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The plant sterol guggulsterone attenuates inflammation and immune dysfunction in murine models of inflammatory bowel disease

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5 The plant sterol guggulsterone attenuates inflammation
6 and immune dysfunction in murine models of inflammatory bowel disease
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10 **Short title**

11 Guggulsterone and intestinal inflammation
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28 **Disclosure**

29 Non conflict of interest
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32

33 **Author contribution**

34 Andrea Mencarelli, has carried out animal studies, ELISA kit and flow cytometry
35 and the manuscript writing
36
37

38 Barbara Renga carried out quantitative RT-PCR experiments
39

40 Giuseppe Palladino carried out histological analysis
41

42 Stefano Fiorucci and Eleonora Distrutti: designed the study and participate to the
43 manuscript writing
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Abstract

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Inflammatory bowel diseases (IBD) are chronic inflammatory and relapsing diseases of the gut that may manifest as either Crohn's disease (CD) or ulcerative colitis (UC). CD and UC are immunologically different diseases characterized by exacerbated Th1 and Th2 response. T-cell resistance against apoptosis contributes to inappropriate T-cell accumulation and the perpetuation of chronic mucosal inflammation. In the present study we have investigated the effect exerted by guggulsterone (GS) a plant derived steroid isolated from the gum resin of the *Commiphora mukul* tree, in two models of intestinal inflammation induced in mice by *trinitro-benzene sulfonic acid* (TNBS) and *oxazolone*. We provided evidence that E-GS protects mice against development of sign and symptoms of colon inflammation. E-GS effectively attenuated the severity of wasting disease and the fecal score and colon inflammation as assessed by measuring the macroscopic- and microscopic-damage scores. Administration Z-GS failed to ameliorate colon inflammation in TNBS-induced colitis and had a partial effect in *oxazolone*-induced colitis. *In vitro*, mechanistic studies carried out using CD4+ cells isolated from the intestinal *lamina propria* demonstrate that GS effectively regulates the function of effector T cells by modulation cell signaling activation pathway caused by CD3/CD28. The net biological effects resulting from exposure to GS includes attenuation of generation of Interleukin-2 and -4 and interferon- γ as well as T cell proliferation. In conclusion, GS as an anti-inflammatory compound with the capacity to prevent and ameliorate T-cell-induced colitis. These data ground the use of GS, a natural cholesterol-lowering agent, in the treatment of chronic inflammatory diseases.

1. Introduction

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2 Inflammatory bowel disease (IBD) is a chronic progressive and destructive disorder
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4 of the gastrointestinal tract that may manifest as either Crohn's disease (CD) or
5
6 ulcerative colitis (UC) [1-4]. There is circumstantial evidence to link the
7
8 pathogenesis of IBD to a dysfunctional interaction between bacterial *microflora* of
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10 the gut and the mucosal immune system. The normal state of immunologic
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12 tolerance to microbial antigens in the gastrointestinal tract is disturbed either by
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14 the presence of a dysregulated mucosal effector T cell population that overreacts
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16 to usual microbial antigens or, alternatively, by the presence of a defective
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18 mucosal *Treg* cell population that under-reacts to usual microbial antigens such
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20 that even normal effector T cells are not properly modulated [5].
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28 CD and UC are immunologically different diseases. Indeed, CD bears all the
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30 stigmata of an exaggerated CD4+ T helper (Th)1 cell response, characterized by
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32 high interferon (IFN)- γ and interleukin (IL)-12, whereas, in UC, the mucosal immune
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34 response is dominated by the production of IL-5 and IL-13 [6,7]. Several factors
35
36 have been implicated in the unrelenting mucosal inflammation of IBD, prominent
37
38 among them being the presence of a persistently elevated number of activated T
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40 cells in the mucosa of CD and UC patients. These T cells display various defects of
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42 proliferation and apoptosis, and these abnormalities are credited with directly
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44 contributing to the pathogenesis of IBD [8]. Thus, therapeutic approaches
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46 inhibiting T cell proliferation such as steroids, azathioprine/6-MP, calcineurin
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48 inhibitors and anti-IL2 and IL-2 receptor or drugs inducing T cell apoptosis such as
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50 tumor necrosis factor(TNF)- α and CD3 monoclonal antibodies and anti-cytokines
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1 strategies (IL-6 and IL-12) responsible of inhibition of apoptotic pathway in T cells,
2 are effective in treating CD and UC [9-14].
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5 Guggulsterone (GS) [4,17(20)-pregnadiene-3,16-dione] is a plant derived
6 steroid isolated from the gum resin of the *Commiphora mukul* tree, termed
7 guggulipid, extensively used in the *Ayurvedic* medicine to treat conditions
8 associated with inflammation such as hyperlipidemia, obesity, and arthritis [15-18].
9
10 The active substances in guggulipid are the pregnane plant sterols cis-
11 guggulsterone (E-GS) and trans-guggulsterone (Z-GS). GS has anticancer potential
12 as indicated by its ability to suppress the proliferation of a wide variety of human
13 tumor cell lines. GS as also been shown to induce apoptosis and reverse
14 chemoresistance [20-23]. The activity of GS has been suggested to be mediated
15 by antagonism of metabolic nuclear receptors [24]. The two stereoisomers of the
16 plant sterol, bind to the farnesoid-x-receptor, the mineralocorticoid receptor, the
17 androgen, the glucocorticoid and progesterone receptors at nanomolar
18 concentrations. However, in cell-based functional cotransfection assays, GSs
19 behave as an antagonist for all these receptors [24]. Agonist activity has been
20 demonstrated for the pregnane-x-receptor and was also demonstrated with
21 estrogen receptor alfa, however the potency is very low [24]. GS exerts potent
22 anti-inflammatory effects by suppressing the activation of the transcription factor
23 NF-kappa B in response to different pro-inflammatory mediators including TNFa
24 and IL-1 β [23-25].
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54 Here, we report that GS was effective in reducing inflammation in two
55 rodent models of colitis in mice. We demonstrated that GS reduced colonic
56 inflammation and suppressed the mediators of adaptive immunity. Mechanistic
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1 studies carried out using CD4+ cells derived from intestinal lamina propria
2 demonstrate that GS effectively regulates the function of effector T cells.
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2. Methods and Materials

2.1. Animals:

BALB/c and SCID mice, 8–10 weeks of age, were obtained from Harlan Nossan (Udine, Italy). Mice were housed under controlled temperatures (22°C) and photoperiods (12:12-hour light/dark cycle). The mice were allowed unrestricted access to standard mouse chow and tap water. They were allowed to acclimate to these conditions for at least 5 days before inclusion in an experiment. Protocols were approved by the University of Perugia Animal Care Committee.

2.2. Reagents

Purified myeloperoxidase (MPO), tri-methylbenzidine, dichlorofluorescein diacetate, trinitro-benzene sulfonic acid, oxazolone and Z-guggulsterone were obtained from Sigma-Aldrich (Milan, Italy). E-guggulsterone were purchased from Steraloids (Newport, R.I.)

2.3. Experimental Procedures

2.3.1. Induction of Colitis

Mice were lightly anesthetized by intraperitoneal injection of 100 μ l of ketamine/xylazine solution (Mix 0.6 ml of ketamine, 100 mg/ml, 0.4 ml of xylazine, 20 mg/ml and 4 ml of saline) per 10 g body weight and then administered intrarectally (i.r.) with the haptening agents: TNBS (1 mg/mouse) or oxazolone (1.5 mg/mouse) dissolved in ethanol 50%, via a 3.5 French (F) catheter equipped with a 1-ml syringe. The catheter was advanced into the rectum for 4 cm and then the haptening agent was administered in a total volume of 150 μ l. To ensure distribution of the agent within the entire colon and cecum, mice were held in a vertical position for 30 seconds. Control mice were administered by an ethanol solution using the same technique. GSs were dissolved each day in 100 mM DMSO, diluted in methylcellulose 1% and administered intraperitoneally at the final volume of 200 μ L/mouse. TNBS/oxazolone group mice received the vehicle alone (1% methylcellulose in a final volume of 200 μ L/mouse) every day. In a further set of experiments mice were administered prednisolone 10

1 mg/kg/day i.p. In all protocol studies, mice were monitored for the appearance
2 of diarrhea, loss of body weight, and overall mortality. Five days after
3 TNBS/oxazolone administration, surviving mice were sacrificed, colons were
4 removed and immediately snap-frozen on liquid nitrogen and stored at -80°C until
5 use. The macroscopic appearance was analyzed considering the presence of
6 indurations, edema, thickness and evidence of mucosal hemorrhage. Grading
7 was performed in a blinded fashion.
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14 **2.3.2. Histological Grading of Colitis**

15 For histological examination, tissues were fixed in 10% buffered formalin
16 phosphate, embedded in paraffin, sectioned, and stained with Hematoxylin and
17 eosin (H&E). Histology images were captured by a digital camera (SPOT-2;
18 Diagnostic Instruments Inc, Burroughs, MI) and analyzed by specific software (Delta
19 Sistemi, Rome, Italy). The degree of inflammation on microscopic cross sections
20 was graded semiquantitatively from 0 to 4 (0, no signs of inflammation; 1, very low
21 level; 2, low level of leukocyte infiltration; 3, high level of leukocyte infiltration, high
22 vascular density, and thickening of the colon wall; and 4, transmural infiltration, loss
23 of goblet cells, high vascular density, and thickening of the colon wall). Grading
24 was performed in a blinded fashion.
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39 **2.3.3. Real-Time PCR**

40 Quantization of the expression level of selected genes was performed by
41 quantitative real-time PCR (qRT-PCR). Total RNA were obtained from small colon
42 pieces (50 mg) and isolated with TRIzol reagent (Invitrogen, Milan, Italy),
43 incubated with DNase I and reverse-transcribed with Superscript II (Invitrogen)
44 according to manufacturer specifications. For real-time PCR, 100 ng of template
45 was used in a 25- μl reaction containing a 0.3 μM concentration of each primer and
46 12.5 μl of 2x SYBR Green PCR Master Mix (Bio-Rad Laboratories, Hercules, CA). All
47 reactions were performed in triplicate using the following cycling conditions: 2 min
48 at 95°C , followed by 50 cycles of 95°C for 10 s and 60°C for 30 s using an iCycler iQ
49 instrument (Bio-Rad Laboratories). The mean value of the replicates for each
50 sample was calculated and expressed as cycle threshold (C_T). The amount of gene
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1 expression was then calculated as the difference (ΔC_T) between the C_T value of
2 the sample for the target gene and the mean C_T value of that sample for the
3 endogenous control (GAPDH). Relative expression was calculated as the
4 difference ($\Delta\Delta C_T$) between the ΔC_T values of the test and control samples for each
5 target gene. The relative level of expression was measured as $2^{-\Delta\Delta C_T}$. All PCR
6 primers were designed using the software PRIMER3-OUTPUT using published
7 sequence data obtained from the NCBI database.
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14 Mouse primers were as follows:

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17 mGAPDH: CTGAGTATGTCGTGGAGTCTAC and GTTGGTGGTGCAGGATGCATTG;

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20 mL-2: AACTCCCCAGGATGCTCAC and CGCAGAGGTCCAAGTTCATC;

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23 mL-4: CCTCACAGCAACGAAGAACA and ATCGAAAAGCCCCGAAAGAGT;

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26 mL-6: CCGGAGAGGAGACTTCACAG and TCCACGATTCCCAGAGAAC;

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29 mL-10: GCTGGACAACATACTGCTAACC and CTGGGGCATCACTTCTACCA;

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32 mINF γ : GCGTCATTGAATCACACCTG and GACCTGTGGGTGTTGACTC;

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35 mTNF α : ACGGCATGGATCTCAAAGAC and GTGGGTGAGGAGCACGTAGT

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38 mTGF β : TGGCTTCAGCTCCACAGAGA and TGGTTGTAGAGGGCAAGGAC.
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41 **2.4. Purification of colon lamina propria CD4⁺ cells**

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44 Colonic lamina propria T cells were isolated from colon mice (N=10) as previously
45 described [26]. In brief, after excision of all visible lymphoid follicles, colons were
46 washed in calcium- and magnesium-free HBSS and treated with 1 mM EDTA in PBS
47 for 20 min to remove the epithelium. The tissue was then digested with type IV
48 collagenase (Sigma) for 20 min in a shaking incubator at 37°C; this step was
49 repeated twice. The released cells were then layered on a 40%–100% Percoll
50 gradient (Pharmacia, Uppsala, Sweden) and spun at 1,800 rpm to obtain the
51 leukocyte-enriched population at the 40%–100% interface.
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56 An enriched lamina propria CD4⁺ T-cell population was obtained by negative
57 selection with magnetic beads (magnetic-activated cell sorting [MACS]; Miltenyi
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1 Biotech, Auburn, CA). The purity of the cell preparations (>95%) was determined by
2 flow cytometry (Coulter Epics XL; Beckman Coulter, Fullerton, CA). LAMINA
3 PROPRIA CD4⁺ T cells were then suspended in complete medium (RPMI 1640, 10%
4 heat-inactivated fetal calf serum, 3 mmol/L L -glutamine, 10 mmol/L HEPES buffer,
5 10 µg/mL penicillin, 100 U/mL streptomycin, and 0.05 mmol/L 2-mercaptoethanol)
6 and cultured at a concentration of 2 × 10⁶ cells/ml.
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11 12 13 **2.4.1. Cytokine production and T cell proliferation** 14

15 Lamina propria CD4⁺ T cells were obtained from mice 5 days after TNBS or
16 oxazolone administration. The cells were placed for 48 hours into uncoated
17 culture wells (to measure production by unstimulated cells) or into wells containing
18 immobilized murine anti-CD3ε 5 µg/mL MAb (clone 145-2C11; Pharmingen) and 5
19 µg/mL soluble anti-CD28 antibody (clone 37.51; Pharmingen) alone or in
20 combination with GS (10µM). At the end of incubation culture supernatants
21 were harvested and assayed for cytokine concentration by specific enzyme-
22 linked immunosorbent assay kits from Biosource (Invitrogen, Milan, Italy). For
23 assessment of cell proliferation cells were pulsed for 6 h with [³H] thymidine (0.5
24 µCi/well), harvested on glass fiber filters and radioactivity counted (in cpm) by a
25 liquid scintillation system.
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39 **2.5. Measure of pJNKS, pP38 and pAKT(ser473) activity** 40

41 Lamina propria CD4⁺ T cells were obtained from mice sacrificed 5 days after TNBS
42 or oxazolone administration. The cells (2 × 10⁶ /mL) placed for into uncoated
43 culture wells (naive cells) or into wells containing immobilized murine anti-CD3ε 5
44 µg/mL mAb (clone 145-2C11; Pharmingen) and 5 µg/mL soluble anti-CD28
45 antibody (clone 37.51; Pharmingen) alone or in combination with GS (100-10 µM).
46 GS, were prepared freshly in 10mM DMSO and subsequently serial dilution were
47 made in complete medium. Cells were harvested at pre-specified time points
48 and JNKs1/2 (pThr183/Tyr185), p38MAPK(pTyr180/182) and pAKT(ser473)
49 determination by specific enzyme-linked immune-sorbent assay kits from
50 Biosource (Invitrogen, Milan, Italy). The data were represented µg of protein.
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2.6 Measurement of apoptosis and reactive oxygen species by T cells

The percentage of apoptotic nuclei of lamina propria CD4⁺ T cells incubated with GSs was assessed by using the propidium iodide (PI) technique [27] using a flow cytometer (Coulter Epics XL).

Generation of intracellular reactive oxygen species (ROS) was measured by loading the cells with the fluorogenic probe dichlorofluorescein diacetate (DCFH-DA). DCFH-DA is deacetylated in cells, where it can react quantitatively with intracellular radicals, mainly H₂O₂, to be converted to its fluorescent product, 2',7'-dichlorofluorescein, which is retained in the cells and thus provides an index of cell cytosolic oxidation. At the end of the incubation periods, 20 μmol/L of DCFH-DA was added for 30 minutes at 37°C, and analyzed by using a flow cytometer. The results were expressed as delta of increase of mean fluorescence intensity compared to cells treated with anti-CD3 anti-CD28 alone.

2.7. Flow cytometric measurement of mitochondrial membrane potential

Integrity of mitochondrial membrane was assessed by measuring the mitochondrial membrane potential ($\Delta\psi_m$) by flow cytometry using the aggregate-forming lipophilic cation JC-1 as a substrate. After eliminating small (ie, noncellular) debris, 50,000 events were collected for each analysis. Results are expressed either as the mean aggregate fluorescence alone (red).

2.8. Cytochrome C release and caspase activity

Migration of cytochrome c into the cytosol is a measure of mitochondrial membrane injury. To obtain cytosolic fractions, lamina propria-derived CD⁺ cells were washed twice in ice-cold PBS, resuspended in cold buffer (1.28 M NaCl, 50.0 mM KCl, 50.0 mM MgSO₄, 13.0 mM CaCl₂, 0.5 M HEPES, 1.0 mM phenylmethylsulfonyl fluoride, 10% vol 2.5 M sucrose, 10.0 mM 1,4-dithiothreitol, 1.0 mM reduced glutathione, and 1% glycerol) and homogenized mechanically. The total cellular extract was centrifuged at 2000 rpm for 3 minutes at 4°C and the cytosolic supernatant recovered and analyzed for the presence of cytochrome C by Cytochrome c Oxidase Assay Kit (Sigma-Aldrich; Milan, Italy).

1 Caspase 3, 8 and 9 activity was measured by a specific caspase fluorometric
2 protease assay according to the manufacturer's instructions (ApoAlert; Clontech,
3 Palo Alto, CA).
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7 **2.9. Statistical analysis**

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10 All values are expressed as the mean \pm SE of n mice per group. Comparisons of
11 more than 2 groups were made with a 1-way analysis of variance with post hoc
12 Tukey tests. Differences were considered statistically significant if P was <0.05 .
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3. Results

3.1. Anti-Inflammatory effects of E-GS in a Th1-mediated model of colitis

Murine TNBS colitis is thought to be a model of Th1-mediated disease with dense infiltrations of lymphocytes/macrophages in the lamina propria and thickening of the colon wall [28-31]. In order to assess whether GS could exert immunomodulatory activity, mice administered TNBS were treated with E-GS and Z-GS at the dose of 30 mg/kg/day for 5 days. The results of these experiments demonstrates that administration of E-GS effectively attenuates colitis development as measured by assessing local and systemic signs of inflammation. Thus E-GS effectively protected against development of the wasting disease (Figure 1D) (n=8-10, P< 0.05), and appearance and severity of diarrhea measured as a fecal-score, but also attenuated the macroscopic- and microscopic-damage scores (Figure 1A-B-C-E) (n=8-10, P< 0.05 versus TNBS group). In contrast to E-GS, its stereoisomer was significantly less effective. Figure 1F illustrates a representative image of histopathological analysis. Compared with colons of mice given vehicle alone, colons obtained from mice administered TNBS show an extensive cellular infiltrate, submucosal edema, and large areas of epithelial erosion that were reduced by E-GS treatment. Furthermore, the E-GS treatment (30 mg/kg) significantly attenuated the TNBS-induced increase of expression colonic expression of inflammatory and immune mediators including INF γ , IL-2, IL-6, TNF α and TGF β (Figure 2) (n=5, P< 0.05). The effect exerted by E-GS was dose-dependent and was lost in animal administered E-GS at the dose of 5 and 15 mg/kg/day (supplementary figure 1, on line).

3.2. Anti-Inflammatory effects of E-GS in Th2 model of IBD

We have then assessed whether protection exerted by GS against colitis development was maintained in a model of Th2 mediated disease. For this purpose we used the *oxazolone* model of colitis, a model of colon inflammation that shows similarities with human UC [31-33]. Administering *oxazolone*-treated mice with E-GS (30 mg/kg) effectively protected against the development of wasting disease and attenuated colon inflammation as measured by assessing

1 the macroscopic and microscopic scores and neutrophil infiltration (figure 3A-B-
2 C-D-E) (n=8-10, P< 0.05). In contrast to the E-GS, Z-GS (30 mg/kg) exerted no
3 protective effect also in the oxazolone model (Figure 2 A-B-C-D-E) (n=8-10, P<
4 0.05). In addition, E-GS treatment effectively reduced the expression of pro-
5 inflammatory genes IL-2, IL-4, IL-6 and TNF α in the colon of oxazolone treated mice
6 oxazolone (n=5, P< 0.05).
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14 **3.3. E-GS and Innate immunity model of IBD**

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16 Because E-GS administration reduced immune-cellular infiltration in the lamina
17 propria of balb/c mice, experiments were carried out to further define the immune
18 compartment mediating E-GS actions *in vivo*. For this purpose, we tested E-GS (30
19 mg/kg) in T and B cell-deficient SCID mice, in which innate immune cells provide
20 important triggers for TNBS colitis [34]. SCID mice developed TNBS colitis as
21 illustrated in Figure 3, but, in this experimental model, E-GS was unable to revert
22 local and systemic signs of inflammation caused by TNBS administration. These
23 findings suggest that E-GS, at least partially, acts directly on cells of adaptive
24 immunity *in vivo*.
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36 **3.4. E-GS attenuates activation of lamina propria CD4+ cells**

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38 Because results from SCID mice suggest that GSs might act on cells of adaptive
39 immunity we have then carried out a series of *ex vivo* experiments to investigate
40 the functional effects of GSs on *lamina propria*-derived T cells obtained from
41 mice treated with TNBS or oxazolone. Exposure to these chemical agents results in
42 a T cell polarization and generation of CD4+ cell clones with a Th1 or Th2 profile.
43 Exposure of TNBS-primed CD4+ cells to E-GS (10 μ M) attenuated IL-2 and INF γ
44 production caused by anti-CD3 and anti-CD28. In addition, E-GS effectively
45 inhibited IL-2 and IL-4 production by oxazolone-primed cells exposed to the
46 same mixture of activating antibodies (Figure 3A,B,D,E) (n=3, P< 0.05 versus CD3-
47 CD28 group). Furthermore, E-GS, effectively inhibited proliferation of *lamina*
48 *propria* derived CD4+ T cells in both models (Figure 9C,F) (n=3, P< 0.05 versus CD3-
49 CD28 group). In all these experimental settings Z-GS was less effective than its
50 stereoisomer.
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3.5. In Vitro Effect of GS in lamina propria CD4+ cells signalling

The principal intracellular pathway involved in T-cells activation by T cell receptor crosslinking includes JNKs, p38MAPK and Akt/PKB [35-37]. Thus we have investigated whether GS (100-10 μ M) modulates the phosphorylation of p(Thr183/Tyr185)JNKs, p(ser473)AKT and p(Tyr180/182)p38MAPK induced by CD3-CD28 stimulation of lamina propria-derived CD4+ cells obtained from mice treated with TNBS. Figure 5A illustrate that CD3-CD28 induced a 3-5 folds increase of pJNKs. A robust induction of this pattern of phosphorylation was found in cell exposed to GS stereoisomers (100 μ M) (n=3, P< 0.05). Subsequent to dysregulation of JNKs we also observed a p38MAPK hyperphosphorilation that increased progressively until 3 hours (n=3, P< 0.05 versus CD3-CD28 group). GS-stereoisomers at lower doses (10 μ M) had no effect on pJNKs/p38MAPK levels. In addition we found that p(ser473)AKT phosphorylation was markedly inhibited by exposure to Z-GS, 10 and 100 μ M, (Figure 5) (n=3, P< 0.05 versus CD3-CD28 group). The total quantity of JNKs, AKT and p38MAPK did not change in the indicated time frame (data not shown).

3.6. GS drives lamina propria CD4+ T cells to apoptosis.

We have next investigated whether in addition to its immune-modulatory activity GSs drive lamina propria derived CD4+ cells to apoptosis. For this purpose lamina propria-derived CD4+ cells stimulated with CD3/CD28 agonistic antibodies were treated with two different doses of GS, 10 and 100 μ M for a different amount of time (6-12-24 hours). As shown in Figure 6A, both GS stereoisomers at dose of 100 μ M caused a time-dependent apoptosis of CD4+ cells (figure 6A) (n=5, P< 0.05 versus CD3-CD28 group), with E-GS being more effective in comparison with Z-GS (n=6, P< 0.05). Again insight on the mechanism mediating the pro-apoptotic activity of GSs we have then measured a number of intracellular mediator of apoptosis in TNBS-primed CD4+ cells exposed to CD3/CD28 agonistic in the presence of GS stereoisomers. As illustrated in Figure 6, exposure to these stereoisomers significantly increased ROI generation and mitochondrial dysfunction as measured by assessing mitochondrial membrane potential ($\Delta\Psi_m$)

1 and the transition of Cyt C from the mitochondria to the cytosol. Experiments
2 shown in Figure 6 demonstrate that while GS at low dose has no effect on $\Delta\Psi_m$ in
3 lamina propria CD4+ cells stimulated with CD3-CD28, both stereoisomers caused
4 $\Delta\Psi_m$ collapse. This effect associated with transition of Cyt C from mitochondria to
5 cytosol (n=6, P< 0.05 versus CD3-CD28 group). Again E-GS was more
6 efficacious than Z-GS (n=6, P< 0.05 versus CD3-CD28 group). In addition exposure
7 to GS, high concentrations, resulted in a robust induction of caspase-3, 8 and 9
8 activity (Figure 6B,C) (n=6, P< 0.05 versus CD3-CD28 group). These data indicated
9 GS activates both the extrinsic and intrinsic apoptosis pathway but E-GS was
10 more efficacious inducing mitochondrial damage. The pro-apoptotic effect of GS
11 on CD4+ cells was reversed by exposing lamina-propria derived T cells to
12 SP600125 (1 μ M) a JNKs inhibitor (n=6, P< 0.05 versus CD3-CD28 plus GSs).
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25 **3.7. Comparative effects of E-GS and prednisolone in TNBS induced colitis.**

26 Steroid are commonly used in the treatment of IBD, we have therefore compared
27 the effect of E-GS with that of a commonly used steroid, prednisolone, in the
28 prevention of development of colitis induced by TNBS. For this purpose, we have
29 used a dose of prednisolone of 10 mg/kg previously shown to be effective in
30 protecting mice from colitis induced by TNBS. As shown in figure 7, protection
31 exerted by E-GS (30 mg/kg) was similar to that seen in animals administered
32 prednisolone at the dose of 10 mg/kg (P<0.05 versus TNBS alone for both
33 treatments). The two treatments exerted the same protective activity against
34 systemic and local signs of inflammation caused by TNBS.
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4. Discussion

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4 Identification of active principles and their molecular targets from traditional
5 medicine is an important opportunity for modern medicine. The gum resin from
6 *Commiphora mukul* has been used for centuries in *Ayurvedic* medicine to treat a
7 number of inflammatory disorders. GS has been identified as one of the major
8 active components of this gum resin [20-25]. Modulation of gene expression by GS
9 leads to inhibition of cell proliferation, induction of apoptosis, suppression of
10 invasion and abrogation of angiogenesis [20-25]. Evidence has been provided to
11 suggest that GS is also effective in attenuating tumor cell growth and metastasis
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21 A previous work [39] has demonstrated that GS exerts a protective effect in
22 a model of colon inflammation induced in mice by oral administration of dextran
23 sulfate sodium (DSS). Mechanistic studies carried out to explain the protective
24 effects have shown that GS inhibits ICAM-1 gene expression induced by bacterial
25 endotoxin and IL-1 β -induced through a mechanism that involves inhibition of NF-
26 kappaB transcriptional activity, IkappaB phosphorylation/degradation and NF-
27 kappaB DNA binding activity in intestinal epithelial cells line [39]. However, since it
28 is generally believed that DSS is directly toxic to gut epithelial cells and affects the
29 integrity of the mucosal barrier this model is not suitable for investigating the role
30 of effector T cells, the main mechanism involved in tissue destruction in IBD. The
31 fact that T- and B-cell-deficient C.B-17^{scid} or Rag1^{-/-} mice still develop intestinal
32 inflammation when challenged with DSS support the notion that the adaptive
33 immune system plays a minor role (at least in the acute phase) in this model
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49 Intestinal inflammation induced by TNBS and oxazolone are two
50 immunologically mediated models of IBD. In the TNBS model, inflammation and a
51 T-cell-mediated immune dysfunction develop in response to the exposure to
52 hapten-modified autologous proteins/luminal antigens [31]. Using this model we
53 provided evidence that E-GS protects mice against development of sign and
54 symptoms of colon inflammation. Thus administering mice with E-GS, 15 and 30
55 mg/kg, effectively attenuated the severity of wasting disease and the fecal
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1 score and colon inflammation as assessed by measuring the macroscopic- and
2 microscopic-damage scores. By striking contrast, administration Z-GS at the dose
3 of 30 mg/kg failed to ameliorate colon inflammation. Furthermore, while E-GS
4 treatment significantly attenuated the TNBS-induced increase of expression of
5 INF γ , IL-2, IL-6, TNF α and TGF β mRNAs in the colon, these effects were not
6 reproduced by Z-GS.
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11 Rectal administration of oxazolone allows to study the contribution of the
12 Th2-dependent immune response to intestinal inflammation. E-GS was effective
13 in reducing inflammation induced by oxazolone and reduced systemic and
14 local signs of inflammation including the severity of wasting disease and the
15 colon macroscopic and microscopic scores. This effect was accompanied by a
16 robust attenuation of neutrophil infiltration and tissue expression of
17 proinflammatory cytokines. Again, Z-GS was less effective than its stereoisomer in
18 treating this condition.
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27 To define the immune compartment mediating the effect of GS *in vivo*, we
28 have then tested the effect of GS in a model of colitis induced by TNBS
29 administration to T and B cell depleted SCID mice. Of outstanding interest we
30 found that, in comparison to control mice, E-GS at the dose of 30 mg/kg was only
31 partially effective in reducing inflammation (weight loss score, stool consistency
32 score and mucosal damage score) induced by TNBS administration to SCID mice,
33 strongly suggesting that T (or B) cells could be a target for this agent.
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41 CD4⁺ T lymphocytes mediate a key component of the intestinal mucosal
42 immune defence against pathogens [10]. To gain insights on the mechanisms
43 mediating the anti-inflammatory activity of GS we have therefore tested its
44 effects on effector functions of CD4⁺ cells purified from the intestinal lamina
45 propria. Results of these *in vitro* studies demonstrated that exposure to E-GS
46 causes T cell apoptosis and inhibits T-cells proliferation induced by co-incubation
47 with a mixture of anti-CD3 and anti-CD28 activating antibodies.
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54 T cell receptor stimulation by anti-CD3 activates a family of mitogen-
55 activated protein kinases (MAPKs), that are serine/threonine kinases, that include
56 Jun NH₂-terminal kinases (JNKs) and p38MAPK [36]. While T cell receptor
57 activation by anti-CD3 agonist mAb drives T cell to apoptosis, co-stimulation by
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1 CD28 agonistic antibody recruits phosphatidylinositol-3-kinase (PI3K) inducing
2 the downstream phosphorylation of serine/threonine of Akt/PKB molecule, an
3 event that leads to CD4⁺ cell activation [37]. Previous works [41-43] indicated
4 that activation of JNKs1/2 and p38MAPK and inhibition of AKT/PBK were
5 biomarkers of GS response in cancer cell lines. In the present study we have
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7 provided evidence that exposure of lamina propria-derived CD4⁺ cells to E-GS,
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9 resulted in a fine tuning of these phosphorylation/activating pathways. Thus while
10 exposure to GS results in hyperphosphorylation of Thr183/Tyr185 of JNKs1/2 and
11 Tyr180/182 of p38MAPK, p(ser473)AKT phosphorylation caused by CD3/CD28
12 was significantly inhibited. The net effects resulting from these biochemical
13 changes induced by exposure of lamina propria CD4⁺ to GS could support the
14 observed the induction of apoptosis. This effect was also associated with an
15 increased of ROI generation, mitochondrial membrane depolarization and
16 release of CytC from damaged mitochondria. Activation of the ERK pathway
17 typically confers growth and survival responses, while p38 and JNKs may promote
18 cell death so that the cellular fate in response to any stimulus is a function of the
19 relative levels of activation of each MAPK [44]. The importance of the kinetics of
20 JNKs activation in the induction of apoptosis in T cells is further underscored by the
21 finding that transient JNKs activation by CD3/CD28 co-ligation does not lead to
22 apoptosis whereas a prolonged activation induced by stress stimuli (UV, γ -
23 irradiation, anisomycin, H₂O₂ and TNF α), mediates the activation of both the
24 extrinsic and intrinsic pathway of apoptosis [45-47]. A number of results suggest
25 that GS targets mitochondria to trigger ROI generation, which is highly likely
26 because JNKs1/2 activation and apoptosis induction by this agent are significantly
27 attenuated by overexpression of intramitochondrial Mn-SOD [41,48]. Support to
28 the role JNKs in this model of apoptosis were provided also by experiments with
29 SP600125, a JNKs inhibitor. Thus exposure to this agent reverted apoptosis
30 induced by GS-stereoisomers.

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32 In addition to inhibition of JNKs, exposure of lamina propria-derived T cells to
33 E-GS caused a robust inhibition of p(ser473)AKT phosphorylation. In T cells Akt
34 activation/phosphorylation requires stimulation of both TCR and CD28 [49]. There
35 is considerable evidence that Akt is an important mediator of T cells survival, thus

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2 its inhibition might contribute to the anti-proliferative and pro-apoptotic
3 activities of GS in lamina propria-derived T cells [50]. CD28 costimulation and AKT
4 activation is also important for IL-2 and IFN- γ generation by Th1 polarized T cells,
5 and therefore it might also contribute to attenuation of T cell function,
6 documented in this report [51,52].
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10 T helper cells derived from the lamina propria of mice exposed for 5 days to
11 TNBS or oxazolone, develop a phenotype with a Th1 or Th2 profile. We have
12 demonstrated ex vivo exposure of these cells to E-GS inhibited IL-2 and INF γ
13 production by CD4+ T cells obtained from TNBS treated mice, and IL-2 and IL-4
14 production by CD4+ T cells obtained from oxazolone treated mice. In both cell
15 subtypes exposure to GS inhibited cells proliferation.
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22 Several of the biological effects exerted by GSs on T cells effector function
23 require a concentration of these stero-isomers of approximately 10 μ M. Despite
24 the pharmacokinetic of GSs is only partially known, a study performed in rats has
25 shown that 50 mg/kg bodyweight oral application of GSs resulted in peak plasma
26 concentrations of 3.2 μ M [53]. Because we have administered GS systemically by
27 intraperitoneal injection at the dose of 30 mg/kg, it is likely that GS
28 concentrations used for in vitro studies may reasonably match the plasma
29 concentrations reached in our in vivo experiments.
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37 Here we have compared the pharmacological effects of E-GS with that of
38 prednisolone, a commonly used steroid (4). Steroid represent the front-line therapy
39 of IBD, however their use associates with endocrine and metabolic side-effects
40 that are dose-dependent and related to the length of treatment (4). Here we
41 have provided evidence that in a pre-clinical model of IBD, E-GS gives
42 approximately the same protection of a fairly high dose of prednisolone
43 (4,34,54,55). In contrast to steroids, side effects of GSs are uncommon, although
44 it is reported that skin reaction develop in approximately 15% of patients taking a
45 dose of 75-150 mg/day of the compound (56).
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55 In summary, we have provided evidence that GSs exerts anti-inflammatory
56 activities in mouse models of colitis by targeting, among other mechanisms,
57 lamina propria T cells. On the light that therapeutic approaches inhibiting T cell
58 proliferation such as glucocorticoids, azathioprine/6-MP and anti-TNF- α antibodies
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are effectively used to treat CD and UC, our results support the notion that GSs might be added to list of compounds that have utility in the treatment of IBD.

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References

1. Loftus EVJ. Clinical epidemiology of inflammatory bowel disease: incidence, prevalence, and environmental influences. *Gastroenterology* 2004; 126:1504–1517.
2. Strober W, Fuss I, Mannon PJ. The fundamental basis of inflammatory bowel disease. *J Clin Invest* 2007; 117: 514–521.
3. Kakazu T, Hara J, Matsumoto T, Nakamura S, Oshitani N, Arakawa T, et al. Type 1 T-helper cell predominance in granulomas of Crohn's disease, *Am J Gastroenterology* 1999; 94:2149–2155.
4. Velayos FS, Sandborn WJ. Positioning biologic therapy for Crohn's disease and ulcerative colitis. *Curr Gastroenterol Rep.* 2007;9:521-7.
5. Bouma G, Strober, W. The immunological and genetic basis of inflammatory bowel disease. *Nat Rev Immunol.* 2003; 3: 521—533.
6. Parronchi P, Romagnani P, Annunziato F, Sampognaro S, Becchio A, Giannarini L, et al. Type 1 T-helper cell predominance and interleukin-12 expression in the gut of patients with Crohn's disease. *Am J Pathol.* 1997; 150:823—832.
7. Xavier RJ, Podolsky DK. Unravelling the pathogenesis of inflammatory bowel disease. *Nature* 2007; 448:427-34.
8. Sturm A, de Souza HS, Fiocchi C. Mucosal T cell proliferation and apoptosis in inflammatory bowel disease. *Curr Drug Targets.* 2008; 5: 381-387.
9. Hanauer SB, Feagan BG, Lichtenstein GR, Mayer LF, Schreiber S, Colombel JF, et al. Maintenance infliximab for Crohn's disease: the ACCENT I randomised trial. *Lancet* 2002; 359:1541–1549.
10. Mudter J, Neurath MF. Apoptosis of T cells and the control of inflammatory bowel disease: therapeutic implications. *Gut* 2006; 56: 293–303.
11. Mannon PJ, Fuss IJ, Mayer L, Elson CO, Sandborn WJ, Present D, et al. Anti-interleukin-12 antibody for active Crohn's disease. *N Engl J Med* 2004;351: 2069–2079.

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12. Ito H, Takazoe M, Fukuda Y, Hibi T, Kusugami K, Andoh A, et al. A pilot randomized trial of a human anti-interleukin-6 receptor monoclonal antibody in active Crohn's disease. *Gastroenterology* 2004; 126: 989–996.
13. Targan SR, Salzberg BA, Mayer L. A phase I-II study: multiple dose levels of visilizumab are well tolerated and produce rapid and sustained improvement in ulcerative colitis patients refractory to treatment with intravenous steroids (IVSR-UC). *Gastroenterology* 2005; 128:A-75.
14. Creed T, Norman M, Probert C, Harvey RF, Shaw IS, Smithson J, et al. Basiliximab (anti-CD25) in combination with steroids may be an effective new treatment for steroidresistant ulcerative colitis. *Aliment Pharmacol Ther* 2003; 18: 65–75.
15. Agarwal RC, Singh SP, Saran RK, Das SK, Sinha N, Asthana OP, et al. Clinical trial of gugulipid—a new hypolipidemic agent of plant origin in primary hyperlipidemia. *Indian J Med Res* 1986; 84:626.
16. Khanna D, Sethi G, Ahn KS, Pandey MK, Kunnumakkara AB, Sung B, et al. Natural products as a gold mine for arthritis treatment, *Curr Opin Pharmacol* 2007; 7:344.
17. Singh RB, Niaz MA, Ghosh S. Hypolipidemic and antioxidant effects of Commiphora mukul as an adjunct to dietary therapy in patients with hypercholesterolemia. *Cardiovasc Drugs Ther* 1994; 8: 659.
18. Urizar NL, Moore DD. GUGULIPID: a natural cholesterol-lowering agent. *Annu Rev Nutr* 2003; 23: 303.
19. Singh SV, Choi S, Zeng Y, Hahm ER, Xiao D. Guggulsterone-induced apoptosis in human prostate cancer cells is caused by reactive oxygen intermediate dependent activation of c-Jun NH2-terminal kinase. *Cancer Res* 2007; 67: 7439–49.
20. Shishodia S, Sethi G, Ahn KS, Aggarwal BB. Guggulsterone inhibits tumor cell proliferation, induces Sphase arrest, and promotes apoptosis through activation of c-Jun N-terminal kinase, suppression of Akt pathway, and downregulation of antiapoptotic gene products. *Biochem Pharmacol* 2007; 74:118–130.

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21. Samudio I, Konopleva M, Safe S, McQueen T, Andreeff M. Guggulsterones induce apoptosis and differentiation in acute myeloid leukemia: identification of isomer-specific antileukemic activities of the pregnadienedione structure. *Mol Cancer Ther* 2005; 4:1982–92.
 22. Singh SV, Zeng Y, Xiao D, Vogel VG, Nelson JB, Dhir R, et al. Caspase-dependent apoptosis induction by guggulsterone, a constituent of Ayurvedic medicinal plant *Commiphora mukul*, in PC-3 human prostate cancer cells is mediated by Bax and Bak. *Mol Cancer Ther* 2005; 4: 1747–1754.
 23. Shishodia S, Aggarwal BB. Guggulsterone inhibits NF- κ B and I κ B α kinase activation, suppresses expression of anti-apoptotic gene products, and enhances apoptosis. *J Biol Chem* 2004; 279: 47148–58.
 24. Burris TP, Montrose C, Houck KA, Osborne HE, Bocchinfuso WP, Yaden BC, et al. The hypolipidemic natural product guggulsterone is a promiscuous steroid receptor ligand. *Mol Pharmacol* 2005; 67: 948-954.
 25. Lee YR, Lee JH, Noh EM, Kim EK, Song MY, Jung WS, et al. Guggulsterone blocks IL-1 β -mediated inflammatory responses by suppressing NF- κ B activation in fibroblast-like synoviocytes. *Life Sci* 2008; 82: 1203-1209.
 26. Van der Heijden PJ, Stok W. Improved procedure for the isolation of functionally active lymphoid cells from the murine intestine. *J Immunol Methods* 1987; 103: 161–167.
 27. Nicoletti I, Migliorati G, Pagliacci MC, Grignani F, Riccardi C. A rapid and simple method for measuring thymocyte apoptosis by propidium iodide staining and flow cytometry. *J Immunol Methods* 1991; 139: 271—279.
 28. Elson CO, Beagley KW, Sharmanov AT, Fujihashi K, Kiyono H, Tennyson GS, et al. Hapten-induced model of murine inflammatory bowel disease: mucosa immune responses and protection by tolerance. *J Immunol* 1996; 157: 2174–2185.
 29. Neurath MF, Fuss I, Kelsall BL, Stüber E, Strober W. Antibodies to interleukin 12 abrogate established experimental colitis in mice. *J Exp Med* 1995; 182: 1281–1290.

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30. Neurath MF, Pettersson S, Meyer zum Büschenfelde KH, Strober W. Local administration of antisense phosphorothioate oligonucleotides to the p65 subunit of NF-kappa B abrogates established experimental colitis in mice. *Nat Med* 1996; 2:998–1004.
 31. Wirtz S, Neufert C, Weigmann B, Neurath MF. Chemically induced mouse models of intestinal inflammation. *Nat Protoc* 2007; 2: 541-546.
 32. Boirivant M, Fuss IJ, Chu A, Strober W. Oxazolone colitis: a murine model of T helper cell type 2 colitis treatable with antibodies to interleukin 4. *J Exp Med* 1998; 188:1929–1939.
 33. Heller F, Fuss IJ, Nieuwenhuis EE, Blumberg RS, Strober W. Oxazolone colitis, a Th2 colitis model resembling ulcerative colitis, is mediated by IL-13-producing NK-T cells. *Immunity* 2002; 17: 629–638.
 34. Fiorucci S, Mencarelli A, Palazzetti B, Sprague AG, Distrutti E, Morelli A, et al. Importance of innate immunity and collagen binding integrin alpha1beta1 in TNBS-induced colitis. *Immunity* 2002; 17: 769-780.
 35. Kane LP, Lin J, Weiss A. It's all Rel-ative: NF- κ B and CD28 costimulation of T-cell activation. *Trends Immunol* 2002; 23: 413-420.
 36. Dong C, Davis RJ, Flavell RA. MAP kinases in the immune response. *Annu Rev Immunol* 2002; 20:55-72.
 37. Kane LP, Weiss A. The PI-3 kinase/Akt pathway and T cell activation: pleiotropic pathways downstream of PIP3. *Immunol Rev* 2003; 192: 7–20.
 38. Shishodia S, Harikumar KB, Dass S, Ramawat KG, Aggarwal BB. The guggul for chronic diseases: ancient medicine, modern targets. *Anticancer Res* 2008; 28: 3647-3664.
 39. Cheon JH, Kim JS, Kim JM, Kim N, Jung HC and Song IS, Plant sterol guggulsterone inhibits nuclear factor-kappaB signaling in intestinal epithelial cells by blocking IkappaB kinase and ameliorates acute murine colitis. *Inflamm Bowel Dis.* 2006; 12:1152-1161.
 40. Dieleman LA, Ridwan BU, Tennyson GS, Beagley KW, Bucy RP and Elson CO. Dextran sulfate sodium-induced colitis occurs in severe combined immunodeficient mice. *Gastroenterology* 1994; 107:1643–1652.

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41. Singh SV, Choi S, Zeng Y, Hahm ER and Xiao D. Guggulsterone-induced apoptosis in human prostate cancer cells is caused by reactive oxygen intermediate dependent activation of c-Jun NH₂-terminal kinase. *Cancer Res.* 2007; 67: 7439-7449.
 42. Shishodia S, Sethi G, Ahn KS and Aggarwal BB. Guggulsterone inhibits tumor cell proliferation, induces S-phase arrest, and promotes apoptosis through activation of c-Jun N-terminal kinase, suppression of Akt pathway, and downregulation of antiapoptotic gene products. *Biochem Pharmacol.* 2007; 74:118-30
 43. Xiao D and Singh SV. z-Guggulsterone, a constituent of Ayurvedic medicinal plant *Commiphora mukul*, inhibits angiogenesis in vitro and in vivo. *Mol Cancer Ther.* 2008; 7:171-80.
 44. Xia Z, Dickens M, Raingeaud J, Davis RJ and M.E. Greenberg, Opposing effects of ERK and JNK-p38 MAP kinases on apoptosis. *Science* 1995; 270:1326-1331.
 45. Faris M, Kokot N, Latinis K, Kasibhatla S, Green DR, Koretzky GA and Nel A. The c-Jun N-terminal kinase cascade plays a role in stress-induced apoptosis in Jurkat cells by up-regulating Fas ligand expression. *J Immunol.* 1998; 160:134-144.
 46. Tobiume K, Matsuzawa A, Takahashi T, Nishitoh H, Morita K, Takeda K and et al. ASK1 is required for sustained activations of JNK/p38 MAP kinases and apoptosis, *EMBO Rep.* 2001; 2: 222-228.
 47. Tournier C, Hess P, Yang DD, Xu J, Turner TK, Nimnual A, Bar-Sagi D and Jones SN. Requirement of JNK for stress-induced activation of the cytochrome c-mediated death pathway, *Science* 2000; 288: 870-4.
 48. Green DR. Overview: apoptotic signaling pathways in immune system, *Immunol. Rev.* 2003; 193: 5–9.
 49. Frauwirth KA, Riley JL, Harris MH, Parry RV, Rathmell JC, Plas DR, Elstrom RL, June CH and Thompson CB. The CD28 signaling pathway regulates glucose metabolism. *Immunity* 2002; 16:769–777.
 50. Kane LP, Shapiro VS, Stokoe D and Weiss A. Induction of NF- κ B by the Akt/PKB kinase. *Curr Biol* 1999; 9:601–604.

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51. Kane LP, Andres PG, Howland KC, Abbas AK and Weiss A. Akt provides the CD28 co-stimulatory signal for upregulation of IL-2 and IFN- γ but not Th2 cytokines. *Nat Immunol.* 2001; 2: 37–44.
52. Ahn KS, Sethi G, Sung B, Goel A, Ralhan R and Aggarwal BB. Guggulsterone, a farnesoid X receptor antagonist, inhibits constitutive and inducible STAT3 activation through induction of a protein tyrosine phosphatase SHP-1. *Cancer Res.* 2008; 68:4406-4415.
53. Verma N, Singh SK and Gupta RC. Pharmacokinetics of guggulsterone after intravenous and oral administration in rats. *Pharm Pharmacol Comm.* 1999; 5: 349.
54. Fiorucci S, Antonelli E, Distrutti E, Del Soldato P, Flower RJ, Clark MJ, Morelli A, Perretti M, Ignarro LJ. NCX-1015, a nitric-oxide derivative of prednisolone, enhances regulatory T cells in the lamina propria and protects against 2,4,6-trinitrobenzene sulfonic acid-induced colitis in mice. *Proc Natl Acad Sci U S A.* 2002; 99:15770-5.
55. Paul-Clark M, Del Soldato P, Fiorucci S, Flower RJ, Perretti M. 21-NO-prednisolone is a novel nitric oxide-releasing derivative of prednisolone with enhanced anti-inflammatory properties. *Br J Pharmacol.* 2000; 131:1345-54.
56. Ulbricht C, Basch E, Szapary P, Hammerness P, Axentsev S, Boon H, Kroll D, Garraway L, Vora M, Woods J; Natural Standard Research Collaboration. Guggul for hyperlipidemia: a review by the Natural Standard Research Collaboration *Complement Ther Med.* 2005; 13: 279-90.

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2 **Figure Legends:**
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5 **Figure 1:** Early administration of E-GS (30 mg/kg) protects against the development
6 of TNBS-induced colitis in mice. Colitis was induced by intrarectal instillation of 1 mg
7 of TNBS per mouse, and animals were killed 5 days after TNBS administration. GS-
8 stereoisomers were administered intraperitoneally daily for 5 days, starting at the
9 same time of intrarectal instillation of TNBS. **(A and B)** The severity of TNBS-induced
10 inflammation (wasting disease and fecal score) is reduced by E-GS administration.
11 Data represent the mean \pm SE of 8–10 mice per group. # $P < 0.05$ vs TNBS. **(C and**
12 **D)** E-GS protects against local signs of inflammation and protects against the
13 increase of macroscopic- microscopic- score induced by intrarectal instillation of
14 TNBS. Data represent the mean \pm SE of 8–10 mice per group. # $P < .05$ vs TNBS plus
15 vehicle group. **(E)** Histologic analysis of colon samples obtained from mice
16 sacrificed 5 days after TNBS. Original magnification 10 \times , H&E staining. TNBS
17 administration causes colon wall thickening and massive inflammatory infiltration
18 in the *lamina propria*. Administering TNBS mice with E-GS (30 mg/kg) attenuates
19 colon thickening and inflammatory infiltration of the mucosa and submucosa.
20 **(F)** Structure of GS E and Z. **(G)**. Reverse-transcription polymerase chain reaction
21 (RT-PCR) analysis of expression of inflammatory mediators in colons obtained 5
22 days after administration of TNBS alone or in combination with GS-stereoisomers.
23 Data represent the mean \pm SE of 5 mice per group. # $P < 0.05$ vs TNBS plus vehicle
24 group.
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46 **Figure 2:** Early administration of E-GS protects against the development of
47 oxazolone-induced colitis in mice. Colitis was induced by intrarectal instillation of
48 1.5 mg of oxazolone and animals were sacrificed 5 days later. GS-stereoisomers
49 were administered intraperitoneally at the dose of 30 mg/kg/day for 5 days,
50 starting at the same time of intrarectal instillation of oxazolone. **(A and B)** E-GS
51 protects against development of wasting disease and fecal score induced by
52 oxazolone. **(C and D)** E-GS and Z-GS administration attenuates the severity of
53 oxazolone-induced inflammation (macroscopic- microscopic- score). *Data*
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1 represent the mean \pm SE of 8–10 mice per group. #*P* < 0.05 vs oxazolone. (E)
2 Histological analysis (original magnification 10 \times , H&E staining) of the colon
3 harvested 5 days after oxazolone administration with or without treatment with
4 GS-stereoisomers. Oxazolone administration alone causes a massive inflammatory
5 infiltration in the subepithelial layer and epithelial cell necrosis. These effects were
6 attenuated by administration of GS stereoisomers (F) Administration of GS-
7 stereoisomers (30 mg/kg) attenuates increases of inflammatory mediators caused
8 by oxazolone. Colon specimens were harvested from mice sacrificed 5 days
9 after oxazolone administration. Data represent the mean \pm SE of 5 mice per
10 group. #*P* < 0.05 vs Oxazolone plus vehicle group.
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21 **Figure 3:** Development of TNBS-induced colitis does not require T and B
22 lymphocytes. SCID mice were treated with 1 mg/mouse of TNBS alone or in
23 combination with E-GS (30 mg/kg) for 5 days. (A, B and C) E-GS 30 mg/kg
24 failed to protect against development of wasting disease and diarrhea and was
25 of limited efficacy on colon macroscopic injury induced by intrarectal instillation
26 of TNBS. Data represent the mean \pm SE of 8–10 mice per group.
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35 **Figure 4:** Effects of GS-stereoisomers on CD4⁺ lamina propria cells obtained from
36 mice administered 5 TNBS or oxazolone. (A, B and C) *Th1 profile*: production of IL-2
37 and INF γ and proliferation of lamina propria CD4⁺ cells obtained from mice
38 administered TNBS and stimulated ex vivo with CD3-CD28 mAbs alone or in
39 combination with GS-stereoisomers (10 μ M). (D, E and F) *Th2 profile*: production of
40 IL-2 and IL-4 and proliferation of lamina propria CD4⁺ cells obtained from mice
41 administered oxazolone and stimulated ex vivo with CD3-CD28 mAbs alone or in
42 combination with GS-stereoisomers (10 μ M). Data represent the mean \pm SE of 4
43 experiments. **P* < 0.05 vs CD3-CD28 group; #*P* < 0.05 vs CD3-CD28 plus Z-GS 10 μ M
44 group.
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56 **Figure 5:** Effect of GS stereoisomers on CD3-CD28 induced phosphorylation of
57 p38MAPK, JNKs and AKT lamina propria CD4⁺ cells obtained from colon mice
58 treated with TNBS. GS (100-10 μ M) modified cell signalling transduction pathway,
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2 at high dose induced hyper-phosphorylation of p38MAPK and JNKs and inhibited
3 AKT activation, whereas E-GS at 10 μ M maintained its ability to prevent AKT
4 phosphorylation. (A) p(Thr183/Tyr185)JNKs levels. (B) p(Tyr180/182)p38MAPK levels.
5 (C) p(ser473)AKT levels. Data represent the mean \pm SE of 3 experiments. * P < 0.05 vs
6 CD3-CD28 group; # P < 0.05 vs CD3-CD28 plus Z-GS 100 μ M group.
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11 **Figure 6:** GS-stereoisomers induce apoptosis of lamina propria-derived CD4+
12 cells. (A) Time course of apoptosis, lamina propria CD4+ T cells were stained with
13 propidium iodide, and analyzed by flow cytometer. Data represent the mean \pm SE
14 of 6 experiments (B) Time course of ROI production, lamina propria CD4+ T cells
15 were stained with H2DCFDA, and analyzed by flow cytometer. Data represent the
16 mean \pm SE of 6 experiments (C) Time course membrane potential ($\Delta\Psi_m$), lamina
17 propria CD4+ T cells were stained with the fluorescent dye JC-1, and analyzed by
18 flow cytometer. Data represent the mean \pm SE of 6 experiments (D) Release of
19 CytC by lamina propria CD4+ T cells after 12 hour of incubation with CD3-CD28
20 alone or in combination with GS-stereoisomers. Data represent the mean \pm SE of 6
21 experiments. (E) GS at 100 μ M induced both extrinsic and intrinsic apoptosis
22 pathway but E-GS was more efficacious to induce mitochondrial damage as
23 demonstrated by major caspase-9 activation and subsequently a robust induction
24 of caspase-3 compared to Z-GS. Data represent the mean \pm SE of 4 experiments
25 (F) Induction of apoptosis by GS-stereoisomers is mediated by activation of
26 JNKs. Pre-treatment of lamina propria CD4+ cells with the JNKs inhibitor, SP600125,
27 attenuates apoptosis induced by GSs. Data represent the mean \pm SE of 6
28 experiments. * P < 0.05 vs CD3-CD28 alone; # P < 0.05 vs GS stereoisomers.
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50 **Figure 7.** Early administration of E-GS (30 mg/kg) and prednisolone (10 mg/kg)
51 protects against the development of TNBS-induced colitis in mice. Colitis was
52 induced by intrarectal instillation of 1 mg of TNBS per mouse, and animals were
53 killed 5 days after TNBS administration. E-GS and prednisolone were administered
54 intraperitoneally daily for 5 days, starting at the same time of intrarectal instillation
55 of TNBS. (A and B) The severity of TNBS-induced inflammation (wasting disease and
56 fecal score) was reduced by pharmacological treatments. Data represent the
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1 mean \pm SE of 8–10 mice per group. #*P* < 0.05 vs TNBS. (**C** and **D**) E-GS (30 mg/kg)
2 and prednisolone (10 mg/kg) protects against local signs of inflammation and
3 protects against the increase of macroscopic-score and myeloperoxidase
4 activity (MPO) induced by intrarectal instillation of TNBS. Data represent the mean
5 \pm SE of 8–10 mice per group. #*P* < 0.05 vs TNBS plus vehicle group.
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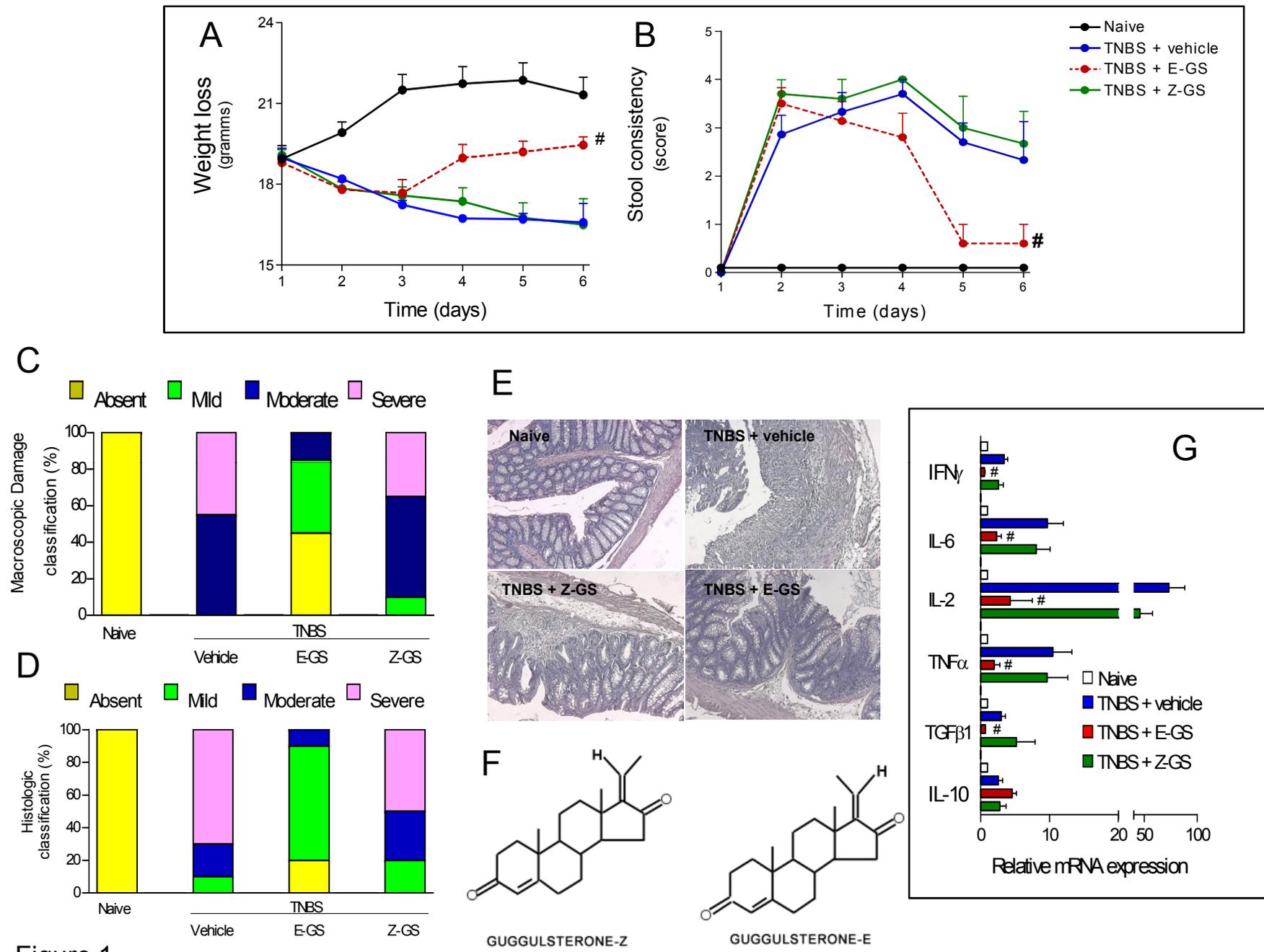


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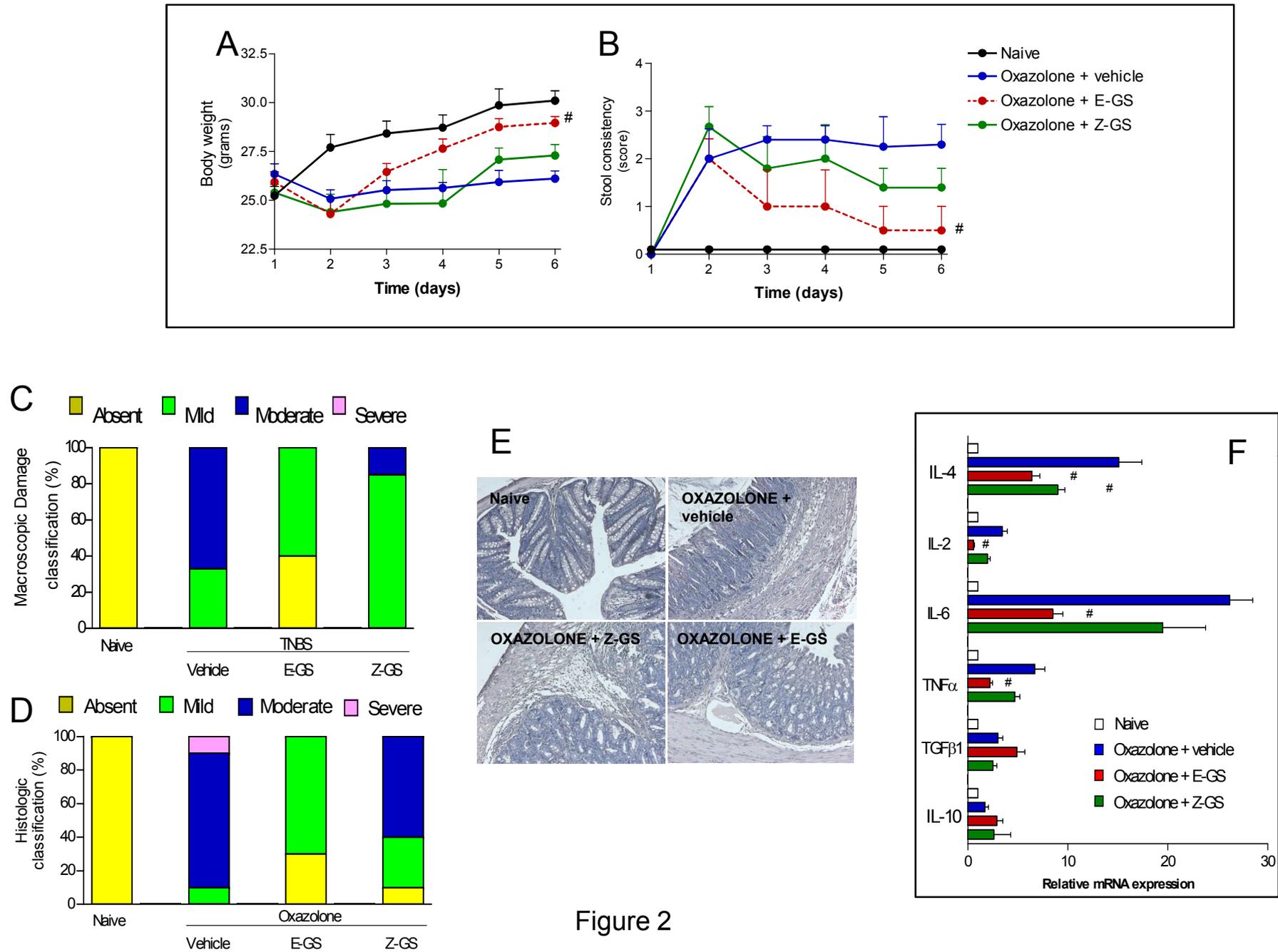


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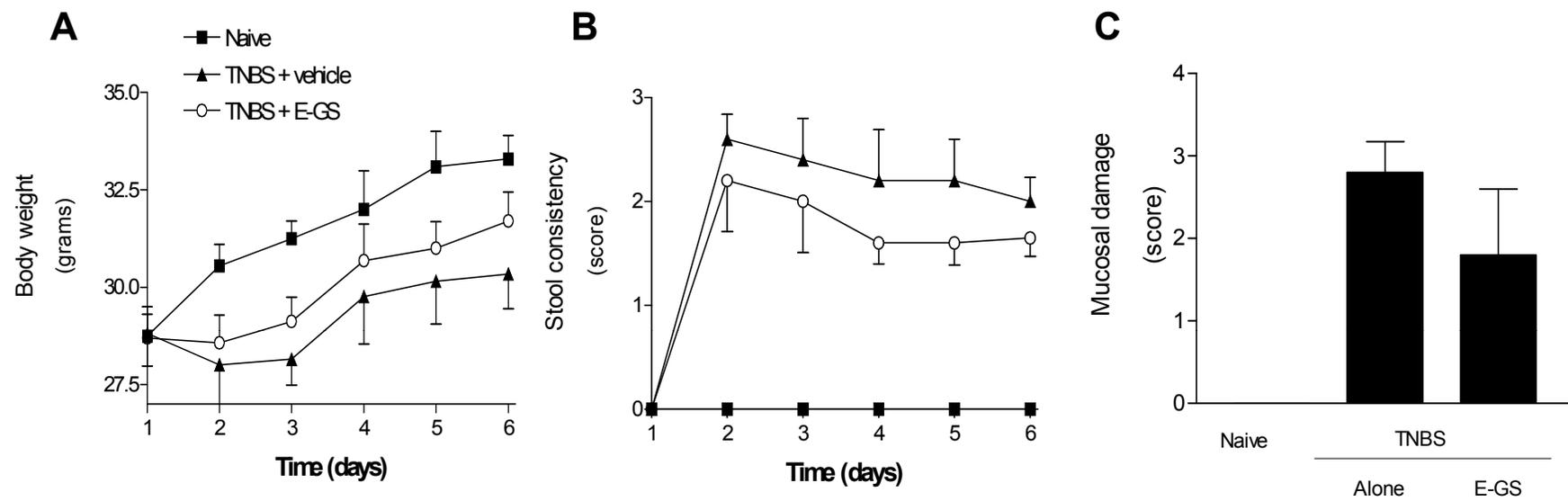


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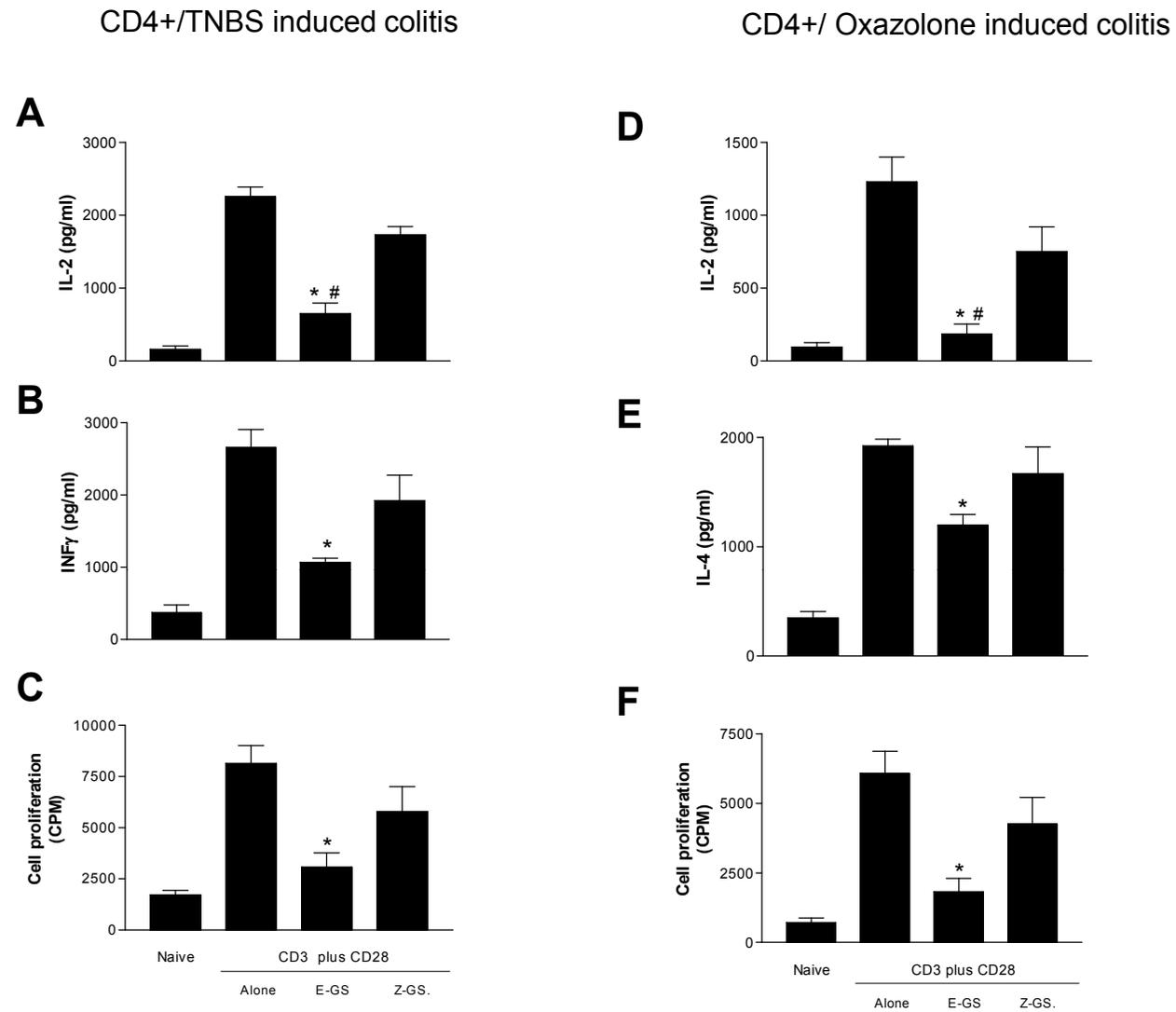


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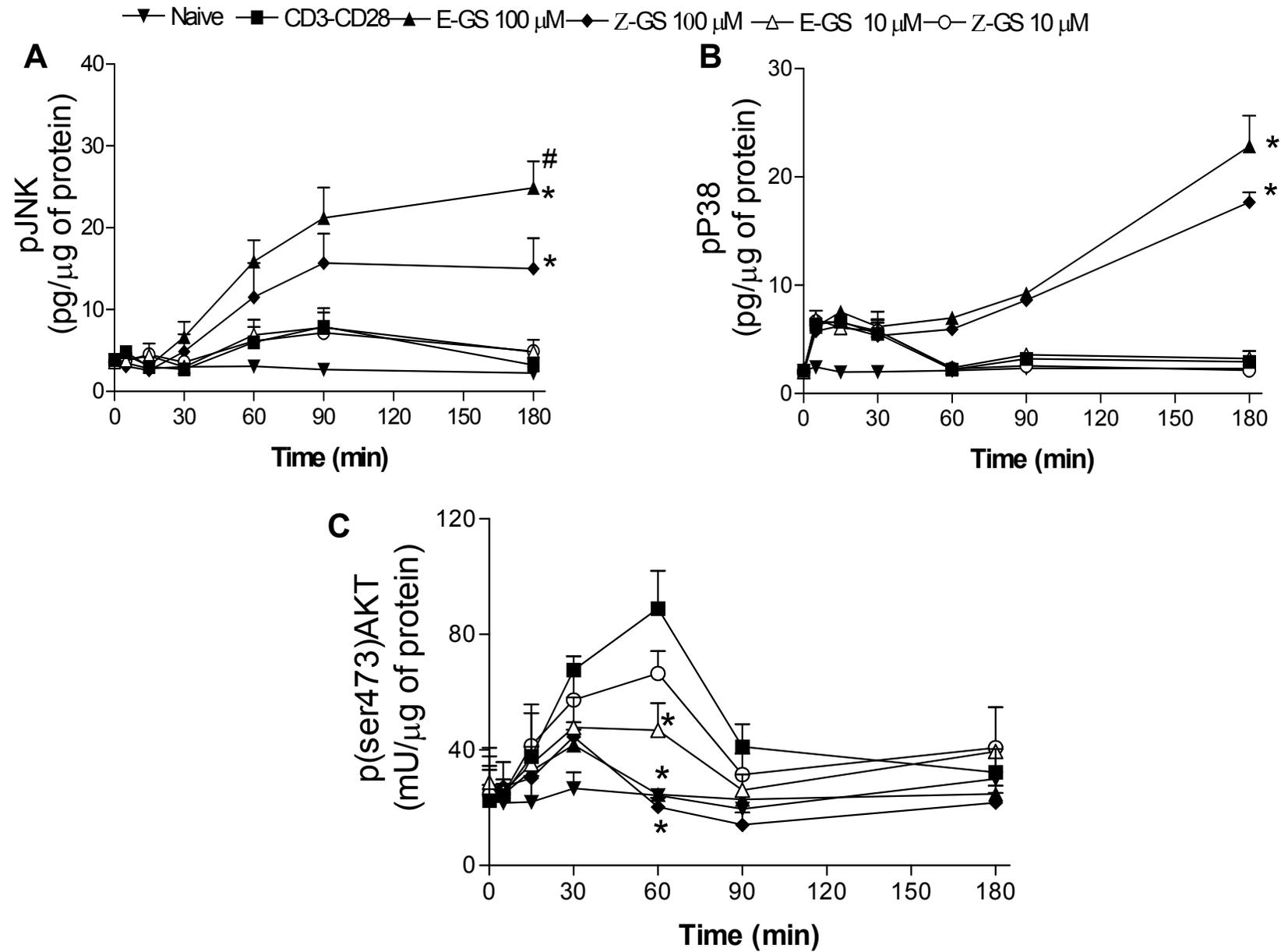


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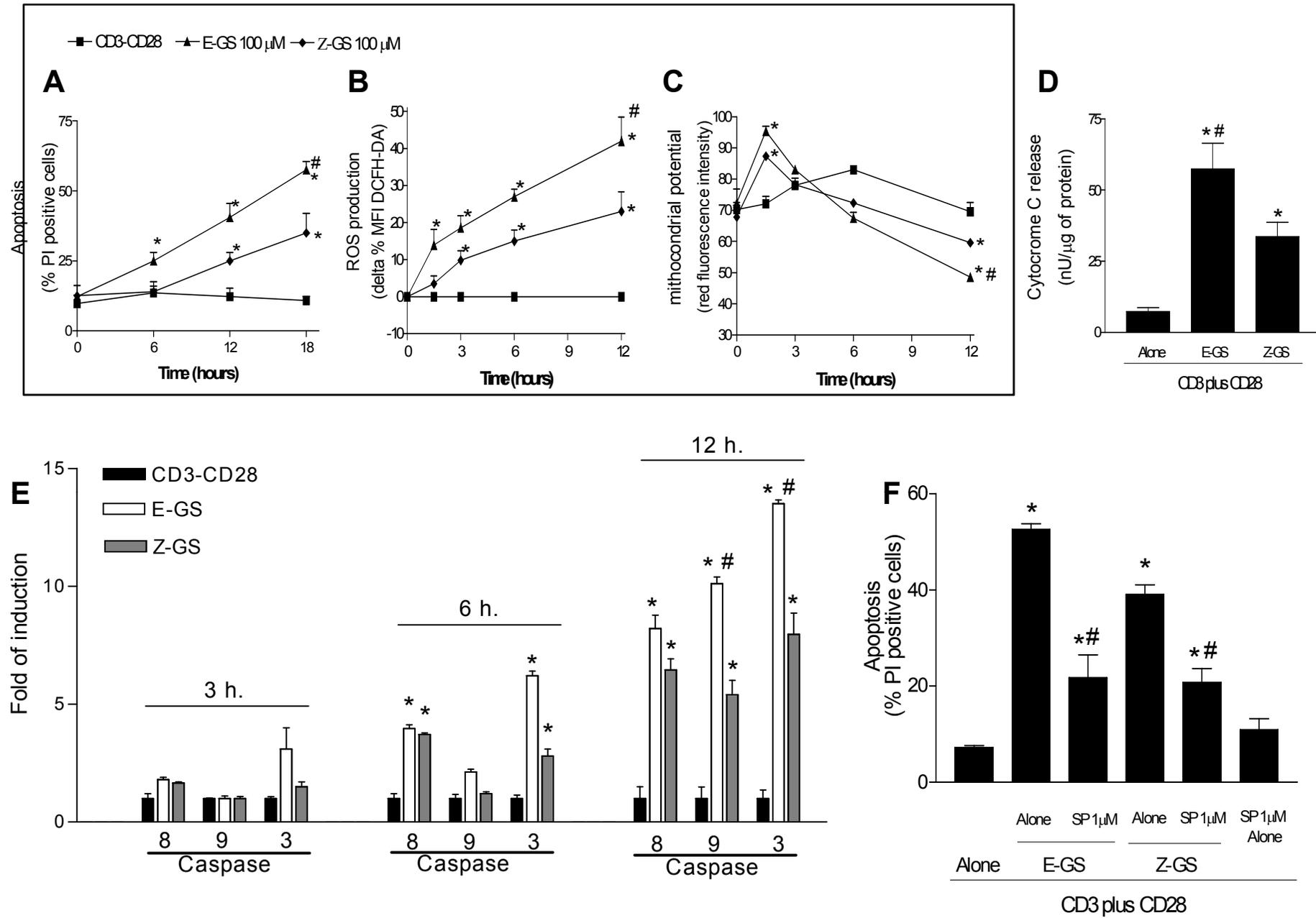
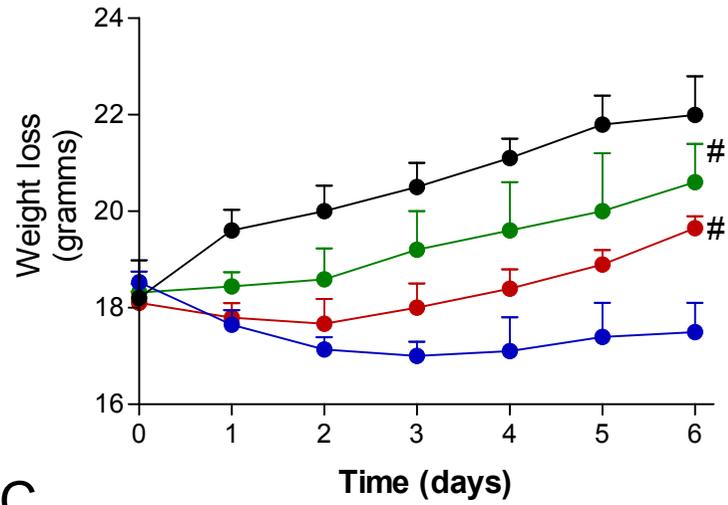


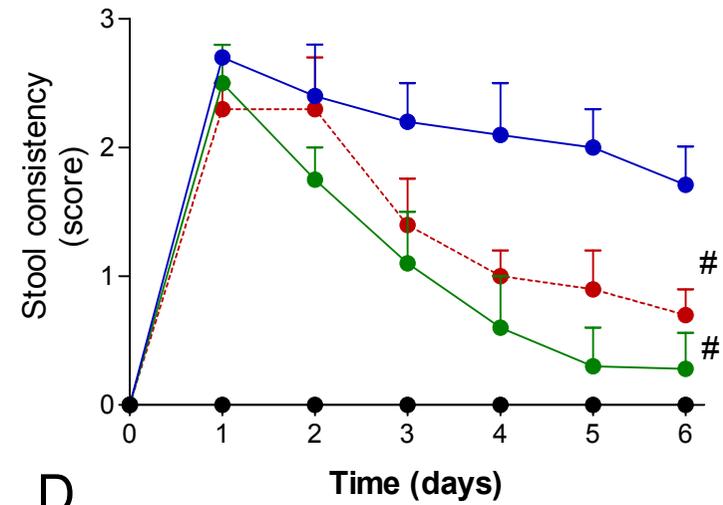
Figure 6

A.

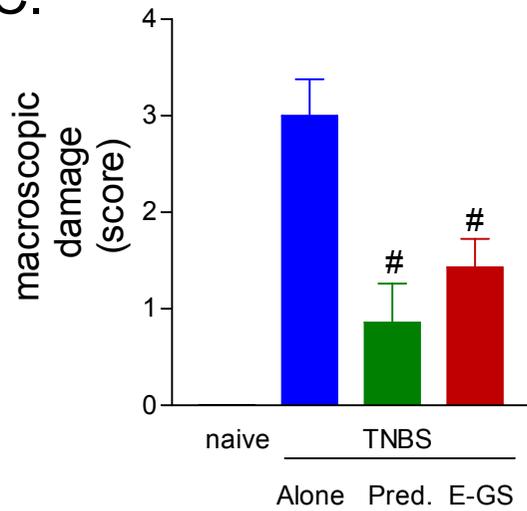
● Naive ● TNBS + Vehicle ● TNBS + Prednisolone 10mg/kg ● TNBS + E-GS 30mg/kg



B.



C.



D.

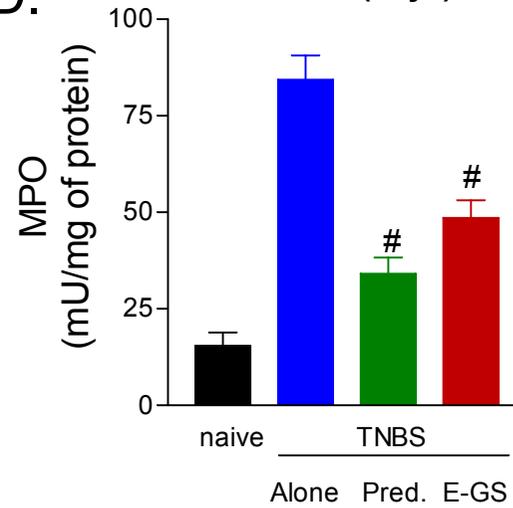
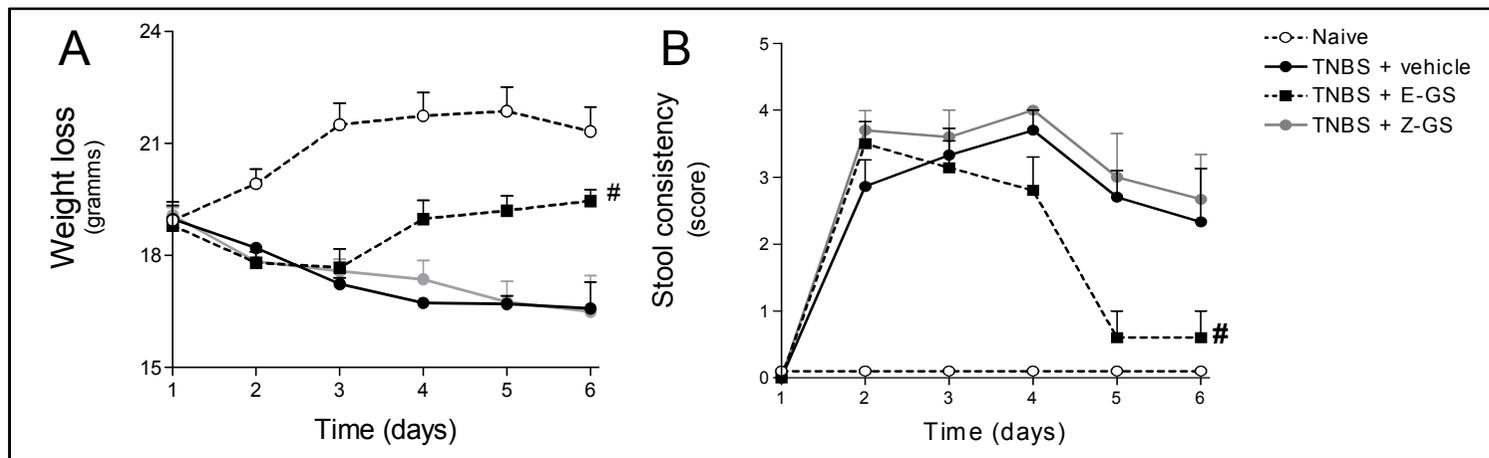
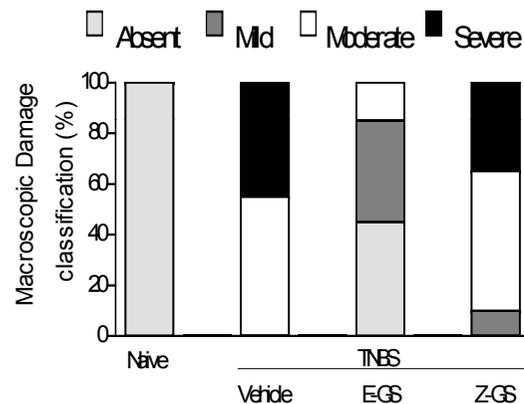


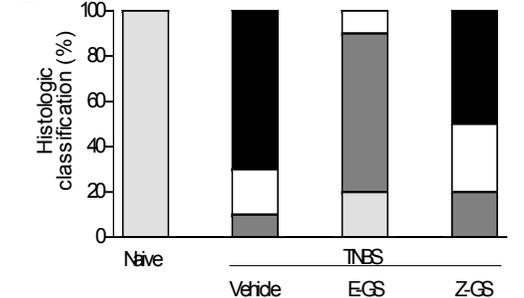
Figure 7



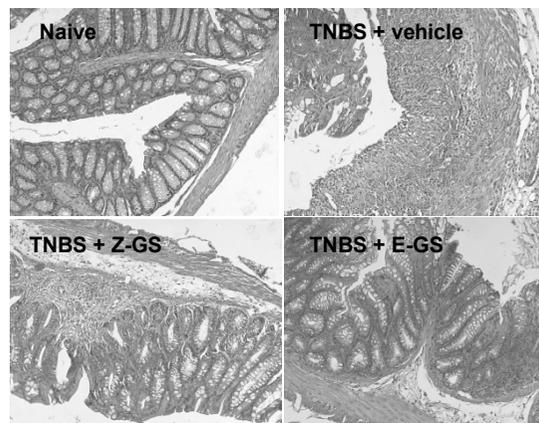
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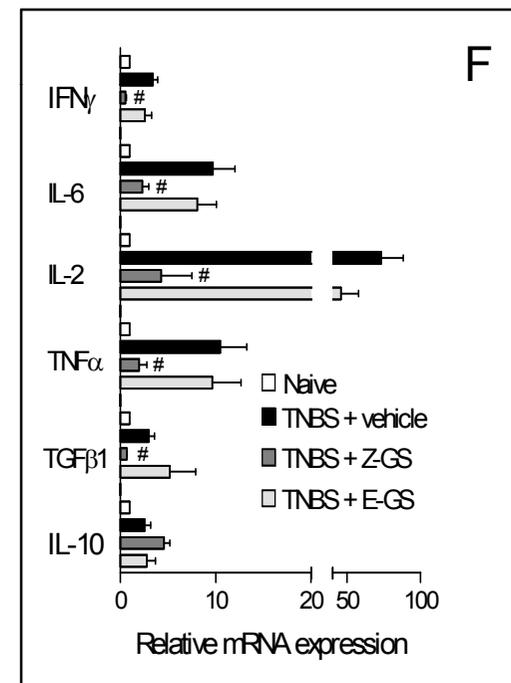


Figure 1

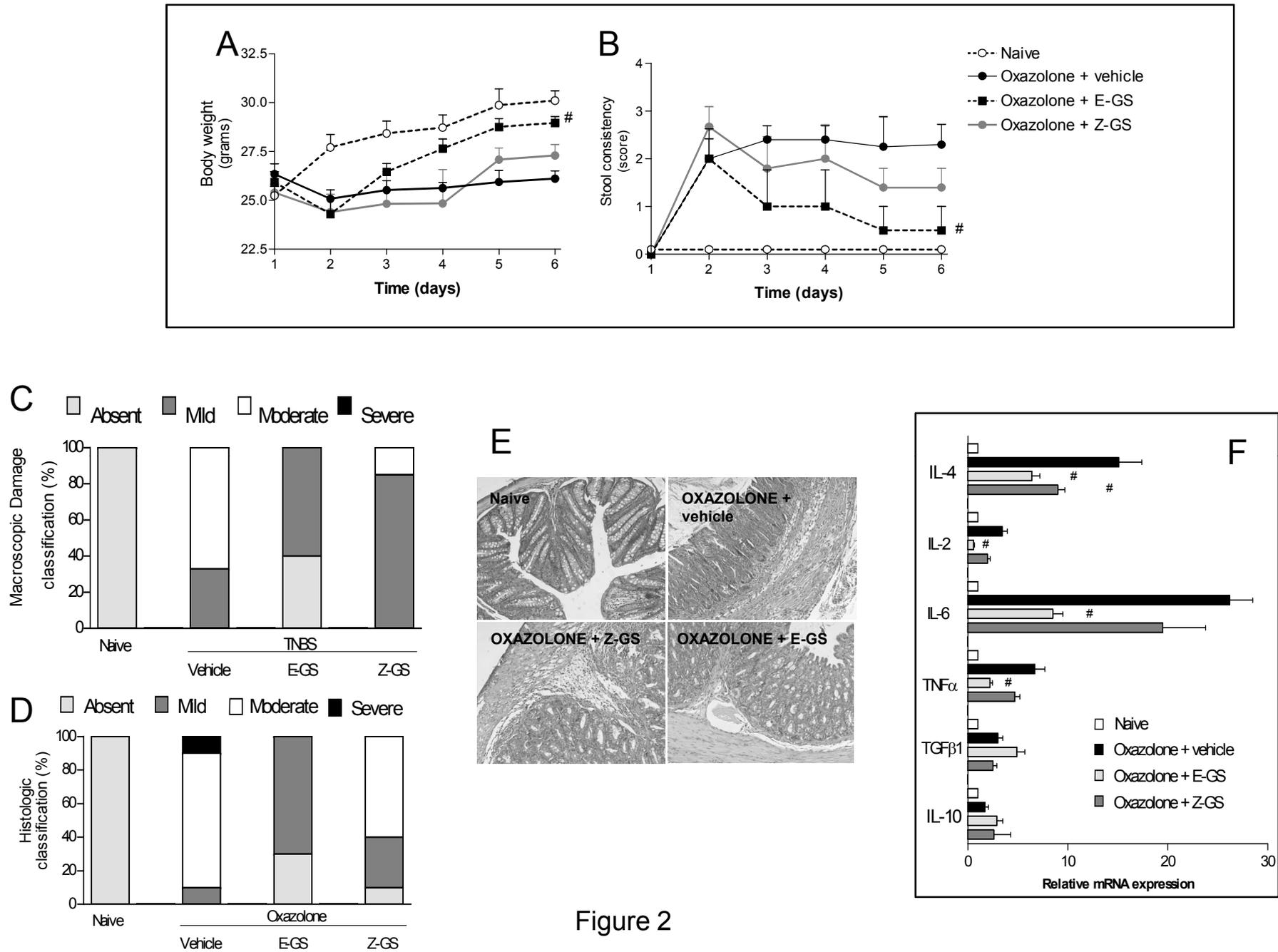


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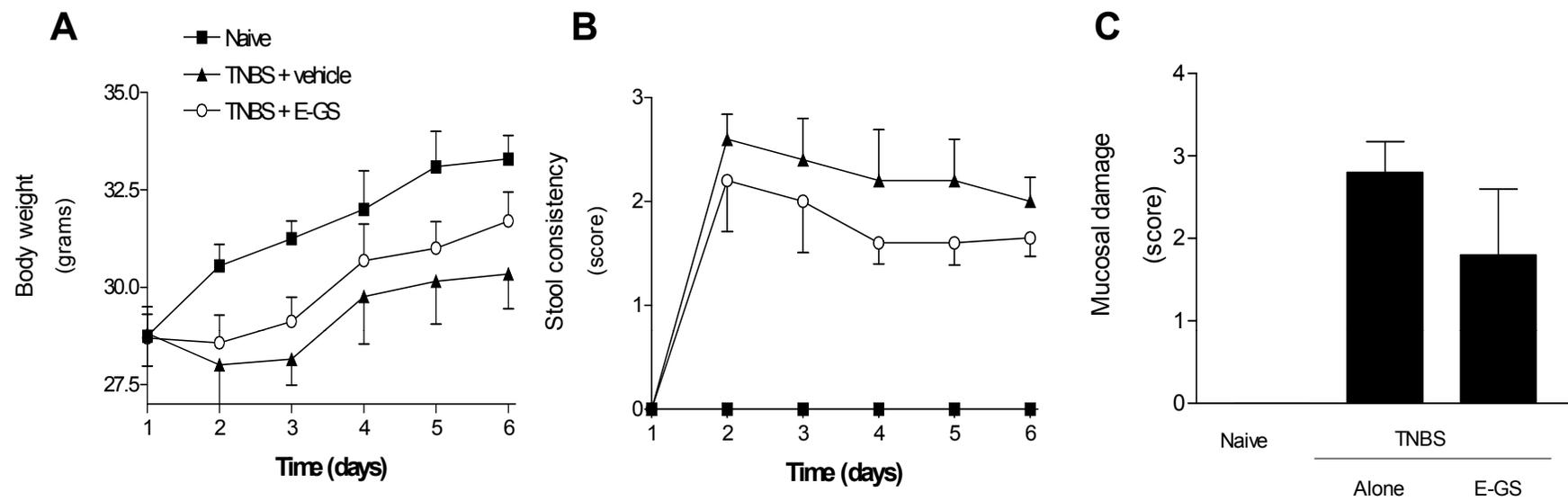


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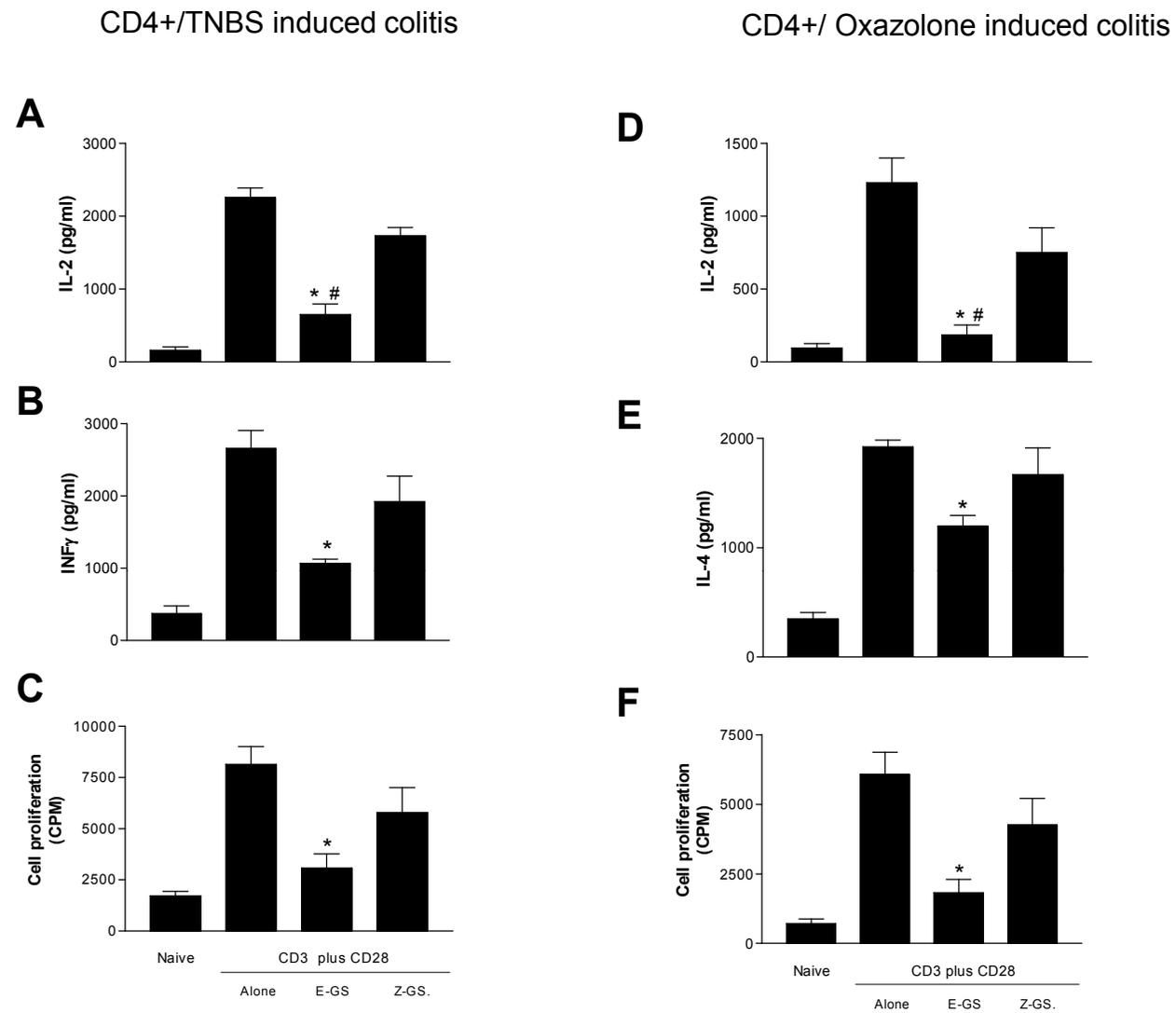


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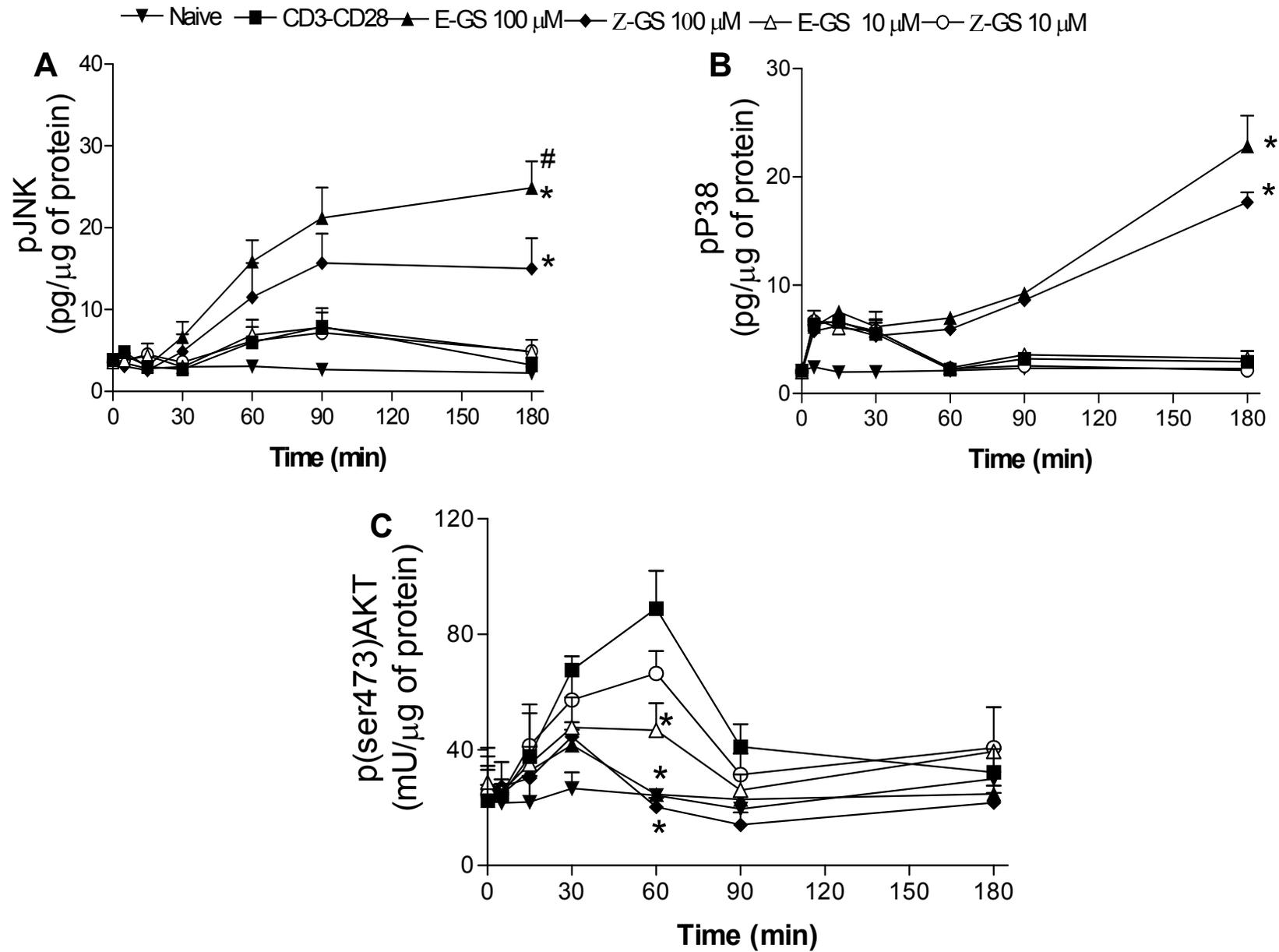


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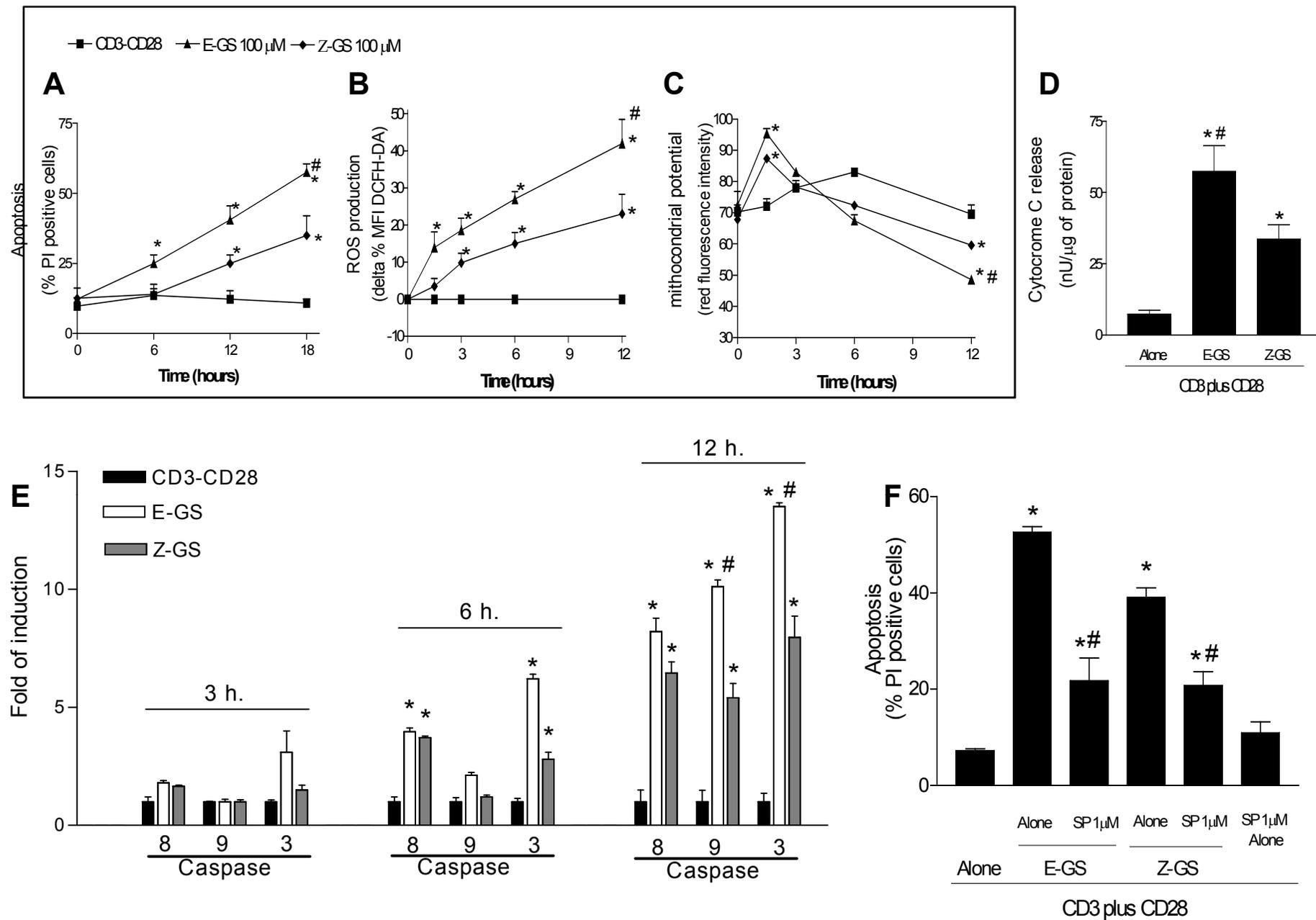


Figure 6

A

--○-- Naive ● TNBS + Vehicle ● TNBS + Prednisolone 10mg/kg ■ TNBS + E-GS 30mg/kg

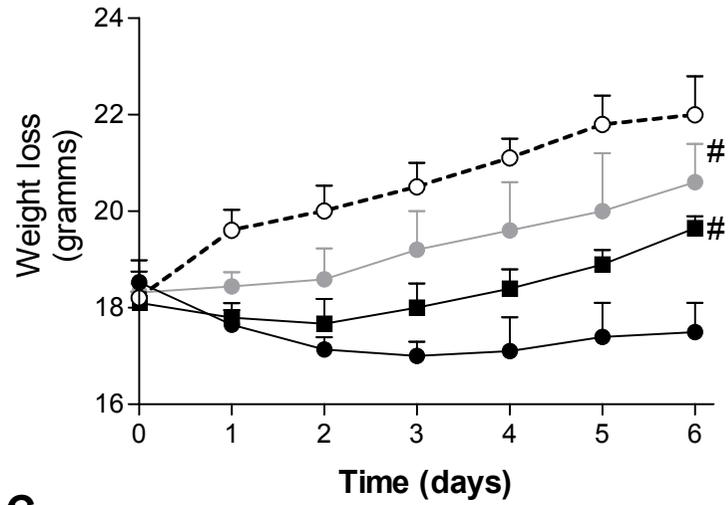
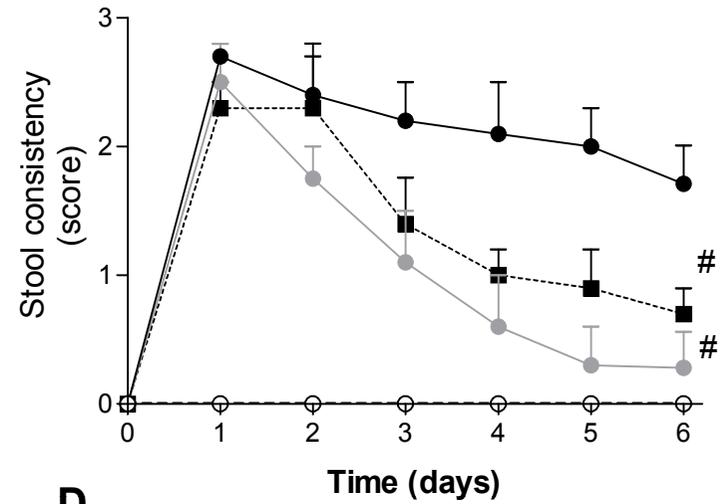
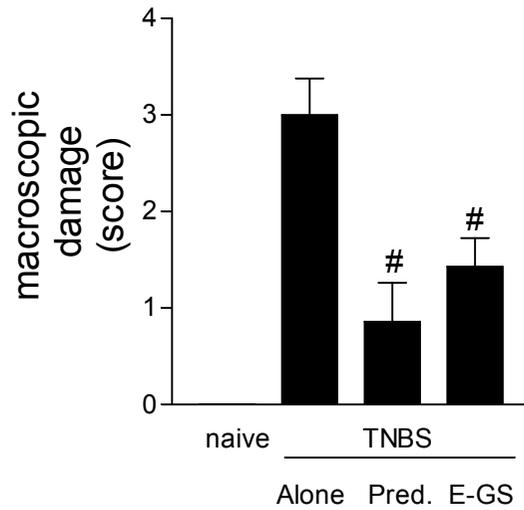
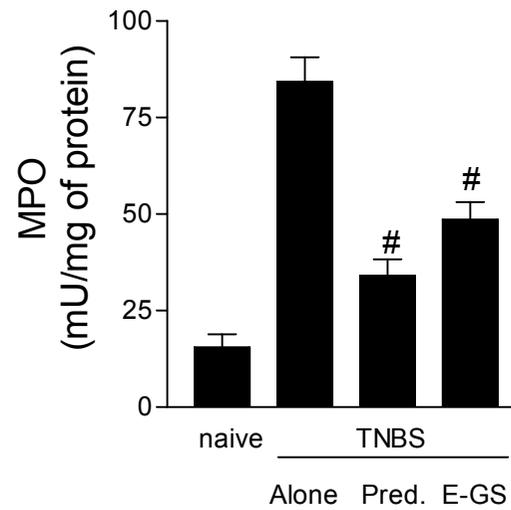
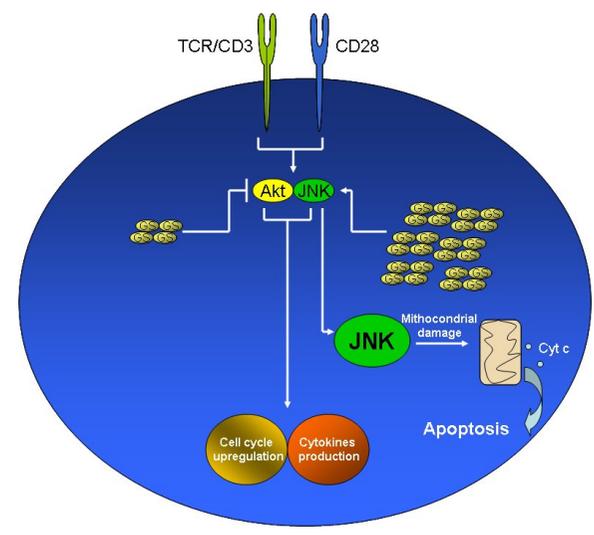
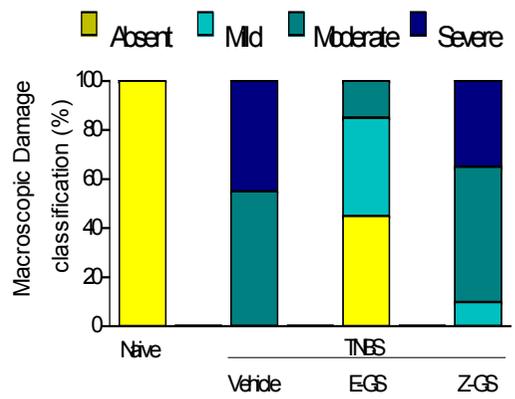
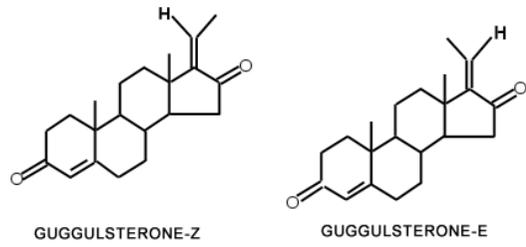
**B****C****D**

Figure 7



Guggulsterone is a plant sterol that exerts immunomodulatory activities in rodent models of T-cell-induced colitis. Guggulsterone directly modulates intracellular pathways in intestinal CD4+ cells