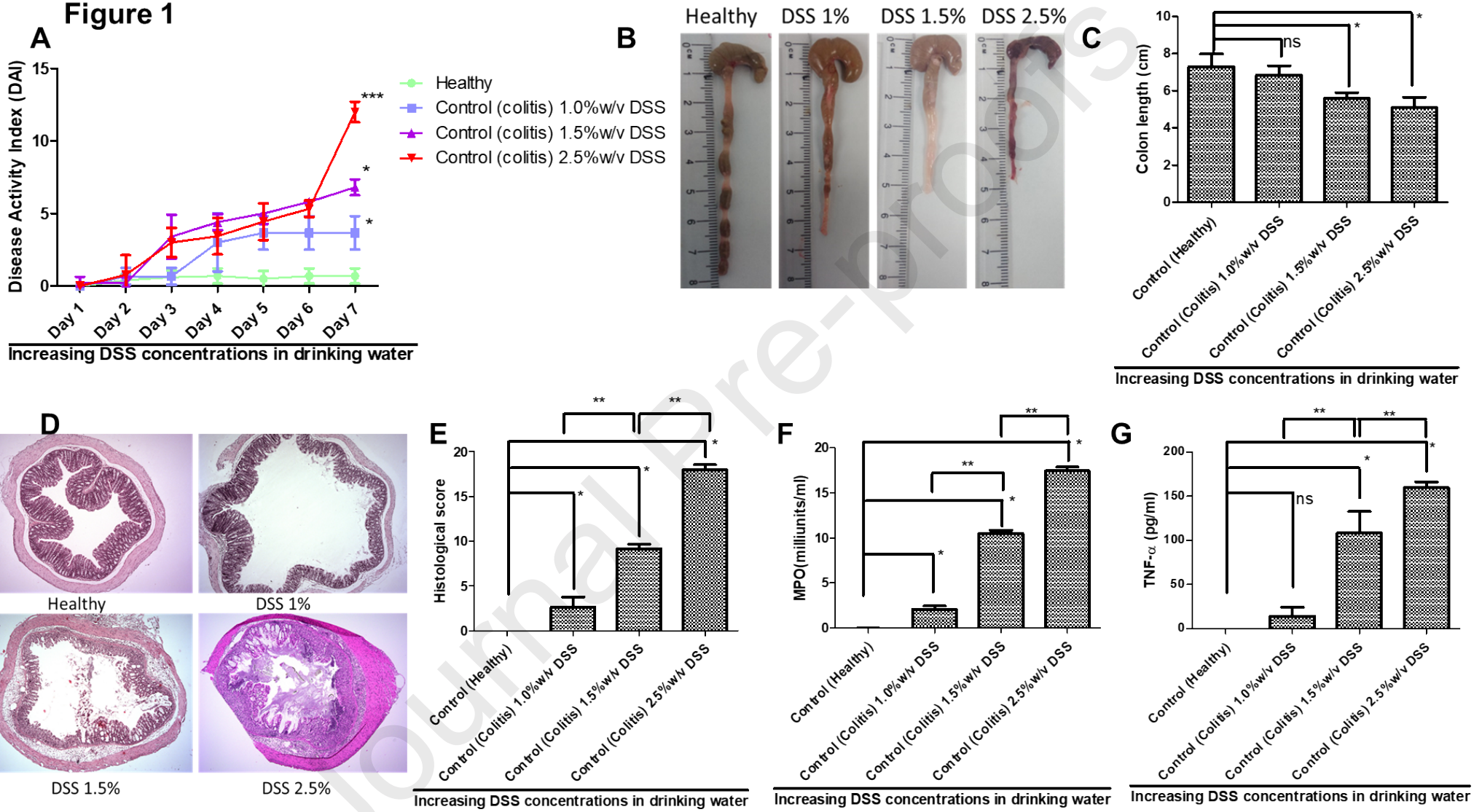


Figure 2 Therapeutic effects of orally administered INF and its particulate formulations in DSS induced moderate colitis mouse model (DSS 1.5%w/v) on **A** Clinical signs of colitis measured as disease activity index (DAI) assessed in control groups: healthy control (light green line), colitis control (pink line), and treatment groups: groups treated with INF (IP) (brown line), INF (OG) (orange line), INF-PU NPs (OG) (medium green line) and INF-PU NPs (OG) (dark green line) **B & C** Colon length in cm from control groups and treatment groups, **D** Changes in Histological features via H&E staining observed in images of colon sections from control groups and treatment groups. **E** Histological scores from control groups and treatment groups, **F** Changes in colonic MPO activity indicating neutrophil infiltration in control groups and treatment groups, and **G** Levels of TNFα secreted in the colon from control groups and treatment groups. Data ($n > 3$) are expressed as mean ± SD. $*p < 0.05$; $**p < 0.01$; $***p < 0.001$; $****p < 0.0001$. Statistical significance was assessed using Student's t-test and one-way ANOVA followed by Tukey multiple comparisons to compare the results

Figure 2

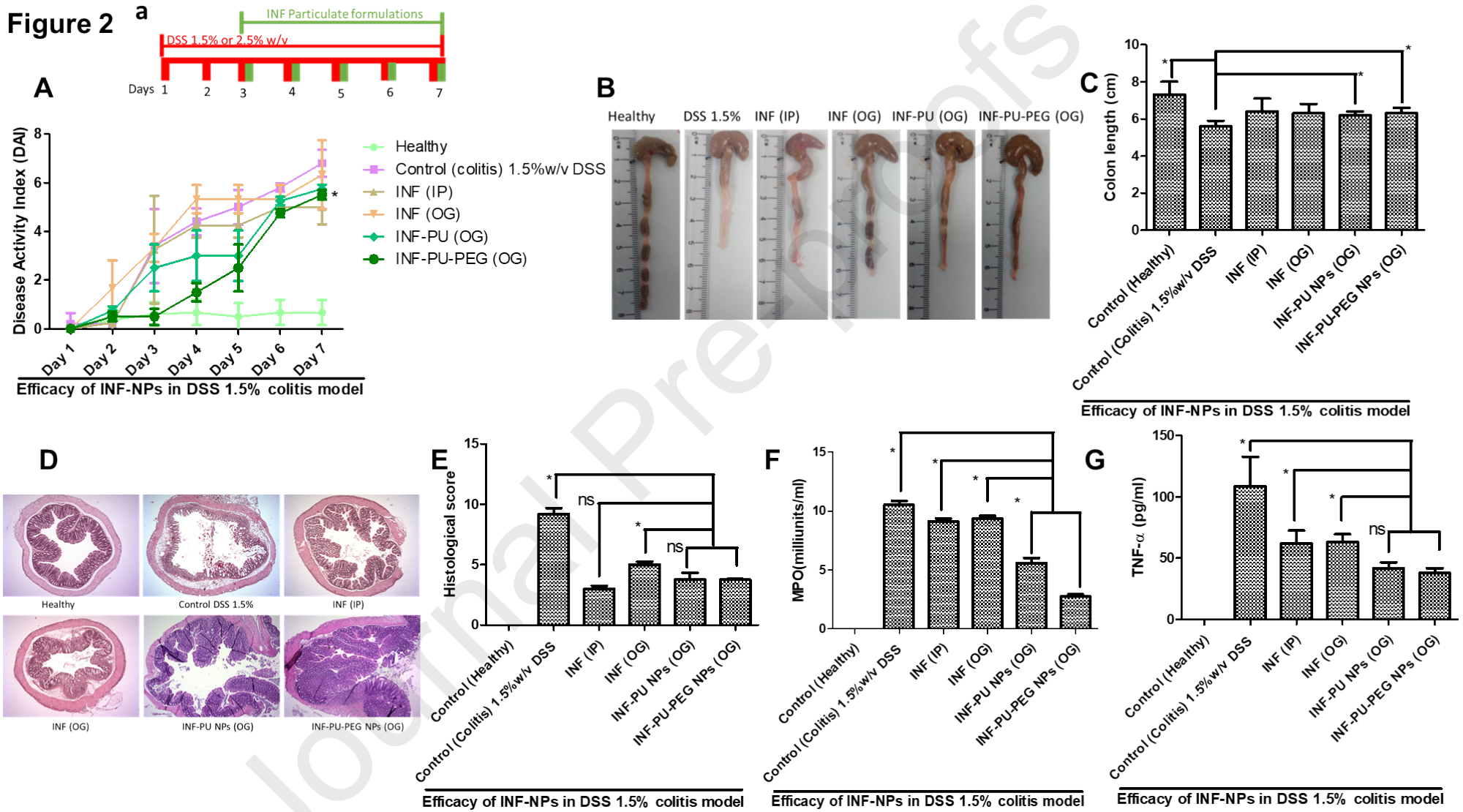


Figure 3 Therapeutic effects of orally administered INF and its particulate formulations in DSS induced severe colitis mouse model (DSS 2.5%w/v) on **A** Clinical signs of colitis measured as disease activity index (DAI) assessed in control groups: healthy control (light green line), colitis control (pink line), and treatment groups: groups treated with INF (IP) (brown line), INF (OG) (orange line), INF-PU NPs (OG) (medium green line) and INF-PU NPs (OG) (dark green line), **B & C** Colon length in cm from control groups and treatment groups, **D** Changes in Histological features via H&E staining observed in images of colon sections from control groups and treatment groups, **E** Histological scores from control groups and treatment groups, **F** Changes in colonic MPO activity indicating neutrophil infiltration in control groups and treatment groups, and **G** Levels of TNFα secreted in the colon from control groups and treatment groups. Data ($n > 3$) are expressed as mean ± SD. $*p < 0.05$; $**p < 0.01$; $***p < 0.001$; $****p < 0.0001$. Statistical significance was assessed using Student's t-test and one-way ANOVA followed by Tukey multiple comparisons to compare the results

Figure 3

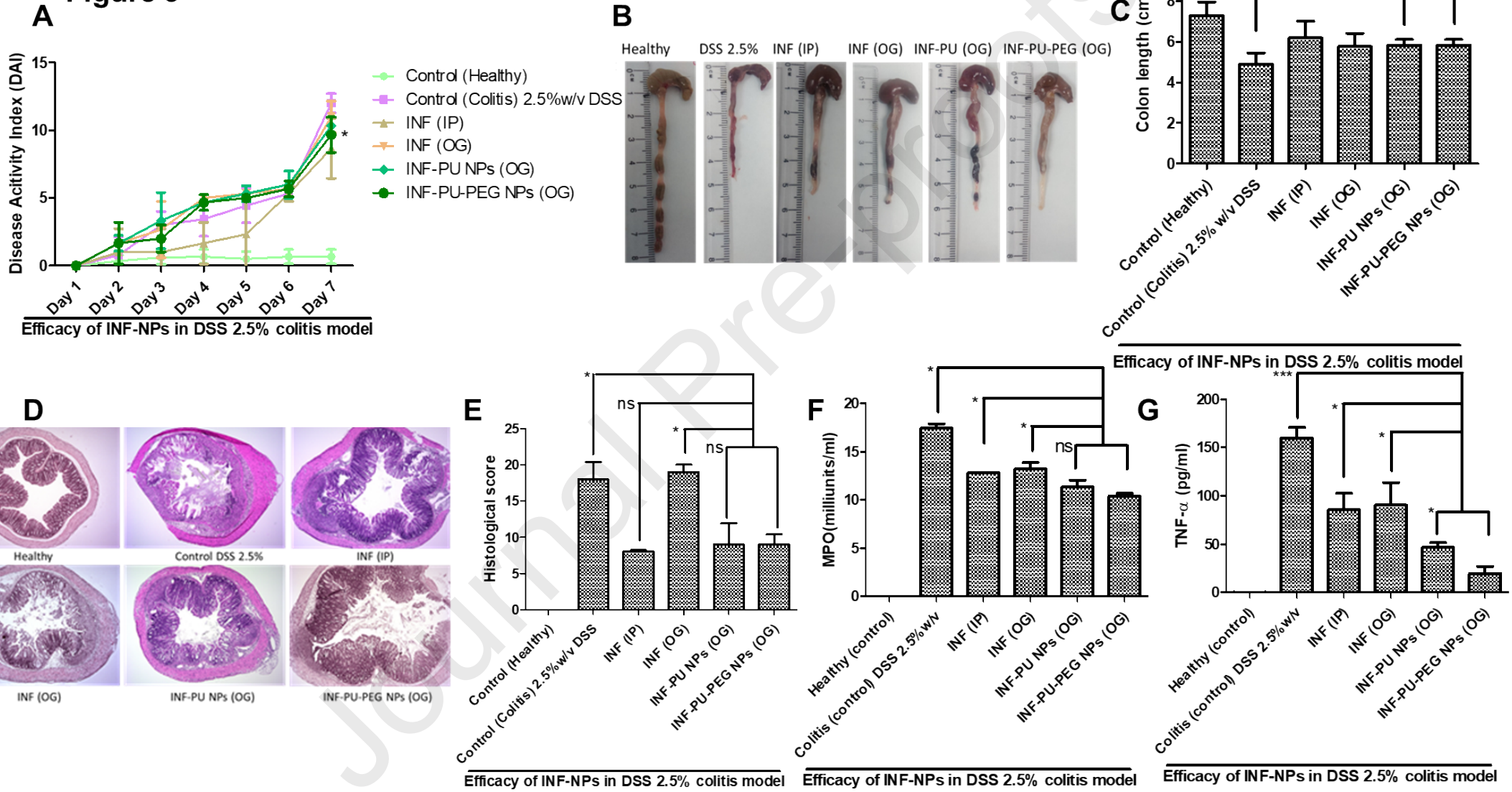
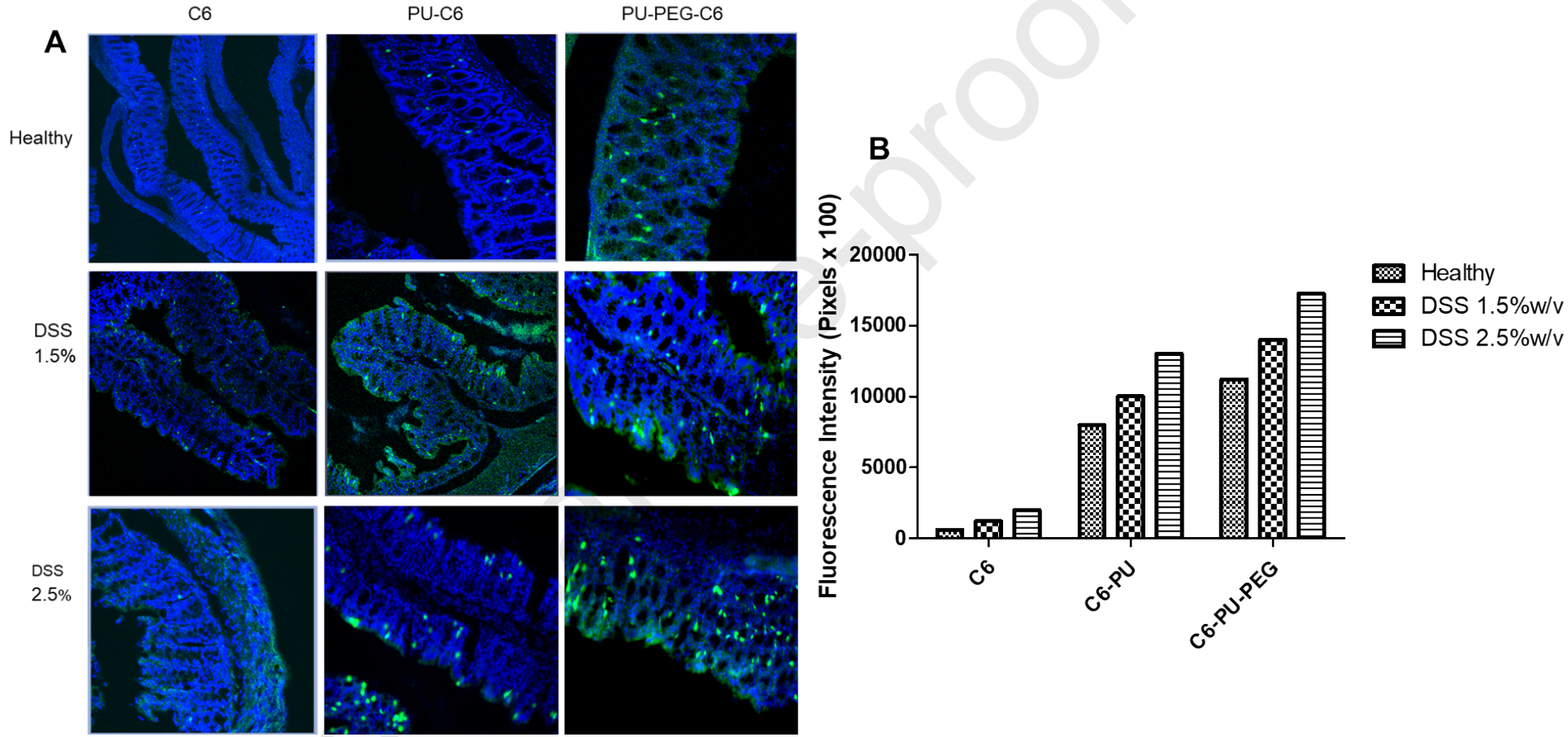


Figure 4 Confocal laser scanning microscopy (CLSM) images showing **A** Localisation of fluorescent, coumarin 6 (C6) and C6 loaded PU and PU-PEG particulate formulations in colon sections from moderate colitis (DSS 1.5%w/v) and severe colitis (DSS 2.5%w/v) groups, **B** Fluorescence pixel intensity calculated by image analysis using R Statistical computing. Data (n > 1) are expressed as mean ± SD

Figure 4



Author contributions

Ritesh M. Pabari: Conceptualisation, Methodology, Investigation, Visualisation, Formal analysis, Original draft preparation, Funding acquisition. Murtaza M Tambuwala: Conceptualisation, Methodology, Formal analysis, Supervision, Writing: reviewing and editing, Natalia Lajczak-McGinley: Methodology, Alaa Aljabali: Formal analysis, Writing: reviewing and editing, Brian Kirby: Supervision, Stephen Keely: Supervision, Zebunissa Ramtoola: Supervision

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: