

Dietary Lactoferrin Does Not Prevent Dextran Sulfate Sodium Induced Murine Intestinal Lymphocyte Death

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Dextran sulfate sodium (DSS) induced intestinal inflammation is characterized by pronounced mucosal and epithelial cell damage. Bovine lactoferrin (bLf), a common dietary protein, influences inflammatory cytokines and intestinal lymphocyte (IL) apoptosis. The objectives of this study were to determine if 1) DSS induces IL necrotic or apoptotic death, 2) dietary bLf affects DSS induced IL death and 3) bLf alters cytokine profiles during DSS induced inflammation. Female C57BL/6 mice were randomized to 2% or 0% bLf diets for 12 d and within diets to 5% or 0% DSS in the drinking water for 4 d after which intestinal histology, IL number, IL apoptosis/necrosis, IL phenotypes, protein levels of pro-inflammatory cytokine (TNF- α) and transcription factor (NF κ B), apoptotic (caspase 3, Bax) proteins, anti-inflammatory cytokine (IL-10) and anti-apoptotic (Bcl-2) protein in IL were evaluated. DSS treatment resulted in shortened intestinal length, decreased body weight and widespread mucosal damage as well as increased IL death as determined by a decreased percentage of viable (PI⁻/ANN⁻, $P < 0.005$) and increased percentage of necrotic/late apoptotic (PI⁺/ANN⁺, $P < 0.05$) and necrotic (PI⁺/ANN⁻, $P < 0.05$) IL. DSS exposure increased caspase 3 ($P < 0.05$) and decreased Bcl-2 ($P < 0.01$) protein levels in mouse IL. Dietary bLf did not influence these cell death outcome measures. However, bLf reduced protein levels of the pro-inflammatory transcription factor, NF κ B, in IL ($P < 0.05$) and was associated with a 34%, albeit non-significant, reduction in TNF- α relative to non-bLf fed mice. DSS treatment increased apoptosis and necrosis of mouse IL and elevated pro-apoptotic and reduced anti-apoptotic protein levels in these cells. Dietary bLf did not influence necrosis or apoptosis of IL but may provide limited protection in the

intestine by affecting the pro-inflammatory transcription factor NF κ B, and potentially, cytokine expression. *Exp Biol Med* 233:1099–1108, 2008

Key words: intestinal lymphocytes; DSS; lactoferrin; apoptosis/necrosis; NF κ B

Introduction

Inflammatory bowel diseases (IBD) are characterized by pronounced destruction of the gastrointestinal mucosa and the epithelial cell barrier (1). Although the etiology of IBD is unknown, alterations in T_H1 and T_H2 cytokine balance coupled with prolonged or excessive immune cell activation to dietary or bacterial antigens are believed to contribute to IBD pathogenesis (2). Inflamed tissue of patients with active IBD contains large quantities of activated immune cells including neutrophils, macrophages and lymphocytes as well as increased concentrations of pro-inflammatory cytokines (1, 3).

Oral administration of dextran sulfate sodium (DSS) is a common method of inducing intestinal colitis in animal models. The mechanism by which DSS induces inflammation likely involves multiple biological pathways including direct cytotoxic effects on epithelial cells (4) and indirect damage due to changes in resident bacteria (5), upregulation of lymphocyte adhesion molecules on intestinal epithelial cells (6) and activation of gut macrophages and T cells (5, 7, 8). DSS induced mucosal injury is characterized by nitrosative damage and, similar to human IBD, increased tissue concentrations of neutrophils, macrophages and activated T cells (4, 7, 8).

Concentrations of inflammatory cytokines (IL-1, IL-6, TNF- α) are increased in patients with active IBD. For example, Isaacs et al. (9) showed elevated mRNA expression of IL-1 and IL-6 in colonic mucosal biopsies of patients with IBD; increased intestinal mRNA of TNF- α was also found in children with active IBD (10). The sources of most cytokines in inflamed tissue are activated macrophages and lymphocytes (3). Similar to human IBD,

This work was supported by grants from NSERC of Canada.

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Received February 11, 2008.
Accepted April 19, 2008.

DOI: 10.3181/0802-RM-53
1535-3702/08/2339-1099\$15.00
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DSS induced intestinal inflammation in mice results in higher concentrations of pro-inflammatory cytokines within the colonic mucosa (7, 8, 11). Moreover, the DNA-binding activity of NF κ B is increased with DSS treatment (12). NF κ B is a transcription factor found inactive in the cytoplasm, which upon activation (e.g., stress signals, pathogens and cytokines such as TNF- α) is relieved of its cytoplasmic inhibitor (I κ B) and translocates to the nucleus where it binds to DNA and activates various pro-inflammatory genes (13). NF κ B is elevated in mice given DSS; addition of oligonucleotides ('NF κ B decoys') that suppress transcription of NF κ B reduces the extent of DSS induced inflammatory damage (14). Thus, decreases in NF κ B could potentially lead to decreased pro-inflammatory (T_H1) cytokine synthesis and less damage to the mucosal barrier.

Cell death can be characterized by two processes distinguished by different cellular morphologies and immunological consequences. Apoptosis, involving cellular shrinking, chromatic condensation, nuclear fragmentation and budding of the plasma membrane, is a genetically coded form of cell suicide with resultant apoptotic bodies engulfed by phagocytes (15). Cell death by apoptosis normally does not recruit lymphocytes or neutrophils, key characteristics of inflammation (16). In contrast, necrotic cell death involves cellular swelling, membrane disruption and release of intracellular contents and involves recruitment of inflammatory cells (17). In DSS induced intestinal inflammation both apoptotic and necrotic damage have been shown to occur for epithelial cells (18); whether DSS induces either or both mechanisms of cell death in other cell populations resident in the gut, such as intestinal lymphocytes (IL), is not known.

Lactoferrin (Lf) is an 80 kDa iron-binding glycoprotein found in neutrophilic granules and various mucosal secretions including milk and colostrums as well as commercially available dairy and whey protein containing products. During periods of physiological stress, dietary Lf may alter cytokine levels with apparent bias to a T_H2 (anti-inflammatory) cytokine profile. For example, dietary administration of bovine lactoferrin (bLf) reduced TNF- α and NF κ B expression in mouse IL following oxidant stress arising from repeated bouts of acute exercise (19). Similarly, Togawa et al. (20) showed reduced TNF- α and NF κ B during DSS induced colitis in the colon of rats fed bLf by gavage. Oral administration of human Lf (hLf) to mice reduced the number of TNF- α producing CD4⁺ T lymphocytes in the colon following DSS treatment (21). Whether dietary bLf alters cytokine profile following DSS treatment in mouse IL is not known. Moreover, bLf has also been shown to influence intestinal cell turnover with reports of increased apoptosis (22) and increased cell viability (23). However, the influence of dietary bLf on IL turnover (apoptosis, necrosis, viability) during periods of DSS induced inflammation has not been empirically characterized.

The purpose of this study was to determine the effect of bLf supplementation in mice given DSS on IL apoptotic and necrotic death and cytokine profile. We hypothesized that DSS induces IL death by both apoptotic and necrotic pathways and that dietary bLf given prior to DSS treatment affects cell death either by enhancing or reducing IL apoptosis and necrosis. We also addressed whether bLf given during DSS induced inflammation in mice decreases T_H1 (TNF- α) or increases T_H2 (IL-10) cytokine protein levels in IL.

Materials and Methods

Animals and Diets. Sixty-three, 3–4 week old, female C57BL/6 mice, obtained from Harlan Sprague Dawley (Indianapolis, IN, USA), were individually housed on a 12/12 h reversed light/dark cycle at 21 $\pm$ 1°C. Mice had *ad libitum* access to tap water and maintenance diet (Laboratory Rodent Chow, PMI Feeds, Richmond, IN, USA) for 2 weeks prior to the start of the study. Following acclimation, mice were randomly assigned by weight to two dietary conditions: bovine lactoferrin (bLf; *n* = 31) or control diet (no bLf; *n* = 32). Diets were prepared to contain either 0% or 2.0% bLf (Erie Foods International, Inc., Erie, IL) while maintaining similar overall protein concentrations (20% total protein in both groups) and were formulated based on a semi-purified AIN 76A standard diet. Concentrations of bLf were based on previous studies showing that 2.0% bLf was sufficient to influence apoptosis as well as cytokine production (19, 22). bLf was 16% iron saturated and greater than 90% pure. Mice had free access to the diets for 12 days prior to sacrifice. All protocols with live animals were approved by the University Animal Ethics Committee according to the principles of the Canadian Council on Animal Care.

DSS Protocol. Within diet conditions, mice were randomized to one of two treatment conditions: 0% DSS (*n* = 31) or 5.0% DSS (*n* = 32) in drinking water. There were a total of 4 groups: no bLf/no DSS (*n* = 16); no bLf/DSS (*n* = 16); bLf/no DSS (*n* = 15) and bLf/DSS (*n* = 16). DSS (MP Biomedicals, Solon, OH; mol wt = 36–50 kDa) was solubilized in tap water at room temperature; mice had *ad libitum* access to DSS containing drinking water for 4 days prior to sacrifice.

Intestinal Lymphocyte Preparation. Mice were sacrificed by sodium pentobarbital (0.06–0.08 ml) overdose; the intestinal compartment, which includes both the small and large intestine, was removed; Peyer's patches and visible fat were dissected out. IL were isolated as previously described (19). Briefly, IL were prepared as single cell suspensions by isolation over a column containing 0.3 g of pre-washed nylon wool, washed and layered over a density gradient medium (Lympholyte-M; Cedarlane Laboratories, Hornby, Ont., Canada) to exclude epithelial cells and remove cellular debris. A sample of IL, which contains both

intraepithelial and lamina propria lymphocytes, was stained with Turk's solution and counted manually by microscopy.

Flow Cytometry. Flow cytometry was used to determine IL apoptosis/necrosis and lymphocyte phenotype distribution. Due to equipment constraints, only a sample of 6–8 mice per treatment condition was included in the flow cytometry analysis. To distinguish between IL populations, an initial acquisition gate was created based on the forward and side scatter properties of a population shown to collect >90% CD45⁺ cells using a CD45 (common leukocyte antigen, clone: 30F11) FITC-conjugated monoclonal antibody (mAb; Miltenyi Biotec, Auburn, CA). Apoptosis and necrosis were determined using FITC-conjugated Annexin V and PI staining, respectively. Annexin V detects externalization of cell membrane associated phosphatidylserine, an early marker of apoptosis, whereas PI binds to DNA following cell membrane disruption and is indicative of necrotic cells (15). Briefly, 1.0×10^5 cells were incubated with 2.5 μ L of both Annexin V-FITC (Pharmingen) and PI (Sigma Chemical) in 100 μ L of Annexin binding buffer (Pharmingen) for 15 min at room temperature in the dark, followed by addition of binding buffer (400 μ L) and analysis by flow cytometry. Individual IL phenotypes were determined by suspending 5×10^5 cells in 100 μ L PBS and incubated with 2.5 μ L of PE-conjugated mAbs (Pharmingen, San Diego, CA, USA) for CD4 (anti-CD4, clone: GK1.5) and CD8 α (anti-CD8 α , clone: 53–6.7) in the dark at 4°C for 45 min. Cells were washed and centrifuged (5 min, 450 g) and the pellet resuspended in 50 μ L Annexin V binding buffer and incubated with 2.5 μ L Annexin V-FITC for 15 min at room temperature in the dark. Following incubation, 400 μ L of binding buffer was added and analyzed for phenotype and apoptosis on a flow cytometer (FACSVantage SE).

Western Blot Analysis of Caspase 3, Bcl-2, Bax, NF κ B, IL-10 and TNF- α . Cytoplasmic and nuclear mouse IL protein samples were prepared and separated by electrophoresis on a 12% SDS PAGE gel and transferred onto a PVDF membrane (Sigma Chemical). Following transfer, membranes were stained with Ponceau S (Sigma Chemical) to confirm quality of transfer and equal loading. Membranes containing the separated proteins (cytoplasmic or nuclear) were incubated for 1 h with primary antibody (1:200 in 10% milk-TBST): Bcl-2 (clone: C-2; mouse anti-human monoclonal IgG₁, mol wt = 28 kDa), caspase 3 (clone: H-227; rabbit anti-human clone IgG, mol wt = 35 kDa), TNF- α (clone: N-19; goat anti-human polyclonal IgG, mol wt = 17 kDa), NF κ B (clone: C-20; rabbit anti-human polyclonal IgG, mol wt = 65 kDa), IL-10 (clone: M-18; goat anti-mouse IgG, mol wt = 15 kDa) or Bax (clone: 5B7; mouse anti-mouse monoclonal IgG₁, mol wt = 23 kDa) (Santa Cruz Biotechnology, Santa Cruz, CA, USA). This was followed by 1 h incubation with secondary antibody: horseradish peroxidase-conjugated anti-mouse (Bcl-2, Bax), anti-rabbit (caspase 3, NF κ B) or anti-goat (TNF- α , IL-10) IgG at a concentration of 1:2000 in 10% milk-TBST. Protein was

determined using ECL or ECL Plus Western blotting detection reagents (Amersham Biosciences, Buckinghamshire, UK) and the ChemiGenius 2 Bio-Imaging System (Cambridge, UK). Each gel contained samples from all 4 treatment groups along with a biotinylated protein ladder to identify the molecular weight of the immunoblotted protein (Cell Signaling Technology, Beverly, MA, USA). For IL-10 and TNF- α blots, recombinant IL-10 and TNF- α standards (mouse IL-10, CL9310R, mouse TNF- α , CL9300TR; Cedarlane Laboratories) were run on each gel.

Histology. Approximately 1 cm sections of distal colon were excised, cleaned of fecal matter, and rinsed with PBS. Samples were fixed in 10% formalin and embedded in paraffin wax. Following sectioning, samples were stained with Hematoxylin and Eosin (H&E) and viewed under light microscopy at $\times 40$ magnification. Samples were masked as to group allocation during visualization.

Statistical Analysis. Data were analyzed as a 2×2 analysis of variance design with diet (two levels: no bLf, bLf) and DSS (two levels: no DSS and DSS) as the independent factors using SPSS (Version 15; Chicago, IL, USA). $P < 0.05$ was accepted as being significantly different from chance alone. Significant interaction effects were further examined by Student's t test analysis within the treatment conditions. All values are expressed as group means $\pm$ SEM.

Results

Effect of DSS and bLf on Mouse Body Weight and Food Intake. Mice did not differ by treatment condition (diet $\times$ DSS) in their average body weights at the start of the experiment: no bLf/no DSS 18.8 ± 0.5 g; no bLf/DSS 18.9 ± 0.5 g; bLf/no DSS 18.1 ± 0.5 g; bLf/DSS 19.6 ± 0.5 g. In contrast, there was a significant effect of DSS, but not diet, on the weight of animals at sacrifice ($F_{1,59} = 12.45$, $P < 0.001$; Table 1). Mice given DSS had significantly lower body weights than those not given DSS ($P < 0.05$). Cumulative food intake did not differ among the treatment groups over the 12 days of feeding: no bLf/no DSS 40.9 ± 1.4 g; no bLf/DSS 40.0 ± 1.3 g; bLf/no DSS 42.5 ± 0.9 g; bLf/DSS 40.1 ± 1.1 g.

Effect of bLf and DSS on Mouse Intestinal Morphology and Disease Indicators. There was a significant effect of DSS on the length of the intestinal compartment (both the small and large intestine) ($F_{1,59} = 6.61$, $P < 0.01$; Table 1) with reduced length in DSS treated mice. As a result of DSS treatment, 30 of 32 mice had visual rectal bleeding and all animals had visual blood within the intestine. No rectal bleeding was observed in mice not given DSS and bLf did not decrease any bleeding. H&E stained colon sections (Fig. 1) indicated structural damage arising from DSS treatment. Figure 1C and 1D illustrate mucosal damage and crypt disruption following DSS treatment compared to the controls not given DSS (Fig. 1A and 1B). Further, in mice given DSS, ablation of crypts was observed

Table 1. Dextran Sulfate Sodium and Bovine Lactoferrin Effects on Mouse Intestinal Inflammatory Indicators (Final Body Weight and Intestinal Compartment Length) and Lymphocyte Counts

Treatment condition	Final body weight (g)	Intestine length (cm)	IL count (10^7)
No DSS	19.93 $\pm$ 0.25	42.92 $\pm$ 0.38	4.29 $\pm$ 0.77
DSS	18.45 $\pm$ 0.32**	41.47 $\pm$ 0.41 ^{##}	4.41 $\pm$ 0.78
No bLf	19.05 $\pm$ 0.32	41.73 $\pm$ 0.48	3.71 $\pm$ 0.35
bLf	19.31 $\pm$ 0.31	42.65 $\pm$ 0.47	5.01 $\pm$ 0.42*

** $P < 0.001$ vs. no DSS, ^{##} $P < 0.05$ vs. no DSS, * $P < 0.05$ vs. no bLf. See text for details of statistical analysis.

whereas intact crypts characterized mice not challenged with DSS.

Effect of DSS and bLf on Mouse IL Counts, Cell Death and Phenotypes. There was a significant diet effect on IL counts ($F_{1,59} = 5.49$, $P < 0.05$; Table 1). This was due to higher cell numbers in mice fed bLf compared with controls. There was no significant main effect of DSS nor was there an interaction between diet and DSS on mouse IL counts.

Figure 2 shows the apoptosis and necrosis flow cytometry results for mouse IL. There were significant effects of DSS on the percentage of PI^+/ANN^- (necrotic) ($F_{1,27} = 7.65$, $P < 0.05$; Fig. 2A), PI^+/ANN^+ (late apoptotic) ($F_{1,27} = 4.35$, $P < 0.05$; Fig. 2B) and PI^-/ANN^- (viable) ($F_{1,27} = 11.34$, $P < 0.005$; Fig. 2C) mouse IL. Diet condition did not significantly affect these outcome measures.

Figure 3 shows the flow cytometric results for $CD4^+$ IL and for expression of the apoptotic marker Annexin V within this subset. There was a significant interaction effect between DSS treatment and diet in the % $CD4^+$ IL ($F_{1,23} = 4.46$, $P < 0.05$; Fig. 3A). This interaction effect was due to a decrease in the percentage of $CD4^+$ IL in the bLf/DSS vs. the no bLf/DSS groups ($t_9 = 2.41$, $P < 0.05$; Fig. 3A) and a decrease in the bLf/no DSS vs. the no bLf/no DSS groups ($t_{11} = 2.92$, $P < 0.01$; Fig. 3A).

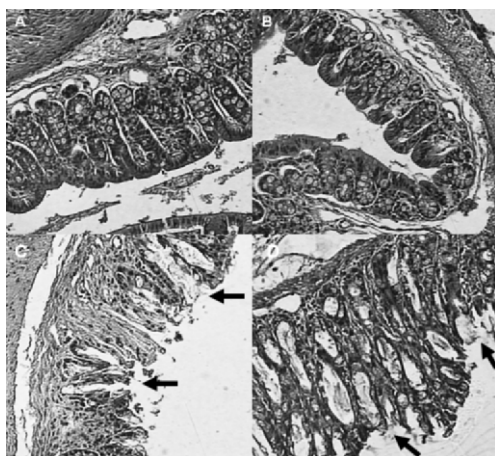


Figure 1. Hematoxylin and Eosin stained tissue sections of mouse colon viewed by light microscopy under $\times 40$. (A) no bLf/no DSS, (B) bLf/no DSS, (C) no bLf/DSS, (D) bLf/DSS. Arrows indicate crypt disruption.

There was a similar interaction effect between DSS and diet on the percentage of mouse $CD4^+/ANN^+$ IL (apoptotic CD4) ($F_{1,23} = 5.03$, $P < 0.05$; Fig. 3B). This interaction reflected a decrease in the percentage of $CD4^+/ANN^+$ IL in the bLf/DSS vs. the no bLf/DSS groups ($t_9 = 1.79$, $P < 0.05$, Fig. 3B). Subsequently, there was also a significant main effect of DSS on the percentage of $CD4^+/ANN^-$ (viable CD4) ($F_{1,23} = 14.04$, $P < 0.001$; Fig. 3C), as these cells decreased following DSS treatment.

Figure 4 illustrates flow cytometric results for $CD8\alpha^+$ IL and for expression of the apoptotic marker Annexin V within this subset. There were no main or interaction effects of diet and DSS on the percentage of $CD8\alpha^+$ IL. There was, however, a trend toward an increase in the percentage of $CD8\alpha^+$ IL as a result of DSS treatment (no DSS: $53.77 \pm 1.85\%$, DSS: $60.86 \pm 3.75\%$, $P = 0.085$). There was a significant main effect of DSS on $CD8\alpha^+/ANN^+$ IL (apoptotic $CD8\alpha$) ($F_{1,23} = 11.67$, $P < 0.01$; Fig. 4A): mice given DSS had a lower percentage of $CD8\alpha^+/ANN^+$ IL as compared to those not receiving DSS. There was also a significant main effect of DSS on the percentage of non-apoptotic $CD8\alpha^+$ IL (i.e., $CD8\alpha^+/ANN^-$) ($F_{1,24} = 4.96$, $P < 0.05$; Fig. 4B) with a higher percentage observed in mice receiving DSS.

Effect of DSS and bLf on NF κ B, IL-10, TNF- α .

Western blotting results for NF κ B, IL-10 and TNF- α concentrations in mouse IL are shown in Figure 5. There was a significant effect of diet on NF κ B protein levels in mouse IL ($F_{1,43} = 5.37$, $P < 0.05$; Fig. 5A), an effect due to lower NF κ B levels in mice fed bLf supplemented diets. There was no significant effect of DSS or interaction between diet and DSS on NF κ B levels in mouse IL. Protein levels of TNF- α , a pro-inflammatory cytokine, in mouse IL was not affected by diet or DSS. Nevertheless, we observed a 34% decrease in TNF- α levels in IL of mice fed bLf compared to no bLf controls (no bLf: 1.3 ± 0.18 , bLf: 0.91 ± 0.090). Protein levels of IL-10, an anti-inflammatory cytokine, in mouse IL were significantly affected as an interaction of diet $\times$ DSS exposure ($F_{1,33} = 6.78$, $P < 0.05$; Fig. 5B). This interaction reflected increased IL-10 concentrations in the no bLf/DSS ($t_{17} = -1.95$, $P < 0.05$) and bLf/no DSS ($t_{15} = -1.95$, $P < 0.05$) groups compared with the no bLf/no DSS control group and a decrease in IL-10 in the bLf/DSS group ($t_{16} = 1.79$, $P < 0.05$) compared to the bLf/no DSS group.

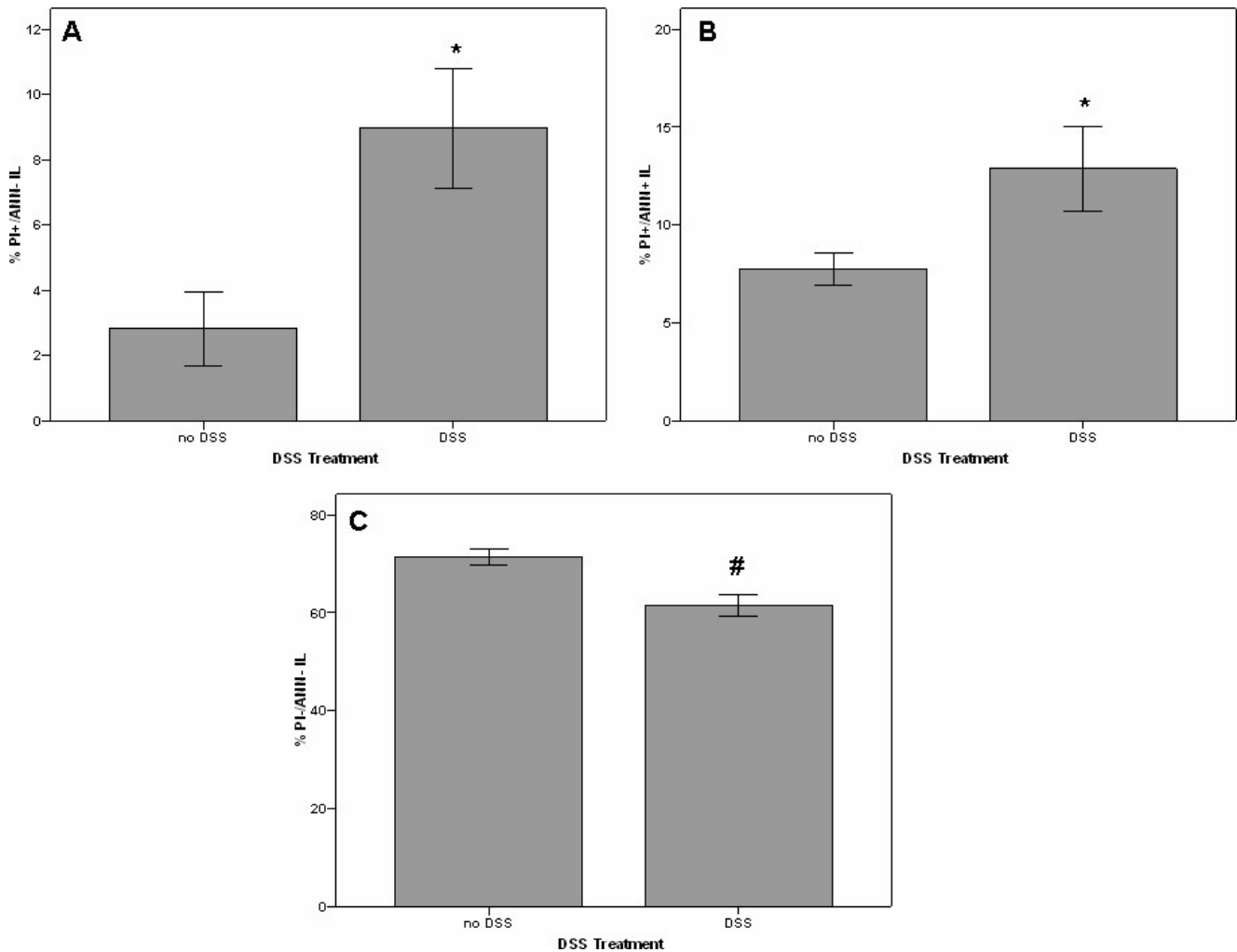


Figure 2. % PI and Annexin V⁺ mouse IL by DSS treatment condition. (A) necrotic mouse IL, (B) late apoptotic mouse IL, (C) viable mouse IL. * $P < 0.05$, # $P < 0.005$. See text for details of statistical analysis.

Effect of DSS and bLf on Bax, Bcl-2, and Caspase 3. Figure 5C shows the effects of DSS on the concentrations of the pro-apoptotic protein caspase 3 in mouse IL ($F_{1,37} = 6.37$, $P < 0.05$), an effect due to higher caspase 3 expression in DSS challenged animals relative to no DSS controls. bLf, either alone or interacting with DSS, did not significantly influence protein levels of caspase 3 in mouse IL. As shown in Figure 5D, there was a significant effect of DSS on the concentration of the anti-apoptotic protein Bcl-2 ($F_{1,54} = 7.60$, $P < 0.01$), with decreased Bcl-2 concentrations following DSS treatment. There was no change in the concentration of the pro-apoptotic protein, Bax, as a function of diet, DSS treatment or as an interaction between diet and DSS in mouse IL (data not shown).

Discussion

The objectives of this study were threefold: 1) to characterize the effects of DSS, an inducer of inflammatory damage in gut mucosa and epithelium, on mouse IL death, 2) to determine if bLf affected mouse IL death during DSS

challenge and 3) to determine if bLf affected protein levels of mouse pro- and anti-inflammatory cytokines (TNF- α and IL-10) and pro- and anti-apoptotic proteins (caspase 3, Bax, Bcl-2) in IL during DSS exposure. A novel finding of this study was that mice subjected to 5% DSS for 4 days had increased death of IL as measured by the expression of Annexin V and PI as well as increased pro-apoptotic caspase 3 and decreased anti-apoptotic Bcl-2 protein levels in IL. Our study is also the first to report that bLf reduced the IL concentration of NF κ B, an important transcription factor in the synthesis of pro-inflammatory cytokines such as TNF- α .

Administration of 5% DSS in drinking water for 4 consecutive days resulted in marked structural intestinal damage, intestinal and rectal bleeding, shortened intestinal length and reduced body weights in mice. These findings suggest active inflammation in the bowel and agree with those structural (reduced colon length, crypt destruction, epithelial cell ulceration) and disease indicators (rectal bleeding, lower final body weight) induced by DSS in mice

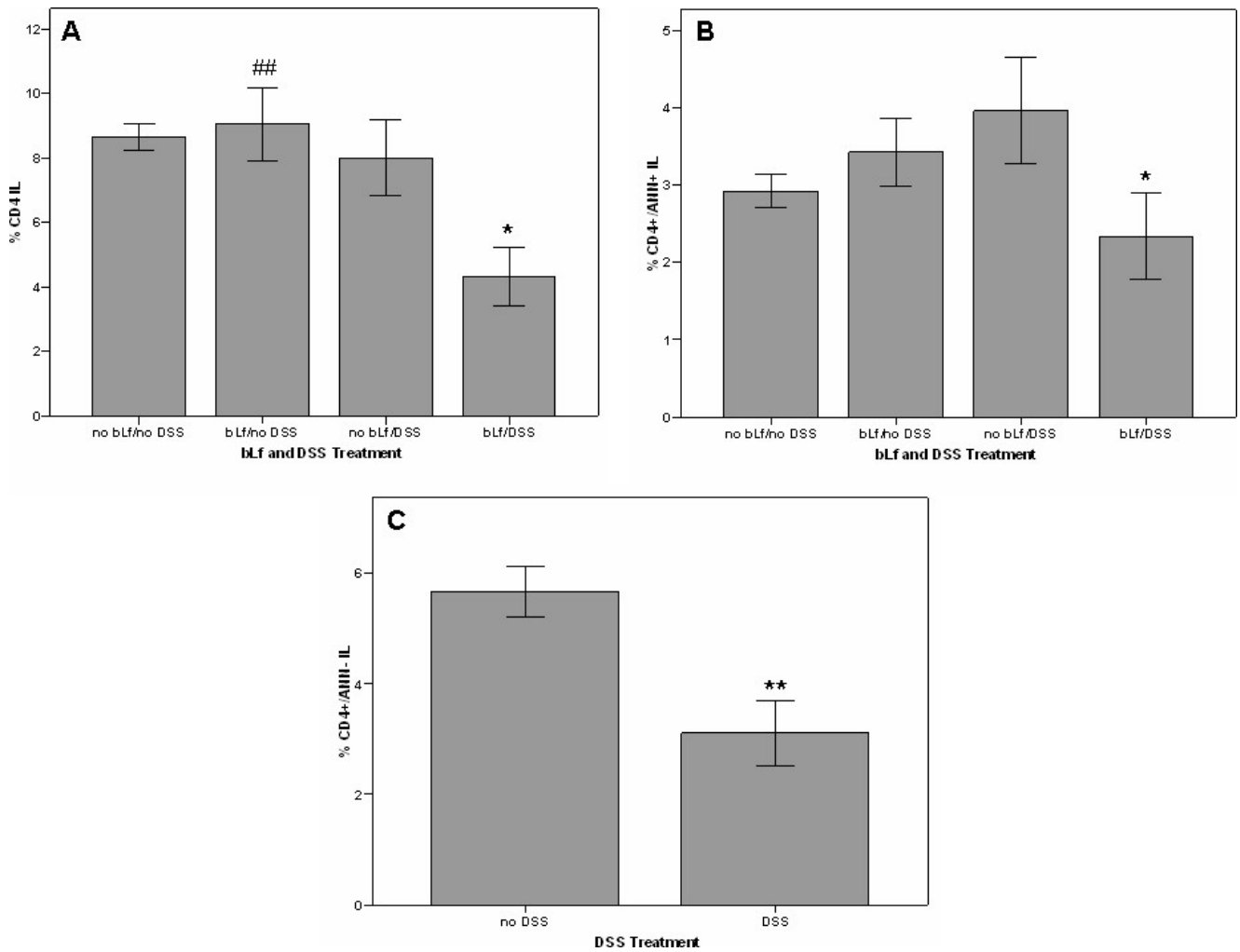


Figure 3. (A) % CD4⁺IL, * $P < 0.05$, ^{##} $P < 0.01$, (B) % CD4⁺/Annexin V⁻, * $P < 0.05$, (C) CD4⁺/Annexin V⁻, ** $P < 0.001$, of mice by DSS and bovine lactoferrin treatment conditions. See text for details of statistical analysis.

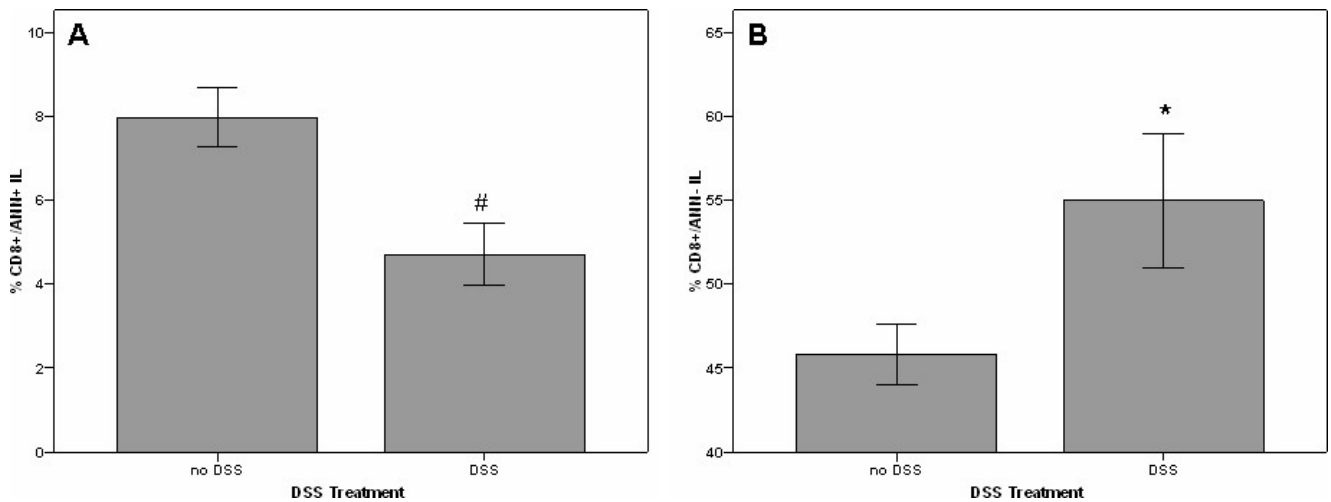


Figure 4. % CD8⁺/Annexin V⁺ (A), and % CD8⁺/Annexin V⁻ (B) of mouse IL by DSS treatment conditions. [#] $P < 0.01$, * $P < 0.05$. See text for details of statistical analysis.

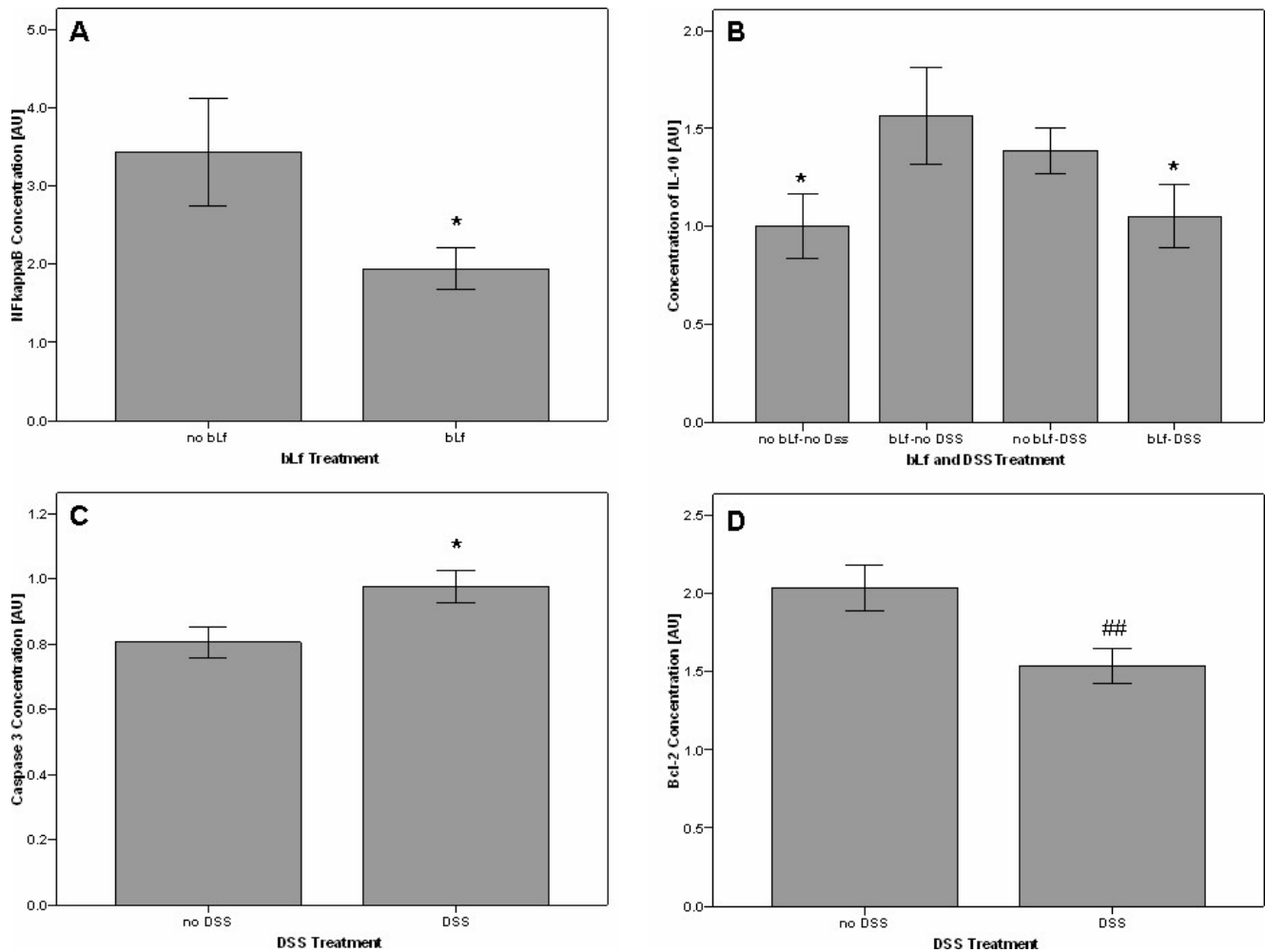


Figure 5. Concentration of apoptotic and inflammatory proteins in mouse IL by DSS and bovine lactoferrin treatment. (A) NFκB, * $P < 0.05$, (B) anti-inflammatory IL-10, * $P < 0.05$, (C) pro-apoptotic caspase 3, * $P < 0.05$, (D) anti-apoptotic Bcl-2, ## $P < 0.01$. Values are expressed as arbitrary densitometric units [AU]. See text for details of statistical analysis.

reported by others (4, 5, 11, 24). In this present study, dietary bLf did not affect disease activity or reduce structural damage in the intestine. These observations are in contrast with those of Haversen et al. (25) showing that mice given 2 mg of human lactoferrin (hLf) twice daily for 7 d during 5% DSS treatment had less colonic length shortening, mucosal damage and fecal occult blood. The reasons for this discrepancy between our findings and those of Haversen and colleagues are not known but may reflect differences in the type of Lf (bovine vs. human), the total concentration of Lf to which the mice were exposed (800 mg vs. 28 mg), the duration of Lf exposure (12 d vs. 7 d), or mode of administration (diet vs. sublingual).

IL isolated from animals given DSS exhibited high degrees of necrotic cell death. The percentage of late apoptotic/necrotic (ANN⁺/PI⁺) IL and necrotic (PI⁺/ANN⁻) IL increased whereas viable (ANN⁻/PI⁻) IL decreased following DSS treatment. DSS induced necrotic death of IL has not been previously reported. However, erosion of the intestinal mucosa has been suggested to occur as a result of

necrosis (18). Dieleman et al. (26) demonstrated that DSS reduced the viability and growth of a colonic epithelial cell line as measured by PI staining and [³H] thymidine incorporation. Thus, given the anatomic proximity of IL to the intestinal epithelium, it seems likely that DSS acts in a direct cytotoxic manner on IL similar to epithelial cells.

Dietary bLf did not affect DSS induced IL death which may be related to the mechanism of action and magnitude of inflammatory damage of DSS. First, if DSS is directly cytotoxic to IL a primary mechanism by which bLf could be protective would be by interaction and neutralization of DSS. There is, however, no evidence in the literature to support a neutralization role for bLf on DSS. Second, the inflammatory response in the bowel induced by DSS is extensive: any potential benefit provided by bLf (at least in the concentration and exposure provided in this study) may be insufficient to compensate for the large pathophysiological changes resulting from DSS exposure. Although bLf primes toward an anti-inflammatory cytokine profile (19, 20, 27) and decreases the extent of oxidative stress (23), this

benefit may not be able to overcome the array of DSS induced disease sequelae including disruption in membrane barrier function and potential exposure of commensal bacteria, immune activation, infiltration of neutrophils, macrophages and lymphocytes, increased nitrosative and oxidative stress and increased pro-inflammatory cytokine production (4, 5, 7, 8, 11, 28).

Death of IL by DSS was largely necrotic; nevertheless, DSS induced apoptosis cannot be completely dismissed given the increased expression of PI^+/ANN^+ , the increased protein levels of caspase 3, and the decreased levels Bcl-2 in mouse IL. Indeed, apoptotic cells that are not recognized by phagocytes undergo secondary (or apoptotic) necrosis, subsequent to apoptosis, a process characterized by the appearance of the PI^+/ANN^+ phenotype (15, 29). Given the dynamic kinetic process of apoptosis and the cross-sectional nature of the study, we were unable to fully differentiate between apoptotic and necrotic IL death induced by DSS. Increases in both measurement parameters indicate that both processes were likely occurring in IL and is consistent with Renes et al. (18) who observed apoptosis and necrosis within the colonic epithelium of DSS treated rats. Future studies, such as sampling at different time points and morphological analysis of IL, will be needed to further clarify the mechanism of DSS induced IL death.

Dietary bLf administration did not affect the concentration of apoptotic related proteins, a finding which is consistent with its inability to influence IL death during DSS induced inflammation. Although bLf has been shown to affect apoptotic related parameters such as mitochondrial membrane permeability (30) and apoptotic proteins such as Bcl-2 (19), the inability of bLf to affect either caspase 3 or Bcl-2 in the present study likely reflects the extent and severity of the DSS induced inflammatory response. Administration of dietary bLf in mice for 12 days resulted in an increase in the total number of IL, similar to other reports (19, 31). However, DSS treatment did not influence overall IL numbers despite an increase in IL death. We suggest that this can be explained by the increase in the percentage of CD8^+ IL with DSS treatment. Increases in CD3^+ T cells (32), T and B lymphocytes (subsets not identified) (4) and activated T lymphocytes (7, 8) in the colonic mucosa of mice following DSS treatment have been reported. In this study, the observed percent increase in CD8 cells, with no change in IL number, may reflect mobilization of this subpopulation from other lymphoid compartments in response to the DSS induced intestinal inflammation. In support of this hypothesis are the following observations: 1) there is a decreased number of $\text{CD8}\alpha^+$ T cells in the spleen coupled with an approximate three fold increase in peripheral blood lymphocytes following DSS treatment in mice (33), 2) there is an increased number of 'mature phenotype' thymocytes following DSS treatment in mice, a phenomenon attributed to preparation for mobilization to other compartments (34) and 3) there is an increased expression of IL adhesion molecules in mouse intestinal

epithelial cells following incubation with DSS (6). Taken together, the percent increase in $\text{CD8}\alpha$ T cells in mouse intestine may reflect lymphocyte recruitment from other pools in the periphery and may contribute to the maintenance of total IL numbers following DSS treatment. Alternatively, the percent increase in $\text{CD8}\alpha$ cells may only reflect a loss of another lymphocyte population (e.g., B cells). However, given the consistent total IL numbers between DSS and non DSS treated mice, this interpretation seems less likely (i.e., a cell population must replace the dying cells).

During inflammation cytokines play central roles in dictating the extent, duration and direction of the immune response. Our results suggest a shift towards an anti-inflammatory response with increased IL-10 (independent of DSS exposure environment) and a trend of decreased $\text{TNF-}\alpha$ following bLf administration. bLf given through the diet alters the ratio of $\text{TNF-}\alpha/\text{IL-10}$ in mouse IL to favor anti-inflammatory responses following a known physiological stressor (repeated bouts of exhaustive exercise with associated oxidant stress) (19). Togawa et al. (20, 27) reported that bLf administered by gavage increased IL-10 and decreased $\text{TNF-}\alpha$ following DSS and TNBS treatment in rat colon, respectively. Thus, a potential benefit of bLf supplementation may involve a shift in the $\text{T}_\text{H}1$ and $\text{T}_\text{H}2$ cytokine balance to favor anti-inflammatory responses.

Treatment of mice with DSS was associated with increased IL-10 concentrations in IL (no bLf/no DSS vs. no bLf/DSS), a finding which may seem paradoxical given the $\text{T}_\text{H}1$ suppressing, anti-inflammatory role of this cytokine. However, increased IL-10 production following DSS exposure may be an adaptive response to reduce the extent of inflammation. In support of this hypothesis are the findings of Egger et al. (11) who demonstrated increased mRNA of several pro-inflammatory cytokines (e.g., $\text{TNF-}\alpha$, $\text{IFN-}\gamma$, IL-1) as well as increased IL-10 mRNA in the colonic mucosa of DSS treated mice. Increased IL-10 mRNA was also found in mucosal biopsies of patients with active Celiac Disease (35). Haversen et al. (25) reported increases in IL-10 producing cells in the colonic mucosa of mice subjected to DSS, which were decreased following oral hLf administration; this reduction was related to a hLf associated down-regulation of DSS induced inflammation. We report a decrease in IL-10 following dietary bLf administration in mouse IL within DSS treatment (bLf/no DSS vs. bLf/DSS), which may reflect a lessening of the inflammatory stress. Therefore, increased IL-10 production may be a physiological compensatory mechanism in response to DSS and the addition of bLf may attenuate the need for this compensation.

Dietary bLf was associated with decreased protein levels of the pro-inflammatory transcription factor NF κ B in mouse IL. Reduced NF κ B may subsequently result in less pro-inflammatory cytokine production by immune cells. In support of this are the following observations: first, following LPS stimulation, binding of NF κ B to the TNF-

α promoter in monocytes was inhibited by Lf (21). Second, Togawa et al. (27) demonstrated reduced phosphorylation of I κ B indicating decreased NF κ B activation in rat colon following gavage administration of bLf in rats with TNBS induced colitis; third, dietary bLf reduced both NF κ B and TNF- α expression in mouse IL following oxidant stress accompanying acute, repeated exercise (19). These findings suggest that bLf may reduce the extent of inflammation by altering the T_H1/T_H2 balance acting through decreased NF κ B protein levels.

In summary, 5% DSS treatment for 4 days in female C57BL/6 mice was associated with marked inflammatory damage within the intestinal compartment. DSS resulted in necrotic death of IL as well as increased protein levels of the apoptotic protein caspase 3 and decreased expression of the anti-apoptotic Bcl-2 protein. DSS treatment also induced a non-significant increase in the percent of CD8 α IL indicative of an activated immune response. Finally, dietary bLf did not provide any observable benefit in disease activity measurements nor did it influence DSS induced IL death. Nevertheless, bLf may offer some limited intestinal protection by decreasing the levels of the pro-inflammatory transcription factor NF κ B. However, the physiological impact of lower NF κ B levels in IL on the actual synthesis of pro-inflammatory cytokines (IL-1, IL-6, TNF- α) will need to be determined experimentally.

The authors gratefully acknowledge the advice of Dr. RP Bird and the technical assistance of J. Guan with these experiments.

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