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Immunomodulatory Therapeutic Effect of Glatiramer Acetate on Several Murine Models of Inflammatory Bowel Disease

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Running title

Glatiramer acetate suppresses two animal models of colitis

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Nonstandard abbreviations used in this paper: IBD, inflammatory bowel diseases; DSS, dextran sulfate sodium; GA, glatiramer acetate; Th, T-helper; TNF-α, tumor necrosis factor-α; IFN-γ, interferon-γ; TGF-β, transforming growth factor β; IL-10, interlukin-10; CE, colon extract; MLN, mesenteric lymph nodes.

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Abstract

Inflammatory bowel disease (IBD) is characterized by detrimental immune reactivity in the gut and imbalance between pro-inflammatory and anti-inflammatory reactivity. In an attempt to down regulate colitis we investigated the effect of the immunomodulator glatiramer acetate (GA, Copaxone, Copolymer 1), on two murine models of IBD - chemically induced and spontaneous. Acute experimental colitis of different levels of severity was induced in C57BL/6 mice by dextran sulfate sodium (DSS) administered orally at different concentrations and frequencies. It was manifested in weight loss, intestinal bleeding and diarrhea, as well as by macroscopic and microscopic colon damage. GA treatment led to amelioration of all these pathological manifestations resulting in improved long-term survival. Moreover, even when colitis was induced by three cycles of DSS in this highly susceptible mouse strain, as well as in BALB/c mice which exhibit a chronic disease pattern, a substantial reduction in disease activity and mortality was obtained. GA treatment induced a beneficial effect also in a spontaneous model of colitis developed in the C3H/HeJBir IL-10 deficient mice. The detrimental pro-inflammatory response manifested by proliferation, TNF-α and IFN-γ expression was modulated by GA, while the regulatory anti-inflammatory TGF-β and IL-10 cytokines response was elevated. This was demonstrated on the level of protein secretion in splenocytes and local mesenteric lymphocytes, in the response to syngeneic colon extract and in the overall response to anti-CD3, as well as on the level of mRNA expression in the colon.

Introduction

Inflammatory bowel disease (IBD) is a generic classification for a group of inflammatory disorders of the gastrointestinal tract, characterized by intestinal inflammation and mucosal damage. An inadequate activation of the intestinal immune system involving mainly CD4 Th1 cells, and imbalance between pro-inflammatory and anti-inflammatory reactivity, play a pivotal role in the pathogenesis of IBD (Shanahan, 2001). It has thus been established that inflammatory mediators such as tumor necrosis factor-α (TNF-α), and interferon-γ (IFN-γ), produced by infiltrating CD4 T-cells and macrophages, exacerbate the disease, whereas regulatory cytokines such as transforming growth factor-β (TGF-β) and interlukin-10 (IL-10) provide a beneficial effect and their intestinal level may ultimately determine whether an immune response to a gut antigen is detrimental or innocuous (Strober et al., 1997; Rogler and Andus, 1998).

Current medical treatments for IBD rely on the use of non-specific anti-inflammatory agents and immunosuppressive drugs that cause severe side effects, and in significant percentage of the patients they do not induce long-term benefit (Sandborn and Targan, 2002, Baert et al., 2004). Based on the immunopathological nature of IBD, novel strategies have been proposed in an attempt to deviate the CD4 pathogenic T-cells from Th1 to Th2 phenotype. However, the newly designed medical interventions employed hitherto exhibited limited effect (Sandborn et al., 2005) or considerable toxicity with narrow therapeutic window (Kwon and Farrell, 2005). There is therefore a need for new, well-tolerated therapies that effectively induce remission and alter the natural course of the disease.

The synthetic copolymer glatiramer acetate (GA, Copaxone, Copolymer 1), an approved drug for the treatment of multiple sclerosis, is very well tolerated with high safety profile (Arnon and Sela 2003). GA has been shown to be effective in several animal models including experimental autoimmune encephalomyelitis (EAE) and immune rejection (Aharoni et al, 2001). Studies on the mechanism of action of GA revealed that it exerts its therapeutic activity by immunomodulating the immune response at different levels of specificity. Thus, it was demonstrated that GA binds promiscuously and with high affinity to various class II major histocompatibility (MHC) molecules of murine and human origin, and can even displace antigens from the MHC antigen-binding groove (Fridkis-Hareli, 1994). This competition for MHC binding can hinder presentation of other antigens and consequently lead to inhibition of various pathological effector functions. In addition, GA was shown to be a potent inducer of Th2/3-cells that secrete high amounts of regulatory substances such as IL-10 and TGF-β, but not Th1 inflammatory cytokines, (Aharoni et al, 1997). Moreover, GA treatment leads to deviation of the immune reactivity from Th1 to Th2 biased cytokine profile in both experimental animals and humans (Aharoni et al, 1998; Neuhaus et al, 2000). In view of these immunomodulating activities of GA and the Th1-related immunopathological nature of IBD, it was of interest to test whether GA can be effective in the suppression of IBD animal models. Indeed recently it was demonstrated that GA treatment ameliorates the various pathological manifestations of one experimental IBD model - trinitrobenzene sulfonic acid (TNBS)-induced colitis, administered rectally, in various mice strains (Aharoni et al, 2005; Gur et al, 2005).

None of the currently IBD models constitutes a faithful equivalent for the human diseases. It is therefore essential to evaluate the effect of any candidate drug in several IBD models. Widely used is the dextran sulfate sodium (DSS) colitis, induced by DSS administration in the drinking

water, which leads to many of the events presumed to initiate and sustain human IBD (Boismenu and Chen, 2000). This model allows the generation of variable disease forms, of acute and chronic nature, depending on the mouse strain or on the dose and frequency of DSS administration. Spontaneous models offer further advantage since both environmental factors and genetic susceptibility contribute to the pathological immunoreactivity causing IBD in humans (Shanahan, 2001). One such model was developed by transferring a genetically disturbed IL-10 allele into the IBD susceptible C3H/HeJBir substrain. The resulting C3H.IL10$^{-/-}$ mice develop an early non-remitting severe inflammation of both the cecum and the colon (Mahler and Leiter, 2002). In the present study we investigated the effect of GA in these two colitis models. We wish to report that GA treatment significantly ameliorated the various pathological manifestations of acute as well as chronic DSS-induced colitis and that it also led to a beneficial effect on spontaneous colitis in the C3H/HeJBir IL-10 deficient mice. Moreover, GA modulated the detrimental pro-inflammatory immune response provoked by the pathological process, while enhancing the secretion of beneficial regulatory anti-inflammatory cytokines.

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Materials and Methods

Mice. C57BL/6 and BALB/c mice were purchased from Harlan (Jerusalem, Israel). Female mice, 8-10 week of age weighing 18-21gr., were kept under specific pathogen free (SPF) environment and used for the induction of DSS colitis. C3H.IL10$^{-/-}$ mice, originated from C3H/HeJBir (Mahler and Leiter, 2002) were kindly provided by E.H. Leiter, from the Jackson Laboratory, and maintained under conventional (non-pathogen free) conditions. C3H.IL10$^{-/-}$ males, 3-4 month old, were used. All experiments were approved by the Institutional Animal Care and Use Committee.

Induction of DSS colitis. Mice were randomized into treatment groups of 6-12 animals, with identical average body weight in each group. Acute colitis in C57BL/6 mice was induced by giving dextran sulfate sodium (DSS), mol wt 36,000-50,000 (MP Biomedicals, LLC, Eschwege Germany) in acidified drinking water, 2-2.5% (wt/vol), for 4-5 days. Chronic colitis was induced by applying three DSS cycles of 5 days with intervals of 5-7 days of plain drinking water. In C57BL/6 mice 1.5% DSS with one week interval after the first DSS exposure and 5 days interval after the second exposure, and in BALB/c mice 5% DSS, for 5 days, with 5 days intervals, were given. We did not observe differences in the water consumption between the various experimental groups (7.0-7.5 ml per day per mouse), excluding the possibility of volume-associated deviation.

Assessment of DSS colitis. In all animals, body weight, rectal bleeding, stool consistency, and survival were monitored daily. Intestinal bleeding was followed by using Hemoccult test (Beckman Coulter, Inc., Fullerton, CA) as well as by observation of bleeding signs on the anus or gross bleeding. The daily disease activity index (DAI) was calculated by grading on a scale of

0 to 4 the following parameters: change in weight (0, ≤1%; 1, 1-5%; 2, 5-10%; 3, 10-15%; 4, >15%), intestinal bleeding (0, negative; 4, positive) and stool consistency (0, normal; 2, loose stools; 4, diarrhea). The combined scores were then divided by 3 to obtain the final disease activity index. 10-30 days following disease induction mice were sacrificed, the large intestine was collected and evaluated for colon length and microscopic colonic damage.

Microscopic scoring. Proximal, medial, and distal portions of colon and cecum were fixed in 10% phosphate-buffered formalin. Paraffin-embedded sections were stained with hematoxylin and eosin. The evaluation was based in part in that used elsewhere (Dieleman et al., 1998) and adapted to the pathology manifested in our laboratory. The degree of histological damage and inflammation was graded in a blinded fashion by an expert pathologist (O. Brenner). The following manifestations were included in the evaluations: the amount of inflammation (0, none; 1, mild; 2, moderate; 3, severe; 4, accumulation of inflammatory cells in the gut lumen), distribution of lesions (0, none; 1, focal; 2, multifocal; 3, nearly diffuse; 4, diffuse), depth of inflammation and layers involved (0, none; 1, mucosa only; 2, mucosa and submucosa; 3, limited transmural involvement; 4, transmural), nature of mucosal changes (0, none; 2, more degeneration; 3, more necrosis). The overall histological score was the sum of the four manifestations (maximum score 15). In C3H.IL10$^{-/-}$ mice, degeneration and necrosis were not included (maximum score 12).

Glatiramer acetate. GA, (Copaxone, Copolymer 1) consists of acetate salts of synthetic polypeptides, containing four amino acids: L-alanine, L-glutamate, L-lysine, and L-tyrosine (Arnon and Sela 2003). GA from batch 242990599, with an average molecular weight of

7300kDa, obtained from Teva Pharmaceutical Industries (Petach Tikva, Israel), was used throughout the study.

GA treatment. GA was administrated by one of the following procedures: Oral treatment - 250μg/day in PBS, fed by gastric intubation with an 18-gauge feeding needle. Feedings were performed on days -7, -5, -3, -1, 0, 2, 4 and 6, relative to the day of DSS induction. Parenteral treatment by daily injections – 2 or 1 mg/day, administered subcutaneous (s.c.) in PBS, starting at the indicated day.

Colon extract. Colons of syngeneic normal mice were cut into small strips and homogenized. The homogenate was diluted in PBS, passed through glass wool and filtered (0.2μ). Protein quantity was measured by using a protein assay kit (Bio-Rad, Richmond, CA).

Reactivity assays. Spleens and mesenteric lymph nodes (MLN) cells (0.5×10^6 /well) were cultured with either GA (50μg/ml), colon extract (200μg/ml), immobilized anti-CD3 (5μg/ml), or PBS control, in a final volume of 250μl/well (6 wells for each antigen). For proliferation assay, cells were cultured in RPMI 1640 with 10% FCS, pulsed with 1μCi of [^{3}H] thymidine after 48h incubation, and harvested 12 hours later. For cytokine assay, cells were cultured in serum-free medium (DCCM-1), supernatants were collected after 24 hours for TNF-α and IFN-γ, 48 hours for IL-10 and 72 hours for TGF-β, and tested by ELISA (R&D Systems, Minneapolis, MN). The presence of NO was determined by measuring the amount of nitrite in culure supernatants, using a colorimetric assay as previously described (Kayhan et al, 2003).

Reverse transcription-polymerase chain reaction (RT-PCR). Samples for mRNA isolation were removed from the colons of 3 individual C57BL/6 mice in each of the following groups:

naïve, DSS colitis untreated and treated with GA. Total RNA was isolated and processed according to Edelheit and Meiri 2004. The following sets of oligonucleotides and amplification conditions were used:

TNF-α: (sense) 5'- AGTCCGGGCAGGTCTACTTT -3'

(anti-sense) 5'- GAGGCAACCTGACCACTCTC -3' 60^0/ 30 cycles.

IFN-γ: (sense) 5'- ATCTGGAGGAACTGGCAAAA -3'

(anti-sense) 5'- TGAGCTCATTGAATGCTTGG -3' 63^0/ 35 cycles.

TGF-β: (sense) 5'- TACAGGGCTTTCGATTCAGC -3'

(anti-sense) 5'- CGCACACAGCAGTTCTTCTC -3' 63^0/ 35 cycles.

IL-10: (sense) 5' - TCCTTGGGAAGCAATTGAAG-3'

(anti-sense) 5'- AACTGGCCACAGTTTTCAGG -3' 63^0/ 35 cycles.

t-Bet: (sense) 5'- CTAAGCAAGGACGGCGAATGT -3'

(anti-sense) 5'- GGCTGGGAACAGGATACTGG -3' 60^0/ 35 cycles.

GATA-3: (sense) 5'- GCCTGCGGACTCTACCATAA -3'

(anti-sense) 5'- CAGGGATGACATGTGTCTGG -3' 54.8^0/ 30 cycles.

GAPD-H: (sense) 5'- GTGTTCCTACCCCCAATGTG -3'

(anti-sense) 5'- CTTGCTCAGTGTCCTTGCTG -3' 60^0/ 25 cycles.

The relative mRNA expression to the housekeeping GAPD-H was performed using NIH image software and averaged from the 3 mice in each group.

Statistical analysis. Weight and disease activity differences between GA-treated and untreated groups were analyzed using two-tailed Mann-Whitney U test. Statistics of survival curves were performed by Kaplan-Meier test. Differences in colon length, histological score, proliferation,

Hemoccult assay, and cytokine responses were compared using the two-tailed independent t test. All the tests were performed by the SPSS 10.0 program. The level of significance for all the tests was set at <0.05.

Results

The effect of GA treatment on DSS colitis induced by different regimens of one DSS cycle

To explore the optimal parameters of GA activity, we tested the ability of GA administered by different routes and dosages to ameliorate acute colitis induced by giving DSS (2.5% for 4 days) to the highly susceptible mouse strain C57BL/6. The effect of oral treatment (250μg/feeding, at alternate days, starting 7 days before disease induction), or daily subcutaneous injections of 2 mg/mouse starting either at the day of induction or 2 days after induction and of 1mg/mouse from the day of induction, are depicted in Fig. 1A. Whereas the colitis untreated mice manifested extensive weight loss, starting 4 days after DSS administration reaching 18% by day 7, mice injected daily with GA suffered considerably less weight loss. The most effective dose of GA was 2 mg/mouse, when injections started at the day of DSS administration (only 3.5% weight loss by day 7), but a significant beneficial effect was observed even when GA treatment started two days after disease induction (8% weight loss). Oral administration in a dose previously found effective in another experimental disease model - EAE (250μg/feeding, Teitelbaum et al., 1999), did not induce significant effect in this model (significance analyzed for the differences on day 7).

The beneficial effect of the optimal GA treatment - daily injections of 2 mg/mouse from the day of induction, was further corroborated in DSS colitis of different severity levels i.e. induced by 2% DSS for 5 days or by 2.5% DSS for 5 and 7 days (Fig. 1B). In all three systems the GA treated mice exhibited significantly higher body weights than those of the equivalent untreated mice as analyzed by day 10. The milder regimen (2% DSS, 5 days) led to 27% weight loss and 75% mortality in untreated animals; in contrast, all the GA treated mice subjected to this regimen

survived, exhibiting only moderate weight loss (maximum 9%) and subsequent weight regain up to complete restoration of their original weight. Moreover, when colitis was induced by 2.5% DSS, for 5 or 7 days, all the untreated mice succumbed to the disease by day 11, but GA treatment resulted in the survival of 6/8 and 3/8 mice respectively.

GA activity on the various manifestations of DSS colitis

A summary of 3 additional experiments (total of 25-26 mice per group) in a DSS colitis induced by 2.5% DSS for 5 days, with 30 days follow up, is demonstrated in Fig. 2. In these experiments untreated mice lost 33% of their original body weight (A) and all of them died by day 12 (C). In contrast, GA-treated mice (2 mg/mouse daily starting from day 0) suffered only 10% weight loss ($p=0.005$ for GA treatment in comparison to no treatment 10 days from induction), and by day 14 regained their original weight, and all of them survived till the end of the experiments (1 month after disease induction). Intestinal bleeding, a pathological manifestation of DSS colitis, was followed by using Hemoccult test, as well as by observation of rectal bleeding signs (Fig. 2B). By both methods GA treatment reduced intestinal bleeding. Hence, on day 10, when all the untreated mice suffered from intestinal bleeding, only 43% and 14% of the GA treated mice demonstrated positive Hemoccult test and bleeding signs, respectively. One month after DSS induction intestinal bleeding was rarely observed in the GA treated animals using both methods, when none of the untreated mice survived (Fig. 2C).

An additional macroscopic manifestation of DSS-induced colitis is the reduction in colon length (Fig. 2D). Thus, 30% decrease in colonic length was found in untreated mice in comparison to naive mice, 5 and 10 days after DSS induction. In contrast, only 20% and 15% reduction,

respectively, was obtained in the GA-treated mice. Furthermore, one month after disease induction, when none of the untreated mice survived, colon length of the GA-treated mice was similar to that of normal mice manifesting only minor insignificant decrease of 7% ($p=0.16$). Histological assessment of colonic damage in untreated mice, 10 days after DSS induction, revealed extensive injury i.e. severe inflammation, with nearly diffused distribution, involving mucosa, submucosa and in some cases extending through all intestinal layers (transmural penetration). This was associated with severe disruption of the normal architecture, necrosis and crypt loss, average histological score 13.4, on a scale of 0-15 (Fig. 2E,F). In colons of DSS-induced mice treated with GA and tested on the same day, a small, but statistically significant reduction in the histological damage was observed - average score 11.8. However, by day 30, when none of the untreated mice survived, the colonic damage in the GA treated mice was lower - grade 7, and more conserved glandular structure were revealed, although, widespread leukocyte infiltrations were still present (Fig. 2E,F).

Thus, GA treatment (daily injections of 2 mg/mouse) in an acute colitis model induced by one DSS cycle, resulted in substantial beneficial effect on all pathological manifestations - weight loss, intestinal bleeding, colonic length and histological damage, resulting in improved long-term survival.

The effect of GA treatment on chronic colitis induced by three DSS cycles in different mouse strains

Since IBD is characterized by multiple exacerbations, an additional model was used, in which three DSS cycles were applied, in both the highly susceptible strain C57BL/6 and the less

sensitive strain BALB/c. Indeed, three 5 days cycles of 1.5% DSS with one week interval after the first DSS exposure, and 5 days interval after the second exposure, resulted in 20% body weight loss in C57BL/6, whereas a more vigorous regime, 5% DSS (for 5 days, with 5 days intervals) were needed to obtain 15% weight loss in BALB/c mice.

The effect of GA treatment in these three DSS cycles model on the daily monitored disease activity index (DAI, combined score of body weight, bleeding and stool consistency), as well as on survival is shown in Fig.3. In both mouse strains a daily dose of 2 mg/mouse of GA drastically decreased disease activity and completely prevented mortality in comparison to 33% and 40% mortality in the untreated C57BL/6 and BALB/c mice, respectively. In BALB/c mice, 1 mg/day GA was sufficient, resulting in a suppressive effect similar to that of 2 mg/day, whereas in C57BL/6, 1 mg/day was less effective than 2 mg/day (similar to the results obtained for this strain in the acute one DSS cycle system Fig.1). Using three DSS cycles in C57BL/6 mice, the ability of the optimal GA dose (2mg/day) to ameliorate an established disease was tested. The results indicated that even when treatment was started 10 days following DSS induction, after the mice reached maximal weight lost (30% of their initial body weight), GA treatment still induced beneficial effect manifested in 15% higher body weight and 20% less mortality than the untreated mice (data not shown).

The effect of GA treatment on spontaneous colitis in C3H.IL10$^{-/-}$ mice

The IL-10 deficient transgenic mice C3H/HeJBir IL-10$^{-/-}$ developed in Jackson Laboratories, were shown to develop spontaneous colitis (Mahler and Leiter, 2002). In our laboratory, when

these C3H.IL10$^{-/-}$ mice were maintained in specific pathogen free (SPF) environment, clinical signs were not apparent and histological abnormalities in their intestine were minimal. When the mice were transferred and bred under conventional (non-pathogen free) conditions, disease manifestations were gradually revealed, mainly histologically as inflammation of the cecum and the colon (in males more than in females). Disease was increasingly aggravated in direct relation to the time passed from their SPF departure (Fig. 4, Table 1). Hence five months from transfer most of the 3-4 month old male mice manifested focal or multifocal mild inflammation in the mucosa, average histological score 4 on a scale of 0-12 (Fig. 4A, Table 1, experiment 1). Five month later, multifocal lesions and moderate inflammation in the mucosa and submucosa, average score 6, were observed (experiment 2). One year after transfer of the colony, the entire male population at the age of 3-4 months, had a severe colitis often with transmural involvement, scored 7.6 (Fig. 4C, Table 1, experiment 3). Towards the fourth month of their life the C3H.IL10$^{-/-}$ mice suffered also from intestinal bleeding, as revealed in Hemoccult test (average 1-1.3 from maximum grade of 2, in the different experiments). GA treatment, starting at the age of three months when disease was already established, (daily injections of 2mg/mouse, for one month), ameliorated the spontaneous colitis in C3H.IL10$^{-/-}$ mice at all the levels of severity (Fig. 4 B,D, Table 1). This was manifested in reduction of the histological score - 3 points in the mild disease (experiment 1), and in a smaller reduction - 1.7 and 1.4 in the more severe disease (experiments 2 and 3 respectively). In experiment 3 however, the differences in histological score did not reach statistical significance. GA-treatment almost eliminated intestinal bleeding only 3 from 17 mice were found positive in Hemoccult tests (average grade 0.1), compared to 9 from 10 of the control untreated mice.

Lymphocyte reactivity in GA-treated DSS-induced mice

To explore the consequence of GA treatment on lymphocyte activity in DSS-induced colitis, we studied cell proliferation and their cytokine profile in the DSS-administered C57BL/6 mice, treated with GA, as compared to untreated mice and naïve healthy controls. The reactivity of lymphocytes from local mesenteric lymph nodes (MLN) that are adjacent to the diseased organ, as well as those from spleen cells was analyzed.

The proliferation in response to colonic extract (CE), obtained from normal syngeneic mice, and to the treatment agent GA, is depicted in Fig. 5. A prominent response to colonic extract was demonstrated by MLN cells of DSS-induced mice, i.e. 12-fold over that of naïve controls. This augmented response to CE was restricted to the local MLN, since in spleens of colitis induced mice proliferation to CE was similar to that of naive mice. The local MLN response to CE was significantly suppressed by GA treatment resulting in 32% inhibition. Lymphocytes from GA treated mice, from both spleens and MLN, proliferated in response to GA, the systemic response being considerably higher than the response of the MLN cells.

The overall secretion of two pro-inflammatory (Th1) cytokines - TNF-α and IFN-γ - as well as of two regulatory anti-inflammatory cytokines - TGF-β (Th3) and IL-10 (Th2) - was investigated by stimulating MLN and spleen lymphocytes with anti-CD3 (Fig. 6). Both MLN and spleen cells from mice with DSS colitis secreted elevated amounts of TNF-α (A) and IFN-γ (B), but not TGF-β (C) or IL-10 (D) in response to the broad stimulation by anti-CD3. The most prominent increase (7-fold from naïve controls), was manifested for TNF-α by MLN lymphocytes. GA treatment led to significant reduction of the overall TNF-α and IFN-γ secretion by MLN (43% and 37% inhibition respectively) and spleen lymphocytes (secretion similar to that of naïve

controls). In contrast to its inhibitory effect on the pro-inflammatory cytokines, GA treatment triggered the secretion of the anti-inflammatory cytokines TGF-β and IL-10 (40-fold increase from untreated mice for TGF-β in MLN as well as in the spleen, and 10-fold for IL-10 in MLN). IL-10 secretion in spleens of DSS untreated mice was reduced by half in comparison to healthy controls, and GA treatment restored it to the normal level.

Similar cytokine patterns to those obtained for the broad stimulation to anti-CD3 were found for the restricted responses to colonic extract demonstrated for MLN lymphocytes, shown in Fig. 7. Thus, cells from mice with DSS colitis secreted elevated amounts of TNF-α (A) and IFN-γ (B) in response to CE (2-fold increase from naïve control). This specific response was completely abrogated by GA treatment and thus the level of the cytokines was similar to that of naive mice. The anti-inflammatory response to CE differed between the two tested cytokines, whereas secretion of TGF-β (C) could not be detected in naïve and colitis induced mice, considerable amounts of IL-10 (D) were secreted in response to the colon antigen by all experimental groups. These amounts were decreased in the colitis untreated mice and increased by GA treatment to an even higher level than in naïve controls. Interestingly, decreased IL-10 secretion in untreated mice and elevation by GA was seen also in the background response (with no antigenic stimulation). *In vitro* stimulation by GA did not result in secretion of either TNF-α or IFN-γ in any of the treatment groups. But, cells from GA treated mice responded to GA by extensive secretion of TGF-β and IL-10. Similar secretion patterns i.e. decrease in Th1 and increase in Th2 cytokines were found also in cells originating from spleens (systemic response, data not shown).

It can thus be concluded that concerning both the overall cytokine secretion and the restricted response to specific colonic antigens, GA treatment inhibited the augmented pro-inflammatory

reactivity provoked by the pathological process, while enhancing the secretion of regulatory anti-inflammatory cytokines.

Nitric oxide (NO) is an important mediator involved in the pathogenesis of IBD. We therefore examined the effect of GA treatment on NO secretion in colitis-induced mice (Fig. 7E). *In vitro* stimulation of normal splenocytes with colonic extract resulted in NO secretion (4-fold increase over the unstimulated cells). Spleen cells from DSS-induced mice manifested elevated NO secretion in response to CE (37% above that of naïve mice). GA treatment abrogated this response and resulted in NO levels similar to that observed in normal mice. *In vitro* stimulation of spleen cells with GA did not induce NO secretion in any of the experimental groups.

The effect of GA on cytokine mRNA expression in the colons of DSS-induced mice

Employing RT-PCR on colon mRNA, the levels of pro-inflammatory and anti-inflammatory cytokines in the DSS-induced mice treated by GA were evaluated and compared to untreated colitic mice and to naïve healthy controls. mRNA expression in colons of three representative mice from each group, as well as their relative levels to GAPD-H are demonstrated in Fig. 8. As shown, the expression of the two pro-inflammatory (Th1) cytokines: TNF-α and IFN-γ was markedly increased in the colons of DSS-induced untreated mice - 5.2 and 15 fold over the naïve mice, respectively. GA treatment reduced the colonic expression of the Th1 cytokines. In particular, the augmentation in TNF-α was completely abrogated, so that its expression was similar to that of naive mice. As for the anti-inflammatory cytokines, a marked increase in the colonic expression of TGF-β was induced by GA treatment (5.8 fold from normal, 4.5 fold from

untreated colitic mice). Unlike TGF-β, the level of IL-10 expression was increased in colitis untreated mice in comparison to naïve control, but GA treatment led to a further 2 fold elevation in its expression.

Consistent with the cytokine expression data, mRNA levels of the Th1 transcription factors - T-bet was increased in colons of DSS untreated mice (14 fold from naïve) and GA treatment significantly reduced its level. The expression of the Th2 transcription factors GATA-3, was somewhat increased in untreated colitic mice and GA treatment resulted in additional elevation in its colonic level. Thus, the inhibition of the pro-inflammatory cytokines and augmentation of the regulatory anti-inflammatory responses induced by GA treatment observed initially on the level of protein secretion, were corroborated by the respective mRNA expression in the colon.

Discussion

In the present study, we substantiate the therapeutic potential of GA for the treatment of IBD by demonstrating its effect in both acute and chronic chemically induced as well as spontaneous colitis murine models. GA treatment suppressed the various pathological manifestations of DSS-induced colitis i.e. weight loss, intestinal bleeding and diarrhea, resulting in substantial reduction of disease activity (Figs. 1-3). The colonic damage characteristic to the disease exhibited macroscopically by shortening of the colon, and microscopically by mucosal ulceration, inflammation and crypt damage, were also reduced by GA treatment (Fig. 2D-F). This led to significantly higher long-term survival in GA-treated mice in comparison to untreated mice (Figs. 1-3). The beneficial effect of GA was manifested in several DSS models: 1) acute colitis of different severity levels, induced by one DSS cycle in C57BL/6 mice (Fig. 1B); 2) three DSS cycles-induced colitis in this susceptible strain (Fig. 3A); 3) in BALB/c mice, which are less sensitive to DSS, and suffer from chronic disease pattern after exposure to vigorous DSS regime (Fig. 3B). As IBD in humans is characterized by multiple exacerbations, the beneficial effect of GA on colitis induced by three DSS cycles in both strains is of therapeutic significance.

The most effective treatment regimen in the C57BL/6 strain was daily s.c. injections, a dose of 2 mg/mouse, starting at the day of DSS administration, but, even when treatment started two days after induction a significant beneficial effect was observed (Fig. 1A). Moreover, in a therapeutic setting of three DSS cycles when treatment was started after the mice reached maximal weight lost, GA treatment still induced a beneficial effect. A daily dose of 1 mg was less effective than 2 mg in the highly susceptible strain C57BL/6, when either one or three DSS cycles were applied (Figs. 1A, and 3AB). Interestingly in the chronic disease form, in the less susceptible BALB/c

mice, 1 mg/day GA was sufficient dose resulting in a suppressive effect similar to that of 2 mg/day (Fig. 3C,D). Oral administration of GA in a regimen, previously found effective in another experimental disease - EAE (250μg/feeding Teitelbaum et al., 1999), did not induce significant effect in the DSS model in C57BL/6 mice (Fig. 1). The oral route for IBD, besides the practical advantage, has the benefit of specific administration into the diseased organ, so local modulatory mechanisms may be activated in addition to the systemic processes. Indeed, feeding with GA ameliorated TNBS colitis in BALB/c mice, but in highly susceptible strains, in which TNBS induced more intensive disease, oral administration was less effective than the parenteral way (Aharoni et al 2004).

The curative effects demonstrated by GA on acute and chronic DSS colitis, as well as on TNBS induced colitis, are of therapeutic significance, since these widely used models recapitulates many of the events proposed to initiate and sustain human IBD (Boismenu and Yaping 2000). Yet, since IBD is believed to occur in genetically predisposed individuals, the spontaneous colitis model offers additional advantageous (Mahler and Leiter, 2002). In our hands these mice did not exhibit disease manifestations under SPF environment, but gradually developed inflammation of both the cecum (typhlitis) and the colon (colitis) upon transfer to conventional conditions, supporting the involvement of environmental factors as well as genetic susceptibility in the pathological immunoreactivity causing IBD. GA treatment, starting at the age of three month (when disease was already established), ameliorated spontaneous colitis in C3H.IL10$^{-/-}$ mice (Fig. 4, Table 1). It should be noted, that the most prominent effect (reduction of 3 points in the histological index) was obtained in the mild disease, whereas, in the case of severe disease, the histological improvement was smaller. Thus, the demonstration of GA therapeutic activity in two chemically induced models: TNBS colitis (in three mouse strains, ref), and DSS colitis

(acute and chronic, in two mouse strains), as well as in spontaneous colitis (in C3H.IL10$^{-/-}$ mice), indicates that this effect is not restricted to a single strain/model but represents a more general phenomenon.

To further understand the protective activity of GA in DSS colitis we investigated the effect of GA treatment on lymphocyte reactivity. We found that the prominent response to syngeneic colonic extract, in C57BL/6 mice with acute DSS-induced colitis, manifested by local MLN cells adjacent to the diseased organ, was reduced by GA treatment (Fig. 5). This proliferation in response to CE was manifested only by MLN of colitis induced mice and not by MLN of naïve mice or systemically by spleen cells of colitis induced mice. Moreover, the MLN cells were cultures in the presence of irradiated spleen cells from syngeneic naïve mice (as antigen presenting cells) and there was no background response to these cells, suggesting local reactivity towards this colonic extract. It was previously shown that GA binds promiscuously with high affinity to various MHC class II molecules from mouse and human, and even displaces antigens from the MHC groove (Fridkis-Hareli et al, 1994). Such efficient binding to local antigen-presenting cells in the intestine may interfere with the presentation of floral/self antigens and thus hinder pathological T-cell activation. Indeed, in TNBS colitis, in which GA treatment blocked the MLN response to colonic extract (Aharoni et al, 2005), a role of class II molecules as a target site for GA competition with the antigens was demonstrated (Gur et al, 2005). MHC blocking by GA proved to be effective also in other pathological conditions such as MS and EAE (Arnon and Sela, 2003), graft versus host disease and graft rejection (Aharoni et al, 2001).

GA treatment resulted not only in inhibition of the proliferative response, but also in significant reduction in the Th1 cytokines TNF-α and IFN-γ. These prototype pro-inflammatory cytokines

were significantly elevated, systemically - in splenocytes, and locally - in MLN, in colitis untreated mice. Both their overall secretion (in response to anti-CD3) as well as their secretion in response to colonic extract were decreased in the GA treated mice (Figs 6 AB and 7 AB). Inflammatory cytokines play a central role in the pathology of the intestine, amplifying and prolonging inflammation (Rogler and Andus, 1998). Rapidly synthesized and secreted upon stimulation, they induce production of inflammatory mediators such as nitric oxide. Indeed in this study the changes in TNF-α and IFN-γ secretion were associated with parallel variations in NO i.e. elevation upon stimulation with colon extract in DSS colitis untreated mice, and complete abrogation of this response by GA treatment (Fig. 7E).

An essential mechanism by which GA had been shown to induce therapeutic effect, in EAE/MS as well as in immune rejection, is the generation of regulatory T-cells that secrete Th2/3 anti-inflammatory cytokines (Aharoni et al, 1997). In the present study, a systemic and to a lesser extent local proliferation in response to GA was evident (Fig. 5), suggesting the generation of GA specific T-cells in the treated mice. Furthermore, elevation in the anti-inflammatory cytokines - TGF-β and IL-10, on the level of overall secretion (Fig. 6CD) as well as in response to the treatment antigen (Fig. 7CD), was found in the GA treated mice systemically and locally, indicating that GA promoted specific Th2/3 regulatory T-cells in this model as well. Importantly a significant increase in IL-10 secretion was found not only after stimulation by GA but also by colonic extract in the MLN of treated mice (Fig. 7D), suggesting a bystander therapeutic effect similar to that demonstrated for GA in other systems (Aharoni et al, 1998). In the case of EAE, GA-specific cells were even shown to reach the diseased organ (the CNS) and secrete there anti-inflammatory cytokines (Aharoni et al, 2003).

Regulatory cytokines are the key factor in maintaining gut homeostasis. In particular TGF-β and IL-10 may ultimately determine whether an immune response to gut antigen is detrimental or innocuous (Strober et al., 1997). Hence, in two models of Th1 mediated murine colitis alleviation of disease was shown to be strictly associated with increased numbers and up regulation of TGF-β producing cells (Neurath et al., 1996 and Powrie et al., 1996). In humans as well, mucosal T-cell unresponsiveness to luminal antigens is mediated by TGF-β and its production in lamina propria mononuclear cells and in T-cells isolated from CD patients is significantly reduced (Del Zotto et al., 2003). Elevation in TGF-β induced by GA treatment was found in additional IBD model, TNBS induced colitis (Aharoni et al., 2005). It is therefore likely that TGF-β play an important role in the inhibitory effect of GA in IBD. The role of IL-10 for gut homeostasis has been demonstrated by the development of colitis in IL-10 deficient mice (Rogler and Andus, 1998). The ability of GA to suppress colitis even in the IL-10 deficient mice (Table 1) could be attributed to compensatory activity of other mechanisms activated by GA treatment e.g. competition for MHC binding, inhibition of TNF-α and IFN-γ secretion, and elevation in TGF-β.

The augmented pro-inflammatory reactivity provoked by the pathological process and its modulation by GA treatment, as well as the ability of GA to increase the regulatory anti-inflammatory pathway, were demonstrated in DSS colitis on the level of protein secretion in response to broad and specific antigens, for the periphery and for local lymph nodes. Similar secretion patterns were obtained after GA treatment in TNBS induced colitis for TNF-α and TGF-β (Aharoni et al, 2005). In this study these results were corroborated on the level of mRNA expression in the diseased organ – the colon (Fig. 8). Hence, mRNA expression of the two pro-inflammatory cytokines: TNF-α and IFN-γ were increased in colons of DSS-induced untreated

mice and GA treatment decreased their level, while inducing a significant elevation in the colonic mRNA of the anti-inflammatory cytokines TGF-β and IL-10. These conclusions are supported by the decrease found in the Th1 transcription factor T-bet and the increase in the Th2 transcription factors GATA-3, in the colons of the GA treated mice. These factors have a critical role in the pathogenesis and the control of IBD by regulating the cytokine balance in mucosal T-cells. Interestingly TGF-β was found to suppress T-bet expression; this is in accordance with the elevated TGF-β and reduced T-bet levels observed after GA treatment (Neurath et al, 2002).

The role of lymphocyte reactivity in the pathogenic process of DSS induced colitis is controversial, since DSS colitis could be induced in the absence of lymphocytes in SCID mice (Dieleman et al., 1998). Still, even in this model, large numbers of activated T-cells are located near diseased segments (Boismenu and Chen 2000) and lymphocyte reactivity may be important in initiating gut inflammation (Pizarro, et al., 2003). The modulatory effect of GA on inflammatory lymphocytes could thus contribute to the suppression of DSS colitis. In addition, GA can alleviate pathological processes by other mechanisms such as its effect on components of the innate immune system, specifically on monocytes and dendritic cells, to stimulate Th2 response (Farina et al., 2005). GA has also been shown to inhibit nuclear factor-kappa B (NF-kB) activation system, resulting in reduced production of inflammatory mediators such as chemokines e.g. RANTES (Li et al., 2001), TNF-α and Nitrite (Kayhan et al., 2003). These activities of GA may play a role in its beneficial effect in IBD.

It should be noted that the cumulative results on the mechanism of action of GA on the one hand and the nature of the T-cell response mediating IBD on the other hand, support its application for the treatment Crohn's disease (CD), which manifest pro-inflammatory immunopathological

features, rather than for ulcerative colitis (UC) which presents a less clear immunological basis involving Th2 immunopathology (Gordon et al, 2005). In conclusion, GA is effective in ameliorating the pathological manifestations in several models of experimental colitis, possibly due to its immunomodulating properties. In view of its high safety profile these results warrants further evaluation of GA treatment for human inflammatory bowel disease in particular for Crohn's disease.

Acknowledgments

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Footnotes

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Legends for Figures

Fig. 1. The effect of various GA treatment modes on acute DSS colitis induced by different regimens of one DSS cycle in C57BL/6 mice**.** A, Amelioration of body weight loss by GA administered by different routes and dosages in colitis induced by 2.5% DSS for 4 days. GA treatment was applied: orally, 250μg/feeding, at alternate days, starting 7 days before disease induction, by daily subcutaneous injections, 2 mg/mouse starting either at the day of induction or 2 days after induction and 1mg/mouse from the day of induction. Each treatment group consisted of 8-10 mice. The weight of the mice injected with 2 mg/mouse from day 0 and 2 were significantly higher than those of the untreated mice ($p = 0.017$ and 0.019 respectively), whereas no significance was found for injection of 1 mg/mouse ($p= 0.073$), or for the oral treatment ($p=0.535$), analyzed on day 7. B, The effect of GA treatment, administered by daily injections, 2 mg/mouse, from the day of disease induction, on body weight loss and survival in colitis of different severity levels induced by: 2% DSS for 5 days, or by 2.5% DSS for 5 and 7 days. In all three systems the weights of GA-treated mice were significantly higher than those of the equivalent untreated mice ($p = 0.017$, 0.019 and 0.042 respectively), analyzed on day 10 .The number of mice that survived till the end of the experiment from each group is shown in brackets.

Fig. 2. The effect of GA treatment on the various manifestations of acute one cycle DSS colitis in C57BL/6 mice**.** Disease was induced by 2.5% DSS for 5 days. GA treatment, 2 mg/mouse, was administered by daily subcutaneously injection, starting from day 0. A, Body weight; B, Rectal bleeding as followed by Hemoccult test, or by observation of bleeding sings; C, Survival; D, colon length; E, histological score. F, Histological appearance of colons from DSS induced

mice. Left - colon of untreated mouse, 10 days after DSS induction, manifests mucosal ulceration, crypt damage and transmural inflammation. Right - colon from GA-treated mouse, 30 days after disease induction, when none of the untreated mice survived, shows conserved or restored mucosal and glandular structure and leukocyte infiltration (H&E, magnification 40x). The data present the combined results from 3 experiments, total of 25-26 mice per group. #Significant effect over naïve control, *significant effect over colitis untreated mice ($p<0.05$). The significance of the body weight differences was analyzed on day 10 ($p = 0.025$ for no treatment in comparison to naïve, and 0.005 for GA treatment in comparison to no treatment). +indicates that none of the mice in the untreated group survived.

Fig. 3. The effect of GA treatment, daily injections of 1mg/mouse or 2 mg/mouse, from the day of disease induction on colitis induced by three DSS cycles. A,B in C57BL/6 mice by three 5 days cycles of 1.5% DSS, with one week interval after the first DSS exposure, and 5 days interval after the second exposure; C,D in BALB/c mice by three 5 days cycles of 5% DSS, with 5 days intervals between cycles. A and C, daily monitoring of DAI (disease activity index, which is the combined score of body weight, rectal bleeding and stool consistency, on a scale of 0 to 4). B and D, survival compared to untreated mice. In both mouse strains 2 mg/mouse GA significantly decreased disease activity and mortality in comparison to untreated mice. The effect of 1 mg/day GA was significant only in BALB/c mice ($P<0.05$).

Fig. 4. Histological manifestations, light microscopy, of colitis in the large intestine of 4 month old male C3H/HeJBir IL-$10^{-/-}$ mice and the effect of GA treatment. A and B, 5 months after transfer of the colony from SPF to conventional conditions. A, Untreated mouse expressing mild colitis with multifocal mucosal and submucosal infiltration of mononuclear cells. The mucosa is

preserved and exhibits mild hyperplasia (histological score 4 from a scale of 12). B, GA treated mouse, 2mg/mouse, exhibits intestinal histology within normal limits (histological score 0). C and D, 12 month after transfer from SPF to conventional conditions, C, Untreated mouse, severe transmural colitis, with extensive mucosal ulceration, necrosis, loss and multifocal coalescing mixed inflammatory infiltration extending and involving the mesentery (histological score 10). D, GA treated mouse, moderate colitis with multifocal mucosal and submucosal mononuclear infiltration and marked hyperplasia. The mucosa lining is preserved (histological scored 5).

Fig. 5. Lymphocyte proliferation in C57BL/6 mice with acute DSS induced colitis. The responses of: A, cells from mesenteric lymph nodes (MLN), or B, spleens of C57BL/6 mice, naive and induced for colitis (2.5% DSS for 5 days), untreated or treated with GA (2 mg/mouse, daily injections, from the day of disease induction). Cells were cultured, 12 days after disease induction, with no antigen, colonic extract (CE 200μg/ml), or GA (50μg/ml). Results of thymidine incorporation are expressed as mean cpm ±1SD of six culture wells and represent one of three similar experiments, using pooled cells from 3-6 mice in each group. #Indicate significant response over naïve control, *indicate significant response over colitis-induced untreated mice, to the same antigen ($p<0.05$).

Fig. 6. The overall cytokine secretion in response to anti-CD3 in C57BL/6 mice induced with acute DSS colitis. The secretion of A, TNF-α; B, IFN-γ; C, TGF-β and D, IL-10 by C57BL/6 mice, naïve, induced for colitis (2.5% DSS for 5 days) untreated, or treated with GA (2 mg/mouse, daily injections, from the day of disease induction). Cells from MLN and spleens were cultured, 10 days after disease induction, with immobilized anti-CD3 (5μg/ml). After 24 hours, supernatants from six culture wells were pooled and cytokines were measured by ELISA in duplicates. Results are expressed as cytokine concentration pg/ml±1SD and represent one of

three similar experiments, using pooled cells from 3-6 mice in each group. #Indicate significant secretion over naïve control, *indicate significant secretion over colitis untreated mice ($p<0.05$).

Figure 7. Cytokine and NO secretion in response to specific antigens in C57BL/6 mice induced with acute DSS colitis. The secretion of A, TNF-α; B, IFN-γ; C, TGF-β and D, IL-10 by C57BL/6 mice, naïve, induced for colitis (2.5% DSS for 5 days), untreated and treated with GA (2 mg/mouse, daily injections, from the day of disease induction). Cells from MLN (TNF-α IFN-γ, TGF-β and IL-10) and spleens (NO) were cultured, 10 days after disease induction, with no antigen, GA (50μg/ml) or colonic extract (CE 200μg/ml). After 24 or 48 hours, supernatants from six culture wells were pooled, cytokines and NO were measured by ELISA in duplicates. Results represent one of three similar experiments, using pooled cells from 3-6 mice in each group. #Indicate significant secretion over naïve control, *indicate significant secretion over colitis untreated mice ($p<0.05$).

Figure 8. RT-PCR analysis of cytokines and transcription factors in colon RNA of C57BL/6 mice induced by DSS for acute colitis (2.5% DSS for 5 days). mRNA expressions of 3 individual mice from the following groups are presented: naïve, colitis untreated, and GA treated (2 mg/mouse, daily injections, from the day of disease induction). The relative (fold increase) expression of TNF-α, IFN-γ, TGF-β, IL-10, T-bet and GATA-3 to the housekeeping GAPD-H mRNA, averaged from the 3 mice in each group is demonstrated in the right panel. #Indicate significant mRNA expression over naïve control, *indicate significant expression over colitis untreated mice ($p<0.05$).

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TABLE 1

The effect of GA treatment on spontaneous colitis in C3H.IL10$^{-/-}$ mice

	Treatment	**N**	**Hemocult**	**Histology**
Experiment 1	**Control** (no treatment)	3	1.2 ± 0.5	4.0 ± 1.0
	GA (daily 2mg/mouse)	6	0.1 ± 0.3 *	1.0 ± 1.6*
Experiment 2	**Control** (no treatment)	2	1 ± 0.0	6.0 ± 0.0
	GA (daily 2mg/mouse)	3	0.1 ± 0.3*	4.3 ± 0.6*
Experiment 3	**Control** (no treatment)	5	1.3 ± 0.2	7.6 ± 1.3
	GA (daily 2mg/mouse)	8	0.1 ± 0.1 *	6.2 ± 1.0

GA treatment by daily subcutaneous injections, 2mg/mouse, for one month, was administered to three month old male C3H/HeJBir IL-10$^{-/-}$ mice with established disease. A vehicle control group, (2 mice injected daily with 0.1ml PBS) in experiment 1, exhibited disease manifestations identical to the control untreated group. Experiments 1, 2 and 3 were performed five, ten and twelve month respectively after the transfer of the C3H.IL10$^{-/-}$ colony from SPF to conventional conditions. N indicates the number of animals in a group. * indicates significant decrease in GA-treated mice versus untreated mice in the same experiment ($p<0.05$).

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Figure 1

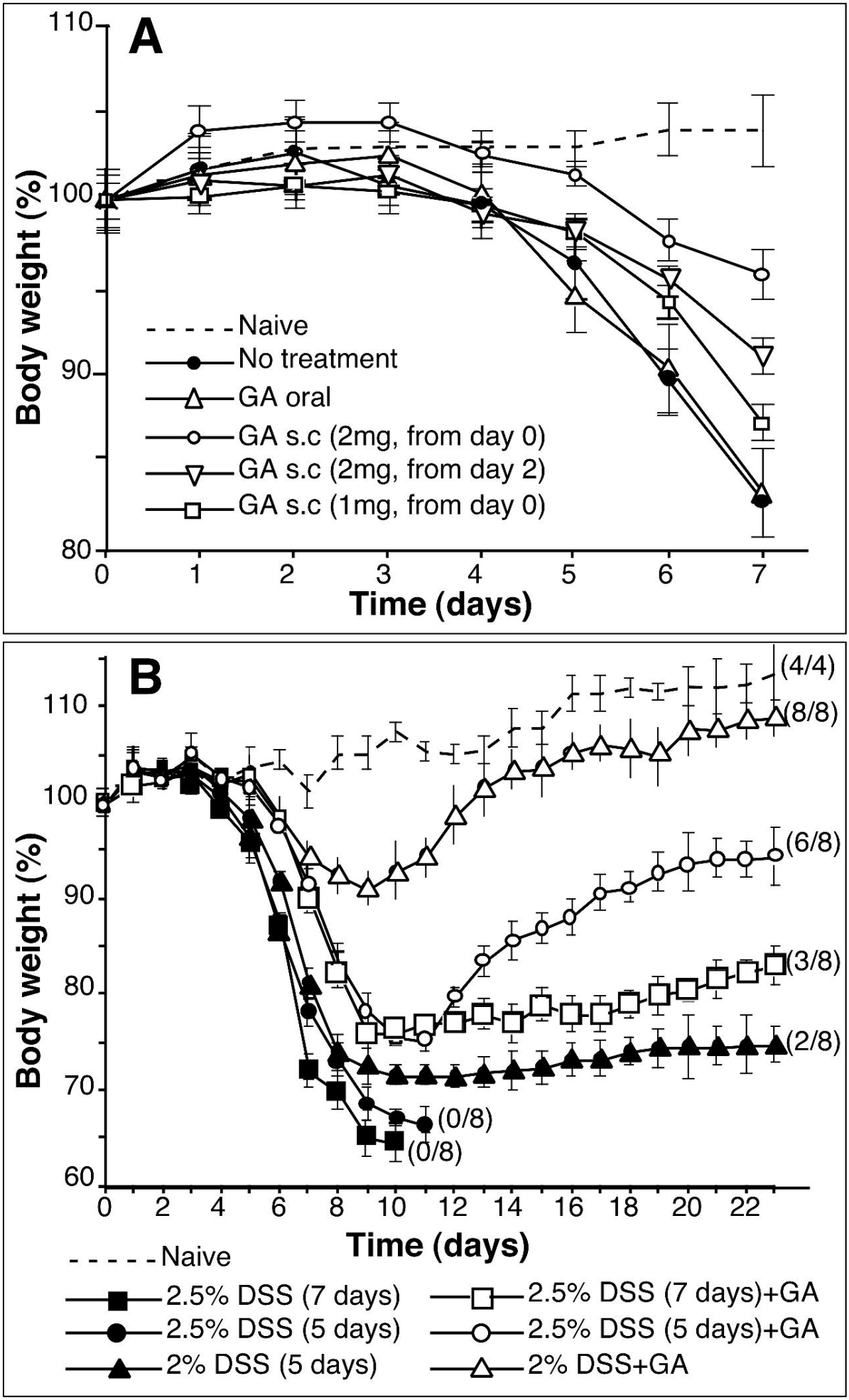

Figure 2

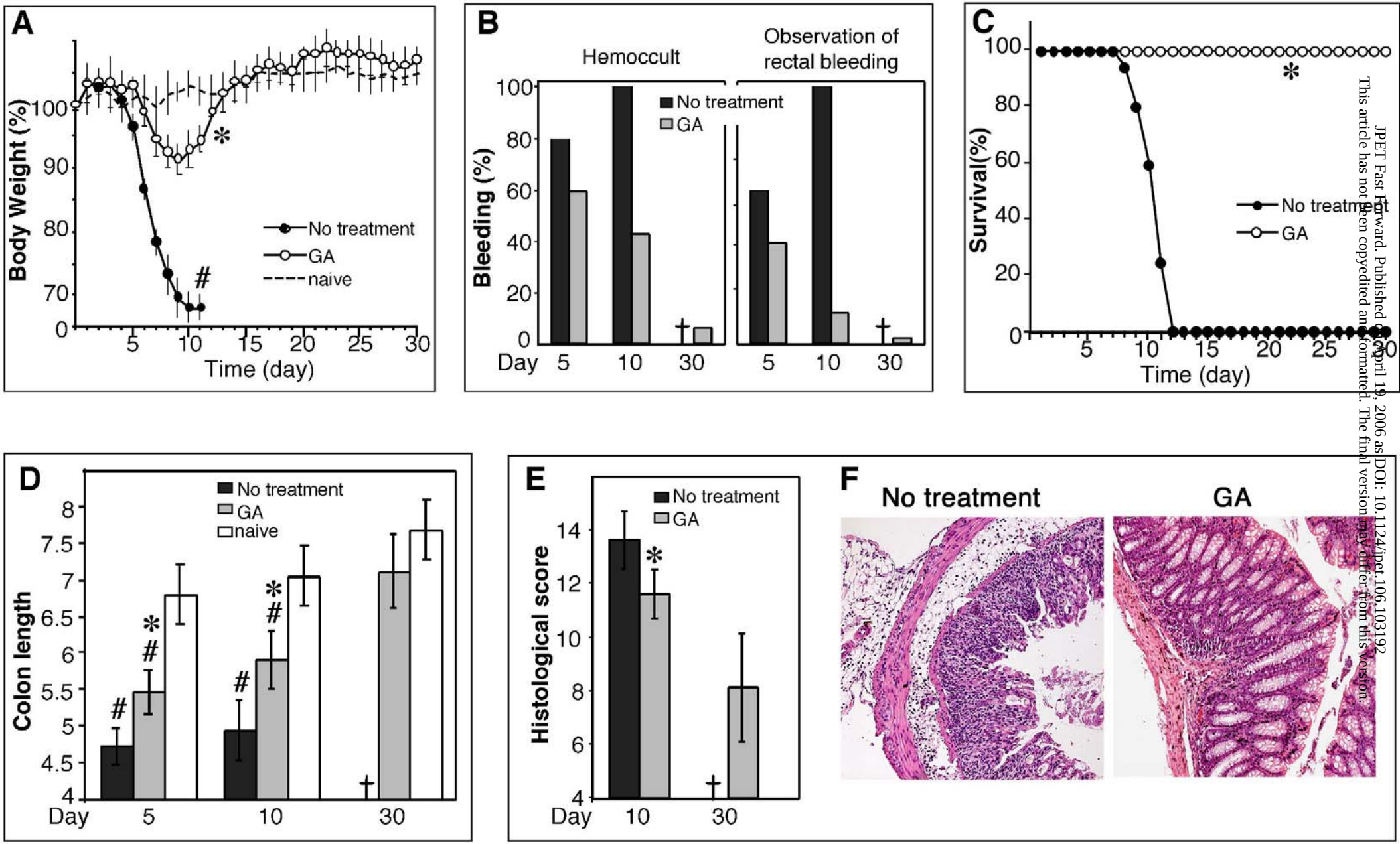

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Figure 3

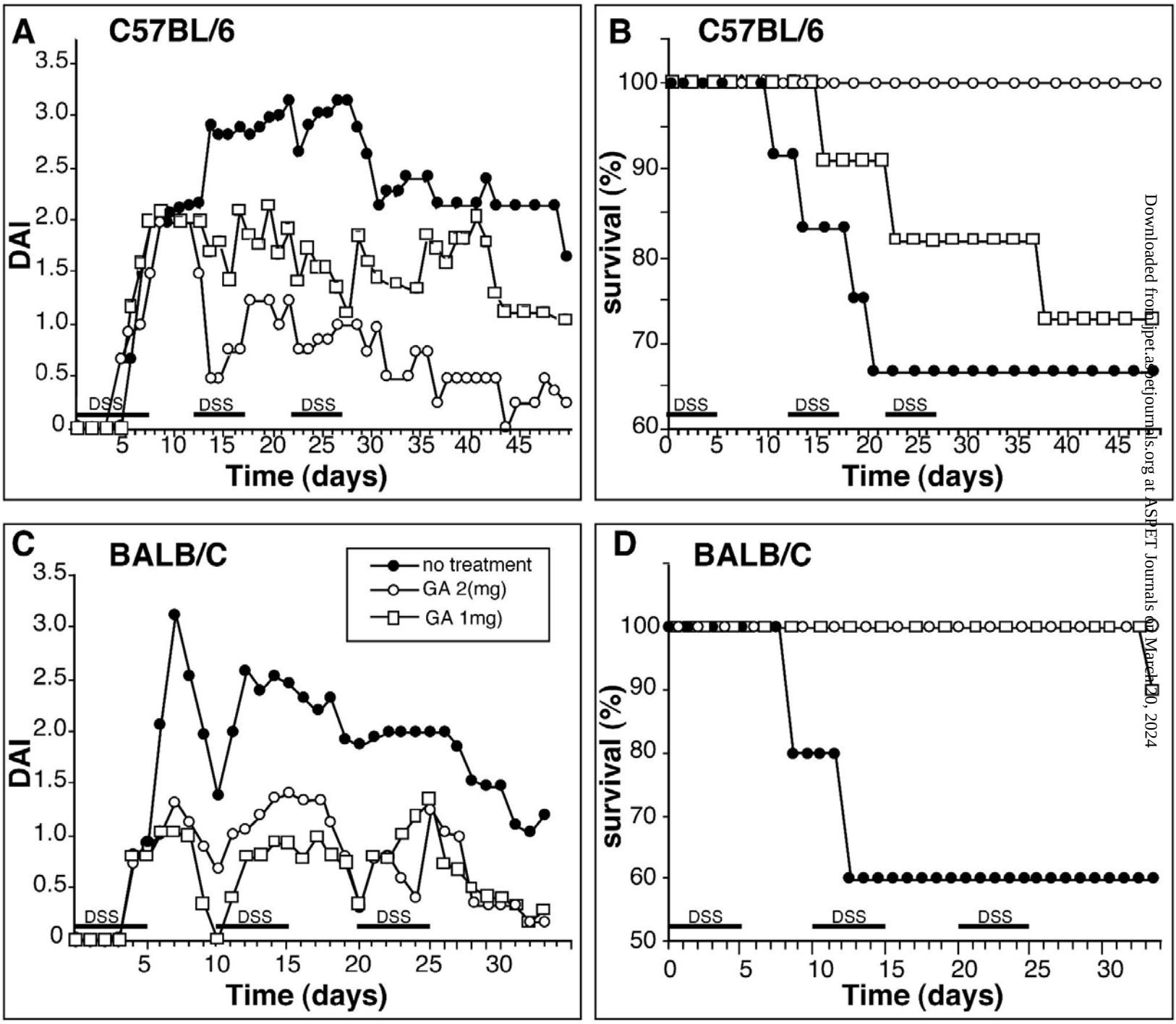

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Figure 4

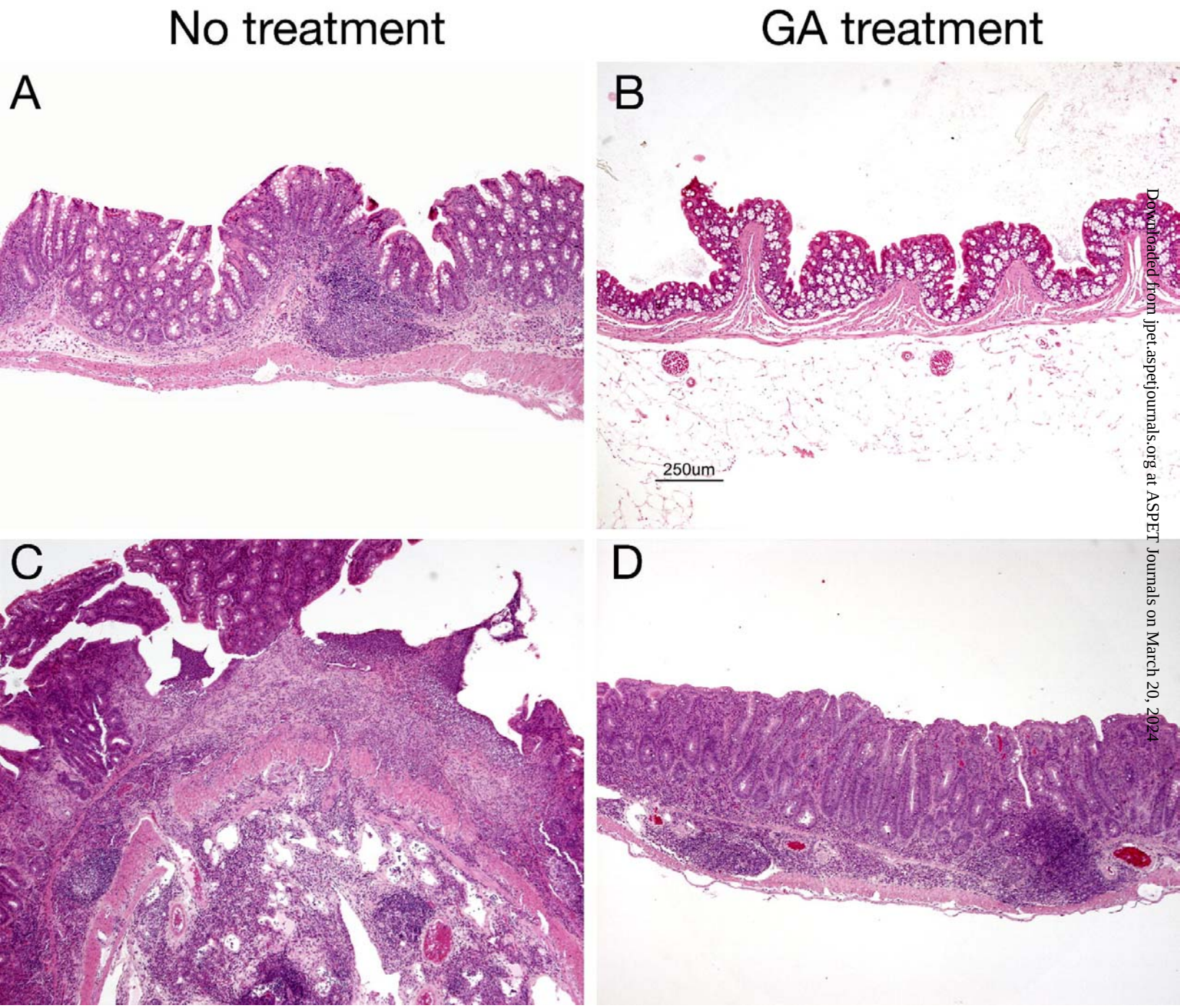

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Figure 5

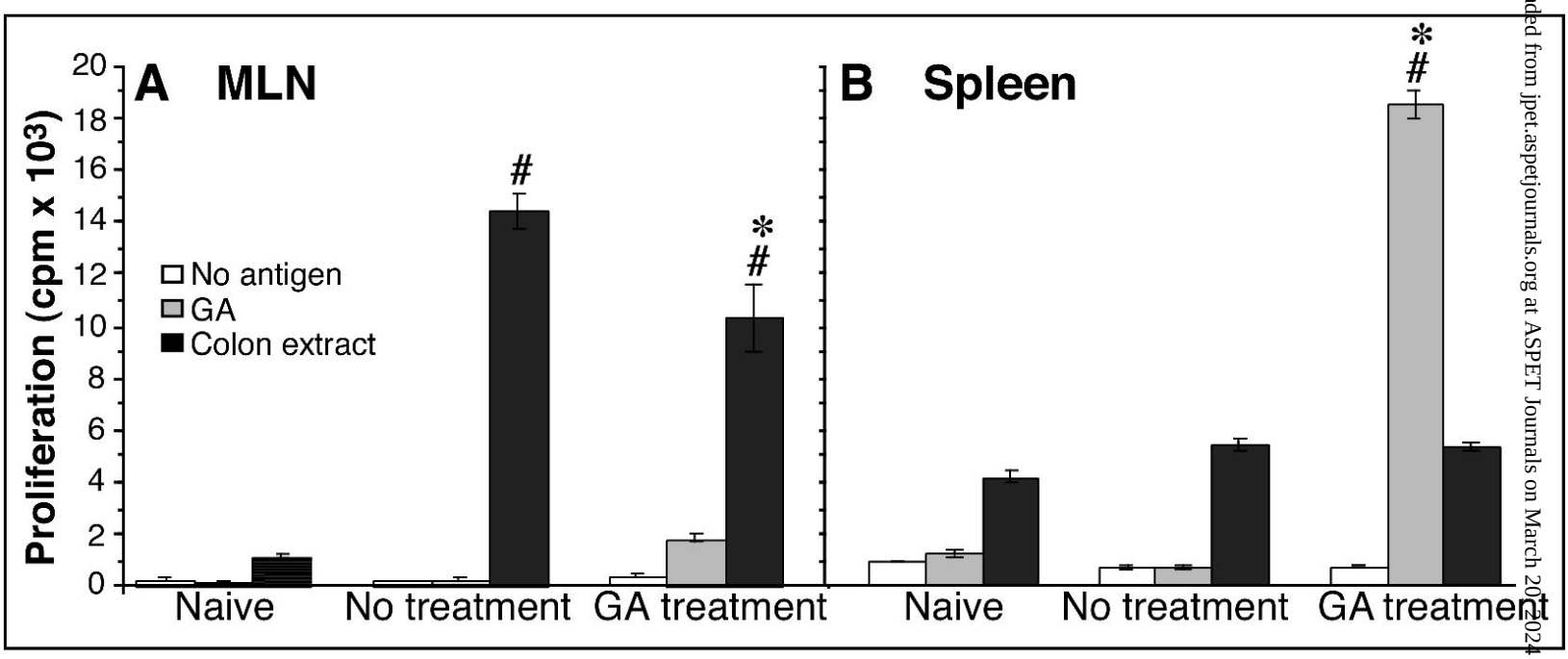
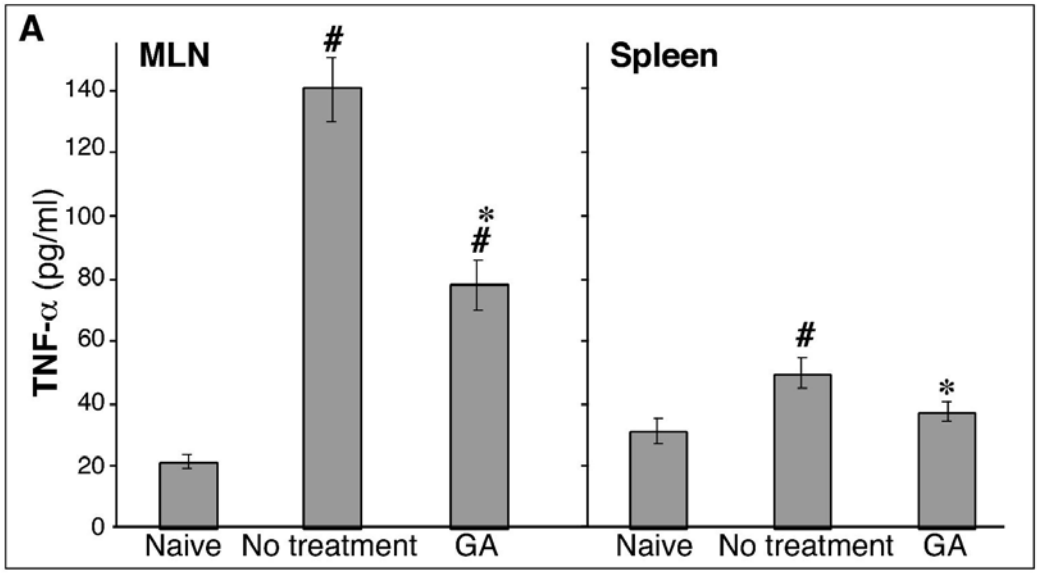
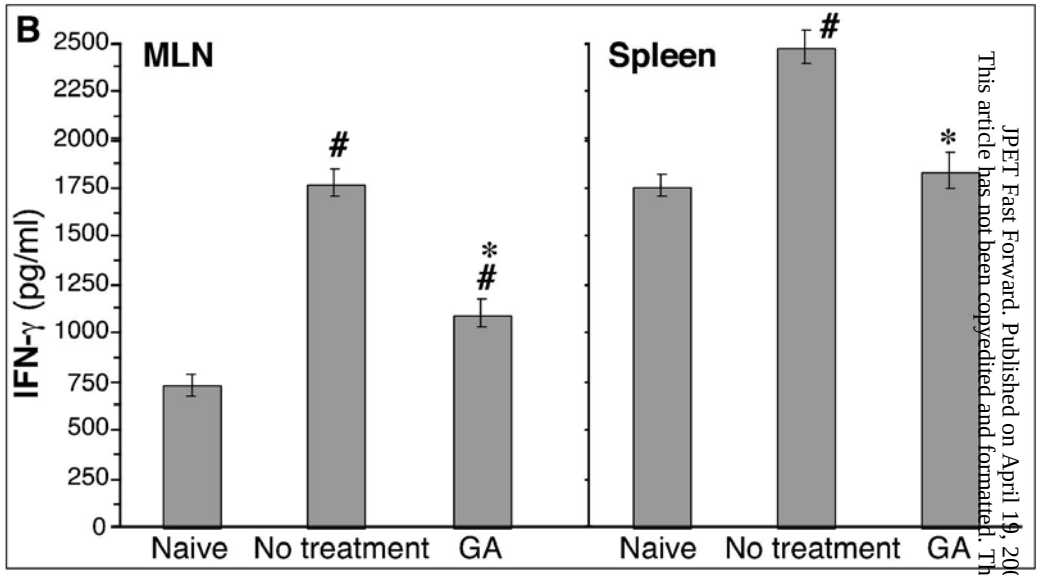
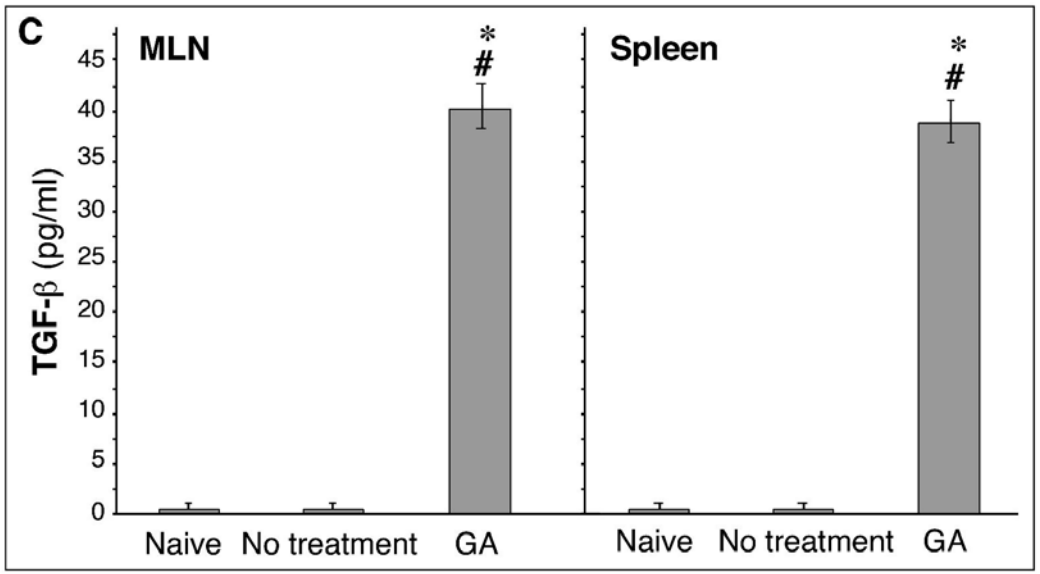


Figure 6







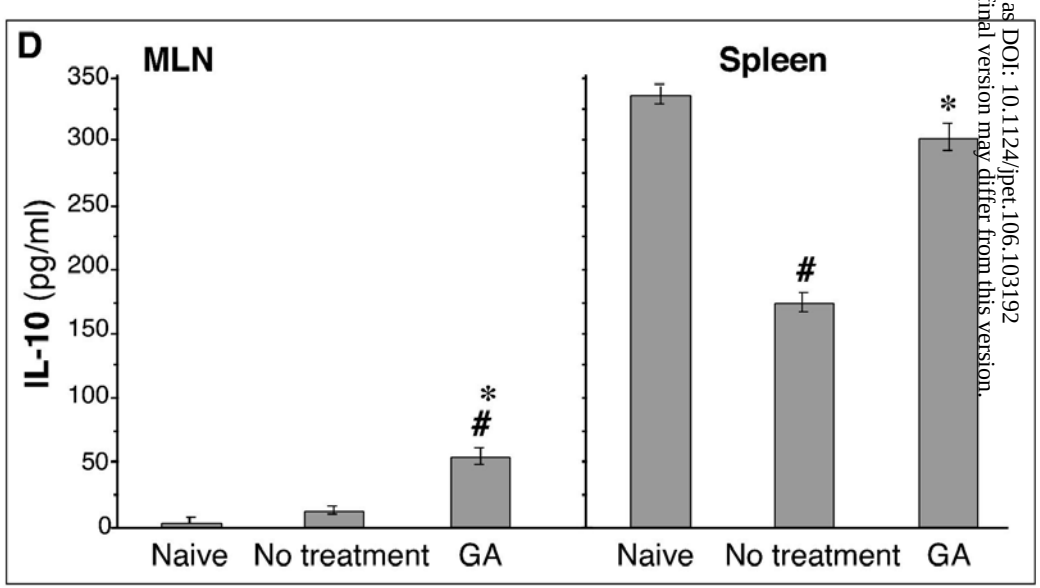


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Figure 7

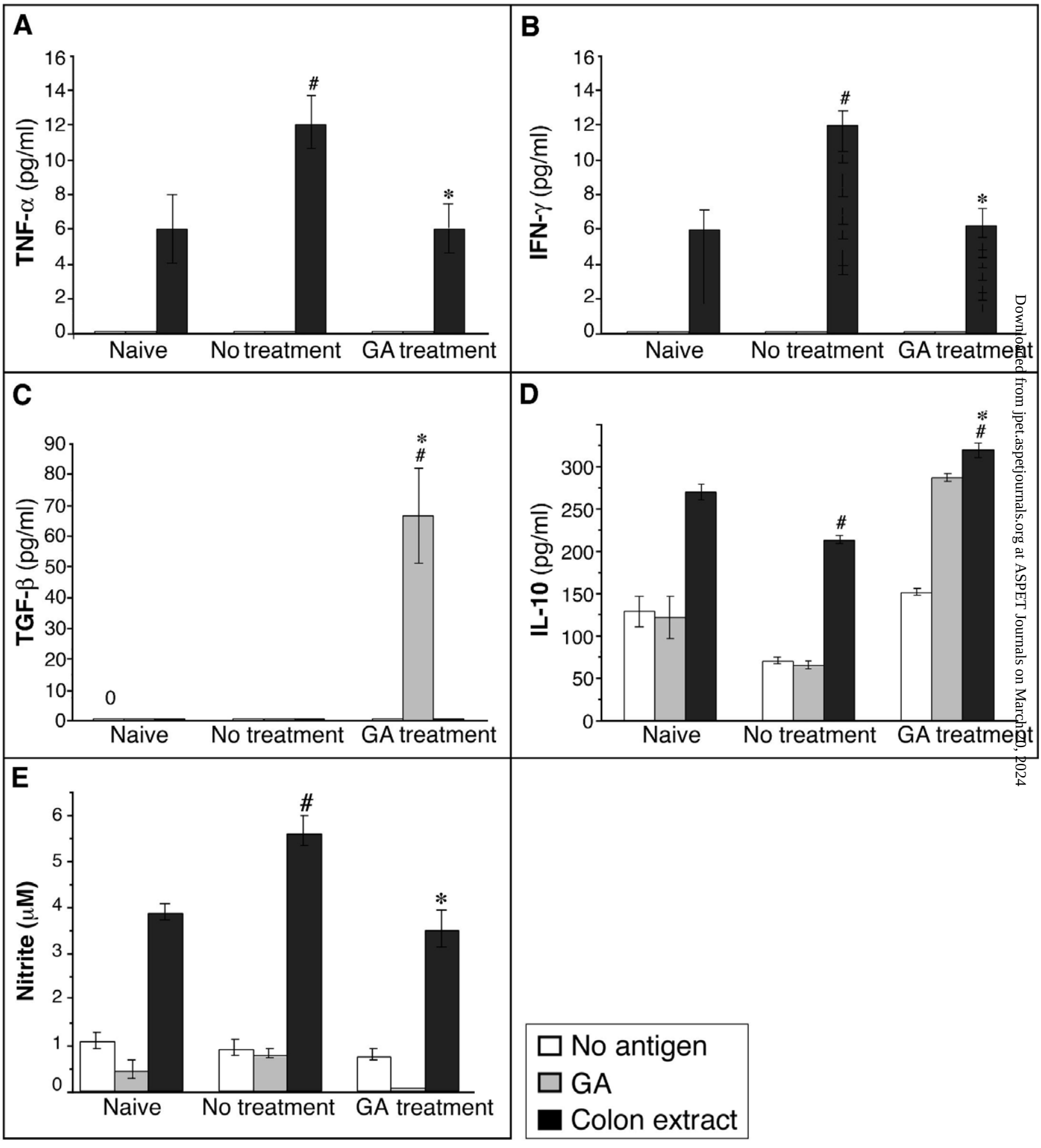

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Figure 8