CARDIFF UNIVERSITY
PRIFYSGOL CAERDYDD

ORCA – Online Research @ Cardiff

This is an Open Access document downloaded from ORCA, Cardiff University's institutional repository:https://orca.cardiff.ac.uk/id/eprint/111838/

This is the author's version of a work that was submitted to / accepted for publication.

Citation for final published version:

McLean, Mairi H., Andrews, Caroline, Hanson, Miranda L., Baseler, Walter A., Anver, Miriam R., Senkevitch, Emilee, Staniszewska, Aleksandra K., Smith, Christopher, Davies, Luke C., Hixon, Julie, Li, Wenqeng, Shen, Wei, Steidler, Lothar and Durum, Scott K. 2017. Interleukin-27 is a potential rescue therapy for acute severe colitis through Interleukin-10–Dependent, T-Cell–independent attenuation of colonic mucosal innate immune responses. Inflammatory Bowel Diseases 23 (11) , pp. 1983-1995. 10.1097/MIB.0000000000001274

Publishers page: http://dx.doi.org/10.1097/MIB.0000000000001274

Please note:
Changes made as a result of publishing processes such as copy-editing, formatting and page numbers may not be reflected in this version. For the definitive version of this publication, please refer to the published source. You are advised to consult the publisher's version if you wish to cite this paper.

This version is being made available in accordance with publisher policies. See http://orca.cf.ac.uk/policies.html for usage policies. Copyright and moral rights for publications made available in ORCA are retained by the copyright holders.

Interleukin 27 is a potential rescue therapy for acute severe colitis via interleukin-10 dependent, T cell independent attenuation of colonic mucosal innate immune responses

Mairi H McLean[1,2], Caroline Andrews[1], Miranda L Hanson[1], Walter A Baseler[1], Miriam R Anver[3], Emilee Senkevitch[1], Aleksandra K Staniszewska[1,2], Christopher Smith[1,2], Luke C Davies[1], Julie Hixon[1], Wenqeng Li[1], Wei Shen[1], Lothar Steidler[4], Scott K Durum[1]

[1]Cancer and Inflammation Program, Center for Cancer Research, National Cancer Institute, Frederick, Maryland, 21702, USA; [2]School of Medicine, Medical Sciences & Nutrition, University of Aberdeen, Scotland, AB25 2ZD, UK; [3]Pathology/Histotechnology Laboratory, Laboratory Animal Sciences Program, Leidos Biomedical Research, Inc., Frederick National Laboratory for Cancer Research, Frederick, Maryland, 21702, USA; [4]Intrexon Actobiotics N.V., 9052 Zwijnaarde, Belgium

Short title; IL-27 attenuates colonic innate immune responses

* Correspondence Dr. Scott K Durum

National Cancer Institute, Laboratory of Molecular Immunoregulation, Bldg 560, 1050 Boyles Street, Frederick, MD 21702-1201

Tel: +1-301-846-1545, Fax: +1-301-846-6752

Email: durums@mail.nih.gov

Conflicts of Interest and Source of Funding

Lothar Steidler is a shareholder of Intrexon Corporation and employee of Intrexon Actobiotics N.V. The other authors have declared that no conflict of interest exists.

This work was supported in part by the Intramural Research Program of the National Institutes of Health, National Cancer Institute, as well as a fellowship funded by Crohn's and Colitis Foundation of America (MHM) and a grant from the Broad Medical Research Foundation. This project has been funded in whole or in part with Federal funds from the National Cancer Institute, National Institutes of Health, under Contract No. HHSN261200800001E (MRA). The content of this publication does not necessarily reflect the views or policies of the Department of Health and Human Services, nor does mention of trade names, commercial products, or organizations imply endorsement by the U.S. Government.

Abstract

Background; IBD patients with acute severe colitis face systemic anti-TNF biologic rescue therapy or colectomy, if treatment with intravenous steroids fail. Interleukin (IL)-27 is a cytokine with an immunosuppressive role in adaptive immune responses. However, the IL-27 receptor complex is also expressed on innate immune cells, and there is evidence that IL-27 can impact the function of innate cell subsets, although this particular functionality in vivo is not understood. Our aim was to define the efficacy of IL-27 in acute severe colitis and characterize novel IL-27 driven mechanisms of immunosuppression in the colonic mucosa.

Methods; We assessed oral delivery of *Lactococcus lactis* expressing an IL-27 hyperkine on the innate immune response *in vivo* in a genetically intact, non-infective acute murine colitis model induced by intra-rectal instillation of 2,4,6-Trinitrobenzenesulfonic acid in SJL/J mice.

Results; IL-27 attenuates acute severe colitis through reduction of colonic mucosal neutrophil infiltrate associated with a decreased CXC chemokine gradient. This suppression was T cell-independent and IL-10-dependent, initially featuring enhanced mucosal IL-10. IL-27 was associated with a reduction in colonic pro-inflammatory cytokines and induced a multifocal strong positive nuclear expression of phosphorylated STAT-1 in mucosal epithelial cells.

Conclusion; We have defined novel mechanisms of IL-27 immunosuppression towards colonic innate immune responses in vivo. Mucosal delivery of IL-27 has translational potential as a novel therapeutic for IBD and is a future mucosal directed rescue therapy in acute severe inflammatory bowel disease.

Keywords; interleukin-27, cytokine, IBD, colitis

Introduction

Inflammatory bowel disease is a progressive relapsing remitting inflammation of the gastrointestinal tract [1] and is increasing in incidence globally [2]. Biologic therapy has revolutionized patient management [3, 4]. Despite this and the use of other immunosuppressant drugs, there remains a significant cohort of patients with refractory or relapsing disease, or those that cannot tolerate available treatments. The systemic nature of current immunosuppressant therapies is associated with an appreciable risk of significant adverse events and carries a financial burden. Novel, safer, and more affordable treatment strategies are required for maintenance therapies.

Acute severe colitis requires hospital admission for intravenous steroid therapy and close monitoring, due to potential life threatening complications such as perforation and toxic dilatation [5-7]. Up to 15% of patients present with acute severe disease. Clinical and laboratory severity markers predict the need for emergent colectomy and 85% of patients with >8 stools/day or 3-8 stools/day and raised CRP >45mg/L on day 3 need urgent consideration of escalated medical therapy with rescue infliximab anti-TNF biologic therapy or intravenous ciclosporin, or surgery. If medical therapy fails by day 7 or life threatening complications occur, emergency colectomy with ileostomy is required as a lifesaving treatment. This clinical need for life saving and life changing surgery unfolds rapidly and can be associated with post-operative psychosocial morbidity, especially in adolescent and young adult patients [8-10].There is a need for novel rescue therapies for the treatment of acute severe colitis.

We previously demonstrated that oral delivery of *Lactococcus lactis (L. lactis)* expressing an IL-27 hyperkine (LL-IL-27), composed of both EBI3 and p28 subunits, a linker molecule and secretary peptide, is immunosuppressive in murine chronic enterocolitis induced by $CD4^{+}CD45Rb^{hi}$ T cell transfer [11]. In this T cell driven colitis model, representing adaptive immune responses, LL-IL-27 led to significant histological improvement and survival advantage, through induction of IL-10 derived from intra-epithelial mucosal T cells. We also described an immunosuppressive action on acute DSS colitis [11], although the

mechanism was not explored. We then hypothesized that IL-27 may exert an immunosuppressive role in innate immune responses.

IL-27, a heterodimeric cytokine composed of EBI3 and p28 subunits, is secreted from antigen presenting cells and signals via a heterodimeric receptor complex composed of the widely expressed Gp130, and the specific IL-27Rα [12-14]. IL-27 was initially considered pro-inflammatory through promotion of Th1 responses [15]. There is now appreciation that IL-27 has a wide functional repertoire, including profound anti-inflammatory effects through promotion of IL-10 secreting Tr1 regulatory T cells and inhibition of Th2 and Th17 responses [12-14]. Knowledge of IL-27 functionality has largely come from T cell biology, and its role in the adaptive immune response is well described [12-14]. However, the IL-27 receptor complex is also expressed on other immune cell types, including granulocytes [16] and macrophages [17], indicating that the functional capabilities of IL27 may be even wider and in particular IL27 may play a role in mediating innate immunity. Indeed, IL-27 can impact the function of innate cell subsets, including macrophages [18], neutrophils [16, 19], and dendritic cells [20-22]. It has been shown that IL-27 influences innate responses to bacterial challenge in the context of systemic sepsis in genetic manipulation models such as IL-27Rα knock-out murine strains. [23,24]. To date, there is no report on the immunosuppressive mechanism of IL27 in acute inflammation of the colon driven by innate cells in a genetically intact, non-infective environment.

Here, by demonstrating an immunosuppressive effect of IL-27 on the innate, rather than adaptive, immune response *in vivo* in a genetically intact, non-infective acute murine colitis model induced by intra-rectal instillation of 2,4,6-Trinitrobenzenesulfonic acid (TNBS), we offer novel mechanistic insights into the biology of IL-27 in vivo. Our data highlights the immunosuppressive role of IL-27 in innate immune responses offering translational potential as a novel rescue therapy in acute severe colitis.

97

Materials and Methods

Study approval - Animal experiments were conducted under approved protocols by the NCI Animal Care and Use Committee, in keeping with federal regulations governing care and use of animals in biomedical research. Frederick National Laboratory is accredited by AAALAC International and follows the Public Health Service Policy for the Care and Use of Laboratory Animals. Animal care was provided in accordance with the procedures outlined in the "Guide for Care and Use of Laboratory Animals" (National Research Council; 2011; National Academies Press; Washington, D.C.).

Animals – Experiments were performed on 6-10 week old male SJL/J, B6 $Rag^{-/-}$ (01BJ2 - B6.129S7-Rag1), and B6 IL-$10^{-/-}$ (01IL6 - IL-10 GFP/C) mice, maintained within the NCI-Frederick animal facility. Mice were fed normal chow, had free access to water and exposure to a 12 hourly light/dark cycle.

Acute colitis induction –100 µl TNBS (Sigma, ~1M in H2O, stored at -20°C, 92823) in 45% ethanol vehicle was administered intra-rectally to isoflurane anaesthetized mice with a 3cm flexible catheter. The administered dose was optimized within our facility and dependent on the sensitivity of the mouse strain; 2mg for SJL/J, 4mg for B6/$Rag^{-/-}$ and 6mg for B6/IL-$10^{-/-}$. The mice were held vertically for > 30secs post instillation to ensure retention. Mice were given supportive care (wet food/ice chips/heat pad) throughout the protocol. Prior to colitis induction, mice were fasted to solid food overnight and given access to 5% sucrose water.

Bacterial handling and administration – *Lactococcus lactis* (*L. lactis*) expressing IL-27 (LL-IL-27) and *L. lactis* empty vector control (LL-C) were prepared as described previously [11]. Briefly, *L. lactis* strain MG1363 was used for bacterial preparations. A bacterial research bank was prepared and IL-27 secretion confirmed by IL-27p28 ELISA (R&D systems, M2728) before storage in 50% glycerol at -80°C. For oral administration, stock bacteria were cultured 1:1000 in Difco M17 broth (BD Biosciences,218561) supplemented with 0.5% glucose and 5mg/ml erythromycin (Sigma, E5389) (GM17E) at 30°C for 16 hours. The bacteria were harvested by centrifugation and resuspended in buffered M9 salt media. Each

mouse was administered 100 µl of this bacterial suspension by oral gavage at 24 hour intervals within the treatment protocol, commencing on the day of colitis induction following recovery from anesthesia.

Clinical assessment of colitis activity – Disease activity index was assessed daily as previously reported [11]. This is a composite score (maximum 12) of three parameters: weight loss from baseline (≤1% 0, 1<≤5% 1, 5<≤10% 2, 10<≤20% 3, >20% 4), consistency of stool (normal pellets 0, soft-semi formed 2, diarrhea 4) and presence of fecal blood (none 0, occult blood positive 2, overt blood 4). Macroscopic colitis score was assessed by colon weight (mg) and length (cm) immediately after harvest. Histological colitis score (maximum 14) was reported by a veterinary pathologist (MRA) based on multiple parameters: severity of inflammatory cell infiltrate (none 0, mild 1, moderate 2, severe 3), goblet cell depletion (none 0, mild 1, moderate 2, severe 3), crypt hyperplasia (none 0, mild 1, moderate 2, severe 3), degree of ulceration of epithelium, (none 0, erosion 1, mild 2, moderate 3, severe 4 ulceration), presence (1) or absence (0) of granulomas.

Measurement of systemic inflammatory response – Blood was collected on day 2 post TNBS instillation in a serum separator tube, centrifuged and the supernatant stored at -80^{o}C for analysis. Serum C-reactive protein (CRP) was measured using ELISA kit (Life Diagnostics Inc, 2210-1) as per instructions.

Gene expression – RNA was extracted from cells or snap frozen distal colon tissue with the RNeasy mini kit (Qiagen, 74104) as per instructions. Quality and yield were assessed by Nanodrop spectrophotometry. cDNA was synthesized using the QuantiTect reverse transcription kit (Qiagen, 205310) with inclusive genomic DNA wipeout buffer. Further details of gene expression assays are available in the supplementary materials.

Total protein extraction and protein expression assays– Total protein was extracted by mechanical homogenization from distal colon tissue in 1X RIPA buffer (Cell signaling 10X, 9806) containing 1:1000 protease inhibitors at 4^{o}C. Following 20 min incubation on ice, tissue homogenates were centrifuged at

14,000rpm for 10 minutes and the supernatant stored at -80ºC for analysis. Total protein concentration was established using the Pierce BCA protein assay kit (23225) as compared to albumin standard. Further details of protein expression ELISA assays are available in the supplementary materials.

Colonic lamina propria cell isolation – Single cell suspension from freshly harvested distal colon tissue was extracted with a lamina propria dissociation kit (Miltenyi Biotec, 130-097-410) on the gentleMACS dissociator as per instructions, incorporating chemical and physical dissociation.

Flow cytometry & cell sorting - Nonspecific staining of cells was blocked by 20 minute incubation with anti-mouse CD16/CD32 Fc block (1:100 dilution) (BD Pharminogen, 553142). Macrophages and neutrophils were identified by cell surface expression of F4/80 and Ly6G using anti-mouse APC-F4/80 (eBiosciences, 17-4801-82, clone BM8) and anti-mouse Pacific Blue-Ly6G (Biolegend, 127611) on 4% paraformaldehyde fixed cells using the BD FACSCanto II flow cytometer. Single stain positive and unstained negative cell controls were included. $F4/80^{+}$ cells were collected using a FACSAria II (BD Biosciences) cell sorter. Data was analyzed using FlowJo software (Tree Star, Inc, Ashland, OR).

Assessment of mucosal inflammatory cell phenotype, proliferation index and phosphorylated STAT-1 response– Immunohistochemistry was performed on 10% NBF fixed, paraffin embedded tissue sections of distal colon. Further details of immunohistochemistry protocols are available in the supplementary materials and methods.

Macrophage culture protocols – Details of bone marrow derived and thioglycollate peritoneal macrophage culture protocols are available in the supplementary materials and methods.

In vitro cell stimulation assay – Cells were stimulated with *E. coli* 0111:B4 derived LPS (Sigma, L4130) from 10mg/ml stock, or 5mM Adenosine 5'-triphosphate disodium sate hydrate (ATP) from 90mM stock (Sigma, A22383-5G), stored at -20ºC. Recombinant mouse IL-27 (NSO expressed, R&D systems, 2799-010/CF) was added to stimulation assay media as appropriate at 100ng/ml. Culture supernatant was collected and stored at -80ºC for analysis.

Statistics - SigmaPlot 11.0 software was used for statistical analysis. Survival curves were analyzed according to the Kaplan-Meier estimator on GraphPad 6. Statistical differences were determined by ANOVA and non-parametric Rank Sum tests, with p values of ≤ 0.05 considered to represent a statistically significant difference between groups. If groups displayed normal variance, Student's t-test was performed (as indicated in the figure legend text). Data is presented as mean +/- SEM.

Results

IL-27 attenuates acute severe colitis in vivo

Intra-rectal instillation of TNBS in SJL/J mice resulted in an acute severe left-sided colitis, with a rapid increase in disease activity index (DAI) within 24 hours. Mice treated with TNBS were included if their DAI was greater than 4 on day 1, reflecting appropriate response to TNBS. Mucosal delivery of LL-IL-27 attenuated TNBS colitis with a decreased DAI and significantly less weight loss from baseline starting weight, compared to *L.lactis* control (LL-C) (Figure 1a & 1b). LL-IL-27 was associated with a significantly lower colon weight and increased colon length (Figure 1c), resulting in a reduced colon weight:length ratio ($p=0.005$).

Oral administration of LL-IL-27 is presumed to act locally since there was no evidence of systemic absorption, as determined by IL-27 p28 ELISA (Figure 1d). Although mucosally-delivered IL-27 was not measurable systemically, we observed a decrease in serum C-reactive protein (CRP) in LL-IL-27 treated mice (Figure 1e). This can be attributed to indirect effects on liver production of CRP, possibly downstream of inflammatory cytokines such as IL-6, as will be discussed.

LL-IL-27 resulted in a significant reduction in total histology colitis score compared to LL-C (Figure 2a & d), although this was surprisingly modest compared to clinical improvement. Assessment of the independent histological parameters of the composite score revealed significant protection against mucosal ulceration in those receiving LL-IL-27 (Figure 2b). We hypothesized that IL-27 may protect the epithelial barrier, shielding the innate immune system from contact with intestinal microbiota. IL-27 was previously shown to increase colonic epithelial cell proliferation, and to promote epithelial wound restitution in vitro [25]. To explore this in vivo, we assessed epithelial cell proliferation by Ki67 immunohistochemistry. Eliciting colitis increased epithelial proliferation by 50% but there was no effect on proliferation index associated with LL-IL-27 (Figure 2c).

Our *in vivo* data shows that oral IL-27 attenuates acute severe colitis when administered shortly after the time of colonic insult. An important question is whether pre-exposure to IL-27 could impact disease induction as a prophylactic effect. Mice were pre-treated with LL-IL-27 for 2 weeks prior to intra-rectal administration of TNBS. This did not affect acute colitis induction (Figure 3), suggesting that an active inflammatory microenvironment is required for IL-27 to suppress inflammation in the colonic mucosa.

IL-27 reduces colonic neutrophil infiltrate associated with decreased CXC chemokine expression

Previous studies have shown that lack of IL-27 signaling increased neutrophil influx to the site of acute bacterial infection in the peritoneal cavity [26] and lung [24]. This raised the question of whether mucosal IL-27 might suppress neutrophil infiltration. To explore this as a potential mechanism, neutrophil and macrophage infiltration was assessed by myeloperoxidase (MPO) and F4/80 immunohistochemistry, respectively. Induction of acute TNBS colitis led to a precipitous increase in mucosal neutrophils ($p=0.001$) and a modest increase in macrophages ($p<0.05$). LL-IL-27 led to a significant reduction in MPO^+ neutrophil infiltration into the colonic mucosa (Figure 4a & Supplementary Figure 1a). $F4/80^+$ macrophage infiltrate did not alter with LL-IL-27 treatment (Figure 4b & Supplementary Figure 1b).

Neutrophils move to areas of acute inflammation via chemokine gradients, therefore expression of several candidate chemokines in the distal colon was assessed. On day 2 post-TNBS instillation, CXCL1 and CXCL2 but not CXCL5 protein was significantly reduced in colitic mice treated with LL-IL-27 compared to LL-C (Figure 4c). CXCL2, a potent neutrophil chemokine, was taken forward in further investigation and at the end of the treatment protocol (day 4 or time of death), the significant suppression persisted (Figure 4d).

Oral IL-27 reduces CXCL2 secretion in ex-vivo colon derived F4/80$^+$macrophage

Macrophages are major producers of chemokines that induce a neutrophil influx [27]. The cellular source of CXCL2 in our acute TNBS colitis model was assessed by selecting F4/80$^+$ cells from distal colon lamina propria cell suspension, on day 2 post-TNBS instillation, cultured for 24 hours to assess CXCL2 response. These colitis derived F4/80$^+$ macrophages secreted CXCL2, and this was significantly reduced in cells exposed to LL-IL-27 in vivo (Figure 4e).

IL-27 does not directly suppress macrophage CXCL2 expression ex vivo

Having shown that IL-27 suppressed chemokine expression by colonic macrophages in vivo, we next evaluated whether this was a direct effect by examining IL-27 responses of different macrophage preparations.

Bone marrow derived macrophage (BMDM) expressed IL-27Rα (Figure 5a) demonstrating their capability to respond to IL-27. LPS stimulation provoked robust CXCL2 secretion. However, addition of recombinant IL-27 (rIL-27) for the 5 hour duration of LPS stimulation did not suppress this response (Figure 5b). To investigate whether the macrophage chemokine response was suppressed by priming with IL-27 prior to LPS challenge, BMDM were cultured in media containing rIL-27 for 48 hours prior to LPS stimulation. Again, exposure to LPS led to CXCL2 secretion and this was not reduced by rIL-27 pre-exposure (Figure 5c).

To evaluate a different macrophage population, thioglycollate-induced peritoneal cells were examined, perhaps better representing the acute inflammatory macrophages in this *in vivo* colitis model. At day 4 post-thioglycollate injection, the majority of cells were F4/80$^+$ macrophages, representing F4/80^{intermediate} inflammatory and F4/80^{high} resident macrophages, co-existing with Ly6G$^+$ neutrophils (Figure 5d). Adherent cells were used as a macrophage enriched population, and expressed IL-27Rα. As

246 in BMDM, LPS induced robust CXCL2 expression and this was not altered by presence of rIL-27 (Figure
247 5e).

248 Overall, LPS induced CXCL2 protein expression in macrophages was not inhibited by rIL-27 in
249 two independent *in vitro* cell culture models, suggesting this is not the direct mechanism for CXCL2
250 suppression in vivo.

251 However, it remained possible that macrophage responses would be site specific to the colon. To
252 explore this, $F4/80^{+}$ cells isolated from murine colon lamina propria were LPS stimulated for 24 hours,
253 with or without rIL-27. As seen in the other macrophage culture models, LPS induced a striking increase
254 in CXCL2 compared to unstimulated cells (Figure 5f). Once again, there was no significant IL-27
255 suppression of CXCL2 production in response to LPS. This supports the hypothesis that mucosal delivery
256 of IL-27 does not directly interfere with the ability of colonic lamina propria macrophages to secrete
257 CXCL2 after an inflammatory stimulus.

258

259 **IL-27 suppression of acute severe colitis is T cell-independent**

260 Since IL-27 inhibits several T cell subsets, we assessed whether T cell mediated
261 immunosuppression could be central to IL-27 effect in acute colitis.

262 TNBS colitis was evoked in Rag1-deficient ($Rag1^{-/-}$) mice that lack mature T and B cells, and
263 responses to LL-C or LL-IL-27 assessed. There was reduced disease severity evoked in the C57BL/6
264 strain of mice due to relative resistance to TNBS (compared to SJL/J strain) [28]. LL-IL-27 led to a
265 significantly improved DAI (Figure 6a). LL-IL-27 treated mice lost significantly less weight initially,
266 though the difference between the groups narrowed during the protocol with a strong trend for
267 improvement in the LL-IL-27 treated group (Figure 6b). CXCL2 protein in distal colon homogenates was
268 significantly inhibited by LL-IL-27 (Figure 6c), and there was a significant reduction in histological

colitis score (Figure 6d). Taken together, these data show that IL-27 mediated inhibition of acute colitis persists in Rag1-deficient mice, implying the mechanism is T cell-independent.

IL-27 immunosuppression in acute severe colitis is IL-10-dependent

It is known that IL-27 can induce the immunosuppressive cytokine, IL-10 [29, 30] from Tr1 T regulatory cells [31], and other cell types, such as macrophages [17]. Our previous data revealed that LL-IL-27 evoked a significant colonic IL-10 response, from $CD4^{+}CD8\alpha$ intra-epithelial T cells, and overall clinical improvement mediated by IL-27 was dependent on production of this cytokine in a chronic enterocolitis model [11]. To evaluate whether IL-10 played a major role in the immunosuppressive function of IL-27 in the context of acute innate cell driven colitis, we employed an IL-10 deficient (C57BL/6 IL-$10^{-/-}$) mouse model in our experimental colitis protocol. There was no significant difference in DAI (Figure 6e) or weight loss (Figure 6f) between mice treated with LL-C versus LL-IL-27. There was no reduction in CXCL2 (Figure 6g) nor total histology score (Figure 6h) associated with LL-IL-27. This suggests IL-27 mediated immunosuppression in acute colitis is IL-10-dependent and CXCL2 reduction is downstream of this IL-10 dependency.

Next, IL-10 protein expression was assessed in distal colon homogenate on either day 2 (Figure 6i) or day 4/time of death post-TNBS instillation (Figure 6j). On day 2, distal colonic IL-10 was increased by 25% in mice exposed to IL-27. Later in the treatment protocol there was no significant difference in mucosal IL-10 detected between colitic mice who received LL-C or LL-IL-27. This suggests that early in disease pathogenesis, *L.lactis* delivered IL-27 hastens the immunoregulatory IL-10 response, increasing IL-10 in the colonic mucosal microenvironment, impacting clinical disease course and outcome.

IL-27 induces phosphorylated STAT-1 in mucosal epithelial cells

To identify the cell type directly impacted by IL-27 treatment, immunohistochemistry was employed to identify STAT-1 phosphorylation in response to orally administered LL-IL-27. Multifocal mucosal epithelial cells and scattered inflammatory cells within the lamina propria and submucosa showed strong positive nuclear labeling for phosphorylated STAT 1 (Figure 7a). This suggests efficacy of LL-IL-27 is a consequence of both immune and non-immune cell mediated effects. In keeping with this, IL-27 was previously shown to promote epithelial wound restitution in vitro [25]. As expected, gavage of LL-IL-27 in healthy non colitic mice did not result in STAT-1 phosphorylation response, again demonstrating that an inflammatory microenvironment is required for LL-IL-27 immunosuppression.

IL-27 significantly reduces pro-inflammatory cytokine profile in vivo

Gene expression of pro-inflammatory cytokines were assessed in distal colon homogenate, namely *Il6, Il1β, Tnf, Il13, Il12, Ifng, Il17a,* and *Il22*, along with the regulatory T cell associated transcription factor *Foxp3.* The majority of these genes were expressed at low abundance or undetectable and not investigated further. *Il6, Il1β,* and *Tnf* were significantly increased in all TNBS treated mice compared to ethanol controls (Figure 7b). *Il6* expression was significantly lower in mice treated with LL-IL-27 compared to LL-C. No differential expression of *Il1β* or *Tnf* was seen with LL-IL-27 treatment.

In contrast, distal colon protein expression of IL6, IL1β and TNF were significantly reduced in colitic mice receiving LL-IL-27 treatment (Figure 7c), highlighting a discrepancy between gene and protein expression for the latter two cytokines. This suggests potential influences on translational or post-translational regulation. Thus we hypothesized that IL-27 may inhibit the inflammasome as a mechanism of immunosuppression. However, there was no impact on expression of distal colon *il-18* or inflammasome components (*Nlrp3, Caspase-1* and *Asc*), nor LPS induced IL1β secretion from *in vitro* macrophage preparations (Supplementary Figure 2), suggesting IL-27 does not directly influence acute colonic inflammasome activation *in vivo*.

316

Discussion

Our data show a novel immunosuppressive activity of the cytokine IL-27 in acute severe colitis, directed toward gut mucosal innate immune responses. Mucosal delivery of IL-27 reduced clinical parameters of colonic disease activity, repressed systemic inflammatory response, and improved colitis histology score. LL-IL-27 improved mucosal ulceration. This is important because mucosal healing is defined by the absence of ulcerated lesions in the gut mucosa during endoscopy, and this represents a primary endpoint in assessing effectiveness of IBD therapy.

The mechanism of IL-27 directed immunosuppression was multifactorial. This data represents first in field evidence of IL-27 evoked reduction in colonic mucosal neutrophil infiltrate, associated with a decreased CXC chemokine gradient. This was T cell independent and IL-10 dependent, with early enhanced mucosal IL-10. IL-27 also significantly attenuated pro-inflammatory cytokine protein expression. Although there was no evidence of direct impact on epithelial cell proliferation, IL-27 provoked an epithelial phosphorylated STAT-1 response. For the first time, we have revealed that IL-27 attenuates acute immune responses in the colon in vivo in a non-infective, genetically intact host.

IL-27 evoked reduction in myeloid cell infiltrate has been reported in different body sites, identified through genetic manipulation of IL-27 receptor signaling. Atherosclerotic prone *Ldlr*$^{-/-}$ mice had attenuated disease with hematopoietic cell IL-27Rα deficiency, mediated partly via reduced plaque chemokine gradient and infiltration of myeloid cells [32]. *Ebi3*$^{-/-}$ mice demonstrated reduced granulocyte flux to acute peritonitis [26]. When IL-27 receptor signaling was interrupted by genetic deficiency or antibody blocking, mice with systemic sepsis secondary to cecal puncture ligation and exposed to a secondary pneumonic bacterial insult, exhibited enhanced neutrophil infiltrate to the lungs This was associated with increased chemokine expression including CXCL2 and pro-inflammatory mediators such as IL-6, TNF, and IL1β [24]. This IL-27 mediated blunting of granulocyte response was deleterious overall and associated with inability to resolve the secondary bacterial infection.

Neutrophils are necessary for efficient resolution of acute microbial attack through a variety of mechanisms [33]. The suppression of neutrophil influx by IL-27 complicates IL-27 therapy for application to human disease. However, LL-IL-27 represents a localized rather than systemic therapy, delivered orally to act directly on the inflamed colonic mucosa, with no evidence of systemic IL-27 absorption as reported here and in our previous publication [11]. The balance of beneficial to deleterious IL-27 inhibition of innate immunity appears site specific. Thus, IL-27 is beneficial in the circumstance of acute non-infective colitis, by limiting granulocyte directed tissue damage, release of pro-inflammatory mediators and activation of downstream adaptive immune responses.

CXCL2 is a potent neutrophil chemokine, secreted mainly by monocytes and macrophages, in response to LPS stimulation [27]. There is existing evidence that IL-27 can modulate the functional capacity of macrophages and other innate cells. It is unclear whether IL-27 effects on innate cells are pro-inflammatory or anti-inflammatory and it appears that the outcome is dependent on stimulus, cell type and surrounding or experimental microenvironment [14], akin to its pleiotropic effect on T cell biology. For example, IL-27 can enhance pro-inflammatory cytokine and chemokine expression from blood derived human monocytes cultured and stimulated in vitro [34,35]. In the gut, host or recipient IL-27R knockout mice, with or without genetic silencing of TCR revealed a role of IL-27-induced, APC-derived, cytokine mediated Th17 responses in T cell driven colitis [36]. Conversely, lung macrophages from IL-27Rα knockout mice exert enhanced pro-inflammatory cytokine and chemokine secretion, along with increased anti-microbial killing capacity [24] exemplifying a suppressive activity of IL-27 on innate immune cells. Iyer [17] revealed macrophage IL-10 secretion in response to LPS or type I interferons is dependent on endogenous IL-27 signaling. Therefore, it was reasonable to speculate that our orally delivered IL-27 may suppress the proinflammatory function of the colonic macrophage population. Indeed, our data suggest that colonic macrophages are a potent source of CXCL2 during acute colitis and this was blunted by in vivo exposure to IL-27. We did not find evidence to support a direct immunosuppressive effect of IL-27 on macrophage chemokine response, despite utilizing three independent ex vivo macrophage culture models including colonic macrophages.

We then assessed the involvement of T cells in the IL-27-mediated immunosuppression in this acute colitis model. IL-27 inhibits Th2 and Th17 responses [13,14], although this was not likely to be prominent in our acute model given the rapid onset of pathology. T cells present in the normal colonic mucosa are part of the 'physiological immunity' that evokes tolerance to luminal antigens and appropriate response to pathogenic insult. IL-27 is known to promote T regulatory cell subsets [31, 37]. However in our model, IL-27 immunosuppression of innate cell driven colonic pathology was T cell independent.

We acknowledge that the direct cell target of orally delivered IL-27 in acute colitis remains elusive. We predict that IL-27 in acute severe colitis acts on epithelial cells and inflammatory cells of unknown phenotype. There are several immune cell types that may, independently or in conjunction, act in response to exogenous IL-27, such as neutrophils, dendritic cells or innate lymphoid cells and although outside the scope of this current study, warrants further investigation to reveal novel biological functions of IL-27. Neutrophils can secrete chemokines and attract an increasing neutrophil influx [38]. IL-27 can inhibit neutrophil ROS production [16, 19], reduce their expression of the surface integrin Mac-1 [16, 19] and provoke short lived IL1β secretion either independently [16] or in response to bacterial challenge [19, 24]. IL-27 receptor deficiency led to enhanced neutrophil intracellular bacterial killing [24]. Neither neutrophil survival nor apoptosis is known to be affected by IL-27 [16]. Another potential cell candidate is dendritic cells (DCs). Human immature DCs exposed to IL-27 displayed reduced antigen presenting capacity [22]. IL-27 provokes DC derived IL-10, and suppresses downstream Th1 and Th17 response [20, 21]. This mechanism impacts disease outcome in CNS autoimmune pathology, with vaccination of IL-27 primed DCs attenuating murine EAE [21]. Another potential cell candidate is colonic epithelial cells, as this cell compartment can secrete chemokines [39, 40] and our results indicate that these cells strongly express phosphorylated STAT1 in response to mucosally delivered LL-IL-27 in vivo.

This data confirms the potential for oral *L. lactis* IL-27 to be a potential rescue medical therapy in acute severe colitis. *Lactococcus lactis* is a non-pathogenic, non-colonizing and non-transmissible food grade bacterium. The use of this bacteria as a mucosal delivery system for therapeutic proteins and

392 vaccines has been reviewed elsewhere [41-43]. We showed previously that orally delivered IL-27 attenuates
393 chronic T cell derived colonic inflammation [11] and now present novel evidence to show efficacy towards
394 acute innate cell derived colonic pathology, highlighting translational impact as a new or adjunct medical
395 treatment strategy in acute severe colitis.

396

Acknowledgements

We thank Kelli Czarra (Laboratory Animal Sciences Program, Leidos Biomedical Research, Inc., Frederick National Laboratory for Cancer Research, Frederick, Maryland, USA) for her assistance with animal studies, and Kathleen Noer and Roberta Matthai of the Flow cytometry core facility, NCI for assistance with cell sorting.

Author contributions

MHM designed and executed experiments, analyzed data, and wrote the manuscript. LS provided *L. lactis* strains. WQL designed the IL-27 construct and assisted with experiments. MRA provided pathology reporting. All other authors assisted with design, execution and analysis of experiments. Specific input with macrophage in vitro culture and flow cytometry (WAB & LCD), preventative assessment (CS), ex vivo colonic cell isolation, pathology, and digital photography (CA) is noted. SKD designed experiments, wrote the manuscript and provided overall direction. All authors edited and agreed on the final manuscript.

Abbreviations; IL-27, interleukin 27; LL-IL-27, *Lactococcus lactis* expressing IL-27; LL-C, *Lactococcus lactis* empty vector control; DAI, Disease activity index;

417 References

418 1 Kaser A, Zeissig S, Blumberg RS. Inflammatory bowel disease. Annu. Rev. Immunol. 2010;28:573–
419 621.

420 2 Molodecky NA, Soon IS, Rabi DM, et al. Increasing incidence and prevalence of the inflammatory
421 bowel diseases with time, based on systematic review. Gastroenterology. 2012;142(1):46-54.

422 3 Ford AC, Sandborn WJ, Khan KJ, et al. Efficacy of biological therapies in inflammatory bowel disease:
423 systematic review and meta-analysis. Am J Gastroenterol. 2011;106(4):644-659.

424 4 Danese S, Colombel JF, Peyrin-Biroulet L, et al. Review article: the role of anti-TNF in the
425 management of ulcerative colitis -- past, present and future. Aliment Pharmacol Ther. 2013;37(9):855-
426 866.

427 5 Fornaro R , Caratto M , Barbruni G , et al. Surgical and medical treatment in patients with acute severe
428 ulcerative colitis. J Dig Dis. 2015 Oct;16(10):558-67

429 6 Macken L , Blaker PA. Management of acute severe ulcerative colitis (NICE CG 166). Clin Med
430 (Lond). 2015 Oct;15(5):473-6. doi: 10.7861/clinmedicine.15-5-473.

431 7 Øresland T, Bemelman WA, Sampietro GM, et al; European Crohn's and Colitis Organisation (ECCO).
432 European evidence based consensus on surgery for ulcerative colitis. J Crohns Colitis. 2015 Jan;9(1):4-
433 25. doi: 10.1016/j.crohns.2014.08.012.

434 8 Carlsson E , Bosaeus I, Nordgren S. What concerns subjects with inflammatory bowel disease and an
435 ileostomy? Scand J Gastroenterol. 2003 Sep;38(9):978-84.

436 9 Knowles SR , Cook SI, Tribbick D. Relationship between health status, illness perceptions, coping
437 strategies and psychological morbidity: a preliminary study with IBD stoma patients. J Crohns Colitis.
438 2013 Nov;7(10):e471-8. doi: 10.1016/j.crohns.2013.02.022

10 Spinelli A, Carvello M, D'Hoore A, Pagnini F. Psychological perspectives of inflammatory bowel disease patients undergoing surgery: rightful concerns and preconceptions. Curr Drug Targets. 2014;15(11):1074-8

11 Hanson ML, Hixon JA, Li W, et al. Oral delivery of IL-27 recombinant bacteria attenuates immune colitis in mice. Gastroenterology. 2014;146(1): 210-221.

12 Stumhofer, JS, Hunter, CA. Advances in understanding the anti-inflammatory properties of IL-27. Immunol Lett. 2008;117(2):123-130.

13 Wojno, ED, Hunter CA. New directions in the basic and translational biology of interleukin-27. Trends Immunol. 2012;33:91–97.

14 Wynick C, Petes C, Gee K. Interleukin-27 mediates inflammation during chronic disease. J Interferon Cytokine Res. 2014;34(10):741-749.

15 Pflanz S, Timans JC, Cheung J, et al. IL-27, a heterodimeric cytokine composed of EBI3 and p28 protein, induces proliferation of naive $CD4^+$ T cells. Immunity. 2002;16:779–790.

16 Li JP, Wu H, Xing W, et al. Interleukin-27 as a negative regulator of human neutrophil function. Scand J Immunol. 2010;72(4):284-292.

17 Iyer SS, Ghaffari AA, Cheng G. Lipopolysaccharide-mediated IL-10 transcriptional regulation requires sequential induction of type I IFNs and IL-27 in macrophages. J Immunol. 2010;185(11):6599-6607.

18 Shimizu M, Ogura K, Mizoguchi I, et al. IL-27 promotes nitric oxide production induced by LPS through STAT1, NF-κB and MAPKs. Immunobiology. 2013;218(4):628-634.

19 Rinchai D, Khaenam P, Kewcharoenwong C, et al. Production of interleukin-27 by human neutrophils regulates their function during bacterial infection. Eur J Immunol. 2012;42(12):3280-3290.

461 20 Wang S, Miyazaki Y, Shinozaki Y, et al. Augmentation of antigen-presenting and Th1-promoting
462 functions of dendritic cells by WSX-1(IL-27R) deficiency. J. Immunol. 2007;179:6421–6428.

463 21 Mascanfroni ID, Yeste A, Vieira SM, et al. IL-27 acts on DCs to suppress the T cell response and
464 autoimmunity by inducing expression of the immunoregulatory molecule CD39. Nat Immunol.
465 2013;14(10):1054-1063.

466 22 Morandi F, Di Carlo E, Ferrone S, et al. IL-27 in human secondary lymphoid organs attracts myeloid
467 dendritic cells and impairs HLA class I-restricted antigen presentation. J Immunol. 2014;192(6):2634-
468 2642.

469 23 Xu F, Liu Q, Lin S, et al. IL-27 is elevated in acute lung injury and mediates inflammation. J Clin
470 Immunol. 2013;33(7):1257-1268.

471 24 Cao J, Xu F, Lin S, et al. IL-27 controls sepsis-induced impairment of lung antibacterial host defence.
472 Thorax. 2014;69(10):926-937.

473 25 Diegelmann J, Olszak T, Göke B, et al. A novel role for interleukin-27 (IL-27) as mediator of
474 intestinal epithelial barrier protection mediated via differential signal transducer and activator of
475 transcription (STAT) protein signaling and induction of antibacterial and anti-inflammatory proteins. J
476 Biol Chem. 2012;287(1):286-298.

477 26 Wirtz S, Tubbe I, Galle PR, et al. Protection from lethal septic peritonitis by neutralizing the biological
478 function of interleukin 27. J Exp Med. 2006;203(8):1875-1881.

479 27 De Filippo K, Dudeck A, Hasenberg M, et al. Mast cell and macrophage chemokines CXCL1/CXCL2
480 control the early stage of neutrophil recruitment during tissue inflammation. Blood. 2013;121(24):4930-
481 4937.

28 Scheiffele F, Fuss IJ. Induction of TNBS colitis in mice. Curr Protoc Immunol. 2002;Chapter 15:Unit 15.19.

29 Awasthi A, Carrier Y, Peron JP, et al. A dominant function for interleukin 27 in generating interleukin 10-producing anti-inflammatory T cells. Nat Immunol. 2007;8:1380–1389.

30 Hunter CA, Kastelein R. Interleukin-27: balancing protective and pathological immunity. Immunity. 2012;37:960–969.

31 Apetoh L, Quintana FJ, Pot C, et al. The aryl hydrocarbon receptor interacts with c-Maf to promote the differentiation of type 1 regulatory T cells induced by IL-27. Nat. Immunol. 2010;11:854–861.

32 Koltsova EK, Kim G, Lloyd KM, et al. Interleukin-27 receptor limits atherosclerosis in Ldlr-/- mice. Circ Res. 2012;111(10):1274-1285.

33 Nauseef WM, Borregaard N. Neutrophils at work. Nat Immunol. 2014;15(7):602-11.

34 Guzzo C, Che Mat NF, Gee K. Interleukin-27 induces a STAT1/3 and NF-kappaB dependent proinflammatory cytokine profile in human monocytes. J Biol Chem. 2010;285(32):24404–24411.

35 Guzzo C, Ayer A, Basta S, et al. IL-27 enhances LPS-induced proinflammatory cytokine production via upregulation of TLR4 expression and signaling in human monocytes. J Immunol. 2012;188(2):864–873.

36 Visperas A, Do JS, Bulek K, et al. IL-27, targeting antigen-presenting cells, promotes Th17 differentiation and colitis in mice. Mucosal Immunol. 2014;7(3):625-633.

37 Pot C, Apetoh L, Awasti A, et al. Induction of regulatory Tr1 cells and inhibition of TH17 cells by IL-27. Semin Immunol. 2011;23:438–445.

38 Kolaczkowska E, Kubes, P. Neutrophil recruitment and function in health and inflammation. Nat Rev Immunol. 2013;13(3):159-175.

504 39 Ohtsuka Y, Lee J, Stamm DS, et al. MIP-2 secreted by epithelial cells increases neutrophil and
505 lymphocyte recruitment in the mouse intestine. Gut. 2001;49(4):526-533.

506 40 Ohtsuka Y, Sanderson IR. Dextran sulfate sodium-induced inflammation is enhanced by intestinal
507 epithelial cell chemokine expression in mice. Pediatr Res. 2003;53(1):143-147.

508 41 Bermúdez-Humarán LG, Kharrat P, Chatel JM, et al. Lactococci and lactobacilli as mucosal delivery
509 vectors for therapeutic proteins and DNA vaccines. Microb Cell Fact. 2011;10 Suppl 1:S4.

510 42 Steidler L, Hans W, Schotte L, et al. Treatment of murine colitis by Lactococcus lactis secreting
511 interleukin-10. Science. 2000;289(5483):1352-1355.

512 43 Steidler L, Rottiers P, Coulie B. Actobiotics as a novel method for cytokine delivery. Ann NY Acad
513 Sci. 2009;1182:135-45.

514

515

Figure Legends

Figure 1. Oral delivery of IL-27 suppresses acute severe colitis. (**a**) LL-IL-27 evokes a decreased disease activity index (DAI) compared to LL-C; 5.8 vs. 8.2 on day 1, 5.3 vs. 8.9 on day 2 and 4.1 vs. 8.2, on day 3, maximum 12, *p=0.001. (**b**) LL-IL-27 led to significantly less weight loss than TNBS+LL-C or TNBS alone, $p<0.01$ and $p<0.05$ on days 2 and 3, respectively. Data in (**a**) and (**b**) combined from 3 separate experiments. n= 14, 21, 25, 25 for ethanol, TNBS, TNBS+LL-C and TNBS+LL-IL-27 groups, respectively. (**c**) Macroscopic colitis score represented by colon weight (mg) and length (cm). Colitis renders the colon shorter and heavier. Both parameters improved with LL-IL-27 vs. LL-C; colon length, p=0.001 and colon weight, p=0.017. Data combined from 2 separate experiments. n= 7, 16, 18, 19 for ethanol, TNBS, TNBS+LL-C and TNBS+LL-IL-27, respectively. (**d**) SJL/J colitic mice (2mg TNBS) receiving daily gavage of LL-IL27 for 4 days (n=10) showed serum IL-27 of 2.9pg/ml, indistinguishable from non-colitic untreated mice (n=8, 4.1 pg/ml), p=0.469 (t-test). (**e**) IL-27 provoked an improved systemic inflammatory response measured by serum CRP (day 2); LL-C vs. LL-IL-27, p=0.019 (t-test). Data from one experiment. n=5, 3, 10, 9, 10 for untreated, ethanol, TNBS, TNBS+LL-C and TNBS+LL-IL-27, respectively. All data (a-e) presented as mean+SEM.

Figure 2. Oral delivery of IL-27 leads to improved histological colitis score and protection against ulceration in acute severe colitis. (**a**) Cumulative histological colitis score based on 5 parameters (degree of inflammatory cell infiltrate/ mucosal ulceration/ crypt hyperplasia/ goblet cell depletion, presence of granuloma) with a maximum of 14. TNBS+LL-C vs. TNBS+ LL-IL-27, p=0.035. (**b**) Degree of distal colonic mucosa ulceration score, graded on a scale of 0-4. 0=none, 1=erosion, 2=mild ulceration, 3=moderate ulceration, 4=severe ulceration. TNBS+LL-C vs. TNBS+ LL-IL-27, p=0.008. Data generated from randomly selected animals from 2 experiments. n=5,5,6,7 for ethanol, TNBS, TNBS+LL-C and TNBS+LL-IL27, respectively. Mean + SEM. (**c**) Epithelial proliferation index was assessed by Ki67 immunohistochemistry on paraffin embedded distal colon sections. n=5/group from 2 experiments. Index derived from number of Ki67 positive cells/total number epithelial cells/ HPF, expressed as percentage.

Mean + SEM % from 5 HPF per sample used in analysis. Colitis evoked a significant increase in proliferation index across all groups compared to ethanol control; p=0.009, 0.001 & 0.002 for TNBS alone, +LL-C and +LL-IL-27, respectively. There was no significant difference between TNBS alone vs. +LL-C vs. +LL-IL-27 (p=0.243), t-test. (**d**) Representative distal colon photomicrographs of each experimental group. There is full-thickness necrosis of the mucosa and marked submucosal inflammation and edema in both the TNBS alone and TNBS+LL-C treated mice. LL-IL-27 treatment significantly reduced mucosal necrosis and decreased inflammatory infiltrates. Slides were optically scanned with an Aperio AT2 digital slide scanner. Hematoxylin and eosin staining.

Figure 3. An active inflammatory microenvironment is required for oral IL-27 to suppress inflammation in the colonic mucosa. Pre-exposure to LL-IL-27 (10 oral gavages over 2 week period) prior to intra-rectal instillation of 2mg TNBS (SJL/J 6-8 week old male mice) did not impact acute colitis induction as measured by (**a**) disease activity index, (**b**) weight loss from starting weight, or (**c**) macroscopic colitis score (colon length and weight). Data presented combined from 2 separate experiments, n=10/group, as mean + SEM; ANOVA on Ranks & Rank Sum test for pairwise comparisons.

Figure 4. Oral IL-27 reduces colonic neutrophil infiltrate associated with decreased CXC chemokine expression. Distal colon inflammatory cell phenotype assessed by immunohistochemistry using (**a**) myeloperoxidase (MPO) and (**b**) F4/80 positivity to identify neutrophils and macrophages, respectively. Data generated from randomly selected animals from 2 experiments. n=5,5,6,6,8 for untreated, ethanol, TNBS, TNBS+LL-C and TNBS+LL-IL-27, respectively. Mean + SEM. (**a**) Treatment with IL-27 led to a significant reduction in MPO^+ neutrophil infiltration into the colonic mucosa (p=0.002, t-test). (**b**) $F4/80^+$ macrophage infiltrate did not alter with LL-IL-27 treatment (p=0.250, t-test). (**c**) On day 2 post TNBS instillation, CXCL1 and CXCL2 but not CXCL5 protein (measured by ELISA

and normalized to mg total protein concentration) were significantly reduced in distal colon homogenate in colitic mice treated with LL-IL-27 compared to LL-C (p=0.026, p=0.038 & p=0.427 (t-test), respectively). Data represents mean + SEM, generated from 2 separate experiments, n=10 /group. (**d**) This IL-27 associated reduction in CXCL2 expression persisted as measured later in the experimental protocol (day 4 or time of death), p=0.001 and p=0.003, compared to TNBS plus LL-C and TNBS alone, respectively. Mean + SEM, generated from one experiment, n=3, 3, 8, 8, 11 for untreated, ethanol, TNBS, TNBS+LL-C and TNBS+LL-IL27, respectively. (**e**) Ex-vivo derived colitis associated lamina propria F4/80^{+} macrophage cells secreted CXCL2 measured by ELISA after 24 hours culture at 37ºC (10^5 cells/200µl), and this was significantly reduced in cells exposed in vivo to LL-IL-27, p=0.001 (t-test). Data generated from 5 pooled distal colon samples per group.

Figure 5. IL-27 does not directly suppress macrophage CXCL2 expression. To investigate the mechanism of IL-27 mediated immunosuppression, chemokine response in bone marrow derived macrophage (BMDM) (**a-c**), thioglycollate induced peritoneal macrophage (TPM) (**d** & **e**) and colonic lamina propria F4/80^{+} macrophage (**f**) were assessed. (**a**) >90% of M-CSF cultured bone marrow derived cells expressed F4/80 and IL-27Rα. (**b**) BMDM were stimulated for 5 hours with various concentrations of LPS as labelled +/- rIL27 (100ng/ml) and CXCL2 measured in supernatant by ELISA. Combined data from several experiments, generated from >3 wells/experimental condition. LPS resulted in a robust CXCL2 response but there was no differential response in association with IL-27, t-test. (**c**) Exposure for >48 hours prior to LPS stimulation did not impact CXCL2 response, t-test. (**d**) Peritoneal cell phenotype day 4 post IP thioglycollate injection. Cells expressed IL-27Rα. (**e**) Adherent cells were stimulated with 50ng/ml of LPS +/- 100ng/ml rIL27, for 5 or 72 hours. Again, LPS resulted in a robust CXCL2 response but there was no differential response in association with IL-27. (**f**) This was also seen in ex vivo colonic lamina propria F4/80+ cells stimulated for 24 hours with 50ng/ml of LPS +/- 100ng/ml rIL27, t-test.

589 Results in C, E & F presented from one experiment, with the same pattern of expression seen in repeat
590 experiment. All data presented as mean + SEM.

591

592 **Figure 6. IL-27 immunosuppression in acute TNBS colitis is T cell-independent and IL-10-**
593 **dependent, evoking early IL-10 secretion in the colonic mucosa. (a-d)** 4mg TNBS instilled intra-
594 rectally into $Rag^{-/-}$ mice. Data combined from 2 separate experiments. n=10/group. There was a
595 significant difference in (**a**) DAI in mice treated with LL-IL-27 compared to LL-C on day 1 and 2,
596 p=0.013 & p=0.040 respectively, t-test, and (**b**) degree of weight loss from baseline on day 1 (p=0.019),
597 then a trend just under significance on days 2&3 (p=0.06), t-test. (**c**) Distal colon homogenate CXCL2
598 protein was significantly reduced with LL-IL-27, p=0.028, (**d**) as was distal colon histology score,
599 p=0.028, t-test. (**e-h**) 6mg TNBS instilled intra-rectally into $IL\text{-}10^{-/-}$ mice. Data combined from 2 separate
600 experiments. n=8/group. (**e**) DAI, p=0.237, 0.903, 0.681, respectively on day 1, 2 & 3, LL-IL-27 vs. LL-
601 C, t-test, (**f**) weight loss from baseline, p=0.727, 0.751, 0.716, respectively on day 1, 2 and 3, LL-IL-27
602 vs. LL-C, t-test. (**g**) CXCL2 protein in distal colon homogenate, p=0.959 and (**h**) distal colon histology
603 score, p=0.608, t-test, were not significantly different in mice treated with LL-IL-27 compared to LL-C.
604 (**i**) On day 2 post TNBS instillation in SJL/J mice, there was a significant increase in distal colon IL-10
605 protein associated with IL-27, p=0.01, t-test. (**j**) Later in the treatment protocol, at day 4 or time of death,
606 this difference in IL-10 protein expression was not apparent, p=0.653. At both time points, n=10/group,
607 combined data from 2 experiments.

608

609 **Figure 7. IL-27 is associated with phosphorylated STAT-1 in colonic epithelial cells and**
610 **significantly reduces pro-inflammatory cytokine profile in vivo.** (**a**) Immunohistochemistry for
611 phosphorylated STAT-1 (Tyr701). Multifocal mucosal epithelial cells and scattered inflammatory cells
612 within the lamina propria and submucosa show strong positive nuclear labeling for phosphorylated

613 STAT-1 in response to oral LL-IL-27. Occasional cells show both strong nuclear and variable
614 cytoplasmic labeling. There is no phosphorylated STAT-1 immunopositivity in colitic mice who received
615 no treatment or treatment with LL-C. Diaminobenzidine chromogen and hematoxylin counterstain. This
616 pattern of expression was seen in all mice per group (n=3). (**b**) Cytokine gene expression profile in distal
617 colon homogenate was assessed by Taqman RT-PCR assays (Applied Biosystems) and analyzed with
618 ddCt method of relative quantification; normalization to endogenous GAPDH control and level of
619 expression compared to ethanol control group. Data, combined from 2 experiments, presented as mean +
620 SEM. *Il6* (p=0.003), *Il1b* (p=0.001) and *Tnf* (p=<0.01) were significantly increased in TNBS treated mice.
621 Expression of *Il6* was significantly reduced with LL-IL-27 compared to LL-C (p=0.036), whereas no such
622 effect was seen for *Il1b* and *Tnf*. n=7, 10-13, 10-15, 10-15, for ethanol, TNBS alone, TNBS+LL-C and
623 TNBS+LL-IL-27, respectively. (**c**) Cytokine protein expression in distal colon homogenate measured by
624 ELISA and normalized to mg total protein. Combined data presented from 2 experiments as mean +
625 SEM. n=5, 8-10, 7-10, 10, for ethanol, TNBS alone, TNBS+LL-C and TNBS+LL-IL-27, respectively.
626 Protein expression of IL-6 (p=0.002), IL-1β (p=0.003) and TNF (p=0.031) were significantly reduced in
627 the distal colon of colitic mice receiving LL-IL-27 treatment compared to LL-C.

628

629

Supplementary Figure Legends_ McLean et al_ Interleukin 27 is a potential rescue therapy for acute severe colitis via interleukin-10 dependent, T cell independent attenuation of colonic mucosal innate immune responses

Supplementary Figure 1 show representative distal colon photomicrographs of $F4/80^+$ and MPO^+ macrophages and neutrophils, respectively in each experimental group as labeled. This was determined by immunohistochemistry with positive staining shown as brown colour on counterstained formalin fixed paraffin embedded tissue sections. TNBS precipitated an increase in macrophages and neutrophils. Colitic mice treated with LL-IL27 had a significantly reduced colonic mucosal neutrophil infiltrate compared to those treated with LL-C ($p=0.002$, t-test). Slides were optically scanned with an Aperio AT2 digital slide scanner.

Supplementary Figure 2. IL-27 does not directly influence acute colonic inflammasome activation *in vivo*. There was no evidence of IL-27 mediated suppression of the NLRP3 inflammasome (*il18, Asc, Nflp3, Caspase-1*) in distal colon homogenate, assessed by Taqman RT-PCR gene expression assays (Applied Biosystems) and analyzed with ddCt method of relative quantification; normalization to endogenous GAPDH control and level of expression compared to ethanol control group (**a** & **b**). n=10/group, t-test. Bone marrow derived macrophage (BMDM) (**c**) and thioglycollate-induced peritoneal macrophage (TPM) (**d**) were stimulated for 5 hours or 72 hours with various concentrations of LPS as labeled, +/- 5mM ATP, +/- rL27 (100ng/ml). IL-1β was measured in supernatant by ELISA. (**c**) Combined data from several experiments, generated from >2 wells/experimental condition. (**d**) Data presented from one experiment, with the same pattern of expression seen in repeat experiment. LPS plus ATP resulted in a robust IL-1β response but there was no differential response in association with IL-27 in either macrophage population.

Mean + SEM. BMDM 100ng LPS dose data passed normality test and analyzed by t-test, all others Rank Sum test.

Figure 1

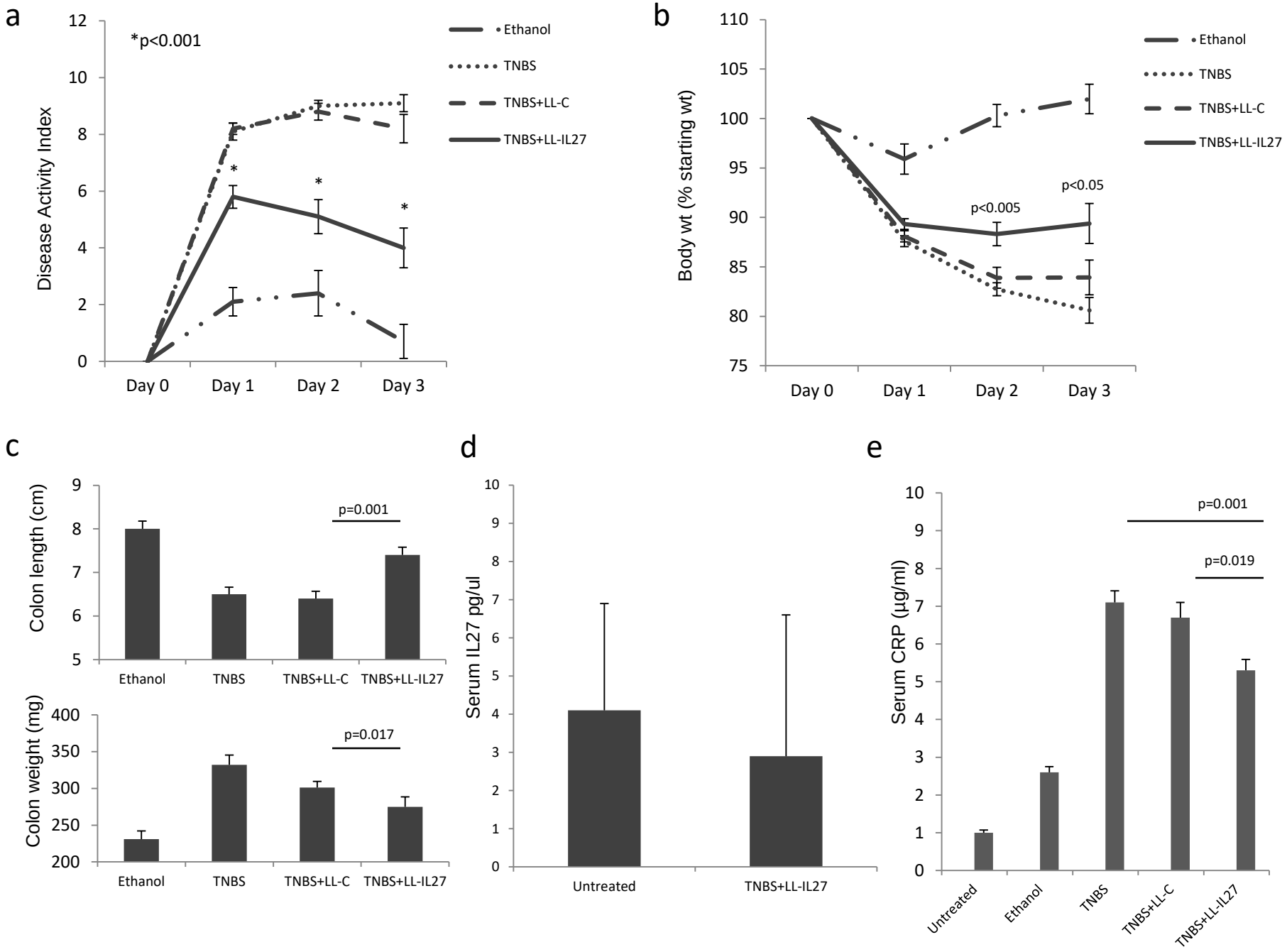

a
*p<0.001
Disease Activity Index
Day 0
Day 1
Day 2
Day 3
Ethanol
TNBS
TNBS+LL-C
TNBS+LL-IL27
b
Body wt (% starting wt)
p<0.05
p<0.005
c
Colon length (cm)
p=0.001
Colon weight (mg)
p=0.017
d
Serum IL27 pg/ul
Untreated
e
Serum CRP (µg/ml)
p=0.001
p=0.019

Figure 2

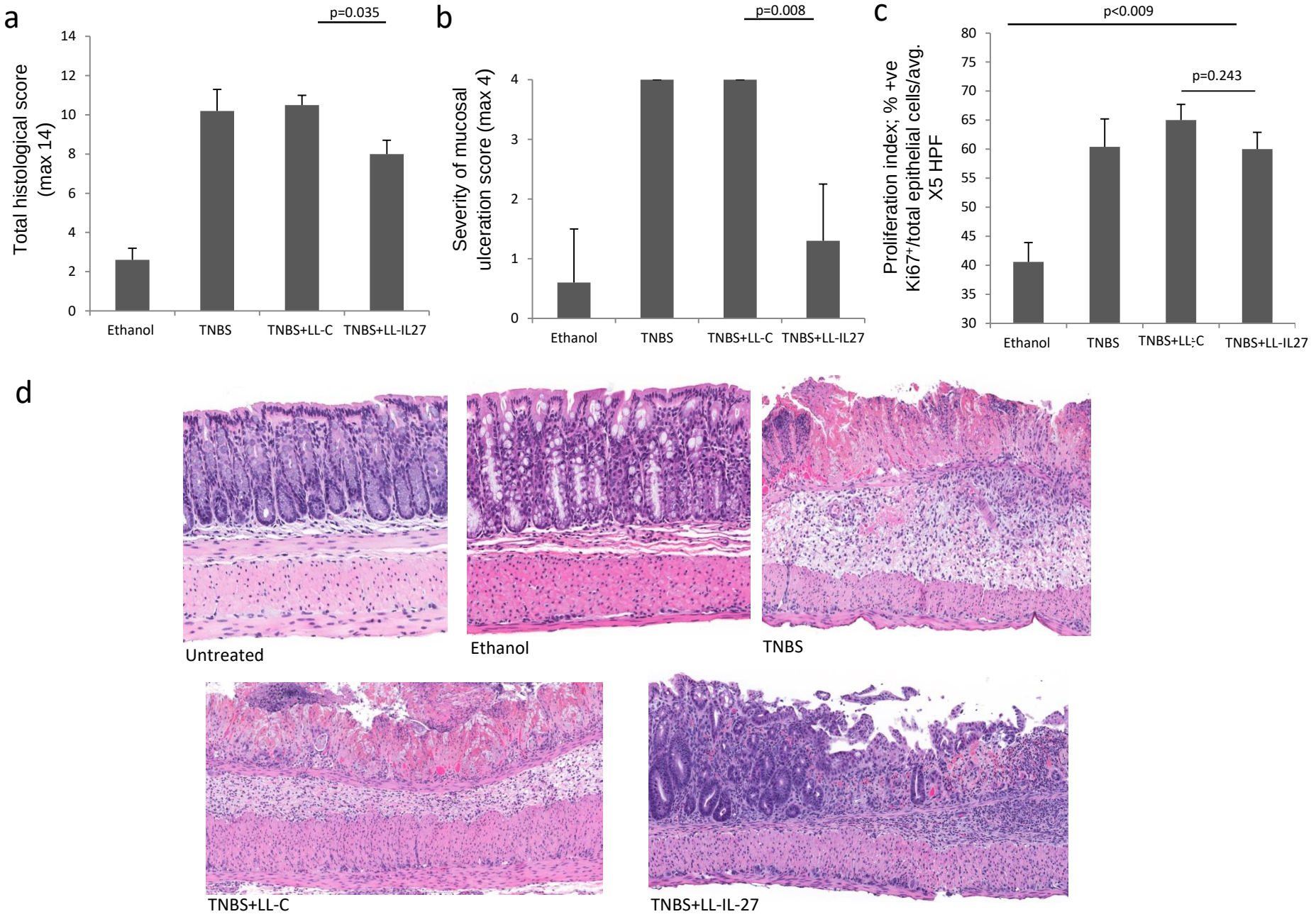

a
p=0.035
Total histological score (max 14)
14
12
10
8
6
4
2
0
Ethanol
TNBS
TNBS+LL-C
TNBS+LL-IL27
b
p=0.008
Severity of mucosal ulceration score (max 4)
4
3
2
1
0
Ethanol
TNBS
TNBS+LL-C
TNBS+LL-IL27
c
p<0.009
p=0.243
Proliferation index; % +ve Ki67+/total epithelial cells/avg. X5 HPF
80
75
70
65
60
55
50
45
40
35
30
Ethanol
TNBS
TNBS+LL-C
TNBS+LL-IL27
d
Untreated
Ethanol
TNBS
TNBS+LL-C
TNBS+LL-IL-27

Figure 3

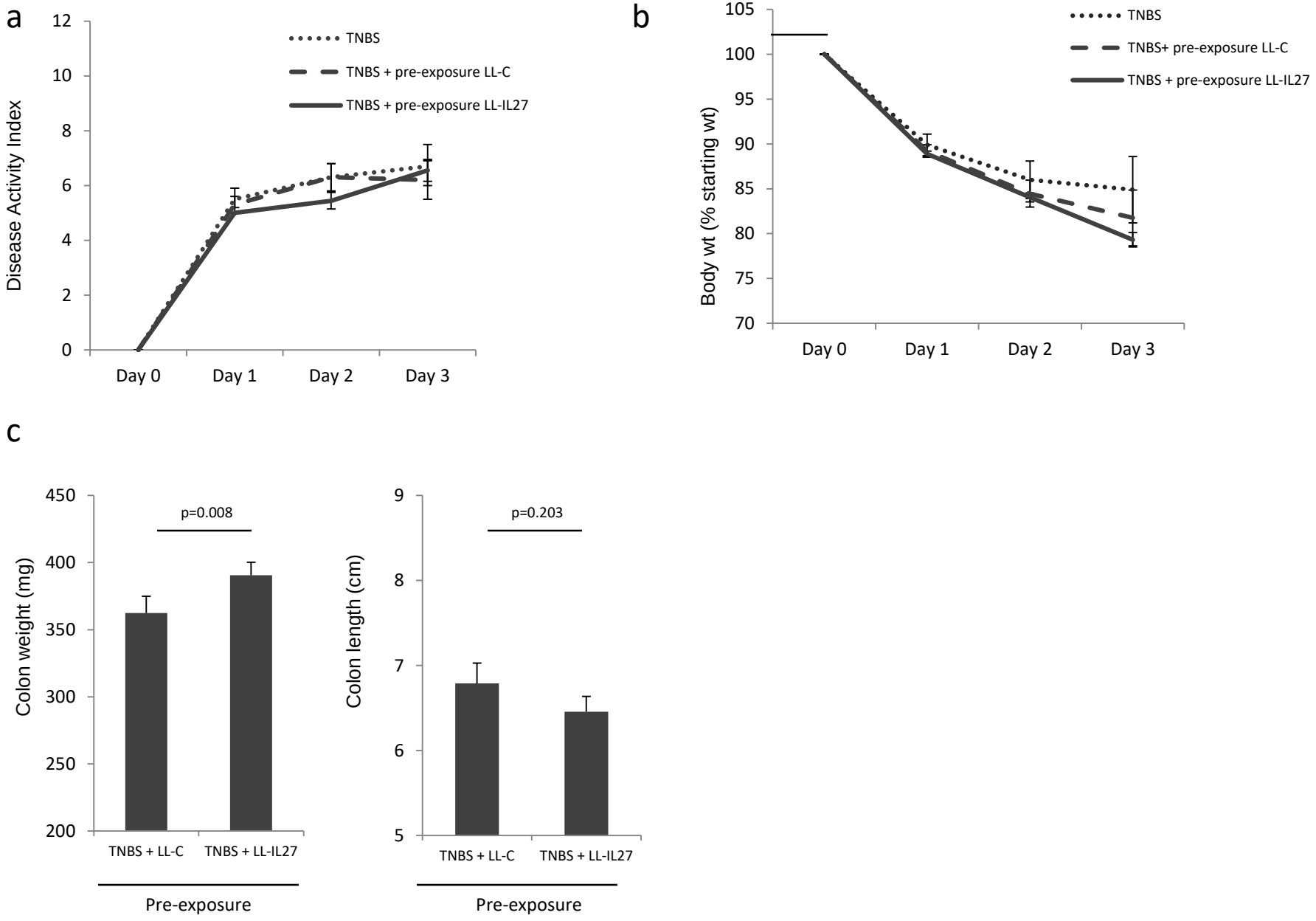
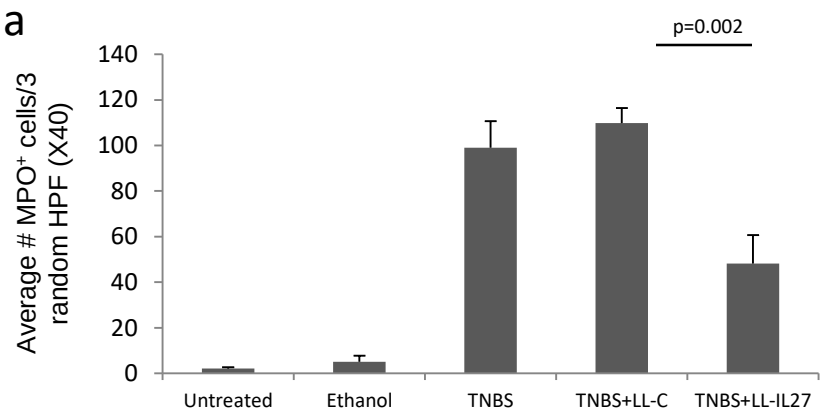
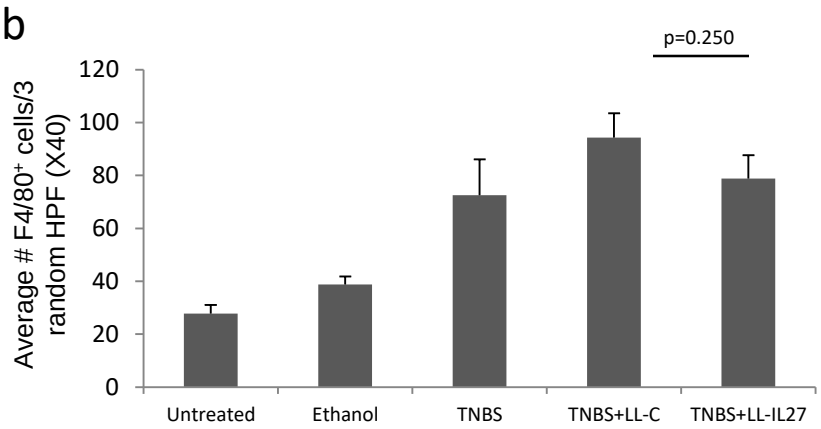
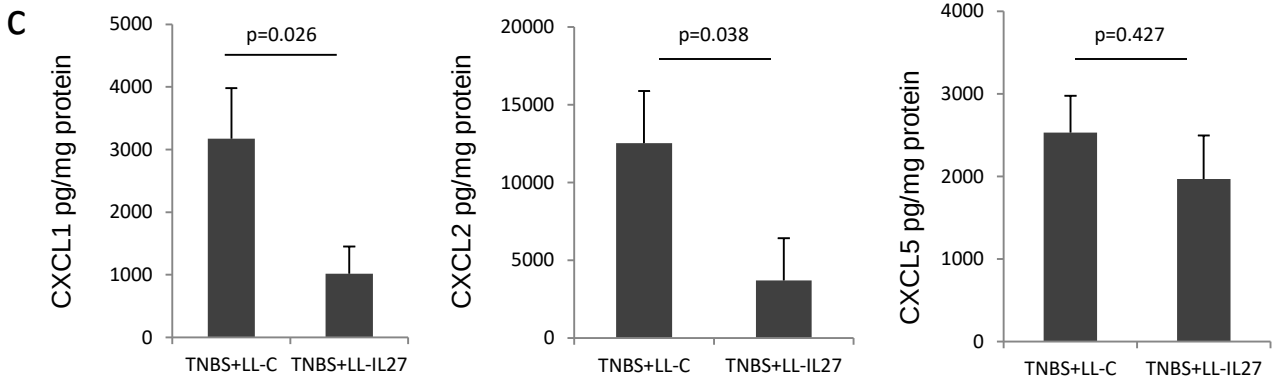
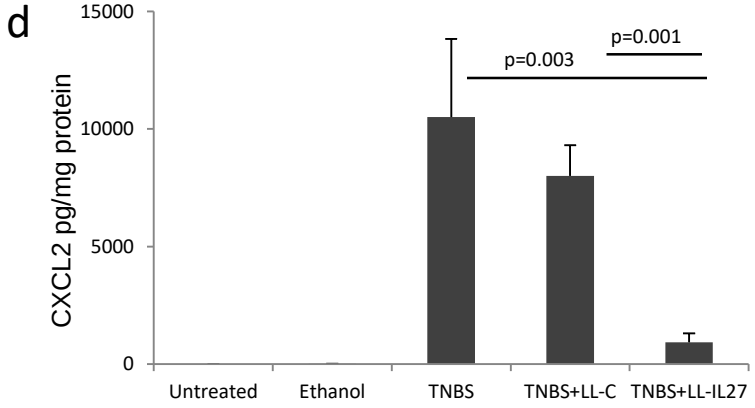

a
Disease Activity Index
TNBS
TNBS + pre-exposure LL-C
TNBS + pre-exposure LL-IL27
Day 0
Day 1
Day 2
Day 3
b
Body wt (% starting wt)
TNBS
TNBS+ pre-exposure LL-C
TNBS + pre-exposure LL-IL27
c
Colon weight (mg)
p=0.008
TNBS + LL-C
TNBS + LL-IL27
Pre-exposure
Colon length (cm)
p=0.203
TNBS + LL-C
TNBS + LL-IL27
Pre-exposure

Figure 4


a
p=0.002
Average # MPO+ cells/3 random HPF (X40)
140
120
100
80
60
40
20
0
Untreated
Ethanol
TNBS
TNBS+LL-C
TNBS+LL-IL27


b
p=0.250
Average # F4/80+ cells/3 random HPF (X40)
120
100
80
60
40
20
0
Untreated
Ethanol
TNBS
TNBS+LL-C
TNBS+LL-IL27


c
CXCL1 pg/mg protein
5000
4000
3000
2000
1000
0
p=0.026
TNBS+LL-C
TNBS+LL-IL27
CXCL2 pg/mg protein
20000
15000
10000
5000
0
p=0.038
TNBS+LL-C
TNBS+LL-IL27
CXCL5 pg/mg protein
4000
3000
2000
1000
0
p=0.427
TNBS+LL-C
TNBS+LL-IL27


d
CXCL2 pg/mg protein
15000
10000
5000
0
p=0.003
p=0.001
Untreated
Ethanol
TNBS
TNBS+LL-C
TNBS+LL-IL27

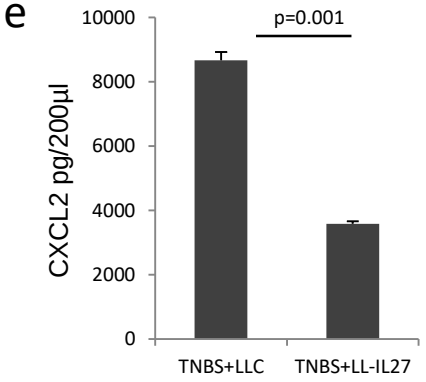

e
CXCL2 pg/200µl
10000
8000
6000
4000
2000
0
p=0.001
TNBS+LLC
TNBS+LL-IL27

Figure 5

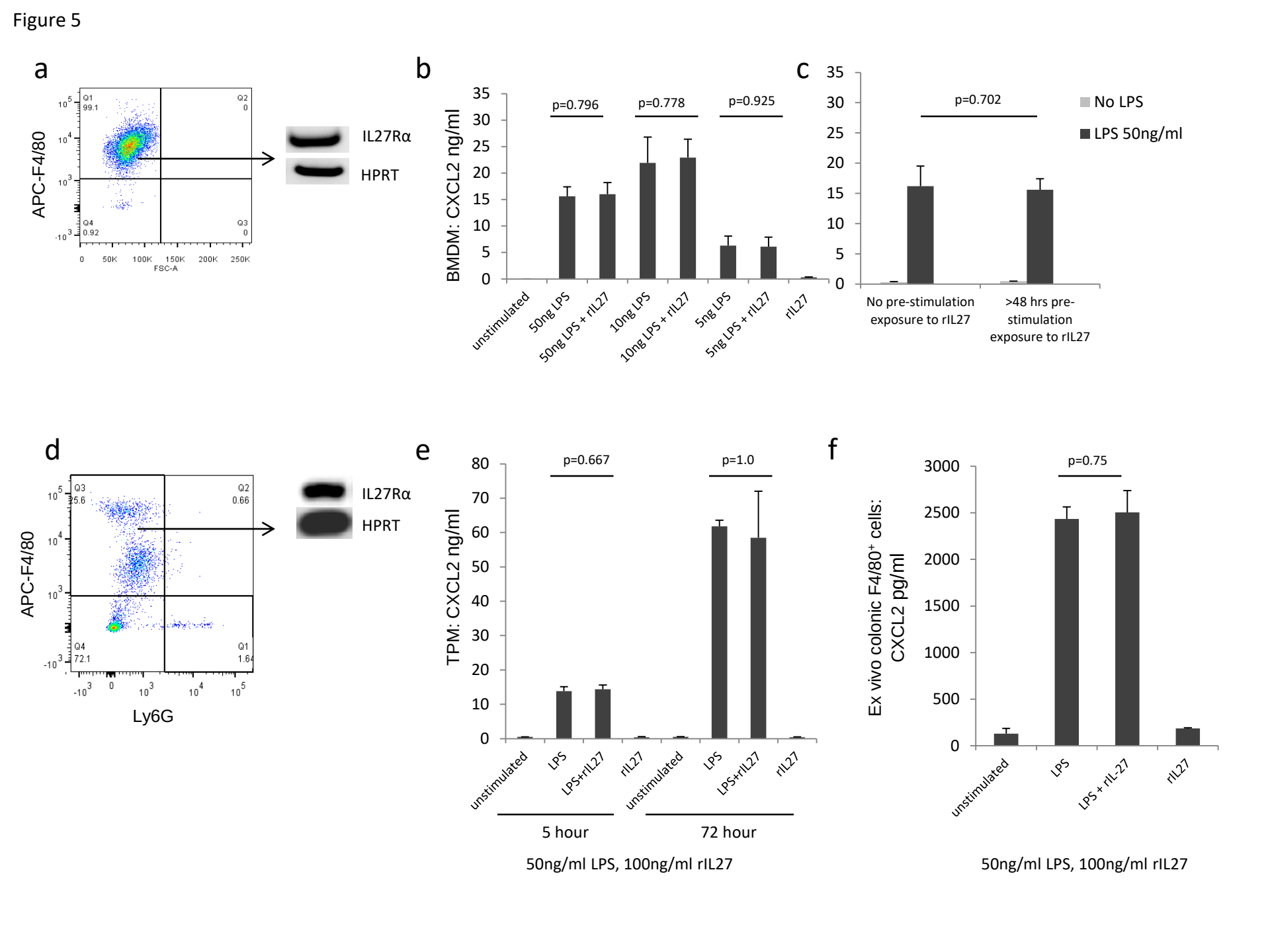

a
APC-F4/80
FSC-A
IL27Rα
HPRT
b
BMDM: CXCL2 ng/ml
p=0.796
p=0.778
p=0.925
unstimulated
50ng LPS
50ng LPS + rIL27
10ng LPS
10ng LPS + rIL27
5ng LPS
5ng LPS + rIL27
rIL27
c
p=0.702
No LPS
LPS 50ng/ml
No pre-stimulation exposure to rIL27
>48 hrs pre-stimulation exposure to rIL27
d
APC-F4/80
Ly6G
IL27Rα
HPRT
e
TPM: CXCL2 ng/ml
p=0.667
p=1.0
unstimulated
LPS
LPS+rIL27
rIL27
5 hour
72 hour
50ng/ml LPS, 100ng/ml rIL27
f
Ex vivo colonic F4/80+ cells: CXCL2 pg/ml
p=0.75
unstimulated
LPS
LPS + rIL-27
rIL27
50ng/ml LPS, 100ng/ml rIL27

Figure 6

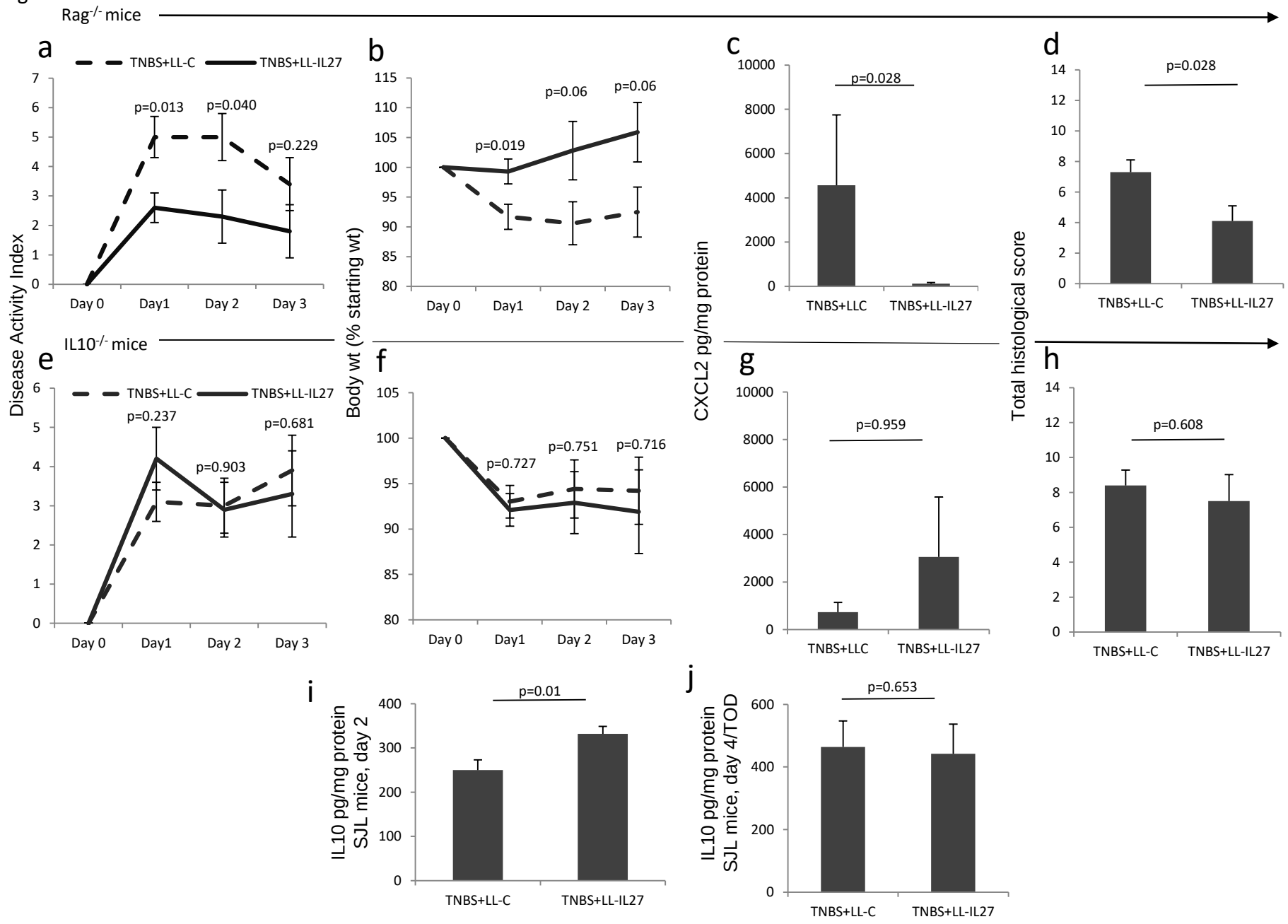
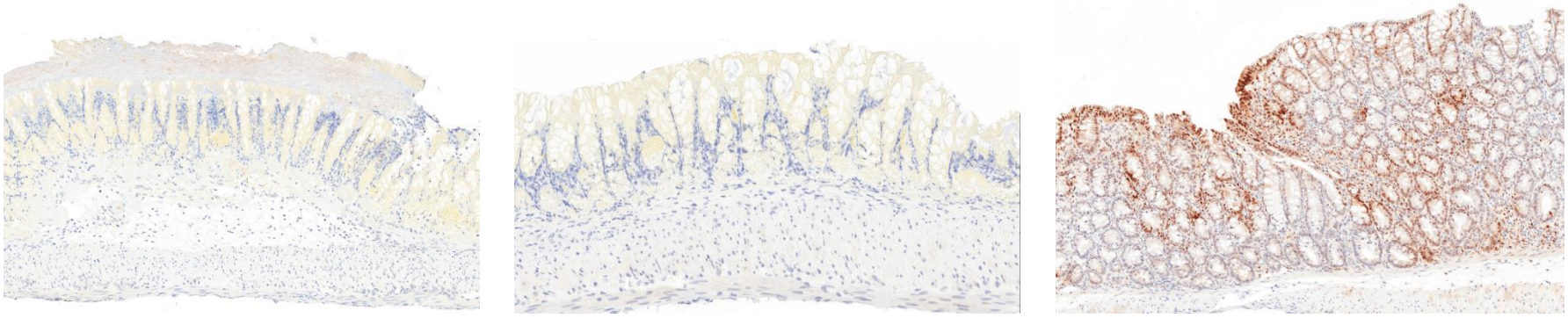
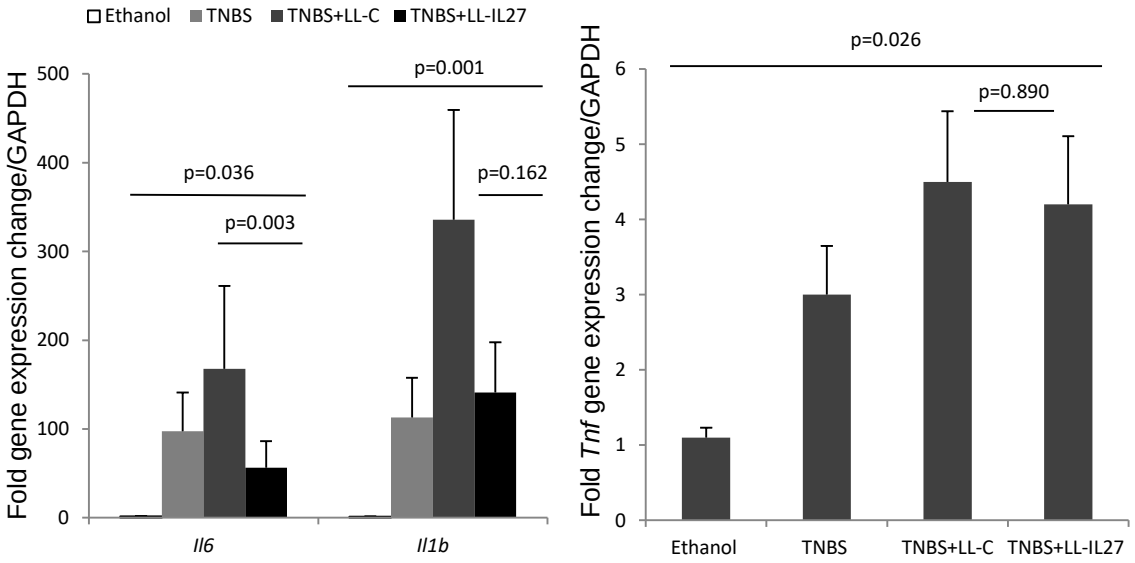

Rag-/- mice
a
TNBS+LL-C
TNBS+LL-IL27
Disease Activity Index
p=0.013
p=0.040
p=0.229
Day 0
Day1
Day 2
Day 3
b
Body wt (% starting wt)
p=0.019
p=0.06
p=0.06
c
CXCL2 pg/mg protein
p=0.028
TNBS+LLC
TNBS+LL-IL27
d
Total histological score
p=0.028
TNBS+LL-C
TNBS+LL-IL27
IL10-/- mice
e
p=0.237
p=0.903
p=0.681
f
p=0.727
p=0.751
p=0.716
g
p=0.959
h
p=0.608
i
IL10 pg/mg protein SJL mice, day 2
p=0.01
j
IL10 pg/mg protein SJL mice, day 4/TOD
p=0.653

Figure 7

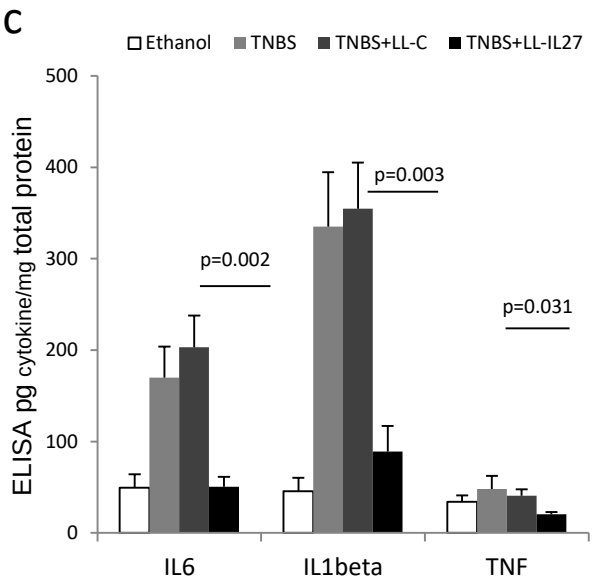
a


TNBS
TNBS+LL-C
TNBS+LL-IL27

b

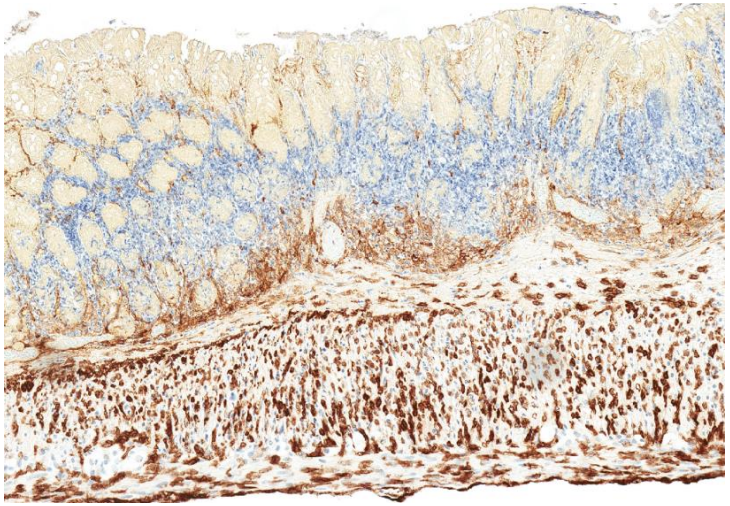

Ethanol
TNBS
TNBS+LL-C
TNBS+LL-IL27
Fold gene expression change/GAPDH
500
400
300
200
100
0
p=0.036
p=0.003
p=0.001
p=0.162
Il6
Il1b
Fold Tnf gene expression change/GAPDH
6
5
4
3
2
1
0
p=0.026
p=0.890
Ethanol
TNBS
TNBS+LL-C
TNBS+LL-IL27

c


Ethanol
TNBS
TNBS+LL-C
TNBS+LL-IL27
ELISA pg cytokine/mg total protein
500
400
300
200
100
0
p=0.002
p=0.003
p=0.031
IL6
IL1beta
TNF

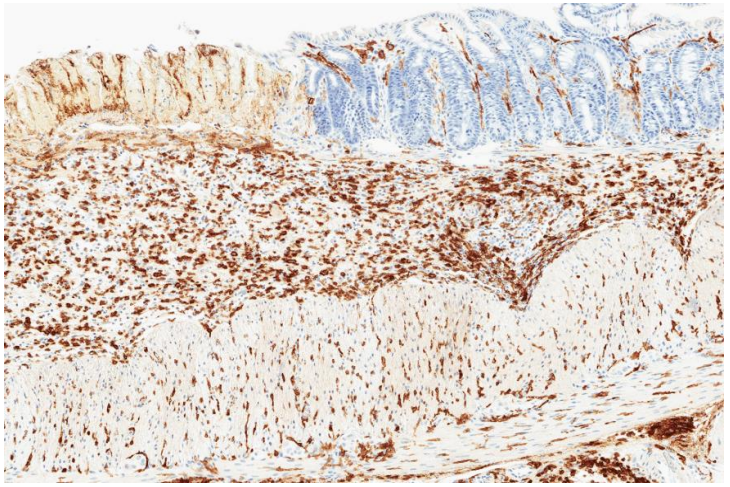
Supplementary Figure 1

a



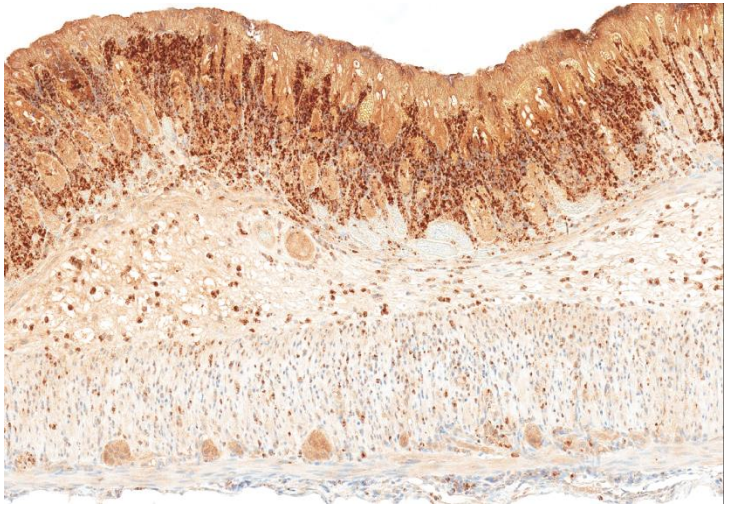
F4/80 TNBS+LL-C

b



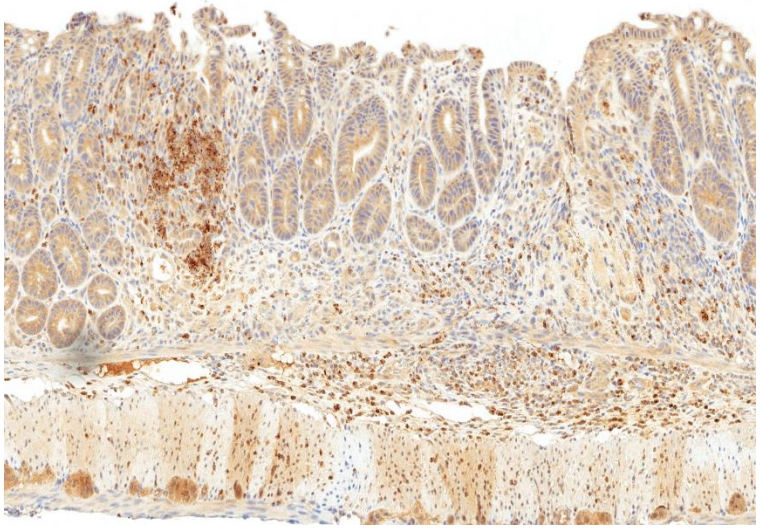
F4/80 TNBS+LL-IL-27

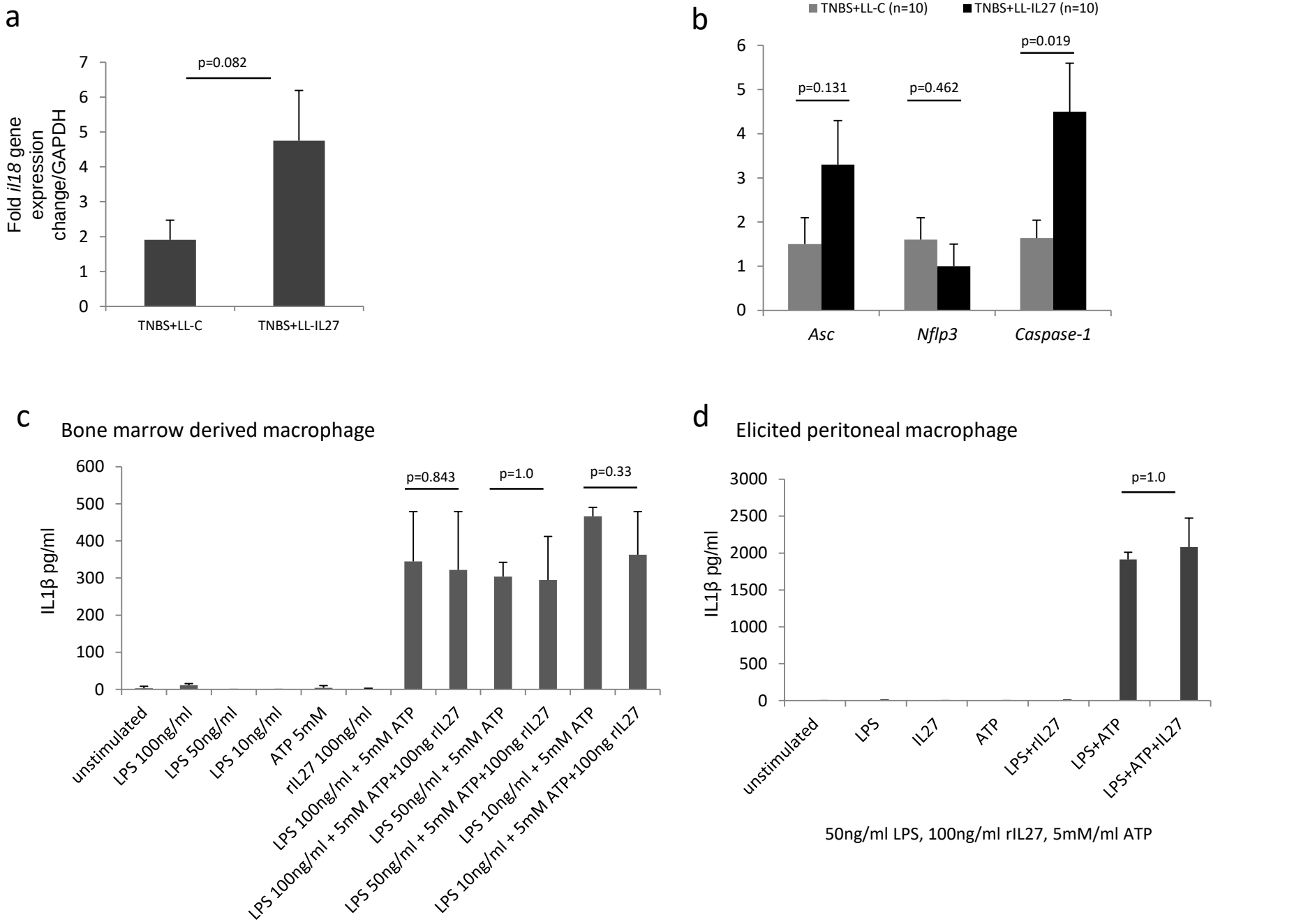
c



MPO TNBS+LL-C

d



MPO TNBS+LL-IL-27

Supplementary Figure 2