



(51) International Patent Classification:

A61K 38/18 (2006.01) A61P 29/00 (2006.01)
A61P 1/00 (2006.01)

(21) International Application Number:

PCT/US2009/034660

(22) International Filing Date:

20 February 2009 (20.02.2009)

(25) Filing Language:

English

(26) Publication Language:

English

(30) Priority Data:

61/029,936 20 February 2008 (20.02.2008) US

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(81) Designated States (unless otherwise indicated, for every kind of national protection available): AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ,

CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States (unless otherwise indicated, for every kind of regional protection available): ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Declarations under Rule 4.17:

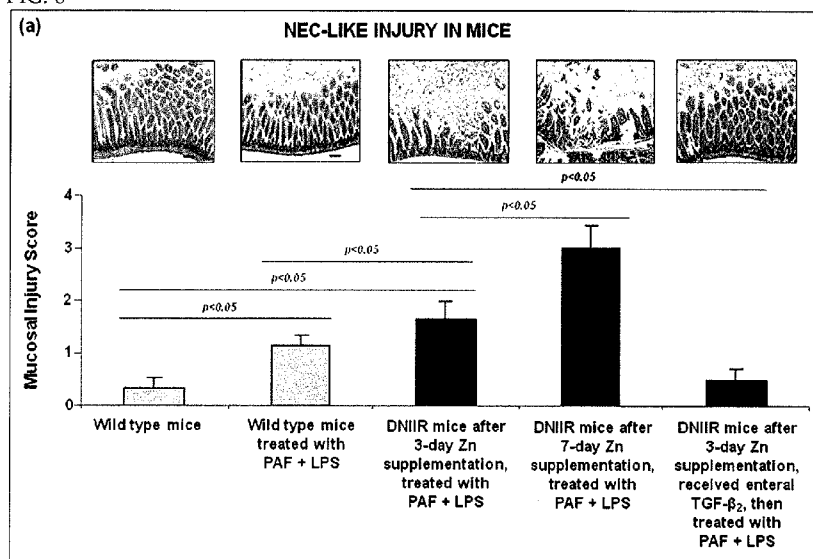
- as to applicant's entitlement to apply for and be granted a patent (Rule 4.17(ii))
- as to the applicant's entitlement to claim the priority of the earlier application (Rule 4.17(iii))
- of inventorship (Rule 4.17(iv))

Published:

- without international search report and to be republished upon receipt of that report (Rule 48.2(g))
- with sequence listing part of description (Rule 5.2(a))

(54) Title: METHODS FOR THE TREATMENT AND PREVENTION OF NECROTIZING ENTEROCOLITIS

FIG. 8



(57) Abstract: The present disclosure provides for the administration of TGF- β or a TGF- β derivative to treat and/or prevent a variety of disease states and/or conditions, characterized by the cytokine response of immature intestinal epithelial cells to mucosal injury, bacterial invasion, bacterial antigens and/or products, including, but not limited to, NEC. Furthermore, the present disclosure provides for a pharmaceutical composition and medicaments comprising a therapeutically effective amount of TGF- β or a TGF- β derivative for use in the methods described herein.

Methods for the Treatment and Prevention of Necrotizing Enterocolitis

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CROSS REFERENCE TO RELATED APPLICATIONS

The present disclosure claims the benefit of US Provisional Patent Application number 61/029,936, filed 02-20-2008.

STATEMENT AS TO FEDERALLY SPONSORED RESEARCH

10 Funding for the work described herein was provided by the federal government, which has certain rights in the invention.

FIELD OF THE DISCLOSURE

The present disclosure relates to the treatment and prevention of necrotizing enterocolitis (NEC) and related disease states and conditions. The disclosure also relates to the treatment and prevention of neonatal NEC and related disease states and conditions. The
15 disclosure further relates to pharmaceutical compositions and medicaments comprising Transforming Growth Factor (TGF)- β for use in the methods of treatment and prevention disclosed herein.

BACKGROUND

20 NEC is an acquired gastrointestinal disease affecting 5–15% of neonates born weighing less than 1500 grams and is a leading cause of morbidity and mortality in these patients. Histopathologically, NEC is characterized by a severe inflammatory response, ischemic changes, and necrosis. Although the etiology of NEC is not fully established, the disease is associated in premature infants with intestinal ischemia, formula feeding, and abnormal
25 bacterial colonization.

Existing epidemiological and experimental evidence indicate that NEC occurs when mucosal injury or altered permeability allow the translocation of gut luminal bacteria into the lamina propria, causing a severe inflammatory response and consequent tissue destruction. However, this patho-physiological model of NEC as an inflammatory reaction to bacterial
30 products is inconsistent with recent observations that gut mucosal cells, such as macrophages, at least in the adult intestine, are profoundly ‘anergic’ to bacterial products. The inflammatory responses of intestinal macrophages in the adult intestine are markedly attenuated due to

various stromal and epithelial cell-derived peptide growth factors and extracellular matrix (ECM) components. Because NEC is seen almost exclusively in premature infants born in the late 2nd/early 3rd trimester of pregnancy, NEC may occur in the premature neonate as the normal mucosal mechanisms of tolerance to bacterial products are developmentally regulated and deficient in the preterm intestine.

The art is currently lacking method for the treatment and prevention of NEC and related disease states and conditions. Due to the severity of NEC and related disease states and conditions and the mortality and morbidity associated with the foregoing, especially in neonates, new methods of treatment and prevention are needed. The present disclosure provides methods for the treatment and prevention of NEC and related disease states and conditions as well as pharmaceutical compositions and medicaments for use in such methods.

DESCRIPTION OF THE DRAWINGS

FIG. 1A demonstrates the severe inflammatory response, ischemic changes, and necrosis often associated with NEC. FIG. 1A (upper panel) shows intra-operative gross morphological appearance of NEC showing an area of infarction. FIG. 1A (lower panel) shows an eosin-hematoxylin photomicrograph showing inflammation (thin arrows). Thick arrows indicate intramural air, which represents trapped gas bubbles from bacterial fermentation and is a pathognomonic finding of NEC.

FIG. 1B shows PCR microarray indicating upregulation of pro-inflammatory cytokines and transcriptional regulators in NEC.

FIG. 1C is data from last 10 years in a neonatal intensive care unit showing that NEC affects extremely preterm infants born during the late 2nd trimester and the early 3rd trimester of pregnancy

FIG. 2A shows immunohistochemical data demonstrating that fetal intestinal macrophages express TNF- α and CD14, unlike macrophages in the adult intestine: Photomicrographs of small intestinal tissue sections (magnification 100x) from (i) 20-wk human fetus (ii) 29-wk premature neonate (iii) full-term neonate and (iv) adult show DAB (brown) staining for HAM56, a pan-macrophage marker. In each panel, insets on the right show higher-magnification (1000x) immunofluorescence photomicrographs show immunoreactivity for HAM56 (green) and TNF- α (red). Lower insets show immunofluorescence staining for HAM56 (green) and CD14 (red), a part of the LPS receptor. Intestinal macrophages in the fetal and premature intestine express TNF- α and CD14, whereas macrophages in the term neonate and adult are negative. Data represent n=3 in each group.

FIG. 2B shows that murine fetal intestinal macrophages respond to LPS to produce TNF- α *in vitro*. Intestinal macrophages from the E15 and E18 murine fetus, unlike those from the term neonate (D1 postnatal) and adult mouse, respond to LPS *in vitro* and produce TNF- α (means $\pm$ SEM). Photomicrographs above the bar diagram show co-localization of F4/80, a murine macrophage marker with TNF- α in the fetal, but not in term neonate (D1 postnatal) and adult murine intestinal macrophages. The inset on the right shows a bar diagram demonstrating that tissue-conditioned media prepared from the adult mouse intestine suppressed LPS-induced TNF- α production (means $\pm$ SEM) in E15 murine fetal intestinal macrophages. Experiments in these figures include data from an n=3 in each group.

FIG. 3A shows fetal tissue-conditioned media (T-CM) suppression of macrophage cytokine production improves with gestational maturation, but remains significantly lower than adult. Bar diagrams show T-CM suppression of TNF- α , IL-6, IL-1 β , and IL-8 production by macrophages (means $\pm$ SEM). Data are representative of 3 independent experiments, each performed with T-CMs derived from 3-5 fetuses in each fetal group and 3 adult tissue samples.

FIG. 3B (B) T-CM suppression of neutrophil chemotactic activity in LPS-stimulated macrophage cultures improves with maturation. Neutrophil chemotactic activity of supernatants from macrophage cultures was measured using a standard fluorescence-based microchemotaxis assay; data are depicted as means $\pm$ SEM of the number of neutrophils migrating towards test samples through a polycarbonate filter. Data are representative of 3 independent experiments, each performed with T-CMs derived from 3-5 fetuses in each fetal group and 3 adult tissue samples; (C) T-CM suppression of LPS-induced NF- κ B activation in macrophages improves with maturation. Unlike T-CMs prepared from adult intestinal tissue, fetal T-CMs did not block LPS-induced NF- κ B activation in macrophages. The bar diagram (means $\pm$ SEM) shows the ratio of phosphorylated: total NF- κ B p65 (rel A). Data are representative of 3 independent experiments, each performed with T-CMs derived from 3-5 fetuses in each fetal group and 3 adult tissue samples

FIG. 4A shows the effect of LPS stimulation on monocyte derived macrophage expression of 23 pro-inflammatory cytokines and chemokines. Bar diagram shows data from a quantitative PCR microarray. Data were normalized against GAPDH and depicted as fold-change above unstimulated macrophages. Data are representative of 3 independent experiments, each performed with T-CMs derived from 3-5 fetuses in each fetal group.

FIG. 4B shows the effect of LPS stimulation on monocyte derived macrophage expression of 23 pro-inflammatory cytokines and chemokines after pre-treatment with TCM derived from 10-14 week fetal intestine. Bar diagram shows data from a quantitative PCR microarray. Data

were normalized against GAPDH and depicted as fold-change above unstimulated macrophages. Data are representative of 3 independent experiments, each performed with T-CMs derived from 3-5 fetuses in each fetal group.

FIG. 4C shows the effect of LPS stimulation on monocyte derived macrophage expression of 23 pro-inflammatory cytokines and chemokines after pre-treatment with TCM derived from 20-24 week fetal intestine. Bar diagram shows data from a quantitative PCR microarray. Data were normalized against GAPDH and depicted as fold-change above unstimulated macrophages. Data are representative of 3 independent experiments, each performed with T-CMs derived from 3-5 fetuses in each fetal group.

FIG. 4D shows the effect of LPS stimulation on monocyte derived macrophage expression of 23 pro-inflammatory cytokines and chemokines after pre-treatment with TCM derived from adult intestine. Bar diagram shows data from a quantitative PCR microarray. Data were normalized against GAPDH and depicted as fold-change above unstimulated macrophages. Data are representative of 3 independent experiments, each performed with T-CMs derived from 3-5 fetuses in each fetal group.

FIG. 5(A) shows TGF- β bioactivity increases with maturation. TGF- β bioactivity was quantified using a luciferase assay to measure TGF- β -mediated activation of the platelet activator inhibitor-1 promoter in mink lung epithelial cells. Data are representative of 3 independent experiments, each performed with T-CMs derived from 3 fetuses in each fetal group and 3 adult tissue samples.

FIG. 5(B) shows T-CM activation of smad signaling in macrophages increases with maturation. Treatment of monocyte-derived macrophages with fetal and adult T-CMs induced the phosphorylation of smad2, a key mediator of the TGF- β -activated signaling pathway. Bar diagram shows densitometric analysis of the blots (means $\pm$ SEM). Data are representative of 3 independent experiments, each performed with a distinct set of T-CMs and utilized three different monocyte donors.

FIG. 5(C) shows T-CM suppression of LPS-induced cytokine production in macrophages was reversed upon neutralization of TGF- β in the conditioned media. Bar diagram shows that T-CMs prepared from human fetal intestinal tissue of different gestational ages suppressed LPS-induced TNF- α (means $\pm$ SEM) production in macrophages in a maturation dependent manner. In some wells, excess neutralizing anti-TGF- β (or isotype control) antibody was added. The gestational age-related suppression of cytokine production (grey bars) was reversed by anti-TGF- β antibody (hatched bars). Data are representative of 3 independent experiments, each performed with a distinct set of T-CMs.

FIG. 5(D) shows the amino acid sequence of human TGF- β_2 , isoform 1 (SEQ ID NO: 1) and isoform 2 (SEQ ID NO: 2).

FIG. 6(A) shows mRNA expression of TGF- β_2 , but not of TGF- β_1 or TGF- β_3 , increases with intestinal maturation. Data are normalized against GAPDH and depicted as fold-change above 10-14 wk fetal intestine (means $\pm$ SEM). Data are representative of 3 independent experiments, each performed with T-CMs derived from 3 subjects in each group.

FIG. 6(B) shows TGF- β_2 immunoreactivity increases with intestinal maturation. TGF- β_2 immunoreactivity (green; open arrow) becomes progressively more prominent in the intestinal epithelium with maturation. Insets with the 12-wk intestine show high-magnification photomicrographs (1000x) of the villus and inter-villus area (V & IVA) and the *muscularis externa* (ME). In other panels, the insets (top to bottom) show high-magnification photomicrographs (1000x) of the villus tip (V), crypt (C), and lamina propria cells (L). Data are representative of 3 different fetuses or adults in each group. The bottom right panel shows that TGF- β_2 immunoreactivity in lamina propria cells co-localizes with α -smooth muscle actin (α -SMA; arrows), indicating that cells expressing TGF- β_2 are of the myofibroblast lineage.

FIG. 6(C) shows concentrations of total and active fractions of TGF- β_2 increased with maturation. All measurements were performed by ELISA and are shown as means $\pm$ SEM. Data are representative of 3 independent experiments, each performed with T-CMs derived from 3-5 fetuses in each fetal group and 3 adult tissue samples.

FIG. 6(D) shows TGF- β_2 is the most important of the three TGF- β isoforms in T-CM downregulation of LPS-induced macrophage cytokine production: Two of the three TGF- β isoforms were immunoprecipitated in separate T-CM aliquots to obtain T-CM derivatives containing only one of the three TGF- β isoforms. The effect of these T-CM derivatives on LPS-induced macrophage TNF- α production is depicted (means $\pm$ SEM). T-CM derivatives containing TGF- β_2 were most effective in suppressing TNF- α production. Data are representative of 3 independent experiments, each performed with T-CMs derived from 3 subjects in each group. The inset shows recombinant TGF- β_2 is the most potent of the three isoforms in suppressing LPS-induced TNF- α production in macrophages. Monocyte-derived macrophages were treated with 0-2000 pg/mL of the three TGF- β isoforms 2 hrs prior to LPS stimulation. Data are representative of 3 independent experiments.

FIG. 7(A) shows NEC is associated with decreased TGF- β_2 expression. TGF- β_2 concentrations in intestinal tissue resected surgically for NEC are lower than the mid-gestation fetal and preterm neonatal intestine. Data show measurements by ELISA (means $\pm$ SEM). TGF- β_2 mRNA expression (top left) and TGF- β bioactivity (top right) in the normal fetal, preterm

neonatal intestine and NEC-affected intestinal tissue are shown. Data are representative of 3 independent experiments, each performed with T-CMs derived from 5 tissue samples of NEC (each from a different patient) and 5 fetal intestinal tissue samples (different fetuses).

FIG. 7(B) shows decreased TGF- β_2 expression in NEC is not due to the non-specific consumption of TGF- β_2 during mucosal inflammation. Unlike in NEC, TGF- β_2 mRNA and protein concentrations are increased in intestinal tissue following intestinal ischemia-perfusion (I/R) injury. I/R injury was induced in 12-day old wild-type mouse pups by first clamping the superior mesenteric artery for 60 min and then releasing the clamp to allow reperfusion for 90 min. Data represent an n = 5 mice in each group.

FIG. 8(A) shows induction of NEC-like intestinal injury by intraperitoneal administration of PAF and LPS in 12-day-old wild type and transgenic DNIIR mice that express a defective, dominant negative TGF- β RII when supplemented with zinc. Three days of zinc supplementation resulted in about 50% expression of the transgene and a partial inhibition of TGF- β signaling, whereas 7 days of zinc supplementation completely abrogated TGF- β signaling. Mice were sacrificed 2 hrs after PAF and LPS administration and mucosal injury was graded on a 4-point scale. Bar diagram (means $\pm$ SEM) shows the severity of mucosal injury in wild type controls, wild type mice after PAF-LPS administration, DNIIR mice after 3 days of zinc supplementation and PAF-LPS, DNIIR mice after 7 days of zinc supplementation and PAF-LPS, and finally, DNIIR mice which received 3 days of zinc supplementation and then 100 ng TGF- β_2 by gavage 2 hrs prior to PAF-LPS. Compared to WT mice, PAF-LPS-induced mucosal injury was more severe in the DNIIR mice, whereas enteral TGF- β_2 protected DNIIR mice against NEC-like mucosal injury. In the absence of zinc supplementation, DNIIR mice were similar to WT controls, whereas zinc supplementation alone had no effect in WT mice. Data represent an n = 5 mice in each group.

FIG. 8B shows TGF- β_2 is expressed in human milk in biologically relevant concentrations but was not detected in infant formula.

FIG. 8(C) shows both type I and II TGF- β receptors are widely expressed in the human fetal and murine intestine (n =3 each). High-magnification photomicrographs (1000x) show immunoreactivity on the epithelium (solid arrow) and cells in the lamina propria (open arrow).

FIG. 9 shows deficient TGF- β activity in the developing intestine predisposes the premature infant to mucosal inflammation. In the adult (schematic representation on the left), epithelial and stromal cell-derived TGF- β downregulates the inflammatory responses of intestinal macrophages to low levels. Thus, exposure to luminal bacteria or their products results in a regulated inflammatory response that is adequate for host defense but does not cause

unnecessary inflammation upon exposure to commensal bacteria. In contrast, in the premature infant (panel on the right), the inflammatory responses of intestinal macrophages are intact because TGF- β expression, and therefore, mucosal tolerance to bacterial products, are deficient. Mucosal injury and bacterial translocation induce an intense inflammatory reaction, which results in widespread tissue damage. Enteral supplementation of recombinant TGF- β_2 represents a therapeutic strategy to correct the developmental deficiency of TGF- β in the intestine, which, in turn, may serve to prevent/ameliorate NEC and related disease states and conditions in neonates.

DETAILED DESCRIPTION

The present disclosure shows that NEC and related disease states and conditions occur in premature infants when mucosal injury and consequent bacterial translocation induce an intense inflammatory reaction. Intestinal macrophages in the developing intestine respond to bacterial products such as LPS and produce inflammatory cytokines and other factors to augment this process. These characteristics contrast with the mature intestine, where bacterial products do not cause mucosal inflammation as a result of extracellular matrix TGF- β_2 -mediated specific differentiation suppresses the inflammatory responses of gut macrophages.

The present disclosure shows that the down regulation of inflammatory responses in the intestinal mucosa is deficient in the fetal intestinal cells because of a developmental deficiency of TGF- β_2 . The present disclosure also shows that TGF- β_2 expression was reduced in tissue samples of NEC to levels lower than expected for the gestational age. Furthermore, transgenic mice deficient in TGF- β_2 signaling showed significantly more severe mucosal injury after administration of PAF and LPS than mice with normal TGF- β_2 signaling. The present disclosure shows that enteral administration of recombinant TGF- β_2 prior to the administration of PAF and LPS protected against mucosal injury in this mouse model.

Therefore, the present disclosure shows that down regulation of inflammatory responses in intestinal cells, such as but not limited to, macrophages, is an effect of TGF- β_2 , and that enteral administration of TGF- β_2 and/or TGF- β_2 derivatives treats and/or prevents NEC-like mucosal injury by correcting the developmental deficiency of gut mucosal tolerance to bacteria and/or bacterial antigens and/or products.

Therefore, in one embodiment the present disclosure relates to methods for treating and/or preventing NEC in a subject in need of such treatment/prevention.

In an alternate embodiment, the present disclosure relates to methods for treating and/or preventing a disease state or condition characterized by, at least in part, un-regulated or improperly regulated intestinal cytokine release in a subject in need of such

treatment/prevention. The disease states and conditions include, but are not limited to, sepsis and chronic infections of the bowel.

In another alternate embodiment, the present disclosure relates to methods for treating a subject having intestinal mucosal damage or preventing such intestinal mucosal damage in a subject in need of such treatment/prevention. The intestinal mucosal damage is caused by, at least in part, NEC or a disease state or condition characterized by, at least in part, un-regulated or improperly regulated intestinal cytokine release. The disease states and conditions include, but are not limited to, sepsis and chronic infections of the bowel.

In a further embodiment, the present disclosure relates to methods for alteration of the cytokine response of immature intestinal cells, such, as but not limited to, immature intestinal epithelial cells, to mucosal injury, bacterial and/or bacterial antigens and/or products so that the cytokine response is matured. In a particular embodiment, a matured cytokine response include a decrease in at least one of the following factors: chemokines having both the C-C and CXC motifs (such as but not limited to CXCL-1, CXCL-5, CXCL-8, CCL-2, CCL-3, CCL-4 and CCL-5), interleukins (such as but not limited to IL-1 α , IL-1 β , IL-1F5, IL-1F7, IL-1F8, IL-1F9, IL-6, IL-8, IL-12 α , IL-12 β , IL-17 α , IL-17 β , IL-18 α , IL-18 β and IL-23 α), and other cytokines (such as but not limited to GM-CSF and TNF α).

In still a further embodiment, the present disclosure provides for pharmaceutical compositions and medicaments containing TGF- β_2 and/or TGF- β_2 derivatives for use in the disclosed methods.

Definitions

As used in this specification, the followings words and phrases have the meanings as defined below, unless otherwise limited in specific instances, either individually or as part of a larger group.

The terms “prevent”, “preventing”, “prevention” “suppress”, “suppressing” and suppression as used herein refer to administering a compound prior to the onset of clinical symptoms of a disease state/condition so as to prevent any symptom, aspect or characteristic of the disease state/condition. Such preventing and suppressing need not be absolute to be useful.

Te terms “treat”, “treating” and “treatment” as used herein refers to administering a compound after the onset of clinical symptoms of a disease state/condition so as to reduce or eliminate any symptom, aspect or characteristic of the disease state/condition. Such treating need not be absolute to be useful.

The term “in need of treatment” as used herein refers to a judgment made by a caregiver that a patient requires or will benefit from treatment. This judgment is made based on

a variety of factors that are in the realm of a caregiver's expertise, and may include the knowledge that the patient is ill as the result of a disease state/condition that is treatable by a compound, pharmaceutical composition or medicament of the disclosure.

5 The term "in need of prevention" as used herein refers to a judgment made by a caregiver that a patient requires or will benefit from prevention. This judgment is made based on a variety of factors that are in the realm of a caregiver's expertise, and may include the knowledge that the patient may become ill as the result of a disease state/condition that is treatable by a compound, pharmaceutical composition or medicament of the disclosure.

10 The term "individual", "subject" or "patient" as used herein refers to any animal, including mammals, such as, but not limited to, mice, rats, other rodents, rabbits, dogs, cats, swine, cattle, sheep, horses, or primates, or humans. The term may specify male or female or both, or exclude male or female.

15 The term "therapeutically effective amount", in reference to the treating, preventing or suppressing of a disease state/condition, refers to an amount of a compound either alone or as contained in a pharmaceutical composition or medicament that is capable of having any detectable, positive effect on any symptom, aspect, or characteristics of the disease state/condition. Such effect need not be absolute to be beneficial.

20 The term "pharmaceutically acceptable salts" is meant to include salts of the active compounds which are prepared with relatively nontoxic acids or bases, depending on the particular substituents found on the compounds described herein. When compounds of the present invention contain relatively acidic functionalities, base addition salts can be obtained by contacting the neutral form of such compounds with a sufficient amount of the desired base, either neat or in a suitable inert solvent. Examples of pharmaceutically acceptable base addition salts include sodium, potassium, calcium, ammonium, organic amino, or magnesium salt, or a
25 similar salt. When compounds of the present invention contain relatively basic functionalities, acid addition salts can be obtained by contacting the neutral form of such compounds with a sufficient amount of the desired acid, either neat or in a suitable inert solvent. Examples of pharmaceutically acceptable acid addition salts include those derived from inorganic acids like hydrochloric, hydrobromic, nitric, carbonic, monohydrogencarbonic, phosphoric, monohydrogenphosphoric, dihydrogenphosphoric, sulfuric, monohydrogensulfuric, hydriodic,
30 or phosphorous acids and the like, as well as the salts derived from relatively nontoxic organic acids like acetic, propionic, isobutyric, oxalic, maleic, malonic, benzoic, succinic, suberic, fumaric, mandelic, phthalic, benzenesulfonic, p-tolylsulfonic, citric, tartaric, methanesulfonic, and the like. Also included are salts of amino acids such as arginate and the like, and salts of

organic acids like glucuronic or galactunoric acids and the like (see, for example, Berge, S. M., et al., "Pharmaceutical Salts", Journal of Pharmaceutical Science, 1977, 66, 1-19). Certain specific compounds of the present invention contain both basic and acidic functionalities that allow the compounds to be converted into either base or acid addition salts.

5 The term "prodrug" is meant to include functional derivatives of the compounds disclosed which are readily convertible *in vivo* into the required compound. Thus, in the methods of treatment of the present disclosure, the term "administering" shall encompass the treatment of the various disease states/conditions described with the compound specifically disclosed or with a prodrug which may not be specifically disclosed, but which converts to the
10 specified compound *in vivo* after administration to the patient. Conventional procedures for the selection and preparation of suitable prodrug derivatives are described, for example, in "Design of Prodrugs", ed. H. Bundgaard, Elsevier, 1985.

Methods of Treatment and Prevention

 The present disclosure describes the use of TGF- β_2 and/or TGF- β_2 derivatives to
15 prevent or treat NEC and/or a disease state or condition characterized by, at least in part, unregulated or improperly regulated intestinal cytokine release in a subject in need of such treatment/prevention. The disease states and conditions include, but are not limited to, sepsis and chronic infections of the bowel.

 In one embodiment, the teachings of the present disclosure provides for treating and/or
20 preventing NEC in a subject in need of such treatment/prevention. The method of treatment/prevention comprises the steps of identifying a subject in need of such treatment/prevention and administering to said subject TGF- β_2 and/or a TGF- β_2 derivative. In a specific embodiment, the TGF- β_2 and/or a TGF- β_2 derivative is administered in a therapeutically effective amount. The TGF- β_2 and/or a TGF- β_2 derivative may be administered
25 by itself or as a part of a pharmaceutical composition or medicament. In one embodiment, the administration of TGF- β_2 and/or a TGF- β_2 derivative inhibits, at least in part, a cytokine response in the intestine to mucosal injury, bacterial invasion, bacterial products, bacterial antigens or combinations of the foregoing. In an alternate embodiment, administration of TGF- β_2 and/or a TGF- β_2 derivative alters the cytokine response of immature intestinal cells, such, as
30 but not limited to, immature intestinal epithelial cells and/or macrophages, to mucosal injury, bacterial and/or bacterial antigens and/or products so that the cytokine response is matured. In a particular embodiment, a matured cytokine response include a decrease in at least one of the following factors: chemokines having both the C--C and CXC motifs (such as but not limited to CXCL-1, CXCL-5, CXCL-8, CCL-2, CCL-3, CCL-4 and CCL-5), interleukins (such as but

not limited to IL-1 α , IL-1 β , IL-1F5, IL-1F7, IL-1F8, IL-1F9, IL-6, IL-8, IL-12 α , IL-12 β , IL-17 α , IL-17 β , IL-18 α , IL-18 β and IL-23 α), and other cytokines (such as but not limited to GM-CSF and TNF α). In a further embodiment, the subject is a premature infant. In still a further embodiment, the subject is a premature infant having a gestational age of 32 weeks or less. In an additional embodiment, the administration is enteral administration.

In another embodiment, the teachings of the present disclosure provides for treating and/or preventing a disease state or condition characterized by, at least in part, un-regulated or improperly regulated intestinal cytokine release in a subject in need of such treatment/prevention. The disease states and conditions include, but are not limited to, sepsis and chronic infections of the bowel. The method of treatment/prevention comprises the steps of identifying a subject in need of such treatment/prevention and administering to said subject TGF- β_2 and/or a TGF- β_2 derivative. In a specific embodiment, the TGF- β_2 and/or a TGF- β_2 derivative is administered in a therapeutically effective amount. The TGF- β_2 and/or a TGF- β_2 derivative may be administered by itself or as a part of a pharmaceutical composition or medicament. In one embodiment, the administration of TGF- β_2 and/or a TGF- β_2 derivative inhibits, at least in part, a cytokine response in the intestine to mucosal injury, bacterial invasion, bacterial products, bacterial antigens or combinations of the foregoing. In an alternate embodiment, administration of TGF- β_2 and/or a TGF- β_2 derivative alters the cytokine response of immature intestinal cells, such as but not limited to, immature intestinal epithelial cells and macrophages, to mucosal injury, bacterial and/or bacterial antigens and/or products so that the cytokine response is matured. In a particular embodiment, a matured cytokine response include a decrease in at least one of the following factors: chemokines having both the C--C and CXC motifs (such as but not limited to CXCL-1, CXCL-5, CXCL-8, CCL-2, CCL-3, CCL-4 and CCL-5), interleukins (such as but not limited to IL-1 α , IL-1 β , IL-1F5, IL-1F7, IL-1F8, IL-1F9, IL-6, IL-8, IL-12 α , IL-12 β , IL-17 α , IL-17 β , IL-18 α , IL-18 β and IL-23 α), and other cytokines (such as but not limited to GM-CSF and TNF α). In a further embodiment, the subject is a premature infant. In still a further embodiment, the subject is a premature infant having a gestational age of 32 weeks or less. In an additional embodiment, the administration is enteral administration.

In another embodiment, the teachings of the present disclosure provides for treating a subject having intestinal mucosal damage or preventing such intestinal mucosal damage in a subject in need of such treatment/prevention. In one embodiment, the intestinal mucosal damage is caused by, at least in part, NEC or a disease state or condition characterized by, at least in part, un-regulated or improperly regulated intestinal cytokine release. The disease states

and conditions include, but are not limited to, sepsis and chronic infections of the bowel. The method of treatment/prevention comprises the steps of identifying a subject in need of such treatment/prevention and administering to said subject TGF- β_2 and/or a TGF- β_2 derivative. In a specific embodiment, the TGF- β_2 and/or a TGF- β_2 derivative is administered in a therapeutically effective amount. The TGF- β_2 and/or a TGF- β_2 derivative may be administered by itself or as a part of a pharmaceutical composition or medicament. In one embodiment, the administration of TGF- β_2 and/or a TGF- β_2 derivative inhibits, at least in part, a cytokine response in the intestine to mucosal injury, bacterial and/or bacterial antigens. In an alternate embodiment, administration of TGF- β_2 and/or a TGF- β_2 derivative alters the cytokine response of immature intestinal cells, such as but not limited to, immature intestinal epithelial cells, to mucosal injury, bacterial invasion, bacterial products, bacterial antigens or combinations of the foregoing so that the cytokine response is matured. In a particular embodiment, a matured cytokine response include a decrease in at least one of the following factors: chemokines having both the C--C and CXC motifs (such as but not limited to CXCL-1, CXCL-5, CXCL-8, CCL-2, CCL-3, CCL-4 and CCL-5), interleukins (such as but not limited to IL-1 α , IL-1 β , IL-1F5, IL-1F7, IL-1F8, IL-1F9, IL-6, IL-8, IL-12 α , IL-12 β , IL-17 α , IL-17 β , IL-18 α , IL-18 β and IL-23 α), and other cytokines (such as but not limited to GM-CSF and TNF α). In a further embodiment, the subject is a premature infant. In still a further embodiment, the subject is a premature infant having a gestational age of 32 weeks or less. In an additional embodiment, the administration is enteral administration.

In another embodiment, the teachings of the present disclosure provide for alteration of the cytokine response of immature intestinal cells, such as but not limited to, immature intestinal epithelial cells, to mucosal injury, bacterial invasion, bacterial products, bacterial antigens or combinations of the foregoing so that the cytokine response is matured. In a particular embodiment, a matured cytokine response include a decrease in at least one of the following factors: chemokines having both the C--C and CXC motifs (such as but not limited to CXCL-1, CXCL-5, CXCL-8, CCL-2, CCL-3, CCL-4 and CCL-5), interleukins (such as but not limited to IL-1 α , IL-1 β , IL-1F5, IL-1F7, IL-1F8, IL-1F9, IL-6, IL-8, IL-12 α , IL-12 β , IL-17 α , IL-17 β , IL-18 α , IL-18 β and IL-23 α), and other cytokines (such as but not limited to GM-CSF and TNF α). The method comprises the steps of identifying a subject in need of such alteration and administering to said subject TGF- β_2 and/or a TGF- β_2 derivative. In a specific embodiment, the TGF- β_2 and/or a TGF- β_2 derivative is administered in a therapeutically effective amount. The TGF- β_2 and/or a TGF- β_2 derivative may be administered by itself or as a part of a pharmaceutical composition or medicament. In one embodiment, the administration

of TGF- β_2 and/or a TGF- β_2 derivative inhibits, at least in part, a cytokine response in the intestine to mucosal injury, bacterial and/or bacterial antigens. In a another embodiment, the patient in need of such alternation is a subject suffering from at risk for NEC a disease state or condition characterized by, at least in part, un-regulated or improperly regulated intestinal cytokine release, such as but not limited to, sepsis and chronic infections of the bowel. In a further embodiment, the subject is a premature infant. In still a further embodiment, the subject is a premature infant having a gestational age of 32 weeks or less. In an additional embodiment, the administration is enteral administration.

Creation and Selection of TGF- β_2 and TGF- β_2 Derivatives

The peptide structures of the three members of the TGF- β family are highly similar. They are all encoded as large protein precursors; TGF- β_1 contains 390 amino acids and TGF- β_2 and TGF- β_3 each contain 412 amino acids, although a second isoform of TGF- β_2 exists comprising 442 amino acids. They each have an N-terminal signal peptide of 19-30 amino acids that they require for secretion from a cell, a propeptide region (called latency associated peptide or LAP), and a 112-114 amino acid C-terminal region that becomes the *mature* TGF- β molecule following its release from the pro-region by proteolytic cleavage. The mature TGF- β protein dimerizes to produce a 25 KDa active molecule with many conserved structural motifs. TGF- β has nine cysteine residues that are conserved among its family; eight form disulfide bonds within the molecule to create a cysteine knot structure characteristic of the TGF- β superfamily while the ninth cysteine forms a bond with the ninth cysteine of another TGF- β molecule to produce the dimer. Other conserved residues in TGF- β are thought to form secondary structure through hydrophobic interactions. The region between the fifth and sixth conserved cysteines houses the most divergent area of TGF- β molecules that is exposed at the surface of the molecule and is implicated in receptor binding and specificity of TGF- β .

The present disclosure contemplates the use of TGF- β_2 and TGF- β_2 derivatives in the methods of treatment and prevention disclosed herein. As defined herein, TGF- β_2 includes isoforms 1 (SEQ ID NO: 1) and isoform 2 (SEQ ID NO: 2), as well as naturally occurring variants of the foregoing; furthermore, the term also includes the full length polypeptide, the polypeptide with the signal sequence (amino acids 1-19 for isoform 1) removed and the polypeptide with the signal sequence and pro-peptide (amino acids 1-302 for isoform 1) removed. The TGF- β_2 may be provided as a homodimer or as a heterodimer with other TGF- β family members (such as TGF- β_1 or TGF- β_3). It is noted that the carboxy terminal regions of isoform 1 and 2 of TGF- β_2 are identical in amino acid content.

As defined herein, TGF- β_2 derivative refers to a TGF- β_2 polypeptide having one or more insertions, deletions or substitutions. In one embodiment, an active fragment of TGF- β_2 is used; in a specific embodiment, the active fragment of TGF- β_2 contains all 9 conserved cysteine residues. In one embodiment, one or more or all of the 9 conserved cysteine residues (amino acids 309, 317, 318, 346, 350, 379, 380, 411 and 413 for isoform 1) are maintained; in an alternate embodiment, amino acids 72, 140 and 241 are maintained. The TGF- β_2 derivative may have an activity that is comparable to or increased (in one embodiment, 50% or more) as compared to the wild-type TGF- β_2 polypeptide activity and as such may be used to increase a TGF- β_2 activity; alternatively, the TGF- β_2 derivative may have an activity that is decreased (in one embodiment, less than 50%) as compared to the wild-type TGF- β_2 activity and as such may be used to decrease a TGF- β_2 activity.

The deletions, additions and substitutions can be selected to generate a desired TGF- β_2 derivative. For example, it is not expected that deletions, additions and substitutions in the non-conserved areas of TGF- β_2 would alter TGF- β_2 activity. Likewise conservative substitutions or substitutions of amino acids with similar properties is expected to be tolerated.

Conservative modifications to the amino acid sequence of any of SEQ ID NOS:1-2, including combinations thereof (and the corresponding modifications to the encoding nucleotides) will produce TGF- β_2 derivatives having functional and chemical characteristics similar to those of naturally occurring TGF- β_2 . For example, a "conservative amino acid substitution" may involve a substitution of a native amino acid residue with a nonnative residue such that there is little or no effect on the polarity or charge of the amino acid residue at that position. Furthermore, any native residue in the polypeptide may also be substituted with alanine.

Conservative amino acid substitutions also encompass non-naturally occurring amino acid residues which are typically incorporated by chemical peptide synthesis rather than by synthesis in biological systems. These include peptidomimetics, and other reversed or inverted forms of amino acid moieties. It will be appreciated by those of skill in the art that nucleic acid and polypeptide molecules described herein may be chemically synthesized as well as produced by recombinant means.

Naturally occurring residues may be divided into classes based on common side chain properties: 1) hydrophobic: norleucine, Met, Ala, Val, Leu, Ile; 2) neutral hydrophilic: Cys, Ser, Thr, Asn, Gln; 3) acidic: Asp, Glu; 4) basic: His, Lys, Arg; 5) residues that influence chain orientation: Gly, Pro; and 6) aromatic: Trp, Tyr, Phe.

For example, non-conservative substitutions may involve the exchange of a member of one of these classes for a member from another class. Such substituted residues may be introduced into regions of the TGF- β 2 derivatives that are homologous with non-human TGF- β 2 orthologs, or into the non-homologous regions of the molecule.

5 In making such changes, the hydropathic index of amino acids may be considered. Each amino acid has been assigned a hydropathic index on the basis of their hydrophobicity and charge characteristics, these are: isoleucine (+4.5); valine (+4.2); leucine (+3.8); phenylalanine (+2.8); cysteine/cystine (+2.5); methionine (+1.9); alanine (+1.8); glycine (-0.4); threonine (-0.7); serine (-0.8); tryptophan (-0.9); tyrosine (-1.3); proline (-1.6); histidine (-3.2); glutamate
10 (-3.5); glutamine (-3.5); aspartate (-3.5); asparagine (-3.5); lysine (-3.9); and arginine (-4.5).

The importance of the hydropathic amino acid index in conferring interactive biological function on a protein is understood in the art (Kyte et al., J. Mol. Biol., 157:105-131, 1982). It is known that certain amino acids may be substituted for other amino acids having a similar hydropathic index or score and still retain a similar biological activity.

15 In making changes based upon the hydropathic index, the substitution of amino acids whose hydropathic indices are within +/- 2 may be used; in an alternate embodiment, the hydropathic indices are with +/- 1; in yet another alternate embodiment, the hydropathic indices are within +/- 0.5.

It is also understood in the art that the substitution of like amino acids can be made
20 effectively on the basis of hydrophilicity. The greatest local average hydrophilicity of a polypeptide as governed by the hydrophilicity of its adjacent amino acids, correlates with a biological property of the protein.

The following hydrophilicity values have been assigned to amino acid residues: arginine (+3.0); lysine (+3.0); aspartate (+3.0+-.1); glutamate (+3.0+-.1); serine (+0.3);
25 asparagine (+0.2); glutamine (+0.2); glycine (0); threonine (-0.4); proline (-0.5+-.1); alanine (-0.5); histidine (-0.5); cysteine (-1.0); methionine (-1.3); valine (-1.5); leucine (-1.8); isoleucine (-1.8); tyrosine (-2.3); phenylalanine (-2.5); tryptophan (-3.4).

In making changes based upon similar hydrophilicity values, the substitution of amino acids whose hydrophilicity values are within +/- 2 may be used; in an alternate embodiment,
30 the hydrophilicity values are with +/- 1; in yet another alternate embodiment, the hydrophilicity values are within +/- 0.5.

Desired amino acid substitutions (whether conservative or non-conservative) can be determined by those skilled in the art at the time such substitutions are desired. For example, amino acid substitutions can be used to identify important residues of TGF- β 2, or to increase or

decrease the affinity of TGF- β 2 with a particular binding target in order to increase or decrease TGF- β 2 activity.

Exemplary amino acid substitutions are set forth in the table 1 below

Table 1			
Original Amino Acid	Amino	Exemplary substitution	Preferred substitution
Ala		Val, Leu, Ile	Val
Arg		Lys, Gln, Asn	Lys
Asn		Glu	Glu
Asp		Glu	Glu
Cys		Ser, Ala	Ser
Gln		Asn	Asn
Glu		Asp	Asp
Gly		Pro, Ala	Ala
His		Asn, Gln, Lys, Arg	Arg
Ile		Leu, Val, Met, Ala, Phe, Norleucine	Leu
Leu		Ile, Val, Met, Ala, Phe, Norleucine	Ile
Lys		Arg, 1,4-diaminobutyric acid, Gln, Asn	Arg
Met		Leu, Phe, Ile	Leu
Phe		Leu, Val, Ile, Ala, Tyr	Leu
Pro		Ala, Gly	Gly
Ser		Thr, Ala, Cys	Thr
Thr		Ser	Ser
Trp		Tyr, Phe	Tyr
Tyr		Trp, Phe, Thr, Ser	Phe
Val		Ile, Met, Leu, Phe, Ala, Norleucine	Leu

5 A skilled artisan will be able to determine suitable derivatives of the polypeptide as set forth in any of SEQ ID NOS:1-2, including combinations thereof, using well known techniques. For identifying suitable areas of the molecule that may be changed without destroying activity, one skilled in the art may target areas not believed to be important for activity. For example, when similar polypeptides with similar activities from the same species
 10 or from other species are known, one skilled in the art may compare the amino acid sequence of TGF- β 2 to such similar polypeptides. With such a comparison, one can identify residues and

portions of the molecules that are conserved among similar polypeptides. It will be appreciated that changes in areas of a TGF- β 2 that are not conserved relative to such similar polypeptides would be less likely to adversely affect the biological activity and/or structure of TGF- β 2. One skilled in the art would also know that, even in relatively conserved regions, one may substitute
5 chemically similar amino acids for the naturally occurring residues while retaining activity (conservative amino acid residue substitutions). Therefore, even areas that may be important for biological activity or for structure may be subject to conservative amino acid substitutions without destroying the biological activity or without adversely affecting the polypeptide structure.

10 Additionally, one skilled in the art can review structure-function studies identifying residues in similar polypeptides that are important for activity or structure. In view of such a comparison, one can predict the importance of amino acid residues in TGF- β 2 that correspond to amino acid residues that are important for activity or structure in similar polypeptides. One skilled in the art may opt for chemically similar amino acid substitutions for such predicted
15 important amino acid residues of TGF- β 2.

One skilled in the art can also analyze the three-dimensional structure and amino acid sequence in relation to that structure in similar polypeptides. In view of that information, one skilled in the art may predict the alignment of amino acid residues of TGF- β 2 with respect to its three dimensional structure. One skilled in the art may choose not to make radical changes
20 to amino acid residues predicted to be on the surface of the protein, since such residues may be involved in important interactions with other molecules. Moreover, one skilled in the art may generate test TGF- β 2 derivatives containing a single amino acid substitution at each desired amino acid residue. The derivatives can then be screened using activity assays known to those skilled in the art and as disclosed herein. Such derivatives could be used to gather information
25 about suitable substitution. For example, if one discovered that a change to a particular amino acid residue resulted in destroyed, undesirably reduced, or unsuitable activity, derivatives with such a change would be avoided. In other words, based on information gathered from such routine experiments, one skilled in the art can readily determine the amino acids where further substitutions should be avoided either alone or in combination with other mutations.

30 Numerous scientific publications have been devoted to the prediction of secondary structure from analyses of amino acid sequences (see Chou et al., *Biochemistry*, 13(2):222-245, 1974; Chou et al., *Biochemistry*, 113(2):211-222, 1974; Chou et al., *Adv. Enzymol. Relat. Areas Mol. Biol.*, 47:45-148, 1978; Chou et al., *Ann. Rev. Biochem.*, 47:251-276, 1979; and Chou et al., *Biophys. J.*, 26:367-384, 1979). Moreover, computer programs are currently

available to assist with predicting secondary structure of polypeptides. Examples include those programs based upon the Jameson-Wolf analysis (Jameson et al., *Comput. Appl. Biosci.*, 4(1):181-186, 1998; and Wolf et al., *Comput. Appl. Biosci.*, 4(1):187-191; 1988), the program PepPlot.RTM. (Brutlag et al., *CABS*, 6:237-245, 1990; and Weinberger et al., *Science*, 228:740-742, 1985), and other new programs for protein tertiary structure prediction (Fetrow et al., *Biotechnology*, 11:479-483, 1993).

Moreover, computer programs are currently available to assist with predicting secondary structure. One method of predicting secondary structure is based upon homology modeling. For example, two polypeptides or proteins which have a sequence identity of greater than 30%, or similarity greater than 40% often have similar structural topologies. The recent growth of the protein structural data base (PDB) has provided enhanced predictability of secondary structure, including the potential number of folds within a polypeptide's or protein's structure (see Holm et al., *Nucl. Acid. Res.*, 27(1):244-247, 1999).

Additional methods of predicting secondary structure include "threading" (Jones, D., *Curr. Opin. Struct. Biol.*, 7(3):377-87, 1997; Suppl et al., *Structure*, 4(1):15-9, 1996), "profile analysis" (Bowie et al., *Science*, 253:164-170, 1991; Gribskov et al., *Meth. Enzym.*, 183:146-159, 1990; and Gribskov et al., *Proc. Nat. Acad. Sci.*, 84(13): 4355

Pharmaceutical Compositions and Modes of Administration

The present disclosure provides for a pharmaceutical composition and medicaments comprising, consisting of or consisting essentially of TGF- β_2 and/or a TGF- β_2 derivative for use in the methods described herein. In one embodiment, the TGF- β_2 and/or a TGF- β_2 derivative is present in a therapeutically effective amount. Such pharmaceutical compositions may be used in the manufacture of a medicament for use in the methods of treatment and prevention described herein. In one embodiment, pharmaceutical compositions may be used in the manufacture of a medicament for treating and/or preventing NEC or a disease or condition characterized, at least in part, by an un-regulated or improperly regulated cytokine response to mucosal injury, bacterial and/or bacterial antigens and/or products. In one embodiment, un-regulated or improperly regulated cytokine response such as, but not limited to, the inappropriate cytokine response of immature intestinal cells, such as, but not limited to, immature intestinal epithelial cells, to mucosal injury, bacteria and/or bacterial antigens and/or products. This inappropriate cytokine response may be caused by the lack of normal developmental cues that would normally regulate such cytokine response in mature intestinal epithelial cells. In one embodiment, the disease or condition is NEC.

The disclosed pharmaceutical compositions and medicaments may also comprise a pharmaceutically acceptable carrier. The compounds of the disclosure are useful in both free form and in the form of pharmaceutically acceptable salts.

5 The pharmaceutically acceptable carriers described herein, including, but not limited to, vehicles, adjuvants, excipients, or diluents, are well-known to those who are skilled in the art. Typically, the pharmaceutically acceptable carrier is chemically inert to the active compounds and has no detrimental side effects or toxicity under the conditions of use. The pharmaceutically acceptable carriers can include polymers and polymer matrices.

10 The compounds described in the instant disclosure can be administered by any conventional method available for use in conjunction with pharmaceuticals, either as individual therapeutic agents or in combination with additional therapeutic agents.

15 The compounds described are administered in therapeutically effective amount. The therapeutically effective amount of the compound and the dosage of the pharmaceutical composition administered will, of course, vary depending upon known factors, such as the pharmacodynamic characteristics of the particular agent and its mode and route of administration, the age, health and weight of the recipient; the severity and stage of the disease state or condition; the kind of concurrent treatment; the frequency of treatment; and the effect desired.

20 A daily dosage of active ingredient can be expected to be about 0.1 to 15 micrograms (μg) per kilogram (kg) of body weight. In one embodiment, the total amount is between about 0.1 $\mu\text{g}/\text{kg}$ and about 1 $\mu\text{g}/\text{kg}$ of body weight; in an alternate embodiment between about 1 $\mu\text{g}/\text{kg}$ and about 5 $\mu\text{g}/\text{kg}$ of body weight; in yet another alternate embodiment between 5 $\mu\text{g}/\text{kg}$ and about 15 $\mu\text{g}/\text{kg}$ of body weight. The above described amounts may be administered as a series of smaller doses over a period of time if desired. As would be obvious, the dosage of
25 active ingredient may be given other than daily if desired.

The total amount of the compound administered will also be determined by the route, timing and frequency of administration as well as the existence, nature, and extent of any adverse side effects that might accompany the administration of the compound and the desired physiological effect. It will be appreciated by one skilled in the art that various conditions or
30 disease states, in particular chronic conditions or disease states, may require prolonged treatment involving multiple administrations.

Dosage forms of the pharmaceutical compositions described herein (forms of the pharmaceutical compositions suitable for administration) contain from about 0.1 μg to about 15 μg of active ingredient (i.e. the compounds disclosed) per unit. In these pharmaceutical

compositions, the active ingredient will ordinarily be present in an amount of about 0.5-95% weight based on the total weight of the composition. Multiple dosage forms may be administered as part of a single treatment.

5 The active ingredient can be administered enterally in solid dosage forms, such as capsules, tablets, and powders, or in liquid dosage forms, such as milk, elixirs, syrups and suspensions. It can also be administered parenterally, in sterile liquid dosage forms. The active ingredient can also be administered intranasally (nose drops) or by inhalation via the pulmonary system, such as by propellant based metered dose inhalers or dry powders inhalation devices. Other dosage forms are potentially possible such as administration
10 transdermally, via patch mechanism or ointment.

In the preferred embodiment, formulations suitable for enteral or oral administration may be liquid solutions, such as a therapeutically effective amount of the compound dissolved in diluents, such as milk, water, saline, buffered solutions, infant formula, and expressed breast milk, other suitable carriers, or combinations thereof. The active ingredients can then be mixed
15 to the diluent just prior to administration. In an alternate embodiment, formulations suitable for enteral or oral administration may be capsules, sachets, tablets, lozenges, and troches. In each embodiment, the formulation may contain a predetermined therapeutically effective amount of the active ingredient, as solids or granules, powders, suspensions and suitable emulsions. Liquid formulations may include diluents, such as water and alcohols, for example, ethanol,
20 benzyl alcohol, propylene glycol, glycerin, and the polyethylene alcohols, either with or without the addition of a pharmaceutically acceptable surfactant, suspending agent, or emulsifying agent. Capsule forms can be of the ordinary hard- or soft-shelled gelatin type containing, for example, surfactants, lubricants, and inert fillers, such as lactose, sucrose, calcium phosphate, and corn starch. Tablet forms can include one or more of the following:
25 lactose, sucrose, mannitol, corn starch, potato starch, alginic acid, microcrystalline cellulose, acacia, gelatin, guar gum, colloidal silicon dioxide, croscarmellose sodium, talc, magnesium stearate, calcium stearate, zinc stearate, stearic acid, and other excipients, colorants, diluents, buffering agents, disintegrating agents, moistening agents, preservatives, flavoring agents, and pharmacologically compatible carriers. Lozenge forms can comprise the active ingredient in a
30 flavor, usually sucrose and acacia or tragacanth, as well as pastilles comprising the active ingredient in an inert base, such as gelatin and glycerin, or sucrose and acacia, emulsions, and gels containing, in addition to the active ingredient, such carriers as are known in the art.

Formulations suitable for parenteral administration include aqueous and non-aqueous, isotonic sterile injection solutions, which can contain anti-oxidants, buffers, bacteriostats, and

solutes that render the formulation isotonic with the blood of the patient, and aqueous and non-aqueous sterile suspensions that can include suspending agents, solubilizers, thickening agents, stabilizers, and preservatives. The compound can be administered in a physiologically acceptable diluent in a pharmaceutically acceptable carrier, such as a sterile liquid or mixture of liquids, including water, saline, aqueous dextrose and related sugar solutions, an alcohol, such as ethanol, isopropanol, or hexadecyl alcohol, glycols, such as propylene glycol or polyethylene glycol such as poly(ethyleneglycol) 400, glycerol ketals, such as 2,2-dimethyl-1,3-dioxolane-4-methanol, ethers, an oil, a fatty acid, a fatty acid ester or glyceride, or an acetylated fatty acid glyceride with or without the addition of a pharmaceutically acceptable surfactant, such as a soap or a detergent, suspending agent, such as pectin, carbomers, methylcellulose, hydroxypropylmethylcellulose, or carboxymethylcellulose, or emulsifying agents and other pharmaceutical adjuvants.

Oils, which can be used in parenteral formulations include petroleum, animal, vegetable, or synthetic oils. Specific examples of oils include peanut, soybean, sesame, cottonseed, corn, olive, petrolatum, and mineral. Suitable fatty acids for use in parenteral formulations include oleic acid, stearic acid, and isostearic acid. Ethyl oleate and isopropyl myristate are examples of suitable fatty acid esters. Suitable soaps for use in parenteral formulations include fatty alkali metal, ammonium, and triethanolamine salts, and suitable detergents include (a) cationic detergents such as, for example, dimethyldialkylammonium halides, and alkylpyridinium halides, (b) anionic detergents such as, for example, alkyl, aryl, and olefin sulfonates, alkyl, olefin, ether, and monoglyceride sulfates, and sulfosuccinates, (c) nonionic detergents such as, for example, fatty amine oxides, fatty acid alkanolamides, and polyoxyethylene polypropylene copolymers, (d) amphoteric detergents such as, for example, alkyl .beta.-aminopropionates, and 2-alkylimidazoline quaternary ammonium salts, and (e) mixtures thereof.

The parenteral formulations typically contain from about 0.5% to about 25% by weight of the active ingredient in solution. Suitable preservatives and buffers can be used in such formulations. In order to minimize or eliminate irritation at the site of injection, such compositions may contain one or more nonionic surfactants having a hydrophile-lipophile balance (HLB) of from about 12 to about 17. The quantity of surfactant in such formulations ranges from about 5% to about 15% by weight. Suitable surfactants include polyethylene sorbitan fatty acid esters, such as sorbitan monooleate and the high molecular weight adducts of ethylene oxide with a hydrophobic base, formed by the condensation of propylene oxide with propylene glycol.

Pharmaceutically acceptable excipients are also well-known to those who are skilled in the art. The choice of excipient will be determined in part by the particular compound, as well as by the particular method used to administer the composition. Accordingly, there is a wide variety of suitable formulations of the pharmaceutical composition of the present invention.

5 The following methods and excipients are merely exemplary and are in no way limiting. The pharmaceutically acceptable excipients preferably do not interfere with the action of the active ingredients and do not cause adverse side-effects. Suitable carriers and excipients include solvents such as water, alcohol, and propylene glycol, solid absorbants and diluents, surface active agents, suspending agent, tableting binders, lubricants, flavors, and coloring agents.

10 The compounds of the present disclosure, alone or in combination with other suitable components, can be made into aerosol formulations to be administered via inhalation. These aerosol formulations can be placed into pressurized acceptable propellants, such as dichlorodifluoromethane, propane, and nitrogen. Such aerosol formulations may be administered by metered dose inhalers. They also may be formulated as pharmaceuticals for
15 non-pressured preparations, such as in a nebulizer or an atomizer.

The formulations can be presented in unit-dose or multi-dose sealed containers, such as ampules and vials, and can be stored in a freeze-dried (lyophilized) condition requiring only the addition of the sterile liquid excipient, for example, water, for injections, immediately prior to use. Extemporaneous injection solutions and suspensions can be prepared from sterile
20 powders, granules, and tablets. The requirements for effective pharmaceutically acceptable carriers for injectable compositions are well known to those of ordinary skill in the art. See *Pharmaceutics and Pharmacy Practice*, J.B. Lippincott Co., Philadelphia, Pa., Banker and Chalmers, Eds., 238-250 (1982) and *ASHP Handbook on Injectable Drugs*, Toissel, 4th ed., 622-630 (1986).

25 Formulations suitable for topical administration include pastilles comprising the active ingredient in an inert base, such as gelatin and glycerin, or sucrose and acacia, as well as creams, emulsions, and gels containing, in addition to the active ingredient, such carriers as are known in the art.

30 Additionally, formulations suitable for rectal administration may be presented as suppositories by mixing with a variety of bases such as emulsifying bases or water-soluble bases. Formulations suitable for vaginal administration may be presented as pessaries, tampons, creams, gels, pastes, foams, or spray formulas containing, in addition to the active ingredient, such carriers as are known in the art to be appropriate.

One skilled in the art will appreciate that suitable methods of administering a compound of the present invention to an patient are available, and, although more than one route can be used to administer a particular compound, a particular route can provide a more immediate and more effective reaction than another route.

5 Results

NEC and related disease states and conditions are associated with severe inflammatory response, increased transcription and expression of inflammatory mediators, ischemic changes, and necrosis often associated with NEC. FIG. 1A (upper panel) shows intra-operative gross morphological appearance of NEC showing an area of infarction. FIG. 1A (lower panel) shows
10 an eosin-hematoxylin photomicrograph showing inflammation (thin arrows). Thick arrows indicate intramural air, which represents trapped gas bubbles from bacterial fermentation and is a pathognomonic finding of NEC. FIG. 1B shows PCR microarray indicating upregulation of pro-inflammatory cytokines and transcriptional regulators in NEC (expressed as fold-change over normal fetal intestine). FIG. 1C is data from last 10 years in a neonatal intensive care unit
15 showing that NEC affects preterm infants born during the late 2nd trimester and the early 3rd trimester of pregnancy

Fetal intestinal macrophages, unlike intestinal macrophages from the adult, respond to LPS and other bacterial products and produce inflammatory mediators, including TNF- α . In the gastrointestinal tract, resident macrophages are the first phagocytic cells of the innate
20 immune system to encounter bacteria and bacterial products that breach the epithelium. Located in the subepithelial lamina propria, these cells play a role in protecting against pathogens and in regulating inflammatory responses to commensal bacteria. Therefore, to investigate the development of mucosal tolerance to bacterial products, human fetal macrophages and adult intestinal macrophages were compared. Consistent with earlier reports,
25 macrophages were identifiable in the human fetal intestine at 10 weeks gestation. Unlike macrophages in the full term neonate (iii) and adult (iv) intestine, macrophages in the fetus (i) and premature (ii) intestine expressed CD14, a key component of the LPS receptor complex, and tumor necrosis factor (TNF)- α (FIG. 2A).

To further investigate these differences, isolated murine fetal and adult intestinal
30 macrophages were studied *in vitro*. Human fetal intestinal macrophages were not available for these studies because of technical difficulties in the standard isolation protocols and immunoselection (lack of specific markers for human intestinal macrophages). Therefore, murine intestinal macrophages, which can be purified by immunoselection for CD11b, were used as a model system. Data from the mouse were consistent with immunohistochemical

analysis on human tissue; unlike macrophages isolated from the neonatal (D1 postnatal) adult mouse intestine (Adult), E15 and E18 fetal intestinal macrophages produced TNF- α upon exposure to LPS *in vitro* (FIG. 2B). These finding shows that macrophages in the developing intestine, in contrast to those in the mature intestine, can produce an inflammatory response upon stimulation with bacterial products

In adult intestine, macrophages are derived from blood monocytes, which differentiate under the influence of various epithelial/stromal cell-derived factors present in the ECM. In response to these factors, the macrophages become downregulated for their inflammatory response to bacterial products and their surface expression of innate immune receptors such as CD14. The biochemical microenvironment seen by monocytes newly recruited to the intestinal mucosa can be simulated *in vitro* by preparing conditioned media from explanted intestinal tissue or from cell-free intestinal stroma. Treatment of monocytes with these intestinal T-CMs *in vitro* induces functional and phenotypic characteristics of intestinal macrophages such as tolerance to bacterial products such as LPS and downregulation of CD14 and other innate immune receptors. To investigate whether the observed differences between pro-inflammatory fetal and non-inflammatory adult intestinal macrophages are secondary to maturational changes in the ECM, murine fetal intestinal macrophages (E15) were treated with T-CMs derived from the adult mouse jejunum. As shown in FIG. 2B (inset), T-CMs from adult intestine downregulated the LPS-induced TNF- α response of E15 fetal macrophages, indicating differential expression of one or more ECM factors in the adult versus fetal intestine that suppress LPS-induced macrophage cytokine production.

To further investigate the development of tolerance to bacterial products in human intestinal macrophages, peripheral blood monocytes from healthy adult volunteers were treated *in vitro* with T-CMs prepared from 10-14, 15-19, 20-24 wks gestation human fetal intestinal tissue and from adult jejunum using: (1) *ex* planted intestinal tissue after removing the epithelial layer to expose the *lamina propria*; (2) intact *ex* planted intestinal tissue without removing the intestinal epithelium; and (3) primary epithelial cells isolated from fetal intestinal tissue. Blood monocytes from healthy adult volunteers were treated with fetal/adult T-CMs *in vitro* prior to LPS stimulation. These T-CMs suppressed LPS-induced cytokine production (TNF- α , IL-6, IL-1 β , and IL-8) in the monocyte-derived macrophages in a dose-dependent fashion (individual doses not depicted in figure), and this suppression increased with maturation (FIG. 3A). Note that while fetal T-CMs showed increasing suppressive effects on LPS-induced cytokine production with maturation, fetal T-CMs were not as effective as adult T-CM in suppressing LPS-induced cytokine production. The suppressive effect of these

conditioned media on macrophage cytokine production was not related to the experimental protocol used for preparing the T-CMs. T-CMs prepared from intact *ex* planted tissue and supernatants from primary epithelial cultures suppressed macrophage cytokine production in a similar maturationally-regulated pattern (data not shown). T-CMs prepared from de-epithelized *ex* planted tissue were used as the default method in subsequent experiments because of low batch-to-batch variation with this method. Fetal intestinal T-CMs contained measurable amounts of IL-8/CXCL8 (but no IL-1 β , IL-6, and TNF- α), which was subtracted as background from the results shown in FIG. 3A.

To further confirm this maturation of intestinal ECM factors, a quantitative PCR array to was used to measure the effect of T-CMs on 23 pro-inflammatory cytokines/chemokines. The suppressive effect of T-CMs derived from 10-14 wk fetal, 20-24 wk fetal, and adult intestinal tissue on LPS-induced macrophage cytokine production increased with maturation (FIGS. 4A-D). In these experiments, T-CMs did not affect total protein synthesis or the synthesis of anti-inflammatory cytokines such as TGF- β and IL-10 by the monocyte-derived macrophages (data not depicted).

The effect of T-CMs on the ability of monocyte-derived macrophages to recruit neutrophils upon LPS stimulation *in vitro* was also measured. Neutrophil chemotaxis was measured by using a previously described fluorescence-based assay (Fox, et al., *Cytokine* 29:135-140, 2005). Similar to the effect on macrophage cytokine production, T-CMs suppression of the chemotactic activity for neutrophils increased with maturation (FIG. 3B). Finally, the anti-inflammatory effect of T-CMs was further investigated by measuring NF- κ B activation (phosphorylation of p65/rel A) in LPS-treated monocyte-derived macrophages. In contrast to T-CMs derived from adult jejunum, fetal T-CMs did not suppress NF- κ B activation in these macrophages (FIG. 3C). The differential effects of fetal vs. adult T-CMs in suppressing LPS-induced neutrophil chemotaxis and NF- κ B activation in macrophages further indicates the presence of one or more ECM factors that are expressed in the adult but not in the fetal intestine that promote the non-inflammatory profile of adult intestinal macrophages.

Although there was not sufficient access to adequate amounts of fresh intestinal tissue from premature and full-term neonates to prepare T-CMs, the suppressive effect of intestinal matrix products was investigated by using tissue lysates of frozen intestinal tissue samples from 20-24 wk fetuses, full-term neonates, and adults on LPS-induced TNF- α production by monocyte-derived macrophages *in vitro*. Intestinal tissue lysates from 20-24 wk fetuses suppressed LPS-induced macrophage TNF- α production from 2.23 ± 0.23 ng/mL to 1.36 ± 0.45 ng/mL, which was significantly higher ($p < 0.05$) than 0.22 ± 0.09 ng/mL with lysates of full-term

intestinal tissue and 0.18 ± 0.05 ng/mL with lysates of adult jejunal tissue. The efficacy of the full-term neonatal and adult intestinal tissues was not significantly different from each other.

In the adult intestinal mucosa, intestinal macrophage differentiation and LPS tolerance result from the effect of TGF- β present in the ECM. To determine the role of TGF- β in gut macrophage differentiation during the fetal period, TGF- β bioactivity in T-CMs was measured using a quantitative luciferase assay for TGF- β activation of the plasminogen activator inhibitor-1 (PAI-1) promoter. As seen in FIG. 5A, TGF- β bioactivity was low in fetal T-CMs and increased with maturation reaching a peak in the adult T-CM. To further investigate role of TGF- β in gut macrophage differentiation during the fetal period, monocyte-derived macrophages were treated with 10-14 week, 15-19 week and 20-24 week fetal T-CMs and adult T-CM and the phosphorylation of Smad2, a key transducer of TGF- β -induced signaling in monocytes/macrophages, was measured. Smad2 phosphorylation correlated with the results of the PAI-1 luciferase bioassay (FIG. 5B), thereby confirming that TGF- β activity increases in the intestinal ECM with maturation. Finally, monocyte-derived macrophages were treated with 10-24 week fetal intestinal T-CMs and excess ($10\text{--}50$ $\mu\text{g/mL}$) neutralizing polyclonal rabbit anti-TGF- β IgG antibody (R&D) was added in some wells. This antibody neutralizes the bioactivity of all three isoforms of TGF- β . Neutralization of TGF- β reversed T-CM suppression of LPS-induced TNF- α (FIG. 5C) and IL-8 production in macrophages. For IL-8, LPS increased macrophage IL-8 production from 0.72 ± 0.01 ng/mL to 4.25 ± 1.46 ng/mL. Fetal intestinal T-CMs from 10-14, 15-19, and 20-24 wks gestation suppressed LPS-induced IL-8 production to 3.49 ± 1.41 (not significant), 2.24 ± 0.02 ng/mL ($p < 0.05$), and 1.15 ± 0.22 ng/mL ($p < 0.05$), respectively. TGF- β neutralizing antibody reversed the suppressive effect of T-CMs on macrophage IL-8 production to 3.60 ± 0.18 (not significant), 3.58 ± 0.23 ($p < 0.05$), and 2.59 ± 0.13 ng/mL ($p < 0.05$) in the three gestational age-based groups, respectively.

To investigate developmental changes in TGF- β expression in the intestine, mRNA expression of the three TGF- β isoforms, TGF- β_1 , TGF- β_2 , and TGF- β_3 was measured in the human fetal (10-24 weeks), neonatal (term newborn), and adult intestine. As shown in FIG. 6A, the expression of TGF- β_2 , but not TGF- β_1 or TGF- β_3 , increased with maturation. These data were supported by immunohistochemical analysis (FIG. 6B). In the 10-14 wk fetal intestine, minimal TGF- β_2 expression was noted in the epithelium but was prominent in the *muscularis externa*. From 15 wks onwards, increasing TGF- β_2 immunoreactivity was noted on epithelial cells and *muscularis mucosae* (MM). TGF- β_2 immunoreactivity became stronger

with maturation in the 20-24 wk fetus, term neonate, and adult. In the mature intestine, TGF- β_2 immunoreactivity was also seen in some lamina propria cells. TGF- β_2 co-localized in these cells with α -smooth muscle actin, indicating that these TGF- β_2^+ cells were myofibroblasts (FIG. 6B). In contrast to TGF- β_2 , immunoreactivity for TGF- β_1 and TGF- β_3 did not change significantly with intestinal maturation (data not shown). The effect of maturation on TGF- β_2 expression was further confirmed by measuring total and active TGF- β_2 in T-CMs by ELISA. As seen in FIG. 6C, both total and active TGF- β_2 increased with maturation.

To determine the relative contribution of the three TGF- β isoforms to the inhibitory effects of T-CMs on the inflammatory properties of macrophages, two of the three isoforms were removed by immunoprecipitation in different aliquots and three different T-CM derivatives were prepared, each containing only one of the three isoforms. When monocyte-derived macrophages were treated with these T-CM derivatives, T-CM derivatives containing only TGF- β_2 were most effective in suppressing LPS-induced TNF- α production (FIG. 6D). T-CMs containing only TGF- β_1 had a smaller suppressive effect on macrophage cytokine production, whereas T-CMs containing TGF- β_3 did not have a significant effect. The effect of TGF- β_1 and TGF- β_2 on macrophage cytokine production was blocked by isoform-specific antibodies (data not shown). IL-8 concentrations were also measured in the same supernatants (data not shown), and the effects were similar to those on TNF- α . Furthermore, when monocyte-derived macrophages were treated with 0-2000 pg/mL of recombinant human TGF- β_1 , TGF- β_2 , and TGF- β_3 for 2 hrs before LPS stimulation, TGF- β_2 was the most potent of the three isoforms in suppressing TNF- α (inset of FIG. 6D) and IL-8 expression (data not shown).

The above data show that intestinal ECM suppression of the inflammatory responses of macrophages is mediated through TGF- β , and that TGF- β_2 is the most important of the TGF- β isoforms for this downregulation of macrophage cytokine production. Because resident macrophages are important regulators of the inflammatory responses in tissues, TGF- β is likely to play an important role in maintaining the absence of inflammation in the normal intestinal mucosa despite exposure to bacteria and bacterial products. In contrast to the normal fetal and adult intestine, inflammation is an important characteristic of NEC. Therefore, experiments were conducted to determine whether NEC is associated with decreased expression and/or bioactivity of TGF- β .

TGF- β expression and bioactivity was compared in surgically removed jejunoileal tissue affected by NEC (n=6; mean gestational age $\pm$ standard deviation = 27 ± 3 wks) with normal margins of small intestinal tissue resected during surgery for intestinal obstruction unrelated to NEC (n=4; atresia 2; functional obstruction in 1, isolated perforation in 1; mean

gestational age $\pm$ standard deviation = 26 ± 2 wks). These data were also compared with measurements in normal fetal intestinal tissue of comparable gestational age (22 ± 0.4 wks). As shown in FIG. 7A, NEC was associated with decreased TGF- β_2 mRNA, TGF- β bioactivity and active TGF- β_2 as compared to both normal intestinal tissue from premature infants as well as the fetal intestinal tissue. The concentrations of active TGF- β_1 were also lower in NEC (104 ± 27 pg/mg in normal preterm intestine and 124 ± 18 pg/mg in normal fetal tissue, vs. 32 ± 10 pg/mg in NEC; $p < 0.05$). The concentrations of active TGF- β_3 were below the lower limit of detection in both normal preterm/fetal tissue and NEC. The bioactivity of TGF- β was decreased in tissue affected by NEC, further confirming the lower concentrations of TGF- β isoforms measured by ELISA.

To determine whether the low TGF- β_2 expression observed in the tissue samples of NEC reflects a non-specific consumption of TGF- β_2 during the acute inflammatory response associated with mucosal injury, TGF- β_2 mRNA and active TGF- β_2 protein was measured in a previously described murine model of intestinal ischemia-reperfusion (I/R) injury, which is associated with a marked inflammatory response (Maheshwari, et al., *Fetal Pediatr Pathol* 23:145-157, 2004). The superior mesenteric artery was clamped for 60 min and then allowed reperfusion by releasing the clamp for 90 minutes. Unlike in NEC, both TGF- β_2 mRNA and protein were increased in intestinal tissue following I/R injury (FIG. 7B), indicating that decreased TGF- β_2 expression in NEC is a specific finding unrelated to possible consumption of TGF- β_2 during mucosal inflammation.

It was next determined whether decreased TGF- β activity (such as in the premature intestine) can increase the severity of the mucosal inflammatory response and NEC-like injury in a murine model. The three isoforms of TGF- β have a quantitatively different but qualitatively similar inhibitory effect on macrophage cytokine production (FIG. 6D, inset), and this redundancy renders deletion/neutralization models for individual TGF- β isoform(s) susceptible to interference from the remaining isoforms. The bioactivity of TGF- β can be accurately manipulated at the receptor level, where all TGF- β signaling requires the presence of TGF- β receptor protein II (TGF- β RII), and several transgenic mice have been described with reduced TGF- β receptor expression (Joseph, et al., *Mol Biol Cell* 10:1221-1234, 1999). In the present study, a transgenic DNIIR mice (Serra, et al., *J Cell Biol* 139:541-552, 1997) was used in which a truncated, kinase-defective TGF- β RII transgene is controlled by a metallothionein-like promoter, MT-DNIIR. In these mice, supplementation with zinc activates the DNA regulatory element and promotes transgene expression; overexpression of the

mutated TGF- β RII inhibits the response to all TGF- β species and thus creates a conditional knock-out model that avoids fetal/neonatal lethality.

In preliminary experiments, it was confirmed that the expression of the TGF- β RII transgene and the inhibition of TGF- β_2 signaling in DNIIR mice correlates with the number of days of zinc administration. Zinc supplementation for 3 days induced a low level expression of the transgene, whereas zinc supplementation for 7 days induced strong expression of the transgene. When DNIIR mice after 3 or 7 days of zinc supplementation were given recombinant TGF- β_2 (100 ng, intraperitoneal), Smad2 phosphorylation in the intestine (and liver) correlated inversely with transgene expression. These data confirmed that after 3 days of zinc supplementation, DNIIR mice develop a partial deficiency of TGF- β effects, whereas 7 days of zinc treatment resulted in a near complete loss of TGF- β signaling. In this study, wild-type mice, mice supplemented with zinc sulfate (subcutaneous, 50 μ g/gm/day) for 3 days were compared with mice supplemented with zinc sulfate for 7 days. NEC-like mucosal injury was induced by intraperitoneal administration of platelet-activating factor (PAF; 50 μ g/kg) and LPS (1 mg/kg) in 12-14 day old pups as described previously (Sun, et al., *Am J Physiol* 273:G56-61, 1997; Hsueh, et al, *FASEB J* 1:403-405, 1987). Appropriate wild type and DNIIR controls were maintained with and without zinc supplementation. Mice were sacrificed 2 hrs after PAF-LPS administration. Mucosal injury was graded on a 4-point scale: grade 0: no injury; grade 1: mild separation of lamina propria; grade 2: moderate separation of submucosa; grade 3: severe separation of submucosa and/or severe edema in submucosa/muscularis; grade 4: transmural injury (Musemeche, et al., *J Pediatr Surg* 26:1047-1049, 1999; Maheshwari, et al., *Fetal Pediatr Pathol* 23:145-157 2004). As seen in FIG. 8A, the deficiency of TGF- β signaling worsened PAF-LPS-induced mucosal injury in a dose-dependent manner.

Unlike infant formula, human milk contains biologically relevant concentrations of TGF- β_2 (FIG. 6B). Furthermore, both TGF- β RI and TGF- β RII components of the heteromeric TGF- β receptor complex are widely expressed in human fetal as well as murine intestine (FIG. 6C). These data suggest that enteral supplementation of TGF- β might be effective as a therapeutic strategy to correct an endogenous deficiency state. Therefore it was determined whether enteral administration of recombinant human TGF- β_2 would prevent mucosal injury in 3-day zinc supplemented DNIIR pups, which are partially deficient in TGF- β signaling. A dose of 100 ng, chosen arbitrarily to provide 10x the estimated dose of breast milk-borne TGF- β_2 received normally by rodent pups in one day, was used (Penttila, et al., *Pediatr Res* 59:650-655, 2006). The use of human TGF- β_2 in murine pups is justified because the two peptides are

nearly identical and human TGF- β_2 has been previously shown to be effective in mice (Penttila, et al., *Pediatr Res* 59:650-655, 2006).

As seen in FIG. 8A, enterally-administered TGF- β_2 protected 3-day zinc-supplemented DNIIR mice against mucosal injury. As expected, TGF- β_2 was not protective in DNIIR mice that had received supplemental zinc for 7 days and therefore had a complete loss of TGF- β signaling (data not shown). These findings show that enteral TGF- β_2 can correct the developmental deficiency of TGF- β in the immature intestine and hence serve as a therapeutic tool to prevent or ameliorate NEC and related disease states and conditions.

The present disclosure provides the first detailed investigation into the normal development of mucosal tolerance to bacterial products in the intestine and also provides a novel pathophysiological model for NEC and related disease states and conditions that depend, at least in part, by un-regulated or improperly regulated intestinal cytokine release that has therapeutic implications. The present disclosure shows that tissue macrophages in the fetal intestine produce an inflammatory response upon stimulation with bacterial products (LPS), which contrast with the LPS-tolerance and non-inflammatory characteristics of intestinal macrophages in the adult. Intestinal macrophages are derived from blood monocytes that are recruited to the lamina propria and undergo specific differentiation under the influence of various ECM components and other bioactive molecules. In the present disclosure, macrophages isolated from the fetal intestine became LPS-tolerant upon treatment with conditioned media prepared from adult intestinal tissue (FIG. 9). Because macrophages in both fetal as well as the adult intestine are derived from monocytes, the present disclosure shows that differences between fetal and adult intestinal macrophages reflects changes in the differentiating microenvironment rather than the intrinsic, programmed differences of two distinct cellular populations. Furthermore, the unique inflammatory attenuation of intestinal macrophages is acquired as a progressive maturational change from the fetal period towards adulthood, further emphasizing the importance of changes in the ECM.

The progressive increase in the ability of fetal intestinal ECM/conditioned media to suppress macrophage inflammatory responses appears teleologically advantageous as a mechanism to prevent unnecessary inflammation from postnatal bacterial colonization and consequent proximity to immunostimulatory bacteria. However, in the event of a preterm delivery during midgestation, colonization of the gut mucosa and exposure to bacterial products before the normal downregulation of these inflammatory pathways can occur may predispose the infant to mucosal inflammation and NEC. The present disclosure shows that mucosal inflammatory responses to bacterial products are likely to be the highest in the

premature intestine than at any other time in life, which explains the occurrence of NEC almost exclusively in premature infants even though altered mucosal permeability and bacterial translocation is common in critically ill patients of all ages.

5 The present disclosures shows for the first time that TGF- β effects on intestinal macrophages can be traced through development and that among the three TGF- β isoforms, TGF- β_2 has the most important role in intestinal macrophage differentiation in humans. The effects of TGF- β in the intestine are interesting when intestinal macrophages are compared to macrophages in other organs such as lung. Although LPS-tolerance is also seen in alveolar macrophages, these cells contrast with intestinal macrophages as LPS-tolerance in alveolar
10 macrophages is a postnatal phenomenon related to the exposure to bacterial flora and represents a secondary state of refractoriness. In the intestine, TGF- β_2 -mediated differentiation represents an endogenous mechanism to suppress mucosal inflammatory responses beginning *in utero*, in anticipation of postnatal bacterial colonization. Although TGF- β is widely expressed in the lung, the differences between intestinal vs. alveolar macrophages can be
15 explained on the basis of spatial differences in expression of TGF- β_2 . Studies from the embryonic/fetal human, murine, and chicken lung consistently show the lack of TGF- β_2 in the alveoli.

We have shown that TGF- β_2 is the most effective of the three TGF- β isoforms in suppressing macrophage cytokine production in the developing intestine. In contrast to TGF-
20 β_1 , the anti-inflammatory effects of TGF- β_2 are not well-characterized because TGF- $\beta_2^{-/-}$ mouse pups die of congenital anomalies during the neonatal period. However, the anti-inflammatory effects of TGF- β_2 have been noted in diverse models such as the systemic inflammatory response syndrome, traumatic brain injury, and T-cell-mediated encephalomyelitis. TGF- β_2 gene polymorphisms have also been associated with an increased risk of atopy. TGF- β_2 is
25 unique among TGF- β isoforms because the absence of the arg-gly-asp (RGD) integrin-binding sequence in its precursor permits activation by mechanisms other than via the $\alpha_v\beta_6$ integrin, which is important in the mature intestine but is expressed at very low levels during the fetal period. Furthermore, phenotypic differences between transgenic mice lacking individual TGF- β isoforms and data from various *in vitro* model systems indicate that the three isoforms of
30 TGF- β may differ in their intracellular signals. TGF- β_2 has a low intrinsic affinity for the type II TGF- β receptor and requires betaglycan, a co-receptor, for optimal receptor activation and signaling. The TGF β_2 -betaglycan dyad is believed to alter the conformation of the TGF- β receptor complex to transduce unique cellular signals and biological effects.

The present disclosure reports for the first time that TGF- β_2 mRNA and protein expression and TGF- β bioactivity is decreased in NEC. TGF- β_2 expression was decreased in NEC to levels even lower than the developmentally 'low' levels of the premature/fetal intestine. Although the role of TGF- β has not been previously investigated in NEC, there is evidence that TGF- β may have a protective effect in experimental colitis. The present disclosure shows that the loss of TGF- β signaling in mice worsened NEC-like mucosal injury, which is consistent with the data from *in vitro* experiments on macrophages and with assays on tissue samples from patients with NEC.

A number of cytokines present in human milk or amniotic fluid survive gastric/proximal intestinal digestion through binding to mucosal receptors, due to deficiency of digestive enzymes during the neonatal period, or because of specific elements in tertiary structure that make these peptides resistant to gastric and enteral proteases. The gastrointestinal mucosa is known to absorb intact proteins and other macromolecules during the fetal/neonatal period, and therefore, some of the TGF- β_2 received by gavage is likely to have been absorbed through the mucosa to reach the macrophages in the lamina propria. Enteral administration of TGF- β_2 , possibly added to premature infant formula or in human milk fortifiers, is biologically plausible because TGF- β_2 is detectable in both amniotic fluid as well as breast milk in significant amounts, indicating that the cytokine is normally swallowed in large amounts by the fetus and the newborn infant. The protective effect of enteral TGF- β_2 against mucosal injury in mice suggests that recombinant TGF- β_2 may have a therapeutic role in the prevention of NEC in premature infants.

Materials and Methods

Human intestinal tissue samples: Human fetal intestinal tissue (11-24 wks, n=25) from elective terminations of pregnancy, adult jejunal tissue from bariatric surgery (n=3), surgically resected intestinal tissue with advanced NEC (n=5; mean gestational age $\pm$ standard deviation=27 $\pm$ 3.7 wks), and unaffected margins from tissues resected for indications other than NEC were obtained after approval by the Institutional Review Boards at University of Alabama at Birmingham and University of New Mexico. Fresh human fetal or adult intestinal tissue was snap-frozen, homogenized in trizol, and processed for preparing T-CMs as described below. Tissue samples from neonates undergoing emergency surgery were homogenized in trizol or snap-frozen for further processing for mRNA or protein measurements.

Real-time PCR: Total RNA was isolated using the acid guanidinium thiocyanate-phenol-chloroform extraction protocol (Invitrogen). First-strand cDNA was synthesized using oligo-dT primers and Moloney murine leukemia virus reverse transcriptase (Invitrogen). Real-

time PCR primers were designed using the Beacon Design software (Bio-Rad, Hercules, CA) and two-step real-time PCR was performed using a SYBR Green protocol described elsewhere. Data were normalized for GAPDH and gene expression was compared between samples by using the $2^{-\Delta\Delta CT}$ method.

5 Immunostaining of tissue sections and cells: Paraffin-embedded tissue sections or cells were immunostained for macrophage markers, TNF- α , CD14, TGF- β_2 , and TGF- β receptors by using our previously reported fluorescenc and diaminobenzindine protocols. Controls slides with no primary antibody, an isotype control, and with competing recombinant cytokine/soluble CD14 were included.

10 Murine intestinal macrophages: Murine intestinal macrophages were isolated using a previously reported protocol (Kamada, et al., *J Immunol* 175:6900-6908, 2005; Kanai, et al., *Gastroenterology* 121:875-888, 2001). Briefly, intestinal tissue was washed with Hanks' balanced-salt solution (HBSS) containing 1 mM DTT (Sigma) to remove any mucus. Tissues were next treated with HBSS containing 1 mM EDTA (Sigma) twice for 20 min each at 37°C,
15 washed thrice, and then incubated in HBSS containing 1 mM collagenase type IV (Sigma) for 2 h at 37°C. Isolated cells were suspended in 40% Percoll (Pharmacia Biotech), layered onto 75% Percoll, and centrifuged at 2000 rpm for 20 min. Cells recovered from the interphase were further purified by immunoselection using CD11b microbeads (Miltenyi Biotec) and then allowed to adhere on polystyrene plates for 1 hr. The purity of adherent macrophages was
20 confirmed as >97% by immunostaining for F4/80, a pan-macrophage marker in mice (E-biosciences).

Tissue-conditioned media: T-CMs from murine and human intestinal tissue were prepared by using a previously reported protocol with minor modifications (Smythies, et al., In *Current Protocols in Immunology*. J.E. Coligan, A.M. Kruisbeek, D.H. Margulies, E.M.
25 Shevach, and W. Strober, editors. New York, NY: Current Protocols. 1-9, 2006). To maximize the exposure of the lamina propria ECM, the intestinal epithelium was removed by enzymatic treatment. Intestinal tissue was washed in HBSS/DTT as above and then treated twice with dispase (each time for 20 min on an agitator). The remaining sub-epithelial tissue was incubated in RPMI (1 ml/gram of tissue, no serum) x 24 hrs at 37°C, 5% CO₂. Exfoliated
30 epithelial cells were cultured in a similar fashion to prepare epithelial-conditioned media. These media were clarified by centrifugation and assayed for LPS (limulus lysate assay, Sigma), protease activity (Sigma), and total protein (BCA, Pierce).

Treatment of monocytes with T-CMs: Monocytes from healthy volunteers were isolated by Ficoll-Hypaque density centrifugation followed by positive immunoselection with CD14

microbeads (Miltenyi) as per our previously described protocol (Maheshwari, et al., *J Leukoc Biol* 80:1111-1117, 2006; English, et al., *J Immunol Methods* 5:249-252, 1974). Cells were allowed to attach (2×10^4 cells/well) in 96-well plates and incubated with T-CM (250, 500, and 1000 μg total protein/mL). After 2 hrs, monocyte cultures were stimulated with 500 ng/mL LPS (pre-determined optimum concentration) for 8 (for mRNA) or 18 hrs (for protein measurements).

Measurement of inflammatory cytokines: TNF- α (human and murine), IL-6, IL-1 β , and IL-8/CXCL8 were measured by ELISA (R&D, Minneapolis, MN) as per the manufacturer's protocol.

Neutrophil chemotaxis: Neutrophil chemotaxis was measured in microchemotaxis chambers using our previously described protocol (Fox, et al., *Cytokine* 29:135-140, 2005). Supernatants from the above macrophage cultures were used in the lower wells of the microchemotaxis chambers (NeuroProbe, Gaithersburg, MD). The number of migrating cells was read off a standard curve generated from known numbers of labeled cells.

NF- κ B activation: Monocytes were treated with T-CMs and LPS as above. NF- κ B p65 phosphorylation was measured using a commercially available kit (SuperArray Biosciences).

Neutralization of TGF- β : Excess (50 $\mu\text{g}/\text{mL}$) neutralizing polyclonal rabbit anti-human TGF- β antibody (R&D) was used to neutralize TGF- β . Appropriate isotype controls were maintained.

Assays for TGF- β : TGF- β_1 , TGF- β_2 , and TGF- β_3 were measured using specific ELISA kits (R&D). Total concentration of each isoform was measured by using the complete protocol including the acid-activation step to convert latent TGF- β to immunoreactive forms. For measuring the active fraction, the acid-activation step was not performed. To measure the TGF- β_2 concentrations in the aqueous fraction of human milk samples, a variation of the standard assay was developed. Standard curves were obtained using standard solutions and milk samples 'spiked' with known amounts of recombinant TGF- β_2 as previously described for other cytokines.

TGF- β bioactivity was measured using a quantitative luciferase assay based on the activation of the PAI-1 gene in stably transfected mink lung epithelial cells (kind gift from Dr. D. B. Rifkin, NYU Medical Center) were grown in 96-well plates. T-CMs equalized for total protein concentration were added to the reporter cells x 16 hrs. The luciferase assay is described elsewhere (Munger, et al., *Kidney Int* 51:1376-1382, 1997). The signal attributable to TGF- β was determined by comparing luciferase activity for each sample in the presence and absence of 15 $\mu\text{g}/\text{mL}$ neutralizing anti-TGF- β antibody.

Smad phosphorylation was measured by western blotting. Monocytes (5×10^6 /well) were treated with T-CMs for 20 min (pre-determined optimum duration) and smad2 (ser423, ser425) phosphorylation was measured using polyclonal anti-phospho-smad 2 and anti-smad 2 (Santa Cruz) with appropriate secondary reagents.

5 The relative importance of the three TGF- β isoforms was determined by immunoprecipitation and removal of two of the three TGF- β isoforms from 20-24 wk T-CMs. Specific monoclonal antibodies (R&D) were used followed by addition of sepharose-immobilized protein A/G (Pierce). Thus, three T-CM derivatives containing only one of the three isoforms, TGF- β_1 , TGF- β_2 , or TGF- β_3 , were obtained. Monocytes were treated with T-
10 CM or one of the three T-CM derivatives and then stimulated with LPS. IL-8 and TNF- α production was measured as above. To determine the potency of the three isoforms in downregulating LPS-induced monocyte cytokine production, monocytes were treated with incremental 0-2000 pg/mL of recombinant human TGF- β_1 , TGF- β_2 , and TGF- β_3 for 2 hrs before LPS stimulation and measured cytokine/chemokine production as above.

15 Mice: Wild type C57B6 mice were procured from a commercial vendor (Jackson Labs, Bar Harbor, ME). DNIIR mice have been described herein. Zinc sulfate was administered (50 μ g/gm/day subcutaneous optimum dose) to DNIIR pups for 3 or 7 days for partial and complete inhibition of TGF- β signaling, respectively. The induction of gut mucosal injury by administration of PAF and LPS has been described herein.

20 Smad phosphorylation was measured in the intestinal tissue by western blotting. DNIIR mice after 0, 3, and 7 days of zinc supplementation were treated with 100 ng recombinant human TGF- β_2 intraperitoneally and euthanized after 1 hr. Intestinal tissues were homogenized in T-PER lysis buffer (Pierce) with protease inhibitors. Smad2 (ser423, ser425) phosphorylation was measured in western blots using polyclonal anti-phospho-smad 2 and
25 anti-smad 2 (Santa Cruz) with appropriate secondary reagents.

Ischemia-reperfusion injury was induced in 10-12 day old wild-type mice by clamping the superior mesenteric artery for 60 and then allowing reperfusion by releasing the clamp for 90 minutes. Mice were euthanized immediately after the experiment to harvest the intestines.

30 The induction of gut mucosal injury by administration of PAF and LPS has been described herein. Ten-12 day old wild type and DNIIR mice were treated intraperitoneally with PAF (50 μ g/kg) and LPS (1 mg/kg). Appropriate wild type and DNIIR controls were maintained with and without zinc supplementation. Mice were sacrificed 2 hrs after PAF and LPS administration and mucosal injury was graded on a 4-point scale.

Statistical analysis: All experiments were performed in at least three independent runs. Statistical analyses were performed using the software package SigmaStat version 5.1 (Systat, San Jose, CA). Experimental data are depicted as means $\pm$ standard errors of mean (SEM). Milk TGF- β_2 concentrations are shown in standard box-plots and summarize the lowest concentration, lower quartile (Q1), median, upper quartile (Q3), and the highest concentration. Group comparisons were done with the Student's *t* test or analysis of variance for parametric and Mann-Whitney/Kruskal-Wallis with appropriate corrections for non-parametric data. A *p* value of 0.05 was accepted as significant.

The disclosure shows and describes only the preferred embodiments of the compounds but, as mentioned above, it is to be understood that the teachings of the present disclosure are capable of use in various other combinations, modifications, and environments and is capable of changes or modifications within the scope of the inventive concept as expressed herein, commensurate with the above teachings and/or the skill or knowledge of the relevant art. The embodiments described hereinabove are further intended to explain best modes known of practicing the invention and to enable others skilled in the art to utilize the invention in such, or other, embodiments and with the various modifications required by the particular applications or uses of the invention. Accordingly, the description is not intended to limit the invention to the form disclosed herein. All references cited herein are incorporated by reference as if fully set forth in this disclosure.

CLAIM

What is claimed:

1. A method for treating or preventing NEC in a subject, the method comprising the step of administering to said subject in need of such treatment or prevention a TGF- β_2 or a TGF- β_2 derivative.
2. The method of claim 1 wherein the TGF- β_2 is present as a homodimer or a heterodimer or the TGF- β_2 derivative is present as a homodimer or a heterodimer.
3. The method of claim 1 wherein the TGF- β_2 consists essentially of amino acids 1-414, 20-302 or 303-414 of SEQ ID NO: 1 or amino acids 1-442, 20-330 or 331-442 of SEQ ID NO: 2.
4. The method of claim 1 wherein the TGF- β_2 derivative is an active fragment of TGF- β_2 .
5. The method of claim 5 wherein the active fragment maintains the 9 conserved cysteine residues.
6. The method of claim 1 wherein the TGF- β_2 derivative contains a conservative amino acid substitution and retains at least 50% of the biological activity of TGF- β_2 as determined by the ability to decrease cytokine expression in immature intestinal macrophages.
7. The method of claim 1 wherein the TGF- β_2 or TGF- β_2 derivative is administered in a therapeutically effective amount.
8. The method of claim 1 wherein the TGF- β_2 or TGF- β_2 derivative is administered by itself or as a part of a pharmaceutical composition or medicament.
9. The method of claim 1 wherein administration of the TGF- β_2 or TGF- β_2 derivative inhibits, at least in part, a cytokine response in the intestine to mucosal injury, bacterial invasion, bacterial products, bacterial antigens or a combination of the foregoing.
10. The method of claim 1 wherein administration of the TGF- β_2 or TGF- β_2 derivative alters the cytokine response of immature intestinal cells to mucosal injury, bacterial invasion, bacterial products, bacterial antigens or a combination of the foregoing so that the cytokine response is matured.
11. The method of claim 10 wherein the immature intestinal cells are an immature intestinal epithelial cells or a macrophage.
12. The method of claim 10 wherein the matured cytokine response include a decrease in at least one of the following: CXCL-1, CXCL-5, CXCL-8, CCL-2, CCL-3, CCL-4, CCL-5, IL-1 α , IL-1 β , IL-1F5, IL-1F7, IL-1F8, IL-1F9, IL-6, IL-8, IL-12 α , IL-12 β , IL-17 α , IL-17 β , IL-18 α , IL-18 β , IL-23 α , GM-CSF or TNF α .
13. The method of claim 1 wherein the subject is a premature infant.

14. The method of claim 1 wherein the subject is a premature infant having a gestational age of 32 weeks or less.
15. The method of claim 1 wherein the administration of the TGF- β_2 or TGF- β_2 derivative is enteral administration.
- 5 16. A method for treating or preventing a disease state or condition characterized by, at least in part, un-regulated or improperly regulated intestinal cytokine release a subject in need of such treatment or prevention, the method comprising the step of administering to said subject a TGF- β_2 or a TGF- β_2 derivative.
- 10 17. The method of claim 16 wherein the TGF- β_2 is present as a homodimer or a heterodimer or the TGF- β_2 derivative is present as a homodimer or a heterodimer.
18. The method of claim 16 wherein the TGF- β_2 consists essentially of amino acids 1-414, 20-302 or 303-414 of SEQ ID NO: 1 or amino acids 1-442, 20-330 or 331-442 of SEQ ID NO: 2.
19. The method of claim 16 wherein the TGF- β_2 derivative is an active fragment of TGF- β_2 .
- 15 20. The method of claim 19 wherein the active fragment maintains the 9 conserved cysteine residues.
21. The method of claim 16 wherein the TGF- β_2 derivative contains a conservative amino acid substitution and retains at least 50% of the biological activity of TGF- β_2 as determined by the ability to decrease cytokine expression in immature intestinal macrophages.
- 20 22. The method of claim 16 wherein the TGF- β_2 or TGF- β_2 derivative is administered in a therapeutically effective amount.
23. The method of claim 16 wherein the TGF- β_2 or TGF- β_2 derivative is administered by itself or as a part of a pharmaceutical composition or medicament.
24. The method of claim 16 wherein administration of the TGF- β_2 or TGF- β_2 derivative
25 inhibits, at least in part, a cytokine response in the intestine to mucosal injury, bacterial invasion, bacterial products, bacterial antigens or a combination of the foregoing.
25. The method of claim 16 wherein administration of the TGF- β_2 or TGF- β_2 derivative alters the cytokine response of immature intestinal cells to mucosal injury, bacterial invasion, bacterial products, bacterial antigens or a combination of the foregoing so that the cytokine
30 response is matured.
26. The method of claim 25 wherein the immature intestinal cells are an immature intestinal epithelial cells or a macrophage.
27. The method of claim 25 wherein the matured cytokine response include a decrease in at least one of the following: CXCL-1, CXCL-5, CXCL-8, CCL-2, CCL-3, CCL-4, CCL-5,

IL-1 α , IL-1 β , IL-1F5, IL-1F7, IL-1F8, IL-1F9, IL-6, IL-8, IL-12 α , IL-12 β , IL-17 α , IL-17 β , IL-18 α , IL-18 β , IL-23 α , GM-CSF or TNF α .

28. The method of claim 16 wherein the subject is a premature infant.

29. The method of claim 16 wherein the subject is a premature infant having a gestational age of 32 weeks or less.

30. The method of claim 16 wherein the administration of the TGF- β_2 or TGF- β_2 derivative is enteral administration.

31. A method for treating a subject having intestinal mucosal damage or preventing such intestinal mucosal damage in a subject, the method comprising the step of administering to said subject a TGF- β_2 or a TGF- β_2 derivative.

32. The method of claim 31 wherein the TGF- β_2 is present as a homodimer or a heterodimer or the TGF- β_2 derivative is present as a homodimer or a heterodimer.

33. The method of claim 31 wherein the TGF- β_2 consists essentially of amino acids 1-414, 20-302 or 303-414 of SEQ ID NO: 1 or amino acids 1-442, 20-330 or 331-442 of SEQ ID NO: 2.

34. The method of claim 31 wherein the TGF- β_2 derivative is an active fragment of TGF- β_2 .

35. The method of claim 35 wherein the active fragment maintains the 9 conserved cysteine residues.

36. The method of claim 31 wherein the TGF- β_2 derivative contains a conservative amino acid substitution and retains at least 50% of the biological activity of TGF- β_2 as determined by the ability to decrease cytokine expression in immature intestinal macrophages.

37. The method of claim 31 wherein the TGF- β_2 or TGF- β_2 derivative is administered in a therapeutically effective amount.

38. The method of claim 31 wherein the TGF- β_2 or TGF- β_2 derivative is administered by itself or as a part of a pharmaceutical composition or medicament.

39. The method of claim 31 wherein administration of the TGF- β_2 or TGF- β_2 derivative inhibits, at least in part, a cytokine response in the intestine to mucosal injury, bacterial invasion, bacterial products, bacterial antigens or a combination of the foregoing.

40. The method of claim 31 wherein administration of the TGF- β_2 or TGF- β_2 derivative alters the cytokine response of immature intestinal cells to mucosal injury, bacterial invasion, bacterial products, bacterial antigens or a combination of the foregoing so that the cytokine response is matured.

41. The method of claim 40 wherein the immature intestinal cells are an immature intestinal epithelial cells or a macrophage.

42. The method of claim 40 wherein the matured cytokine response include a decrease in at least one of the following: CXCL-1, CXCL-5, CXCL-8, CCL-2, CCL-3, CCL-4, CCL-5, IL-1 α , IL-1 β , IL-1F5, IL-1F7, IL-1F8, IL-1F9, IL-6, IL-8, IL-12 α , IL-12 β , IL-17 α , IL-17 β , IL-18 α , IL-18 β , IL-23 α , GM-CSF or TNF α .
- 5 43. The method of claim 31 wherein the mucosal damage is caused by NEC or a disease state or condition characterized by, at least in part, un-regulated or improperly regulated intestinal cytokine release.
44. The method of claim 31 wherein the subject is a premature infant.
45. The method of claim 31 wherein the subject is a premature infant having a gestational age
10 of 32 weeks or less.
46. The method of claim 31 wherein the administration of the TGF- β_2 or TGF- β_2 derivative is enteral administration.
47. A method for altering a cytokine response of immature intestinal cells to mucosal injury, bacterial invasion, bacterial products, bacterial antigens or a combination of the foregoing
15 so that the cytokine response is matured, the method comprising the step of administering to said subject a TGF- β_2 or a TGF- β_2 derivative.
48. The method of claim 47 wherein the TGF- β_2 is present as a homodimer or a heterodimer or the TGF- β_2 derivative is present as a homodimer or a heterodimer.
49. The method of claim 47 wherein the TGF- β_2 consists essentially of amino acids 1-414, 20-
20 302 or 303-414 of SEQ ID NO: 1 or amino acids 1-442, 20-330 or 331-442 of SEQ ID NO: 2.
50. The method of claim 47 wherein the TGF- β_2 derivative is an active fragment of TGF- β_2 .
51. The method of claim 51 wherein the active fragment maintains the 9 conserved cysteine residues.
- 25 52. The method of claim 47 wherein the TGF- β_2 derivative contains a conservative amino acid substitution and retains at least 50% of the biological activity of TGF- β_2 as determined by the ability to decrease cytokine expression in immature intestinal macrophages.
53. The method of claim 47 wherein the TGF- β_2 or TGF- β_2 derivative is administered in a therapeutically effective amount.
- 30 54. The method of claim 47 wherein the TGF- β_2 or TGF- β_2 derivative is administered by itself or as a part of a pharmaceutical composition or medicament.
55. The method of claim 54 wherein the immature intestinal cells are an immature intestinal epithelial cells or a macrophage.

56. The method of claim 54 wherein the matured cytokine response include a decrease in at least one of the following: CXCL-1, CXCL-5, CXCL-8, CCL-2, CCL-3, CCL-4, CCL-5, IL-1 α , IL-1 β , IL-1F5, IL-1F7, IL-1F8, IL-1F9, IL-6, IL-8, IL-12 α , IL-12 β , IL-17 α , IL-17 β , IL-18 α , IL-18 β , IL-23 α , GM-CSF or TNF α .
- 5 57. The method of claim 47 wherein the subject is a premature infant.
58. The method of claim 47 wherein the subject is a premature infant having a gestational age of 32 weeks or less.
59. The method of claim 47 wherein the administration of the TGF- β_2 or TGF- β_2 derivative is enteral administration.
- 10 60. A pharmaceutical composition for treating or preventing NEC, said composition comprising TGF- β_2 or TGF- β_2 derivative.
61. The composition of claim 60 wherein the TGF- β_2 or TGF- β_2 derivative is present in a therapeutically effective amount.
62. The method of claim 60 wherein the TGF- β_2 is present as a homodimer or a heterodimer or
15 the TGF- β_2 derivative is present as a homodimer or a heterodimer.
63. The method of claim 60 wherein the TGF- β_2 consists essentially of amino acids 1-414, 20-302 or 303-414 of SEQ ID NO: 1 or amino acids 1-442, 20-330 or 331-442 of SEQ ID NO: 2.
64. The method of claim 60 wherein the TGF- β_2 derivative is an active fragment of TGF- β_2 .
- 20 65. The method of claim 64 wherein the active fragment maintains the 9 conserved cysteine residues.
66. The method of claim 60 wherein the TGF- β_2 derivative contains a conservative amino acid substitution and retains at least 50% of the biological activity of TGF- β_2 as determined by the ability to decrease cytokine expression in immature intestinal macrophages.

25

30

FIG. 1

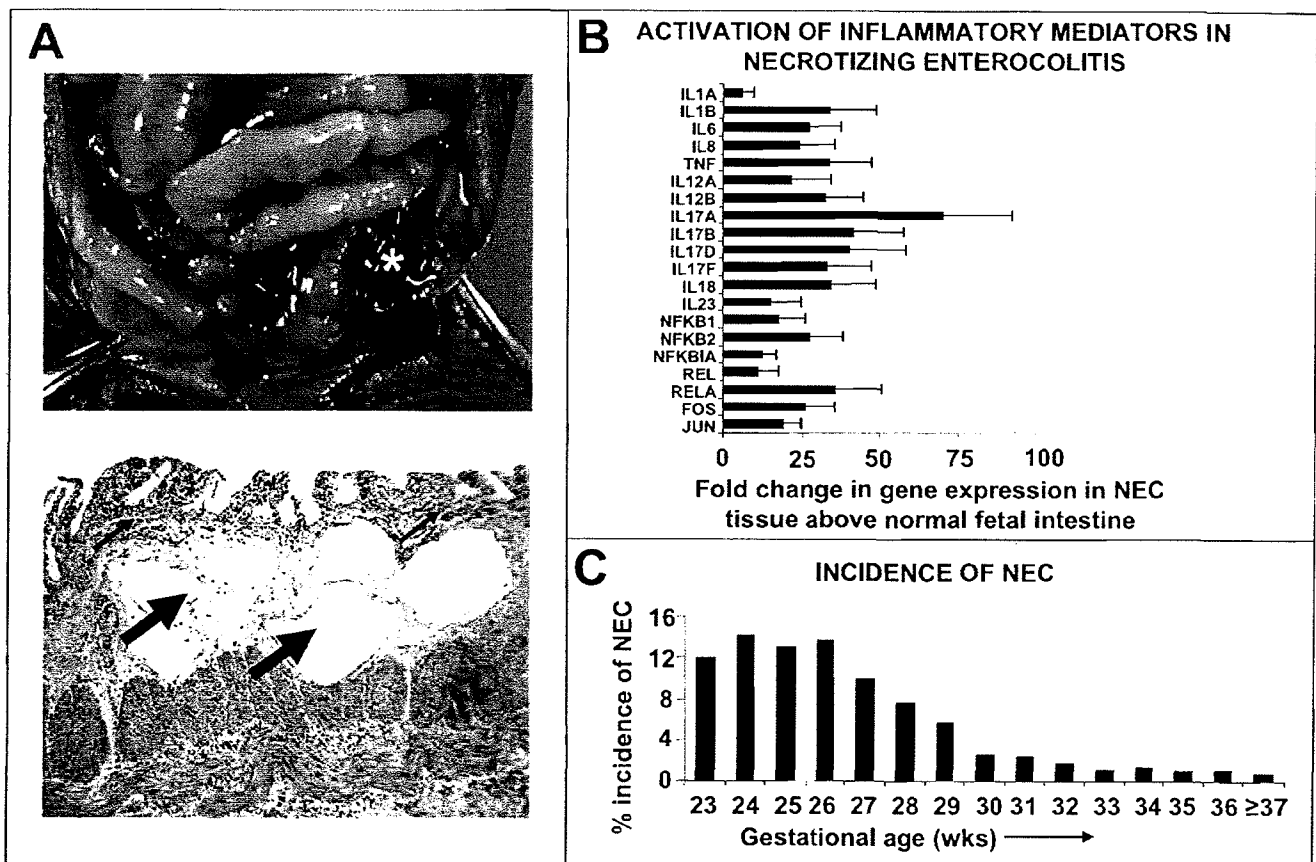


FIG. 2A

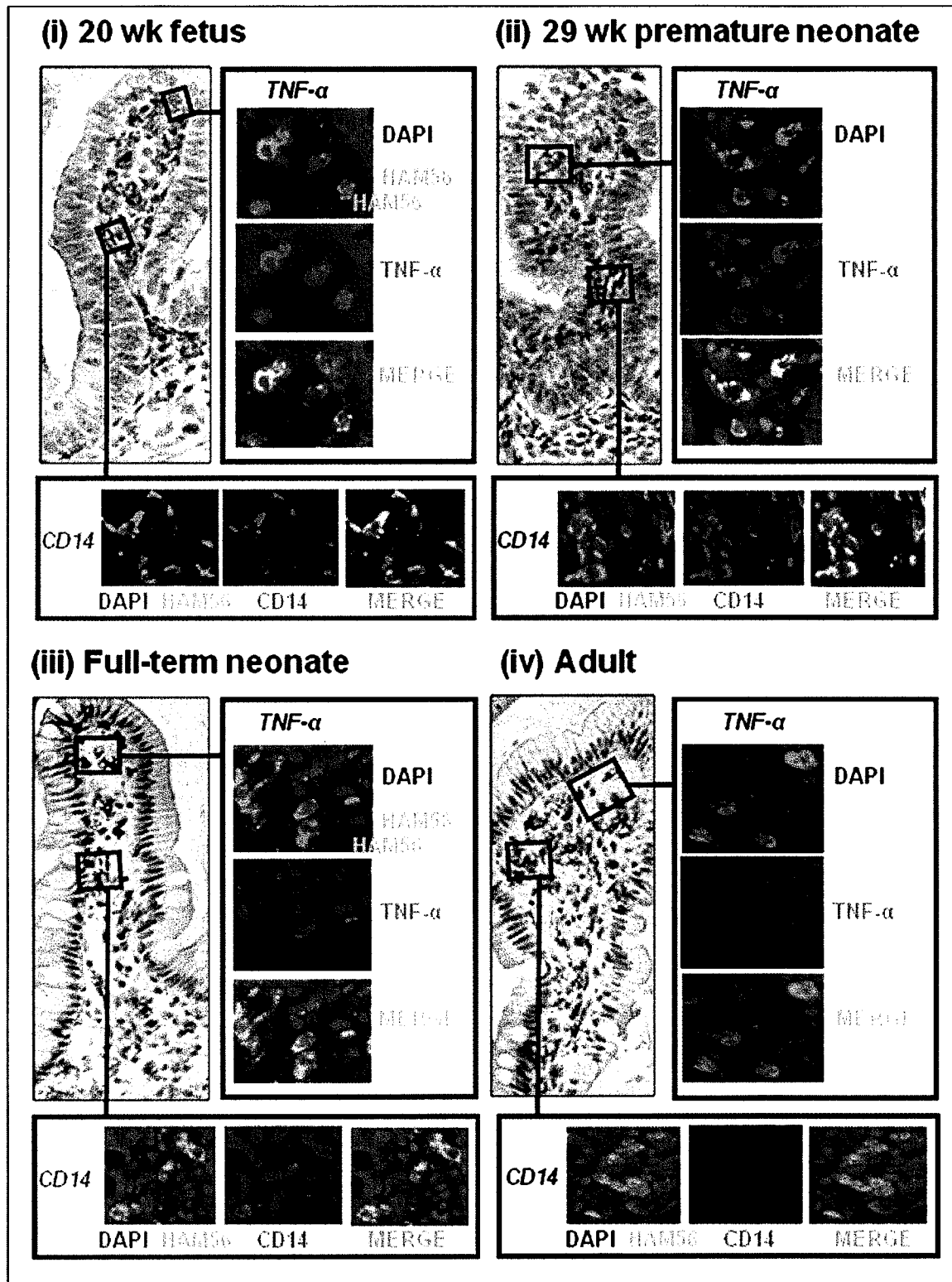


FIG. 2B

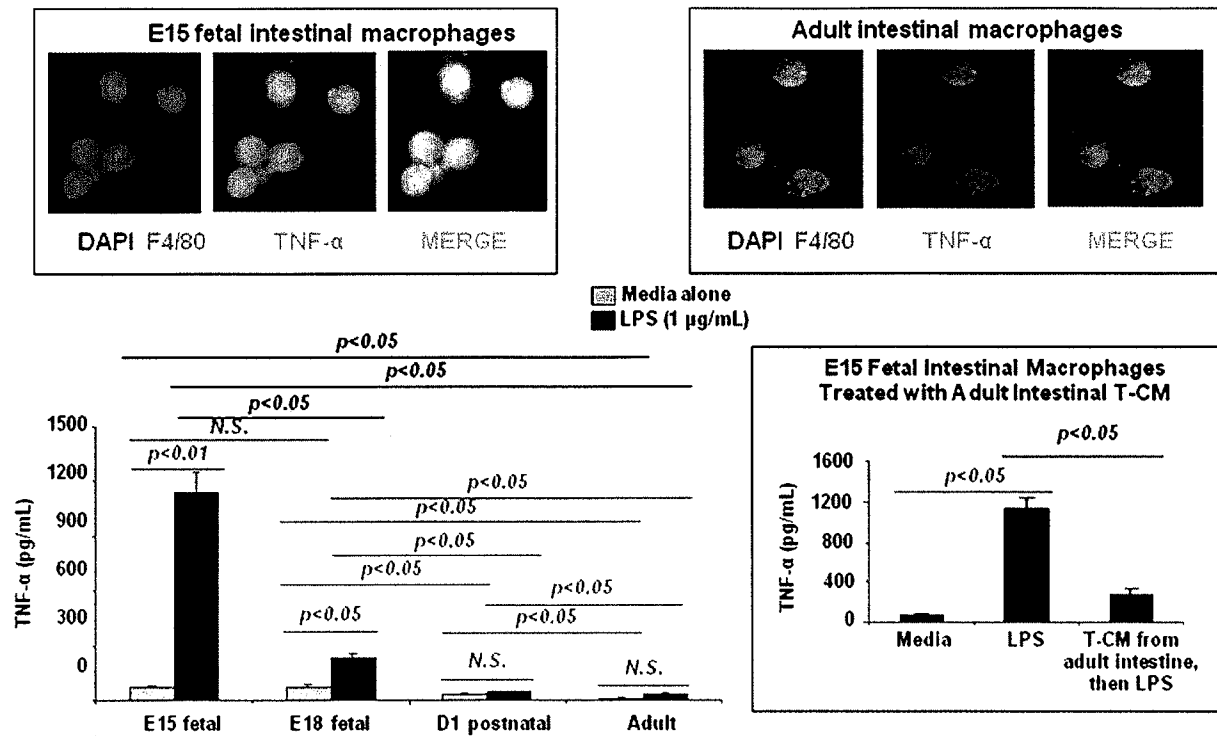


FIG. 3

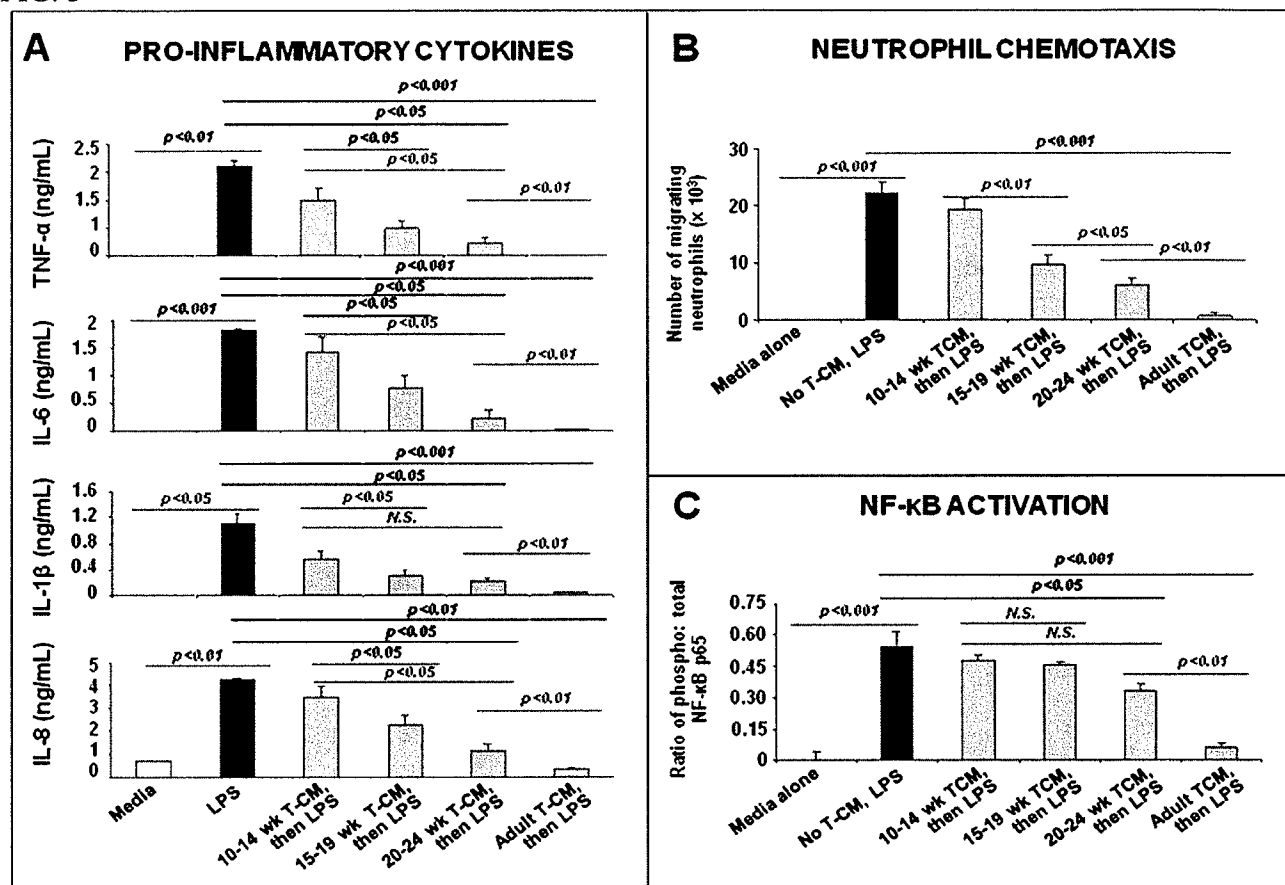


FIG. 4

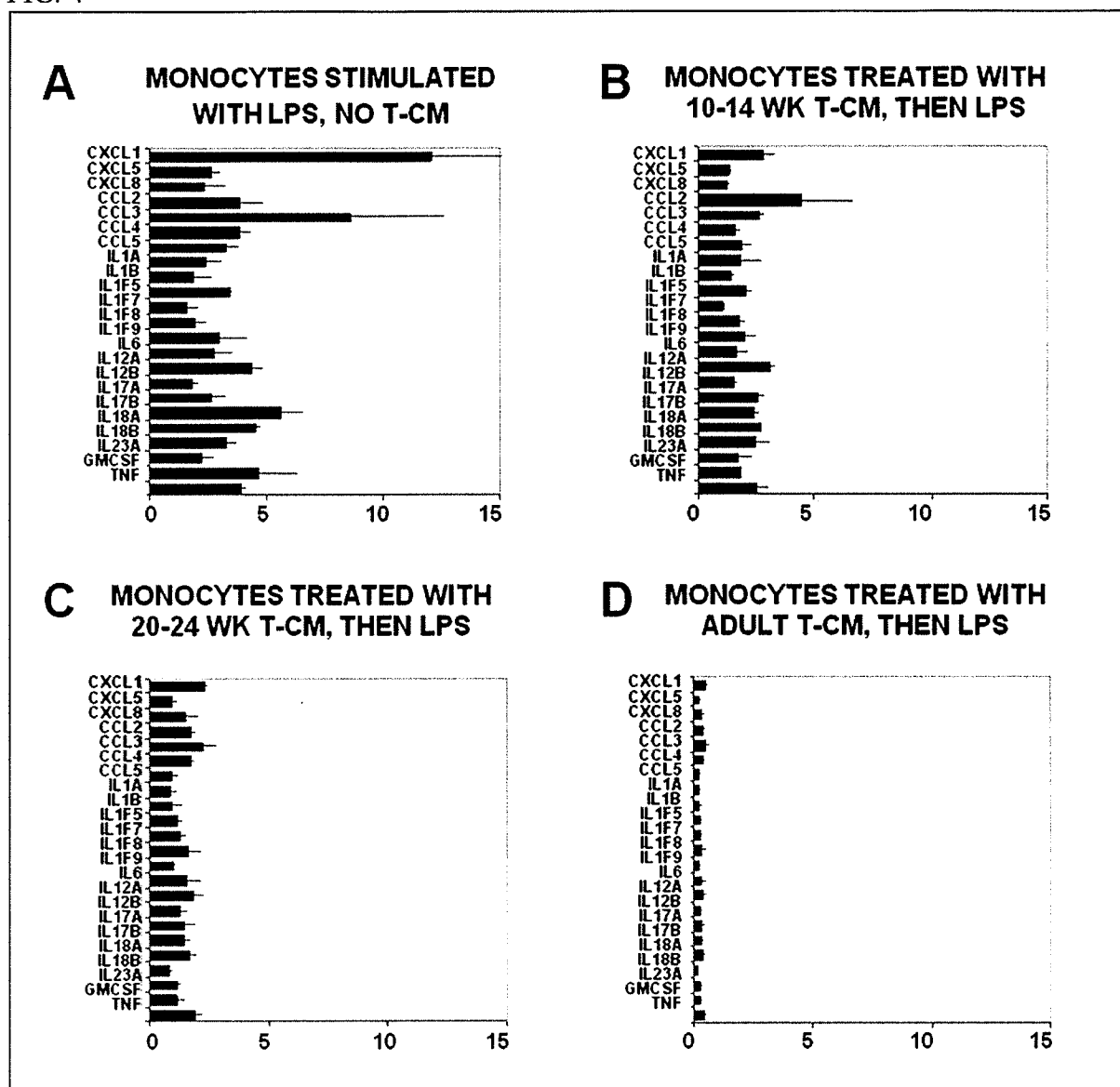
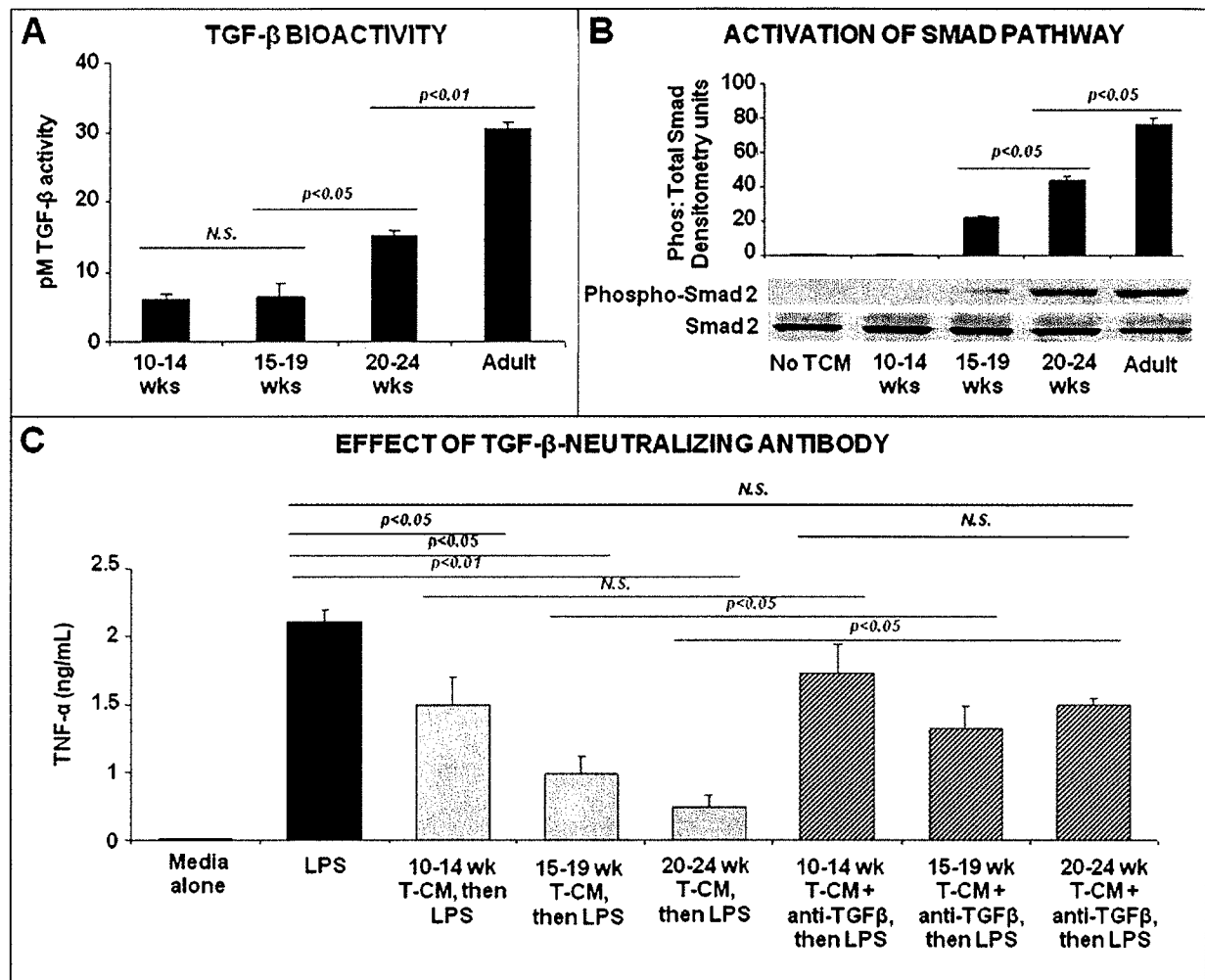


FIG. 5



D

Isoform 1

MHYCVLSAFL	ILHLVTVALS	LSTCSTLDM	QFMRKRIEAI	RGQILSKLKL	TSPPEDYPEP	60
EEVPPEVISI	YNSTRDLLQE	KASRRAAACE	RERSDEEYYA	KEVYKIDMPP	FFPSENAIPP	120
TFYRPFYRIV	RFDVSAMEKN	ASNLVKAEFR	VFRLQNPKEP	VPEQRIELYQ	ILKSKDLTSP	180
TQRYIDSKVV	KTRAEGEWLS	FDVTDVHEW	LHHKDRNLGF	KISLHCPCTT	FVPSNNYIIP	240
NKSEELERAF	AGIDGTSTYT	SGDQKTIKST	RKKNNGKTPH	LLLMLLPSYR	LESQQTNRK	300
KRALDAAYCF	RNVQDNCCLR	PLYIDFKRDL	GWKWIHEPKG	YNANFCAGAC	PYLWSSDTQH	360
SRVLSLYNTI	NPEASASPPC	VSQDLEPLTI	LYYIGKTPKI	EQLSNMIVKS	CKCS	414

Isoform 2

MHYCVLSAFL	ILHLVTVALS	LSTCSTLDM	QFMRKRIEAI	RGQILSKLKL	TSPPEDYPEP	60
EEVPPEVISI	YNSTRDLLQE	KASRRAAACE	RERSDEEYYA	KEVYKIDMPP	FFPSETVCPV	120
VTPSGSVGS	LCSRQSQVLC	GYLDAIPPTF	YRPFYRIVRF	DVSAMEKNAS	NLVKAEFRVF	180
RLQNPKEP	EQRIELYQIL	KSKDLTSP	RYIDSKVVKT	RAEGEWLSFD	VTDVHEWLH	240
HKDRNLGPKI	SLHCPCTTFV	PSNNYIIPNK	SEELEARFAG	IDGTSTYTS	DQKTIKSTRK	300
KNSGKTPHLL	LMMLPSYRLE	SQQTNRKRR	ALDAAYCFRN	VQDNCCLRPL	YIDFKRDLGW	360
KWIHEPKGYN	ANFCAGACPY	LWSSDTQHSR	VLSLYNTINP	EASASPPCVS	QDLEPLTILY	420
YIGKTPKIEQ	LSNMIVKSCK	CS				442

FIG. 6

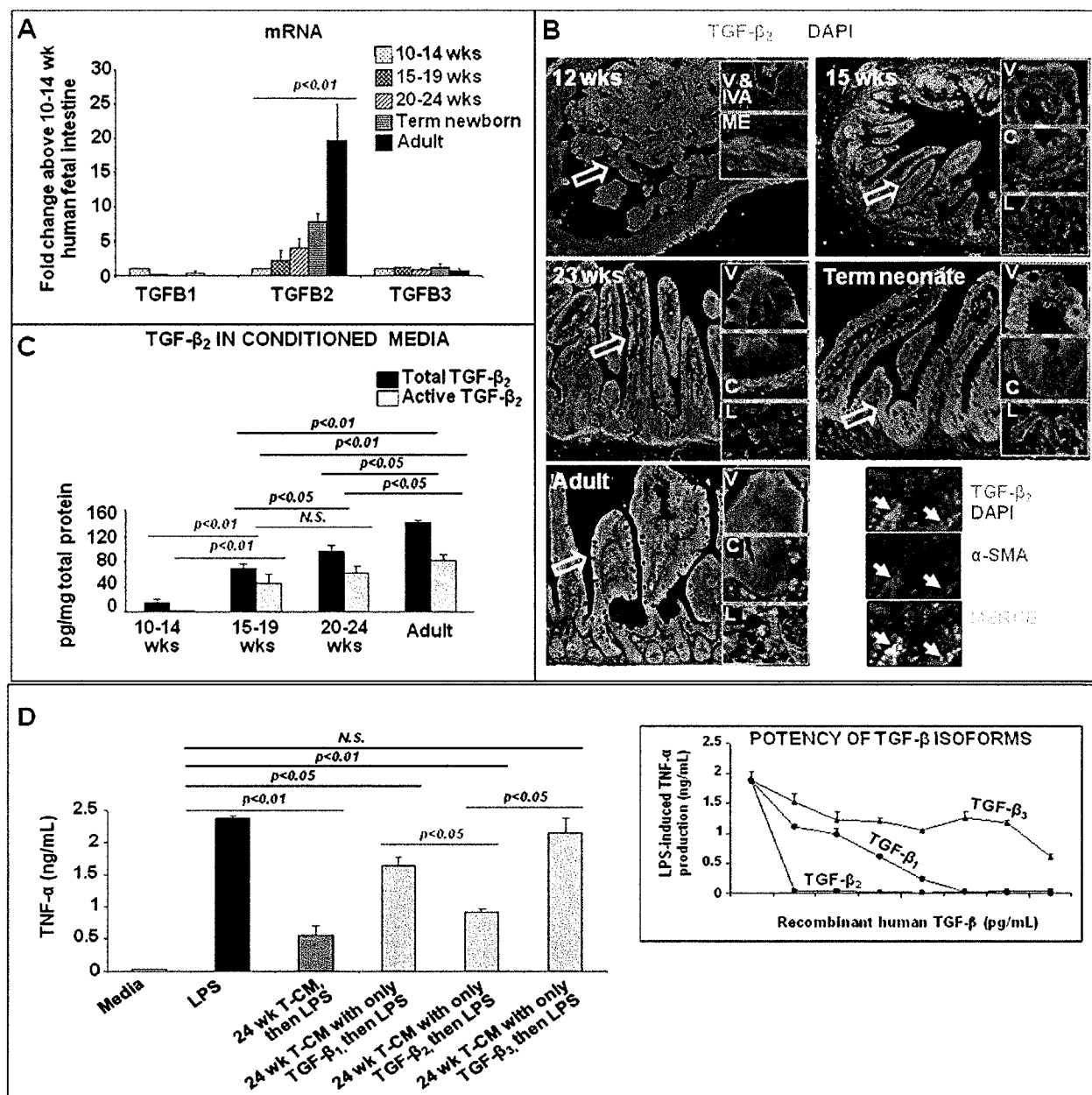


FIG. 7

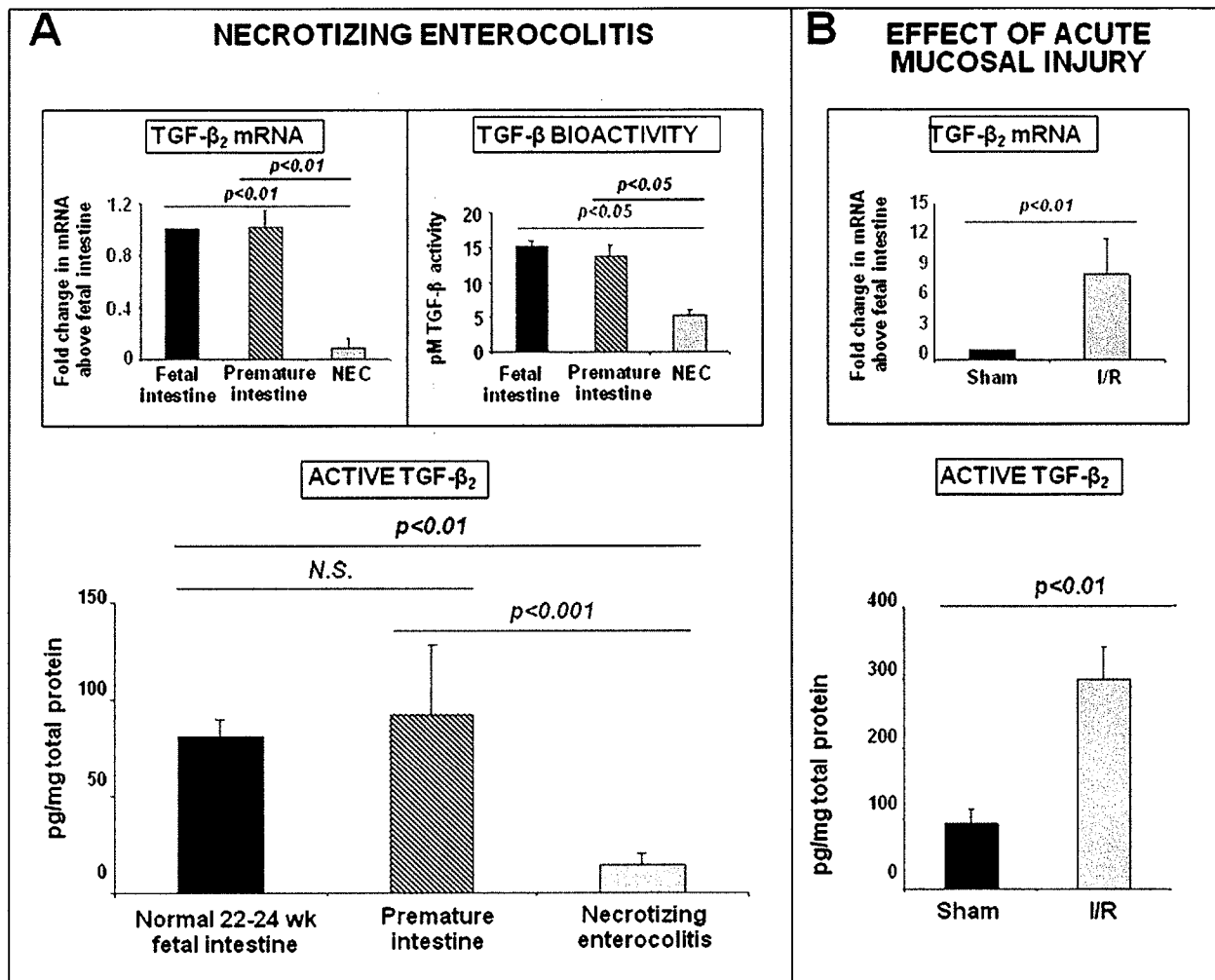


FIG. 8

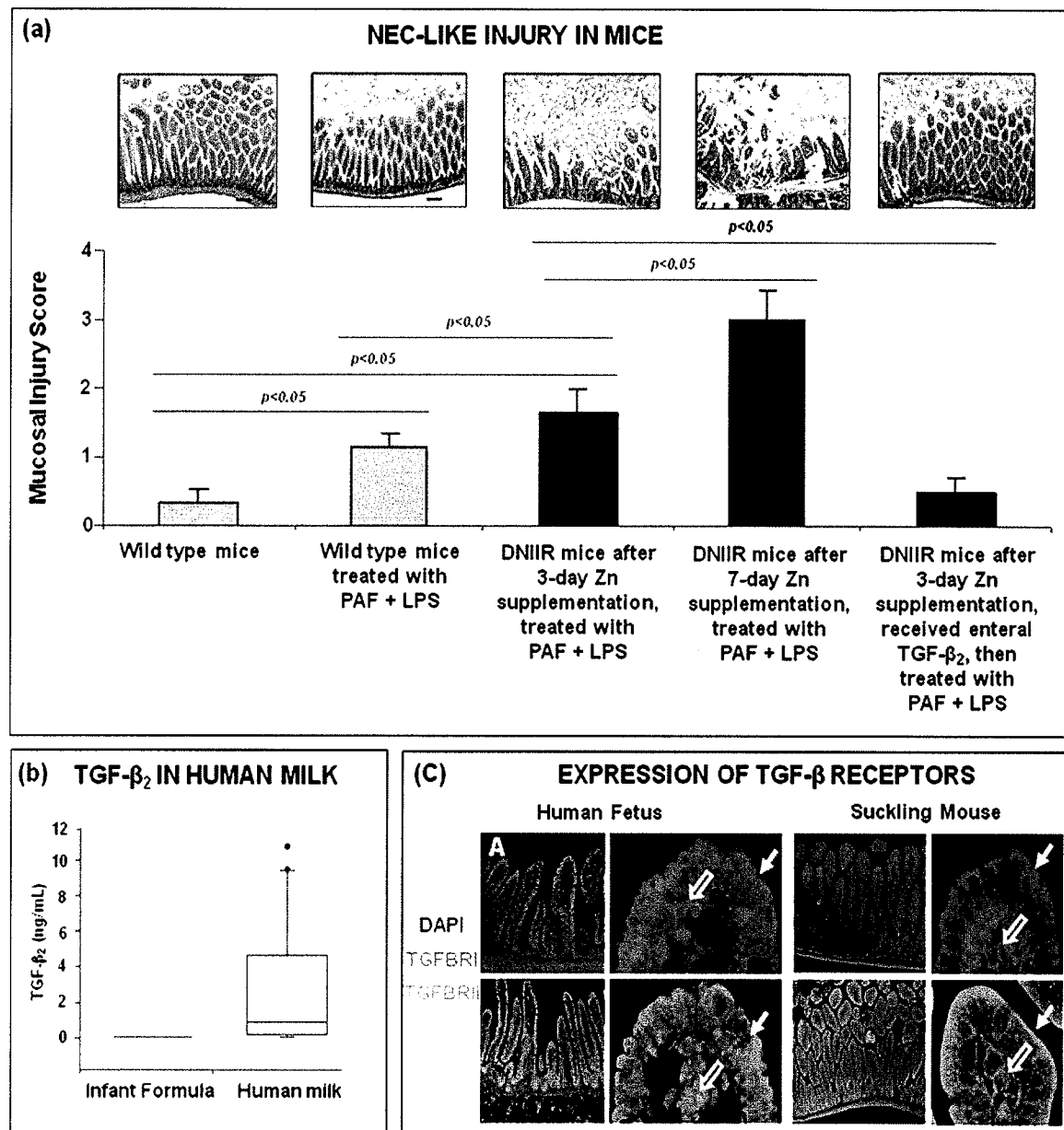


FIG. 9

