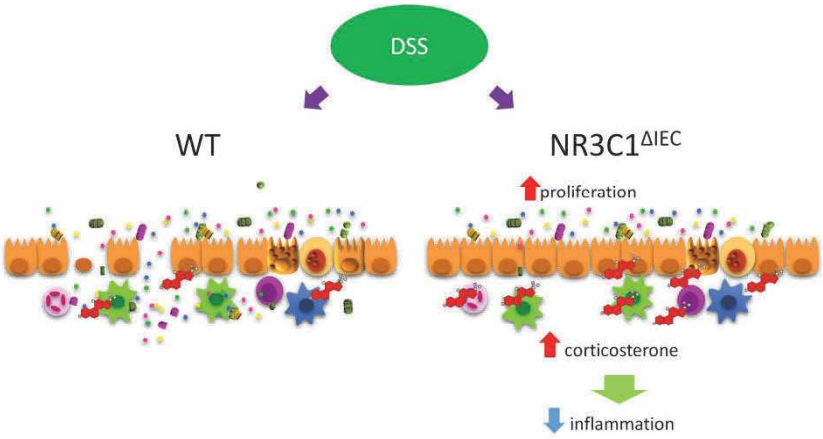

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Epithelial deletion of the glucocorticoid receptor protects the mouse intestine against experimental inflammation

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Running title: NR3C1$^{\Delta IEC}$ mice are protected against colitis.

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Abstract

Background and Purpose.

Glucocorticoids are the first line treatment for the flare-ups of inflammatory bowel disease due to their well-known anti-inflammatory and immunosuppressive actions. Nevertheless, the use of glucocorticoids has some significant limitations. The objective of this study is to investigate whether glucocorticoid epithelial actions contribute to such limitations.

Experimental Approach.

Intestinal epithelium glucocorticoid receptor knockout mice ($NR3C1^{\Delta IEC}$) were treated with dextran sulfate sodium (DSS, 2.5%) to induce colitis. Inflammatory status was assessed by morphological and biochemical methods and corticoid production was measured in colonic explants.

Key Results.

After 7 days of DSS $NR3C1^{\Delta IEC}$ mice exhibited lower weight loss and tissue damage, reduced colonic expression of S100A9, attenuated phosphorylation of STAT3 and a better overall state compared with WT. Ki67 immunoreactivity was also shifted, indicating an effect on epithelial proliferation. A subgroup of mice were treated with budesonide and showed completely prevented budesonide induced weight loss. Epithelial deletion of the glucocorticoid receptor also protected mice in a protracted colitis protocol. Conversely knockout mice presented a worse status compared to the control group at 1 day post DSS, as shown by blood in feces and increased inflammatory parameters. In a separate experiment colonic corticosterone production was shown to be significantly increased in knockout mice at 7 days of colitis but not at earlier stages.

Conclusions and Implications.

The intestinal epithelial glucocorticoid receptor has deleterious effects in experimental colitis induced by dextran sodium sulfate, probably related to inhibition of epithelial proliferative responses leading to impaired wound healing and reduced endogenous corticosterone production.

ABBREVIATIONS: 11βHSD1/2, 11β-hydroxysteroid dehydrogenase type 1 and 2; AP, alkaline phosphatase; DAI, disease activity index; DMEM, Dulbecco's modified Eagle's medium; DSS, dextran sodium sulfate; GC, glucocorticoid; GILZ, glucocorticoid-induced leucine zipper; GR, glucocorticoid receptor; GM-CSF, granulocyte-macrophage

colony stimulating factor; HPA, hypothalamic-pituitary-adrenal; H&E, hematoxilyn & eosin; IBD, inflammatory bowel disease; IEC, intestinal epithelial cells; IFNγ, interferon γ; IHC, immunohistochemistry; KO, knock out; LRH-1, liver receptor homolog 1; MCP, monocyte chemoattractant protein; MPO, myeloperoxidase; NR3C1, nuclear receptor subfamily 3 group C member 1; SF-1, steroidogenic factor 1; STAT3, signal transducer and activator of transcription 3.

1. Introduction

Glucocorticoids (GC) are steroid hormones of great physiological importance which modulate numerous processes such as metabolism, development, response to stress, blood pressure or immunomodulation. The glucocorticoid receptor, NR3C1, is thought to mediate GC actions through genomic and also non-genomic ways (Kumar *et al.*, 2005; Newton, 2014; Ramamoorthy *et al.*, 2016). In the gut GCs exert their well-established antiinflammatory and immunosuppresive actions, but they additionally decrease epithelial proliferation and impair wound healing, among other effects (Ocon *et al.*, 2016). GCs are widely used for the management of intestinal inflammation, mainly inflammatory bowel disease (IBD). In fact, GCs are the drugs of choice for the control of IBD bouts, although resistance is common (25-30%)(Truelove *et al.*, 1978; Munkholm *et al.*, 1994; Tung *et al.*, 2006; Dubois-Camacho *et al.*, 2017). One peculiar aspect is the inability of GCs to prolong the relapse periods (Ford *et al.*, 2011).

The epithelial cells in both the small and large intestine feature a complete GC biosynthetic route, also found in the skin, thymus and brain (Talaber *et al.*, 2013). Intestinal regulation of GC biosynthesis differs significantly from that in the adrenal cortex (Flynn *et al.*, 2018). Adrenal GC production is augmented under the control of the SF-1 transcription factor (Miller *et al.*, 2011), while intestinal GC synthesis requires the expression of LRH-1 (*Nr5a2*) (Mueller *et al.*, 2006). Basal intestinal epithelial GC levels are reportedly low, but rise substantially under inflammatory conditions, an effect that has been linked to TNF signaling (Noti *et al.*, 2010b). In addition, local glucocorticoid levels can be regulated by the 11β-hydroxysteroid-dehydrogenase (11βHSD1 and 11βHSD2) enzymes. 11βHSD2 is responsible of the conversion from the active (corticosterone in mice, cortisol in humans) to the inactive form (11-dehydrocorticosterone in mice, cortisone in humans) while 11βHSD1 catalyzes the opposite reaction.

Our group and other investigators have established that GCs exert deleterious actions in experimental models of colitis, despite the fact that intestinal inflammation is actually dampened (Marrero *et al.*, 2000; van Meeteren *et al.*, 2000; Graffner-Nordberg *et al.*, 2003; Sann *et al.*, 2013; Ocon *et al.*, 2016; Sales-Campos *et al.*, 2017). This mechanism may underlie GC limitations in IBD management such as early resistance or failure to

prolong remission. Because of the unfavorable actions of GC at the epithelial level, we hypothesized that these effects may be due to alteration of mucosal barrier function (Sanchez de Medina *et al.*, 2014). Thus we generated mice with a conditional deletion of the *Nr3c1* gene in intestinal epithelial cells (IECs), i.e. NR3C1$^{\Delta IEC}$ mice (Aranda *et al.*, 2019). NR3C1$^{\Delta IEC}$ mice display mild, self-limiting colitis with systemic involvement which is maximal 1 week after tamoxifen-induced *Nr3c1* deletion. The colonic epithelium shows increased proliferation and mucus production, while the small intestine is not affected, despite showing similar subtotal epithelial gene knockout. In the present study we set out to characterize the response of NR3C1$^{\Delta IEC}$ mice to dextran sulfate sodium (DSS) induced experimental colitis, and the changes observed after treatment of colitic mice with budesonide.

2. Materials and methods

2.1. Reagents

Except where indicated, all reagents, including primers, were obtained from Sigma (Madrid, Spain).

2.2. Animals and in vivo experimental design

C57BL/6J transgenic mice expressing the tamoxifen-inducible CRE recombinase under the control of the villin promoter and carrying lox sequences flanking the GR (*Nr3c1*) gene, referred to in the present paper as NR3C1$^{\Delta IEC}$, were used with their respective controls, referred as WT, as explained previously (Aranda *et al.*, 2019). All the animals were maintained under SPF conditions in air-conditioned animal modules with a 12 h light-dark cycle at the University of Granada Animal Facility (Biomedical Research Center, University of Granada, Granada, Spain). Mice were given free access to autoclaved tap water and standard chow (Teklad global 14% proteins rodent diets, Envigo, Barcelona, Spain). All animal procedures in this study were carried out in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the U.S. National Institutes of Health and were approved by the Animal Experimentation Ethics Committee of the University of Granada (ref. 01-12-14-165).

Nr3c1 deletion was induced with 1 mg tamoxifen (sunflower seed oil/ethanol mixture, 10:1 v v^{-1} i.p.) injected for 5 consecutive days. WT mice received the same treatment. Seven days after the first tamoxifen injection, colitis was induced by adding DSS 2.5% (w v^{-1}) to the drinking water. In some cases budesonide (6 or 60 µg d^{-1}, Molekula, Rimini, Italy) was administered by gavage using 1% methylcellulose as vehicle, control mice receiving only the vehicle.

To study the response of these animals to the first stages of colitis, a 2x2 experiment was carried out. Mice from different genotypes (WT or NR3C1$^{\Delta IEC}$) were randomly assigned to the budesonide group (BD) (6 µg d^{-1}) or vehicle group (n=8). Animals were sacrificed after one day of DSS and budesonide exposure. Other two similar experiments were performed, but in these cases, mice were sacrificed after 7 days of DSS, when colitis was established. One of them was destined to isolation of colonic IECs for analysis (n=8). Mice used in these experiments were approximately 1:1 male:female, with an average weight of 23 g and 21 g, respectively (6-8 wk old).

Additionally, three different DSS (2.5% w v^{-1}) colitis experiments were carried out. First, a survival experiment, in which WT and $NR3C1^{\Delta IEC}$ male mice (20 wk old, 30-33 g, n=10 and 9, respectively) were exposed to DSS for 21 days. Second, a regular colitis experiment similar to the one explained above, with three different euthanizing time points: at 3, 5 and 7 days of DSS exposure (male 15 week old mice, 27 g, n=6 except n=7 for WT groups euthanized at day 5 and 7). Third, a five-day DSS colitis experiment where WT and $NR3C1^{\Delta IEC}$ mice were orally administered 60 µg d^{-1} of BD (30 wk old, 36 g male and 25 g female, n=10 except n=11 for female WT and male $NR3C1^{\Delta IEC}$ mice).

The status of the animals was checked daily for body weight evolution (expressed as a percentage of the initial weight), stool consistency, and the presence of blood in feces (visible or fecal occult blood), corresponding to the signs of the pathology in this model. The Disease Activity Index (DAI) was obtained as the sum of these three factors: body weight loss (0: none; 1:1-5%; 2:5-10%; 3:10-15%; 4: >15%), presence of blood in feces (0-4) and stool consistency (0-4).

Mice were sacrificed by the administration of Ketamine/Xylacine (24 mg mL^{-1} and 1.6 mg mL^{-1} respectively). After that, a blood sample was drawn intracardiacally and spun to obtain plasma, which was snap frozen and kept at −80 °C until measurement.

2.3. Intestinal assessment

The entire colon was removed, gently flushed with pre-cooled PBS and blotted on filter paper, placed on an ice-cold plate, cleaned of fat and mesentery and longitudinally opened to eliminate fecal remains. Each specimen was weighed and its length measured under a constant load (2 g). Two small segments were dissected from the distal zone of the colon and used for histology and RNA isolation. After that, four 3 mm-diameter fragments were destined for culture and exposed to a PBS solution containing 500 U mL^{-1} penicillin, 0.5 mg mL^{-1} streptomycin, 12.5 µg mL^{-1} amphotericin B and 10 µg mL^{-1} gentamycin. The remaining colon tissue was subsequently gently minced and separated in two aliquots for biochemical determinations. The fragments were immediately frozen in liquid nitrogen and kept at -80°C until used.

Separation of intestinal epithelial cells (IECs) was accomplished using the method previously described (Mucida *et al.*, 2007; Weigmann *et al.*, 2007).

Colonic myeloperoxidase (MPO) and alkaline phosphatase (AP) activities were quantitated spectrophotometrically as described (Lopez-Posadas *et al.*, 2011; Martinez-Moya *et al.*, 2012) after tissue homogenization in a Bullet Blender® (Next Advance, Averill Park, NY, USA). Fecal occult blood was measured in stool samples by an adaptation of the method developed by Dr. Donald S. Young (Welch *et al.*, 1983; Ocon *et al.*, 2016).

2.4. Colonic explant culture.

The four colon fragments destined for explant culture were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with charcoal stripped heat-inactivated fetal bovine serum (10% v v^{-1}), 100 U mL^{-1} penicillin, 0.1 mg mL^{-1} streptomycin, 2.5 μg mL^{-1} amphotericin and 2 mM glutamine during 24 h. After that, medium was collected and snap frozen at −80 °C.

2.5 Determination of cytokines by ELISA and Multiplex® assay.

TNF, IFN-γ, GM-CSF, IL-10, IL-17A and MCP-1 levels in medium culture from colon explants were measured by the ProcartaPlex® kit (Affimetrix, eBioscience Inc.). Plasmatic levels of TNF and IL-6 in mouse plasma were measured by Multiplex assay (EMD Millipore Merk, Damstadt, Germany). The lecture was realized by the Luminex 200 (Luminex Corporation) equipment. Corticosterone concentration in colonic explant medium was measured using ELISA (Enzo Life Sciences, Farmingdale, NY) following the manufacturer's instructions.

2.6. Histological Assessment of Colon Damage and Epithelial Proliferation

Distal colon tissue fragments were processed for haematoxylin and eosin (H&E) histology assessment. In brief, samples were fixed in 4% paraformaldehyde (w v^{-1}), deparaffinized, rehydrated, and stained. For immunohistochemistry (IHC), deparaffinized colon samples were incubated with 10 mM citrate buffer for 30 min at 100 ºC, washed in Tris 50 mM NaCl 0.15 M solution for 5 min and incubated with 3% H_2O_2 in methanol for 10 min. Sections were subsequently rinsed with tap water, and IHC was performed using the Vectastain ABC kit (Vector Laboratories, Burlingame, CA, USA). The sections

were blocked for 1 hour and exposed (overnight, 4 ºC) to the primary antibody to Ki67 (1:400; Cell Signaling; Danvers, MA, USA) followed by a biotinylated secondary antibody and an avidin–peroxidase complex (30 min each) and incubation with Dako Real DAB+Chromogen (K5007) for staining development (1 minute). The Ki67 staining was evaluated by a double-blinded analysis. The parameters considered were the intensity (0-3) and diffusion of the staining (1-3), being 1 when it was confined to the crypts and 3 when it was spread through all the epithelium. Images were captured and digitalized using a Leica DMI3000B microscope (Barcelona, Spain) equipped with a Leica DFC420 C Camera.

2.7. Organoid culture.

Jejunum crypts isolation from WT and NR3C1$^{\Delta IEC}$ mice (17 wk old) was accomplished as previously described (Romero-Calvo *et al.*, 2018). Briefly, crypts were cultured in a 1:1 dome of Matrigel® Growth Factor Reduced (GFR) Basement Membrane Matrix, Phenol Red-Free (Corning, New York, USA) and IntestiCult™ Organoid Growth Medium (Mouse) (Stemcell, Vancouver, Canada) following the manufacturer's instructions. When Matrigel® was set, 500 µl of IntestiCult™ were added to each well. After 2-3 organoid passages, the experiment was conducted. Tamoxifen 1 µM was added for 24 h 1 day after the passage. Organoids were treated with TNF (10 ng mL^{-1}) (eBioscences, San Diego, California, USA) and FBS 5% (v v^{-1}). After 4 h of incubation, organoids underwent RNA extraction.

2.8. RNA isolation and quantitative reverse-transcription polymerase chain reaction (RT-qPCR) analysis

Total RNA from tissue was isolated with the RNeasy minikit (Qiagen, CA, USA), while organoid RNA was obtained with QIAzol Lysis Reagent (Qiagen, California, USA). 1 µg of RNA per sample was retrotranscribed using iScript Select cDNA Synthesis kit (Biorad Laboratories, California, USA). Specific DNA sequences were amplified with a Biorad CFX connect real-time polymerase chain reaction (PCR) device (Alcobendas, Madrid, Spain). Primers used are shown in Supplementary Table 1. Results are expressed as 2^{-ddCt} using *Ppib* and *18S* as reference genes.

2.9. Western blot analysis

Colon tissue was homogenized in lysis buffer (0.1% w v^{-1} SDS, 0.1% w v^{-1} sodium deoxycholate, 1% v v^{-1} Triton X-100 in PBS) with protease inhibitor cocktail 1:200 (v v^{-1}) (Sigma, P9599) and phosphatase inhibitor cocktail 1:100 (v v^{-1}) (SC-45045, Santa Cruz, Heidelberg, Germany). Then homogenates were sonicated and centrifuged at 10,000 g/10 min/4 °C. Protein concentration was determined by the bicinchoninic acid assay (Canny *et al.*, 2006). Samples were boiled for 5 min in Laemmli buffer (Biorad), fractionated by SDS–PAGE, and transferred to nitrocellulose membranes (pore size 0.45 μm) (Millipore, Madrid, Spain). After incubation with 5% nonfat milk in TBST (10 mM Tris, pH 8.0, 150 mM NaCl, 0.5% Tween 20) for 60 min, membranes were incubated with the corresponding antibody. The bands were detected by enhanced chemiluminescence (PerkinElmer, Waltham, MA, USA). The primary antibodies were used at a 1:1000 dilution except where indicated, and were obtained from: R&D Systems® (Minneapolis, MN, USA) (S100A9 Ref. AF2065); Cell Signaling (Danvers, MA, USA) (pSTAT3 (1:2000) Ref. 9145, STAT3 Ref.9139, Cyclin D1 Ref. 2978); Hybridoma Bank (Iowa, USA) (Actin (1:500)). The bands were quantified with the National Institute of Health software Image J.

2.10. Data and Statistical Analysis

The data and statistical analysis complies with the recommendations on experimental design and analysis in pharmacology (Curtis *et al.*, 2015). Group mean values and statistical analysis use independent values. Analyses of a subjective nature (i.e. histology) were carried out in a blinded fashion. Results are expressed as mean ± SEM. Differences among means were tested for statistical significance by two-way ANOVA and *a posteriori* Fisher's least significant difference tests. Pairwise comparisons were performed with two-tail t-tests. In the case of survival curves, a Mantel-Cox test was used. All analyses were carried out with the GraphPad Prism 6 (La Jolla, CA, USA). Differences were considered significant at $P < 0.05$.

3. Results

3.1 The lack of intestinal epithelial NR3C1 exacerbates the inflammatory status in the early stages of DSS exposure

Mice were sacrificed after 1 day of DSS exposure, a time point where intestinal lesions are essentially nonexistent in this model, because NR3C1$^{\Delta IEC}$ mice showed signs of premature disease. While WT mice presented an essentially normal colon, weight/length ratio was increased in knockout mice (Figure 1A, B), and infiltration of leukocytes, crypt architecture distortion and thickening were observed (Figure 1C). Bloody feces were noted solely in this group, with fecal occult blood analysis showing substantially higher levels, although without reaching statistical significance, due to the high variability in the budesonide treated group (Figure 1C).

Knockout mice also showed augmented expression of *S100a8* and *S100a9,* encoding the calprotectin heterodimer (Figure 2A). The transcription factor STAT3 can bind directly to the *S100a9* promoter and stimulate its expression, and accordingly increased phosphorylation of STAT3 was noted in NR3C1$^{\Delta IEC}$ mice, which was associated with higher protein levels of S100A9 (Figure 2B). Colonic explant TNF, IFNγ, IL17a, MCP-1, IL10 and GM-CSF production was increased in NR3C1$^{\Delta IEC}$ mice compared to WT mice (Figure 2C). Additionally, the inflammatory status was not limited to the colon, as plasmatic levels of IL6 and TNF were found to be elevated (Figure 2D).

The effect of BD administration (6 µg d^{-1} p.o.) was also assessed. BD had no discernible impact on colonic weight:length ratio, colonic macroscopic/microscopic appearance, or alkaline phosphatase activity (Figure 1 and Supplementary Figure 1). In turn, fecal occult blood was clearly increased in NR3C1$^{\Delta IEC}$ but not WT mice treated with BD (Figure 1D). *S100a8* and *S100a9* overexpression in NR3C1$^{\Delta IEC}$ mice was fully suppressed by BD (Figure 2A). In contrast, the colonic protein levels of S100A9, although increased in NR3C1$^{\Delta IEC}$, were not affected by BD (Figure 2B). BD had no effect on STAT3 phosphorylation (Figure 2B) but generally decreased inflammatory marker release by colonic explants, such as MCP-1 or TNF, with the exception of IL10 and GM-CSF (Figure 2C). Finally, plasma levels of IL6 and TNF were lower in budesonide treated mice ($P>0.05$, Figure 2D).

Thus epithelial suppression of the GC receptor enhances the early response to DSS, resulting in inflammation and hemorrhage. budesonide limits inflammation in this context but further promotes blood loss.

3.2 The absence of intestinal epithelial NR3C1 has a protective role in the late stages of DSS induced experimental colitis

As expected, WT mice started to lose weight substantially by the fifth day of DSS exposure (Figure 3A). NR3C1$^{\Delta IEC}$ mice exhibited initially a worsened status, with visible fecal blood loss and increased body weight loss. However, the situation was reversed after day 5, so that ultimately body weight loss was ~33% lower in this group compared with WT mice. DAI values showed a similar trend, with roughly two-fold increased values in NR3C1$^{\Delta IEC}$ mice compared with the WT group from day 2 to day 4, after which the DAI quickly augmented in WT mice (~7 DAI units in 3 days), while it was more contained in the KO group (~2 DAI units). Consistent with these findings, WT colons presented higher vascularization, rigidity and rectal bleeding, as well as damaged crypts and infiltration, than those lacking the epithelial receptor (Figure 3B and Supplementary Figure 2). There were no differences however in colon weight/length ratio (data not shown).

Colonic myeloperoxidase activity was significantly reduced in knockout mice (Figure 3C). The colonic expression of *S100a8* and *S100a9* as assessed by RT-qPCR was also lower in NR3C1$^{\Delta IEC}$ compared with WT mice ($P>0.05$, Figure 3D), due to a large extent to the distortion introduced by the magnitude of the changes in the BD treated groups (pairwise t-test shows 0.02 and 0.06 values, respectively). At the protein level, this profile was confirmed to be significant for S100A9 in colonic epithelial cell enriched samples, and STAT3 phosphorylation followed the same tendency (Figure 3E). As a rule, the supernatant levels of cytokines/chemokines released by colonic explants in KO mice was <50% of that in WT specimens, with the only exception of GM-CSF (Supplementary Figure 3A). However, statistical significance was achieved only for IL17A. Plasmatic levels of IL-6/TNF were comparable to those measured after one day of DSS exposure in the case of NR3C1$^{\Delta IEC}$ mice, whereas a substantial increase was observed for WT samples (i.e. a ~9-fold and ~4-fold increase, respectively, Supplementary Figure 3B). There were no significant differences between groups at this time point as a result.

In addition, cell proliferation was assessed by Ki67 immunohistochemistry (Figure 4). Staining intensity was comparable between both genotypes (bottom, left), but it was significantly less widespread along the crypt axis in the latter (bottom, right).

Budesonide treatment of WT mice at the dose of 6 μg d^{-1} resulted in a much more pronounced weight loss and higher DAI values than in vehicle treated animals (Figure 3A). There was no major improvement at the histological level. While the release of inflammatory proteins by colonic explants was in several cases lower in the samples obtained from glucocorticoid treated mice, such as TNF, IFN-γ and IL-17A, and plasma TNF and IL-6 exhibited the same trend, none of these apparent changes reached the significance threshold, and levels were comparable for IL-10, MCP-1 and GM-CSF. Further, STAT3 phosphorylation was unchanged by the treatment. In fact, RT-qPCR revealed increased expression of *S100a8* and *S100a9*, although the latter was unconfirmed at the protein level in epithelial cells. In the case of $NR3C1^{\Delta IEC}$ mice, the administration of BD had no effects (Figure 3D, E). Budesonide reduced Ki67 expression in both WT and $NR3C1^{\Delta IEC}$ mice. Ki67 immunoreactivity was more confined to the crypt base region in WT mice, with no change in knockout mice, which already showed a similar pattern without GC treatment (Figure 4). Remarkably, epithelial NR3C1 deletion completely prevented weight loss associated with budesonide treatment (Figure 3A).

Thus epithelial NR3C1 deletion resulted in protection against colitis at 7 days, despite the initial deleterious effect. In this experiment budesonide worsened animal status in WT but not $NR3C1^{\Delta IEC}$ mice, suggesting that epithelial actions underlie the harmful effects of the glucocorticoid.

In order to further establish the protective role of the deletion of the intestinal epithelial NR3C1, WT and $NR3C1^{\Delta IEC}$ mice were exposed to DSS 2.5% for an extended period of time (21 days). This protocol results in aggravation of colitis status beyond the 7^{th} day and is associated with substantial mortality. Hence the lack of the epithelial GC receptor was associated to an almost 50% higher survival rate in absolute terms, corresponding to a 68% lower relative risk (3/10 WT vs 7/9 $NR3C1^{\Delta IEC}$ surviving mice) (Figure 5A).

3.3 Increased corticosterone synthesis by intestinal epithelium in $NR3C1^{\Delta IEC}$ mice correlates with protection from colitis

In order to determine the mechanism accounting for the lower susceptibility of NR3C1$^{\Delta IEC}$ mice to colitis, the production of corticosterone by colonic explants was measured, as intestinal steroidogenesis reportedly has protective effects in colitis (Cima *et al.*, 2004; Coste *et al.*, 2007; Noti *et al.*, 2010a; Kostadinova *et al.*, 2014).

Given the time-dependent effects of epithelial NR3C1 deletion in DSS colitis, corticosterone in colonic explants was measured at three different time points: 3, 5 and 7 days after DSS exposure (Figure 5B). Intestinal steroidogenesis was found to increase overtime in both genotypes, so that it was maximal at day 7. Within this time frame, corticosterone production was higher in WT mice at 3 days, comparable in both groups at day 5, and it was finally increased roughly 2.4 fold in NR3C1$^{\Delta IEC}$ vs. WT explants. In fact, corticosterone production was augmented ~4-fold vs. that observed at day 5 in knockout mice. Thus while colonic steroidogenesis is upregulated by DSS colitis in both groups, NR3C1$^{\Delta IEC}$ mice exhibit a biphasic response characterized by low early output and enhanced late release, a pattern that parallels the inflammatory phenotype in these animals. No differences were noted in plasmatic corticosterone (data not shown).

To investigate the origin of the augmented colonic steroidogenesis in the absence of epithelial NR3C1, the expression of key enzymes of corticosterone synthesis pathway was measured by RT-qPCR. The development of the colitic response was paralleled by an increase in the expression of *Hsd11b1* in both genotypes, without significant changes in *Hsd11b2* (Figure 5C, D). In addition, the colon from knockout animals also presented an augmented expression of *Cyp11a1* and *Star* at the end of the experimental period (Figure 5E, F). Therefore there is a positive correlation of measured genes with corticosterone production of NR3C1$^{\Delta IEC}$ colons at day 7 of DSS exposure.

3.4 Deleterious effects of high dose budesonide in DSS colitis are partially attributable to intestinal epithelial NR3C1 expression.

As shown above, the deleterious effects of budesonide in DSS colitis can be partly prevented by eliminating its epithelial actions, suggesting an improved efficacy:toxicity profile in NR3C1$^{\Delta IEC}$ mice. To verify this hypothesis we tested the effect of high dose (60 µg d^{-1}) budesonide in DSS colitis. This dose has been previously to correspond to the supratherapeutic range of budesonide (Ocon *et al.*, 2016). As anticipated, mice were euthanized early (namely on day 5) for ethical reasons. This was expected due to the aforementioned harmful effects of budesonide in DSS colitis, particularly at high doses.

Significant body weight loss was noted in both groups (Figure 6A), along with significant mortality, which interestingly only affected the females in the WT group (5/11, Figure 6C). Therefore we decided to analyze all results by sex (i.e. 4 groups). Body weight loss was found to be lower in WT male mice compared to the other groups (Figure 6A). DAI was lower in this group as well, albeit without reaching statistical significance (Figure 6B). Female mice exhibited a shorter colon, independently of genotype (Figure 6D, E).

Consistent with the above, the relative colonic expression of *S100a8*, *S100a9*, *Tnf* and *Il6* was highest in female WT mice, with the other groups (i.e. male WT mice and male/female NR3C1$^{\Delta IEC}$ mice) showing generally comparable results (Figure 7A). Male WT mice exhibited a certain tendency for the lowest expression of inflammatory markers, including decreased *S100a8 vs.* male NR3C1$^{\Delta IEC}$. A similar profile was obtained when STAT3 phosphorylation and S100A9 expression were assessed by western blot (Figure 7B). The expression of *Nr3c1* and *Tsc22d3* was higher in male WT than in female WT mice (Supplementary Figure 4). Finally, the proliferative status of the epithelium was assessed, given the potent actions of GCs at this level. Not surprisingly, NR3C1$^{\Delta IEC}$ mice presented an increased expression of *Ccnd1*, as well as protein levels of cyclin D1, while *Myc* expression was unaffected (Fig 7A, B).

Hence WT mice exhibited a dramatic sex-based differential response to high dose budesonide, with females showing a heightened inflammatory state. This low response was reversed in female NR3C1$^{\Delta IEC}$ mice, suggesting that the intestinal epithelial NR3C1 receptor is responsible of the deleterious effects of BD during colitis, at least in female mice.

3.5 NR3C1$^{\Delta IEC}$ organoids exhibit a proinflammatory phenotype

Jejunal organoids were generated from WT and NR3C1$^{\Delta IEC}$ mice and gene deletion was subsequently induced *in vitro*. As expected, expression of the GC receptor almost completely vanished in the latter group (Figure 8A, up, left). Organoid cultures were exposed to TNF in the presence of FBS to activate epithelial cells in an inflammatory fashion. TNF had no effect on GC receptor expression (Figure 8A), but caused an augmented expression of chemokines such as *Cxcl1* and *Ccl2*. This increase was more pronounced in the absence of the GC receptor, in line with its well-known anti-

inflammatory effects. Moreover, the lack of the receptor provoked a hyperproliferative state, characterized by an overexpression of *Myc* and *Ccdn1*, only in basal conditions in the latter case (Figure 8B).

4. Discussion

The response of NR3C1$^{\Delta IEC}$ mice to DSS is clearly time dependent, with enhanced inflammation observed early after DSS administration and protection against inflammation in later stages. Since epithelial *Nr3c1* deletion causes mild inflammation after 7 days of the first tamoxifen administration (Aranda *et al.*, 2019), i.e. only one day earlier than the +1 d in the experiment reported here, the initial inflammatory response may represent simply an overlapping condition. *Nr3c1* knockout intestinal epithelial cells exhibit a proinflammatory phenotype *in vitro*, consistent with our previous observation based on RNAseq analysis of primary epithelial cell samples in basal conditions (Aranda *et al.*, 2019). This factor and the occurrence of barrier defects probably contribute to the initial inflammatory response. It should be noted that the phenotype was comparatively more prominent in DSS exposed mice: bloody feces, microscopically visible epithelial lesions, increased leukocyte infiltration and elevated pro-inflammatory markers.

On the other hand, colitis was attenuated after 7 d of DSS exposure in NR3C1$^{\Delta IEC}$ mice, i.e. the standard protocol, as evidenced by histology, reduced epithelial S100A9 expression and STAT3 phosphorylation, improved DAI, and so forth. Because of the time dependent effects of NR3C1 epithelial deletion, we carried out the survival experiment, which provided further evidence of the deleterious role of this receptor in DSS colitis, as mortality was dramatically reduced in knockout mice. Obviously these differences in phenotype must be attributed to the effects of endogenous GCs, of either systemic or local origin. In this regard, intestinal corticosterone production was found to correlate with protection against colitis. Intestinal steroidogenesis has previously been established to have such protective effects (Cima *et al.*, 2004; Coste *et al.*, 2007; Noti *et al.*, 2010a; Kostadinova *et al.*, 2014). In keeping with this notion, colonic steroidogenesis was maximal at 7 d post-DSS in both WT and NR3C1$^{\Delta IEC}$ mice. However, compared to WT mice, steroidogenesis was upregulated in knockout mice (although depressed initially, possibly adding up to the early phenotype). Based on RT-qPCR analysis, increased corticosterone levels may be attributed to both 11-dehydrocorticosterone precursor conversion (*Hsd11b1*) and augmented synthesis (*Cyp11a1*, *Star*). *De novo* synthesis has been proposed to be more important in the intestine, while precursor conversion plays a major role in the lung (Coste *et al.*, 2007; Hostettler *et al.*, 2012). It should be noted that

precursor conversion is presumably negligible in our culture conditions. The expression of *Cyp11a1* has been reported to increase in experimental colitis (Finnie *et al.*, 1996).

A second possible mechanism at play is related to the effect of NR3C1 knockout on epithelial proliferation. A hyperproliferative state may have a beneficial effect for epithelial repair, which is an important factor in the context of colitis. GCs inhibit epithelial proliferation, and accordingly NR3C1$^{\Delta IEC}$ mice display an enhanced intestinal epithelial proliferative state (Aranda *et al.*, 2019). Increased expression of *Myc* and *Ccdn1* by NR3C1$^{\Delta IEC}$ jejunal organoids is consistent with such observations, although differences were not noted for *Ccdn1* in proinflammatory conditions. Of note, organoids were obtained from a pair of animals per group and maintained for different experiments, thus allowing a reduction in the number of animals used in the study. Ki67 staining of colonic samples revealed a change in the pattern of epithelial proliferation, as it was more restricted to the base of the crypts in NR3C1$^{\Delta IEC}$ mice, with no discernable alteration of the overall signal, possibly due to the confounding effects of colitis.

Our results additionally show that the intestinal epithelial deletion of NR3C1 affects the response of DSS colitis to budesonide. The most striking finding in this regard is the observation that the enhanced weight loss and DAI increase induced by a 'standard' dose of budesonide is essentially prevented in NR3C1$^{\Delta IEC}$ mice, suggesting that epithelial actions account for this deleterious effect. It is important to note that despite the occurrence of these harmful effects, budesonide showed also evidence of benefit in DSS colitis, as shown by reduced MPO at 7 d (Figure 3C), and by a reduction of various inflammatory parameters at 1 d. This is in keeping with previous observations. It is noteworthy that epithelial proliferation was attenuated by BD in both WT and knockout mice, indicating an indirect mechanism. These observations prompted us to test the effects of a high dose of budesonide. We found that NR3C1$^{\Delta IEC}$ mice were protected from death, as no mice died in this group vs. 5 WT mice (all female). In this experiment female WT mice exhibited increased expression of inflammatory markers and weight loss, and additionally tended to have a higher DAI. The favorable phenotype of WT male mice may be related to upregulated expression of *Nr3c1* and of the proximally regulated gene *Tsc22d3* (encoding glucocorticoid-induced leucine zipper, GILZ), suggestive of augmented GC dependent signaling. In experimental rat sepsis males respond to lower dexamethasone doses, consistent with this hypothesis (Duma *et al.*, 2010). As a result

epithelial *Nr3c1* knockout had sex-dependent effects, so that it was protective in female mice, while it was neutral or weakly harmful in males. This may reflect either sex-dependent inflammation or a sex-dependent effect of budesonide. Of note, weight loss was not prevented in this case, suggesting that it is attributable to extraepithelial GC receptors with this high budesonide dose.

In conclusion, deletion of the intestinal epithelial GC receptor has protective effects in DSS colitis (except in the early stages), which may be at least partly attributed to increased local corticosterone production and the lack of deleterious effects at the epithelial level. The therapeutic benefit of budesonide appears to be limited by its epithelial actions. Thus, taken together our results suggest that epithelial GC actions, elicited either by endogenous corticosterone or by exogenous budesonide, have a negative impact in experimental DSS colitis. If this holds true also in human colitis, our study further suggests that GC with diminished effects at this level may be preferable for the management of colitis.

AUTHOR CONTRIBUTIONS.

MAA, CJA, BO and RGP were responsible for acquisition of data and statistical analysis. OMA and FSM did the study concept and design. MAA, OMA and FSM drafted the manuscript. All authors participated in the analysis and interpretation of data and general revision of the paper.

ACKNOWLEDGEMENTS.

The authors thank the helpful comments of Dr. Paloma Pérez (CSIC). This work was supported by funds from the Ministry of Economy and Competitivity, partly with Fondo Europeo de Desarrollo Regional FEDER funds [BFU2014-57736-P, AGL2014-58883-R, SAF2017-88457-R, AGL2017-85270-R] and by Junta de Andalucía [CTS235, CTS164]. BO, CJA and MAA were supported by fellowships from the Ministry of Education. CIBERehd is funded by Instituto de Salud Carlos III.

CONFLICT OF INTEREST

The authors declare no financial or commercial conflict of interest. The authors have received funds and/or support from Amino Up Chemical, Pfizer, Hospira, Sanofi, Biosearch Life, Bioiberica and APC Europe.

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FIGURE LEGENDS

Figure 1. Macroscopic and microscopic appearance of colonic segments after 1 day of DSS administration (n=8). (A) Colonic weight/length ratio. (B) Representative images of the macroscopic appearance of colons. (C) Representative histological sections of colons (hematoxylin & eosin staining). (D) Fecal occult blood in stools. $^{+}P < 0.05$ *vs*. WT.

Figure 2. Colonic and systemic inflammatory state 1 day exposure to DSS (n=8). (A) Relative expression of *S100a8* and *S100a9* in whole colonic tissue assessed by RT-qPCR. *Ppib* and *18S* were used as reference genes. (B) STAT3 phosphorylation expression (left) and S100A9 (right) in colonic tissue by Western Blot. (C) Cytokine release by colon explants by multiplex analysis. (D) Plasmatic levels of IL6 (left) and TNF (right) by multiplex analysis. $^{+}P < 0.05$ *vs*. WT; $*P < 0.05$ *vs* NT. AU: arbitrary units.

Figure 3. Colonic inflammatory state after a 7 day exposure to DSS. (A) Body weight evolution (percentage change, left). DAI (right). Data are representative of two independent experiments (n=5-9 each). (B) Representative images of the macroscopic appearance of colons. (C) Colonic MPO activity (n=5-9). (D) Relative expression of *S100a8* and *S100a9* in whole colonic tissue assessed by RT-qPCR. *Ppib* and *18S* were used as reference genes (n=8). (E) S100A9 expression (left) and STAT3 phosphorylation (right) in colonic IECs enriched samples by Western Blot (n=8). AU= Arbitrary Units. $^{+}$P<0.05 NR3C1$^{\Delta IEC}$ *vs*. WT; *P<0.05 WT BD *vs*. WT; $^{\#}$P<0,05 WT BD *vs*. NR3C1$^{\Delta IEC}$ BD; ^{a}P<0.05 NR3C1$^{\Delta IEC}$ *vs*. NR3C1$^{\Delta IEC}$ BD.

Figure 4. Ki67 immunohistochemistry of colon sections after a 7 day exposure to DSS. Representative images (up). Intensity of staining (bottom, left); Staining diffusion (bottom, right). AU= Arbitrary Units. $^{+}P < 0.05$ *vs*. WT; $*P < 0.05$ *vs* NT.

Figure 5. NR3C1$^{\Delta IEC}$ mice are protected against colitis produced by prolonged DSS exposure and show an increased colitis induced steroidogenic response. (A) Survival curve of control (WT) and NR3C1$^{\Delta IEC}$ male mice exposed to DSS 2.5% for 21 days (n=10 and 9, respectively). (B) Corticorticosterone release by colonic explants measured by ELISA. Data normalized to reference value. Colitis was induced with 2.5% DSS and male mice were sacrificed at day 3, 5 or 7 (n=6, except WT groups euthanized at day 5 and 7

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Figure 6. Effect of high dose (60 µg d^{-1}) budesonide on body weight, DAI, survival, colonic appearance and thickening in colitis induced with 2.5% DSS in NR3C1$^{\Delta IEC}$ and WT mice (n=11 in female WT and male NR3C1 groups and n=10 in the remaining groups). (A) Body weight evolution. (B) Disease Activity Index (DAI). Factors included in DAI are body weight loss, presence of blood in feces and stool consistency. (C) Survival curve. (D) Representative images of the macroscopic appearance of colons. (E) Colon length. $^{+}P < 0.05$ *vs*. WT; $*P < 0.05$ *vs* Female.

Figure 7. Effect of high dose (60 µg d^{-1}) budesonide on colonic inflammatory gene expression in colitis induced with 2.5% DSS in NR3C1$^{\Delta IEC}$ and WT mice (n=11 in female WT and male NR3C1 groups and n=10 in the remaining groups). (A) Relative expression of *Il6*, *S100a8* and *S100a9* (top); *Tnf, Ccnd1* and *cMyc* (bottom) in whole colonic tissue assessed by RT-qPCR. *Ppib* and *18S* were used as reference genes. (B) STAT3 phosphorylation (left) S100A9 (middle) and cyclin D1 expression (right) in colonic tissue by Western Blot. AU= Arbitrary Units. $^{+}P < 0.05$ *vs*. WT; $*P < 0.05$ *vs* Female.

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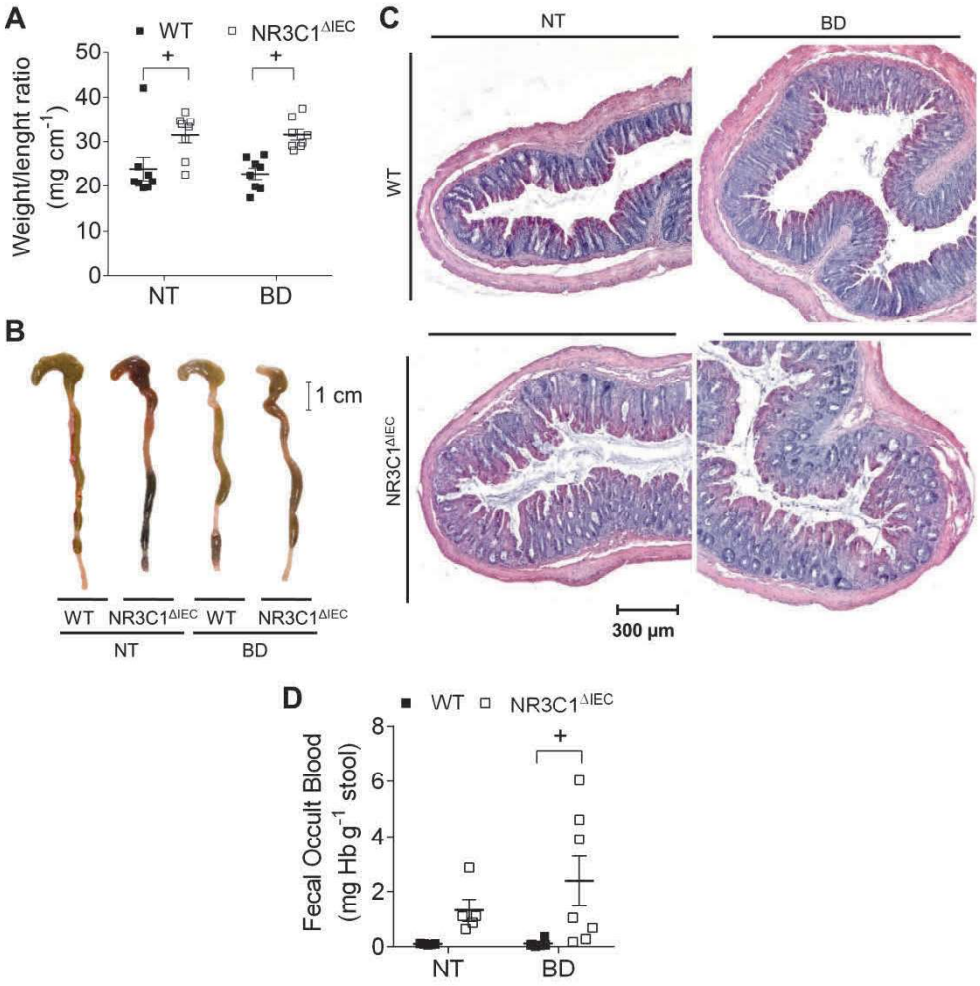

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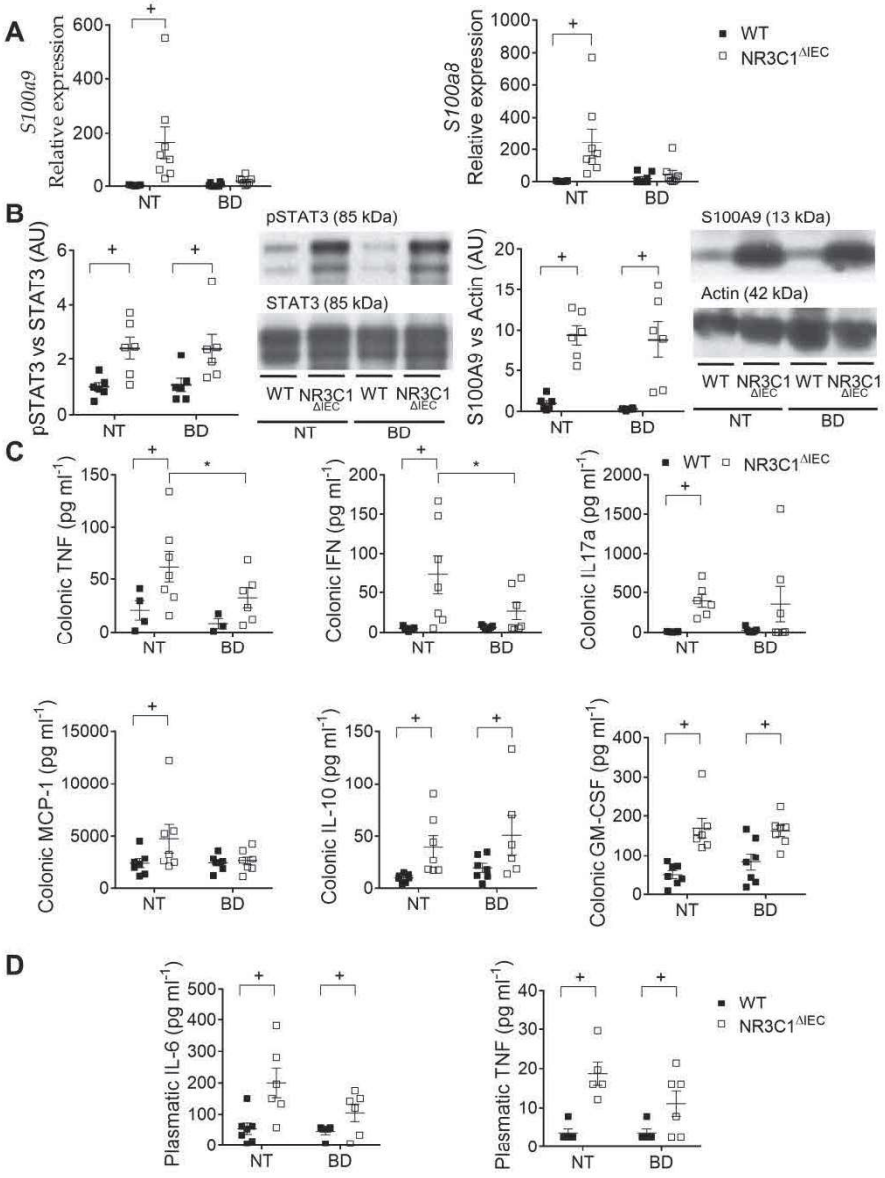

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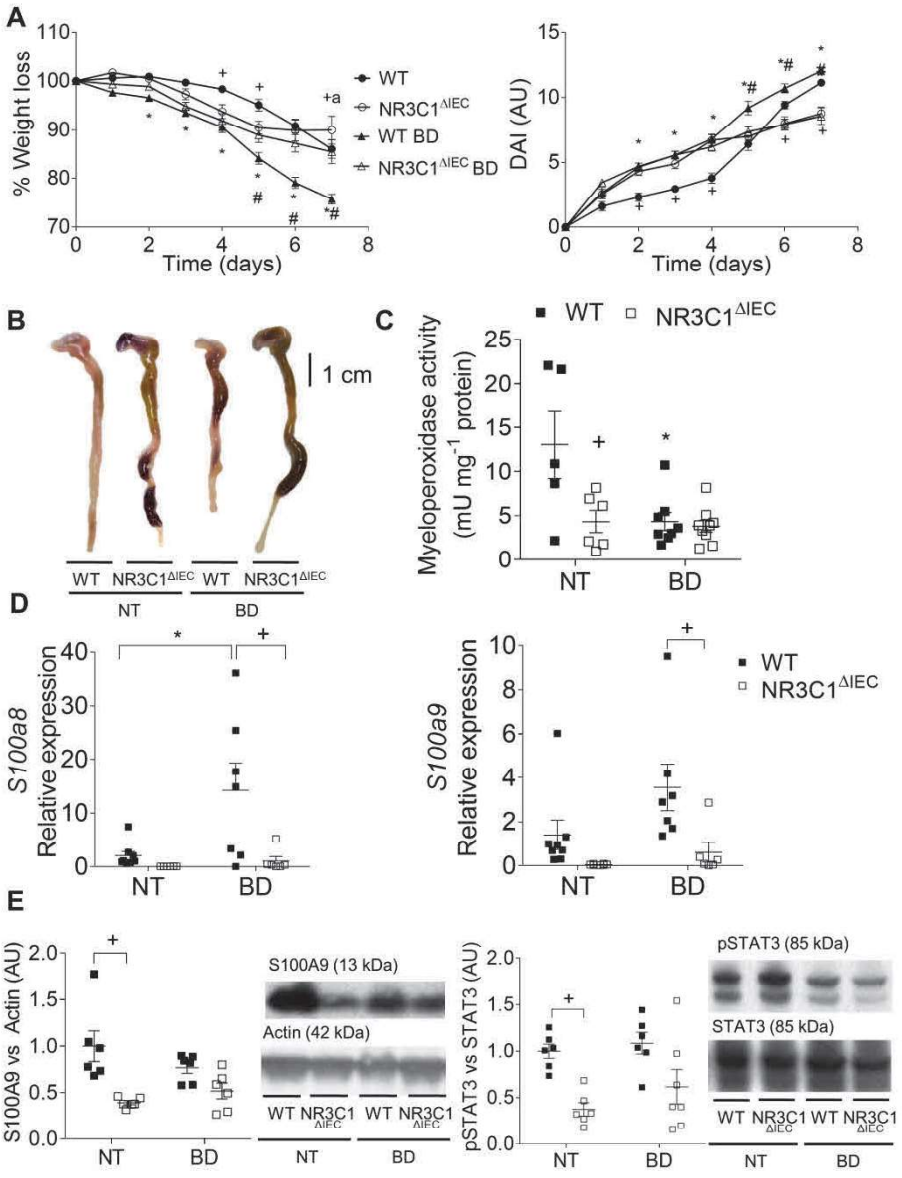

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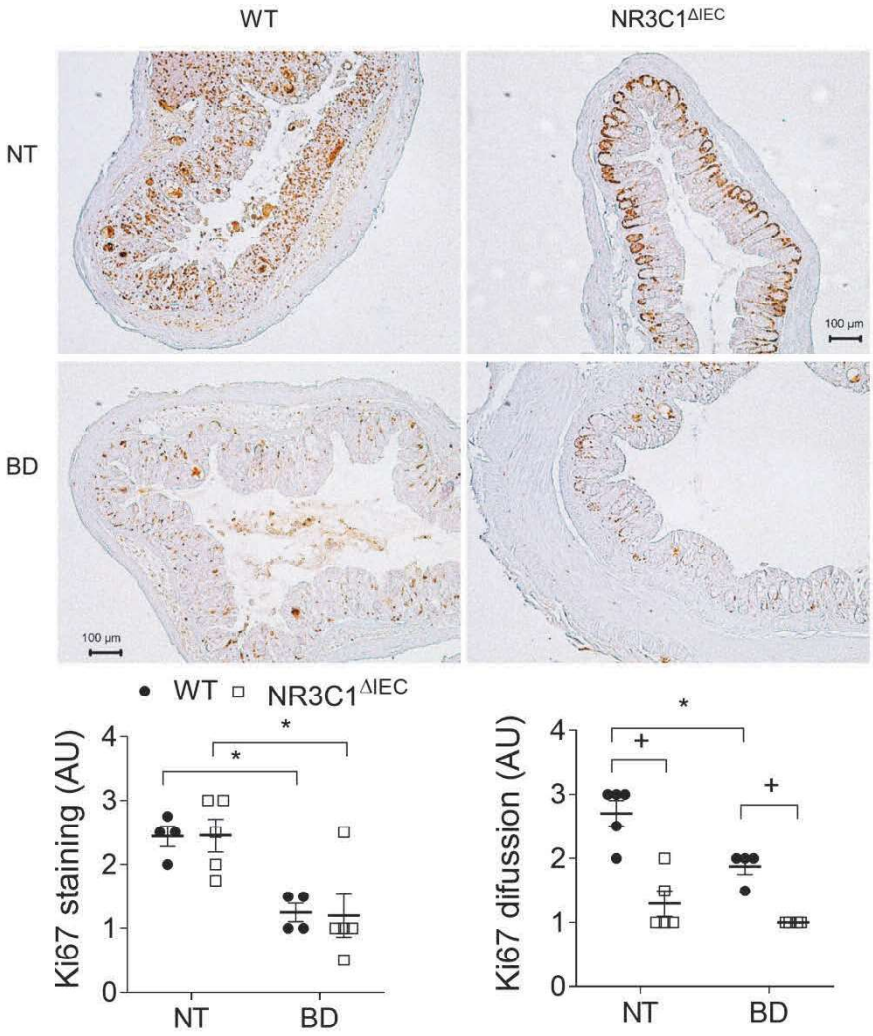

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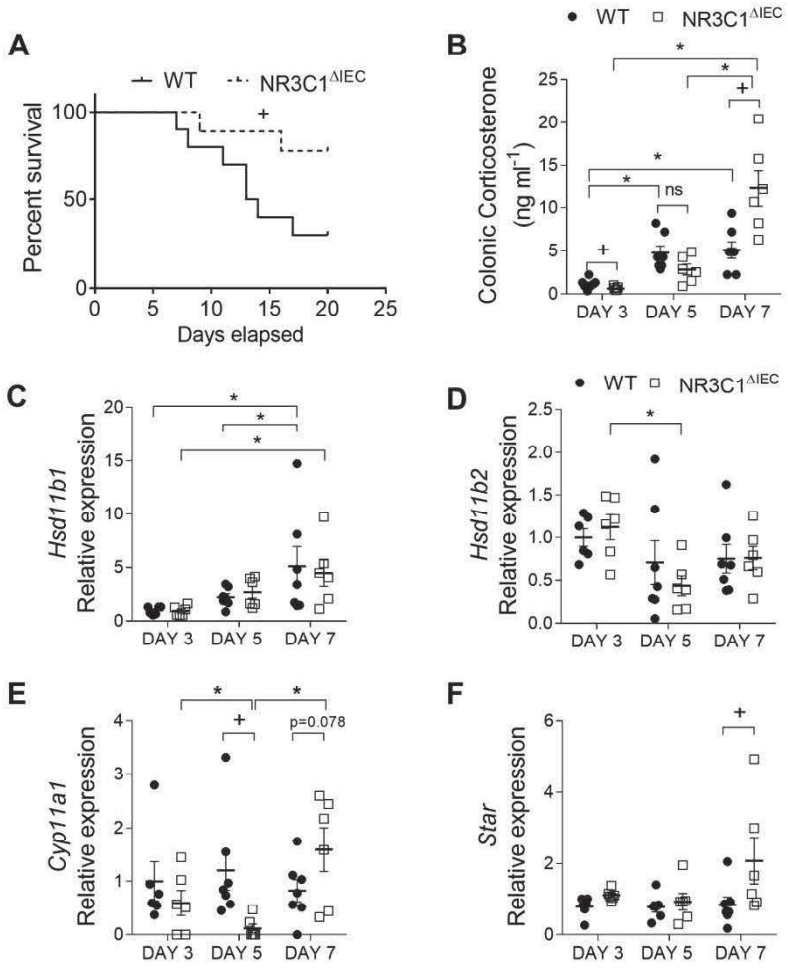

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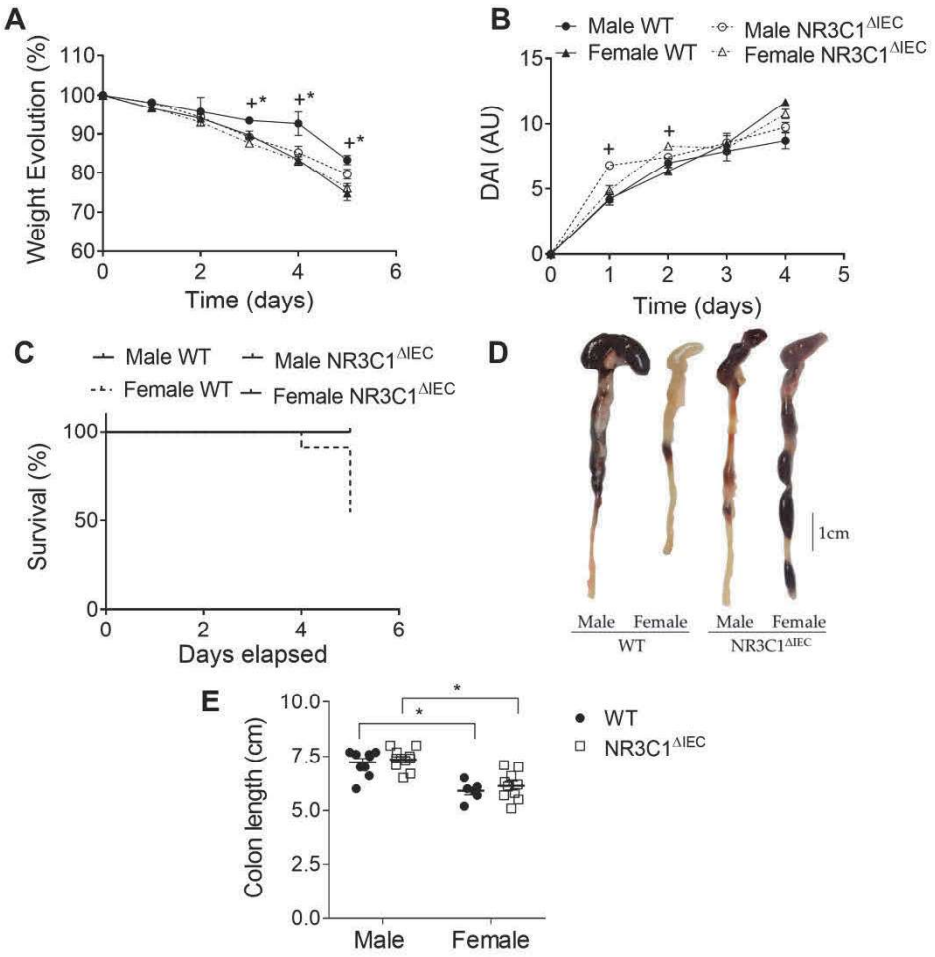

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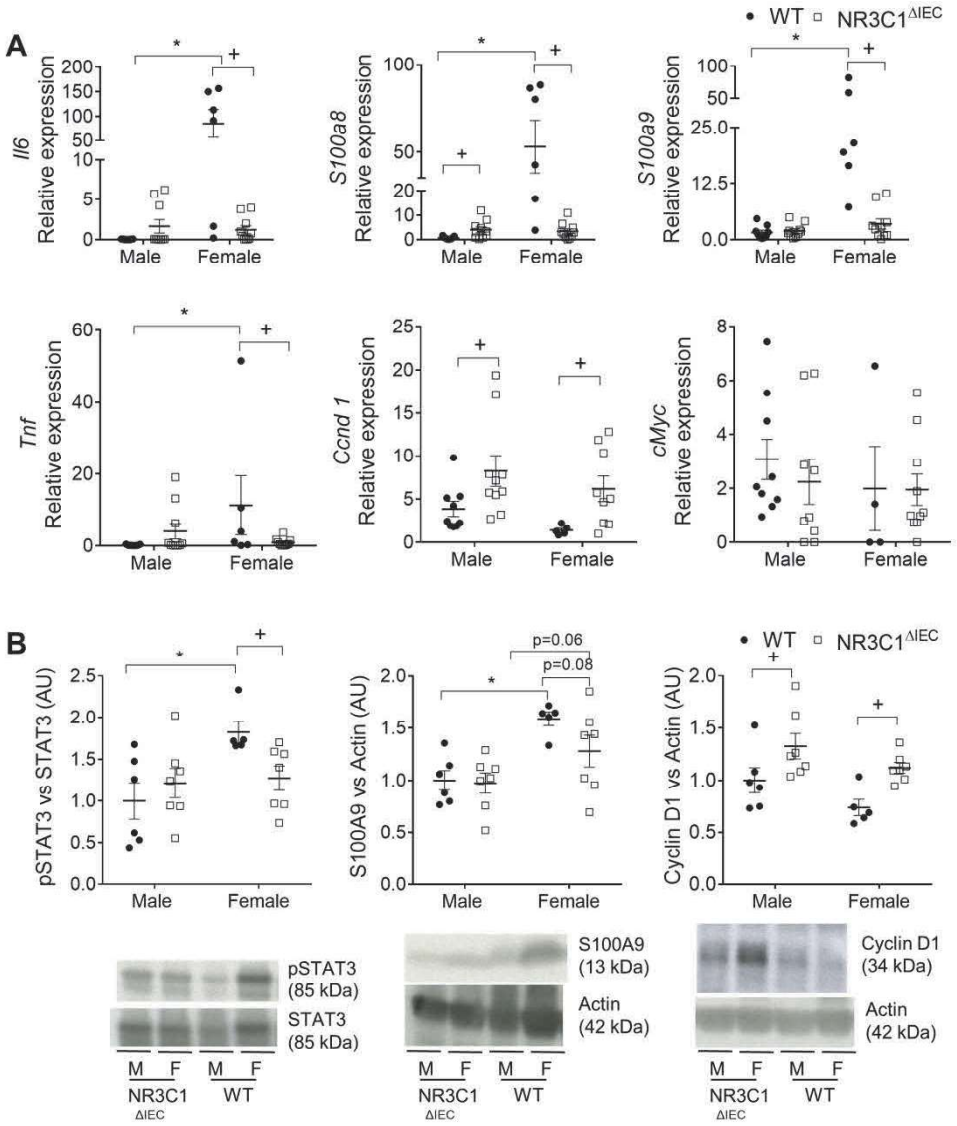

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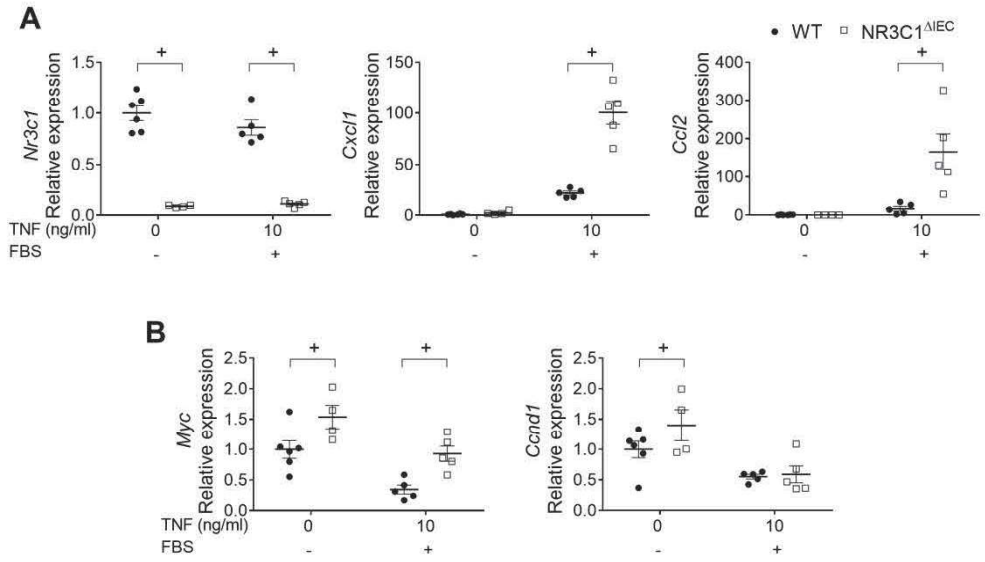

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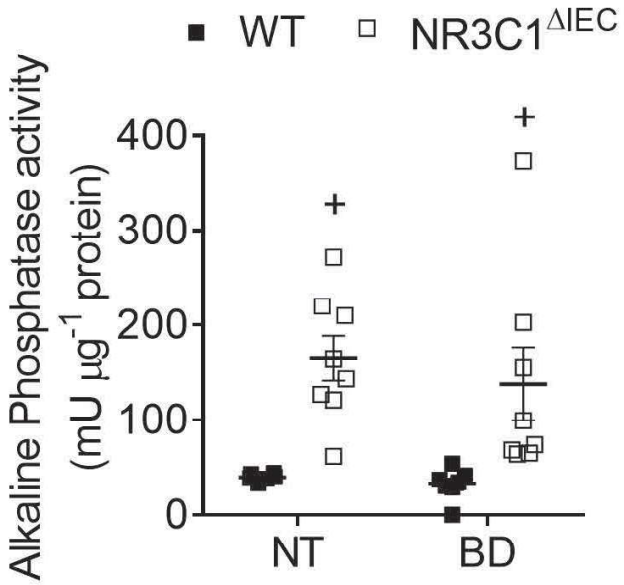
190x140mm (300 x 300 DPI)

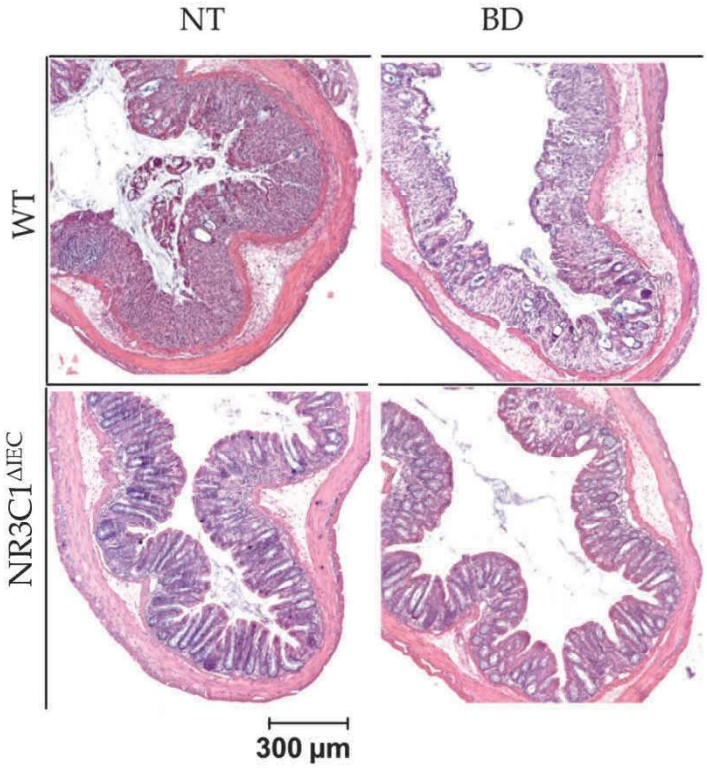
Supplementary Table 1.Primers used in the RT-qPCR analysis.

Gene	Forward 5'-3'	Reverse 3'-5'
18s	TGGTGGAGCGATTTGTCTGG	ACGCTGAGCCAGTCAGTGTACG
Ccl2	CAAGATGATCCCAATGAGTAG	TTGGTGACAAAAACTACAGC
Ccnd1	CTAAGATGAAGGAGACCATTCC	GGTCTGCTTGTTCTCATCC
Cxcl1	CCGAAGTCATAGCCACACTCAAG	ACCAGACAGGTGCCATCAGAG
Cyp11a1	CAGCTGCCTGGGATGTGATTT	ACCAGGGTACTGGCTGAAGT
Hsd11b1	CTCATAGACACAGAAACAGC	TCAAAGGCGATTTGTCATAG
Hsd11b2	CAATAGCACTGCTTATGGAC	CATTAGTCACTGCATCTGTC
Il6	CTCTGGGAAATCGTGGAAAT	TGTACTCCAGGTAGCTATGG
Myc	TTTTGTCTATTTGGGGACAG	CATAGTTCCTGTTGGTGAAG
Nr3c1	CTTCTCTCCTCAGTTCCTAAG	CCAATTCTGACTGGAGTTTC
Ppib	CAAATCCTTTCTCTCCTGTAG	TGGAGATGAATCTGTAGGAC
S100a8	GATGGTGATAAAAGTGGGTG	CTGTAGACATATCCAGGGAC
S100a9	CTTTAGCCTTGAGCAAGAAG	TCCTTCCTAGAGTATTGATGG
Star	GCGGAATATGAAAGGATTAAGG	GTCACTATAGAGTGTTGCTTC
Tnf	CGTGGAACTGGCAGAAGAGG	CAGGAATGAGAAGAGGCTGAGAC
Tsc22d3	CAAGCTGAACAACATAATGC	GCTCAATCTTGTTGTCTAGG



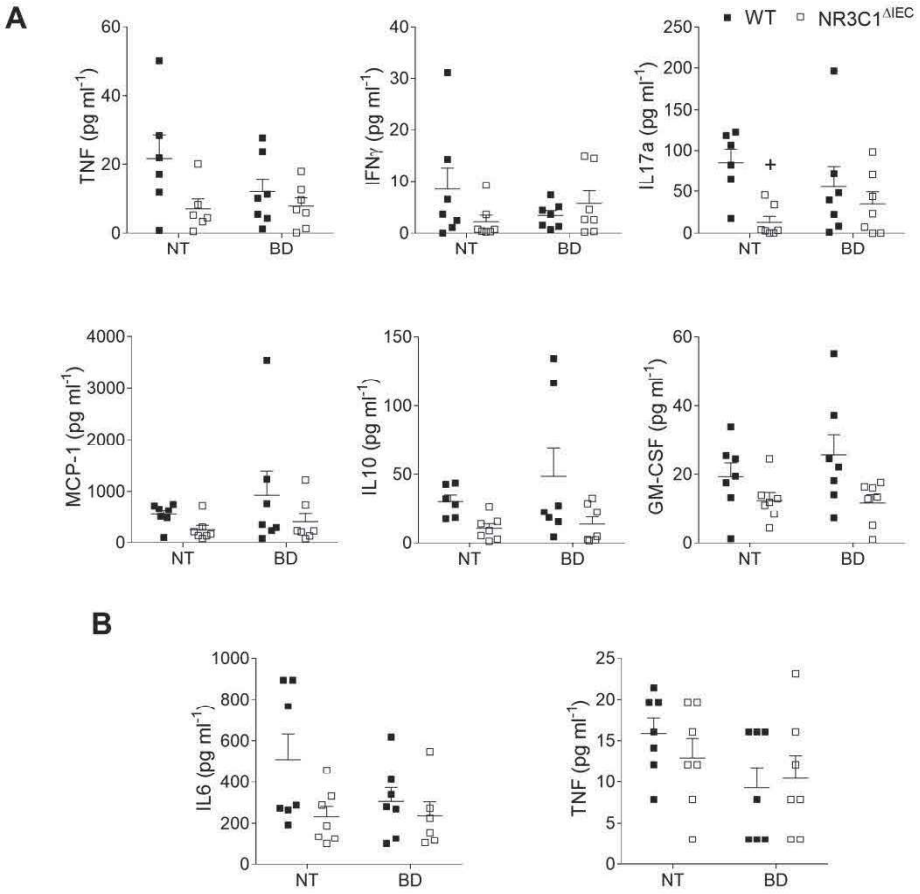
Supplementary Figure 1. NR3C1$^{\Delta IEC}$ and WT mice were exposed to 1 day of DSS 2.5%. Colonic AP activity. Mice were administered 6 ug/day of Budesonide (BD) or not treated (NT) (n=8). $^{+}P < 0.05$ vs. WT.

99x119mm (300 x 300 DPI)



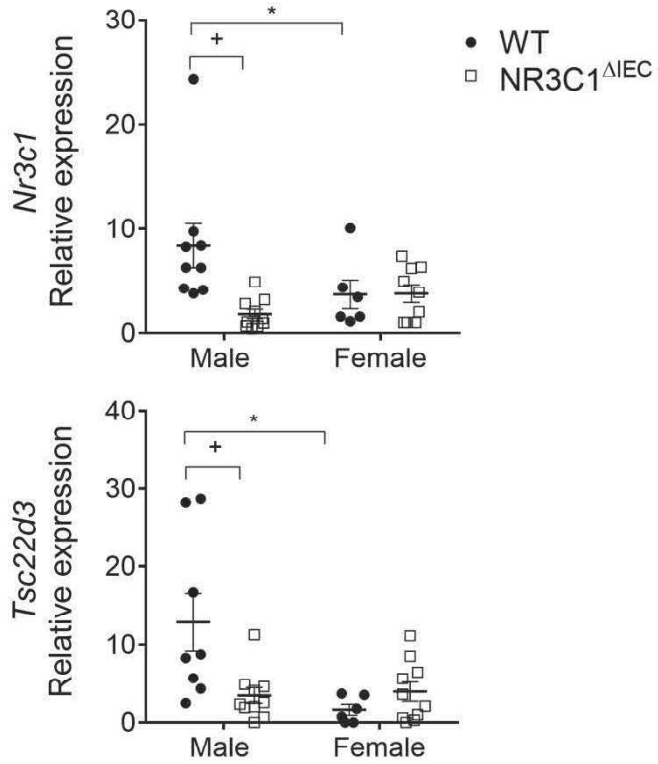
Supplementary Figure 2. NR3C1$^{\Delta IEC}$ and WT mice were exposed to 7 days of DSS 2.5%. Mice were administered with 6 µg/day of Budesonide (BD) or not treated (NT). Representative histological sections of colons (hematoxylin & eosin staining)

140x160mm (300 x 300 DPI)



Supplementary Figure 3. NR3C1$^{\Delta IEC}$ and WT mice were exposed to 7 days of DSS 2.5%. Mice were administered with 6 µg/day of Budesonide (BD) or vehicle (NT, not treated) (n=6-7) A. Cytokine release by colon explants measured by multiplex analysis. B. Plasmatic levels of IL6 (left) and TNF (right) measured by multiplex analysis. $^{+}P < 0.05$ vs. WT; $^{*}P < 0.05$ vs NT.

190x219mm (300 x 300 DPI)



Supplementary Figure 4. NR3C1$^{\Delta IEC}$ and WT mice were exposed to DSS 2.5% and treated with 60 µg/day of Budesonide throughout 5 days (n=9-11). Relative expression of *Nr3c1* (up) *Tsc22d3* (bottom) and in whole colonic tissue assessed by RT-qPCR. *Ppib* and *18S* were used as reference genes. $^{+}P < 0.05$ vs. WT; $^{*}P < 0.05$ vs female.

150x160mm (300 x 300 DPI)