



(51) International Patent Classification:

A61K 38/18 (2006.01) A61P 29/00 (2006.01)
A23L 1/29 (2006.01)

(21) International Application Number:

PCT/US2014/015105

(22) International Filing Date:

6 February 2014 (06.02.2014)

(25) Filing Language:

English

(26) Publication Language:

English

(30) Priority Data:

61/761,433 6 February 2013 (06.02.2013)

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, BN, BR, BW, BY, BZ, CA, CH, CL, 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, IR, IS, JP, KE, KG, 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, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, 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, LR, LS, MW, MZ, NA, RW, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, 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, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

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(54) Title: METHOD AND COMPOSITION FOR PREVENTING NECROTIZING ENTEROCOLITIS AND GASTROINTESTINAL ALLERGIES

(57) Abstract: Disclosed are compositions and methods for treating or preventing necrotizing enterocolitis or gastrointestinal allergies in an infant or toddler.

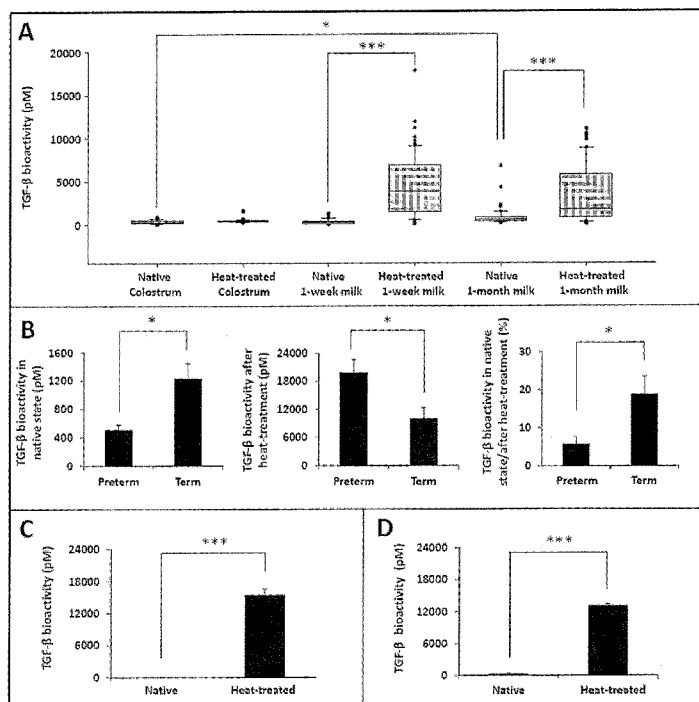


FIG. 1



Declarations under Rule 4.17:

— *of inventorship (Rule 4.17(iv))*

Published:

— *with international search report (Art. 21(3))*

METHOD AND COMPOSITION FOR PREVENTING NECROTIZING ENTEROCOLITIS AND GASTROINTESTINAL ALLERGIES

CROSS-REFERENCE TO RELATED APPLICATIONS

5 This application claims the benefit of US Provisional Application No. 61/761,433, filed February 6, 2013, which is incorporated by reference in its entirety.

STATEMENT REGARDING GOVERNMENT-SPONSORED RESEARCH OR DEVELOPMENT

10 This invention was made with government support under contract number R01 HD059142 awarded by the National Institutes of Health. The government has certain rights in the invention.

BACKGROUND OF THE INVENTION

15 Necrotizing enterocolitis (NEC), a condition seen primarily in premature infants, is the most common and most serious gastrointestinal disorder among hospitalized preterm infants. Despite improvements in clinical care and extensive research leading to many clinical trials, the prognosis for newborns with NEC has not significantly improved over the past 30 years and the mortality rate remains unchanged. Although the etiology of NEC is unclear, current evidence associates
20 NEC with diverse pre- and postnatal factors such as placental insufficiency, chorioamnionitis, gut ischemia, altered bacterial colonization, viruses, and blood transfusions.

 There is a need for compositions and methods of preventing, reducing the risk for, or treating NEC. The present invention addresses that need.

25

SUMMARY OF THE INVENTION

 Accordingly, in certain embodiment, this invention provides a composition comprising human milk, milk from a non-human mammal, or infant formula and one or more neuraminidases. In another embodiment, this invention provides a method
30 for treating or preventing necrotizing enterocolitis or gastrointestinal allergies in an infant or toddler comprising administering to the infant or toddler an effective amount of a composition comprising one or more neuraminidases.

BRIEF DESCRIPTIONS OF THE DRAWINGS

FIG. 1A is a graph of TGF- β bioactivity in native and heat-treated preterm human colostrum and milk collected 1 week and 1 month after delivery; **FIG. 1B** is a graph showing relative TGF- β bioactivity of native and heat-treated preterm and term human milk; **FIG. 1C** shows TGF- β bioactivity in the native and heat-treated donor human milk;

FIG. 1D shows TGF- β bioactivity in the native and heat-treated commercially-available human milk-derived human milk fortifier.

FIG. 2A is a plot showing TGF- β_1 , TGF- β_2 and TGF- β_3 concentrations in preterm and term milk samples; **FIG. 2B** is a non-denaturing PAGE-immunoblot and densitometric scans of the immunoblot showing active and inactive TGF- β_2 in preterm and term milk; **FIG. 2C** shows TGF- β_1 , TGF- β_2 , and TGF- β_3 mRNA expression in the cellular fraction in preterm and term milk; **FIG. 2D** is an immunofluorescence photomicrograph of mammary tissue showing TGF- β_2 immunoreactivity in epithelial cells

FIG. 3A is a plot showing THBS-1 concentrations in preterm milk; **FIG. 3B** is a plot showing THBS-1 concentrations in term milk; **FIG. 3C** is a plot showing expression of MMP-2 and MMP-9 in preterm milk; **FIG. 3D** is a plot showing expression of MMP-2 and MMP-9 in term milk; **FIG. 3E** shows neuraminidase activity in preterm and term milk; **FIG. 3F** is an immunofluorescent photomicrograph of mammary tissue showing neuraminidase-1 immunoreactivity in epithelial cells (green); **FIG. 3G** is a bar diagrams showing mRNA expression of integrin chain α_v (ITGAV), integrin chain β_6 (ITGB6), integrin chain β_8 (ITGB8) and Thy-1/CD90 in cellular fractions of preterm and term milk.

FIG. 4A is a graph showing TGF- β bioactivity in untreated preterm human milk and preterm human milk treated with murine P10 or murine adult intestinal lysates; **FIG. 4B** is a graph showing TGF- β bioactivity in untreated preterm human milk and preterm human milk treated with murine P10 intestinal lysate in the presence or absence of a neuraminidase inhibitor; **FIG. 4C** is a graph showing neuraminidase activity in murine intestine harvested from adults, fetuses on embryonic day 18.5 (E18.5), or pups on postnatal day 2 (P2) and or postnatal day 10 (P10); **FIG. 4D** is an image showing localization of neuraminidase human preterm or term intestine.

FIG. 5A is a graph showing TGF- β bioactivity, measured in MLEC reporter cells, of untreated preterm human milk and preterm human milk treated with *Clostridium*

perfringens neuraminidase (0, 1, and 10 mU; Invitrogen, San Diego, CA). (B) Addition of *C. perfringens* neuraminidase (10 mU) to preterm human milk samples before treating ex planted murine intestinal tissue with these milk samples increased Smad2 phosphorylation in the explants. Immunoblots show phospho- and total Smad2 expression in murine intestinal tissue; **FIG. 5B** is a Western blot (bottom) showing Smad2 and phospho-Smad2 levels in in explanted intestinal tissue from 10-day-old C57/BL6 mouse pups after treatment with untreated preterm human milk and preterm human milk treated with neuraminidase, a densitometric scan (top) of the Western blot, and a graph showing relative expression of the TGF β -R2 mRNA expression in the fetal, neonatal, and adult intestine (inset); **FIG. 5C** is a graph showing that the effect of adding neuraminidase to preterm human milk on suppression of LPS-induced NF- κ B activation, as measured by expression of secreted alkaline phosphatase under the control of an NF- κ B promoter.

FIG. 6A is a graph showing TGF- β bioactivity in preterm human milk in the native state, after heat-treatment, and with added culture supernatants from *L. acidophilus*, *L. rhamnosus*, *L. plantarum* and *L. casei* bacterial cultures (10^9 cfu/mL); **FIG. 6B** is a graph showing TGF- β bioactivity in preterm human milk treated with live, or heat-killed *L. rhamnosus* (10^8 - 10^9 cfu/mL), or with culture supernatants from 24h cultures of *L. rhamnosus* (10^9 cfu/mL, diluted 1:25); **FIG. 6C** is a graph showing TGF- β bioactivity in preterm human milk after treatment with various fractions of *L. rhamnosus* culture supernatants restricted by molecular weight.

FIG. 7A is a graph showing neuraminidase activity in bacterial culture supernatants of *Lactobacillus* species; **FIG. 7B** is a graph showing TGF- β bioactivity in milk treated with *L. rhamnosus* culture supernatants in the presence and absence of a neuraminidase inhibitor; **FIG. 7C** is a graph showing in neuraminidase activity in *L. rhamnosus* culture supernatants and in *L. rhamnosus* culture supernatants enriched by immunoprecipitation using an antibody against the bacterial neuraminidase domain.

FIG. 8A is a graph showing neuraminidase activity in various fractions of *L. rhamnosus* culture supernatants restricted by molecular weight; **FIG. 8B** is a Western blot showing the presences of neuraminidase in the 30-50 kDa fractions of *L. rhamnosus* culture supernatants, and a bar graph showing densitometric data.

FIG. 9 is a graph showing survival of IEC6 cells as a function of exposure to *L. rhamnosus*-derived.

DETAILED DESCRIPTION OF THE INVENTION

Human milk contains biologically-important amounts of TGF- β , with a predominance of the TGF- β_2 isoform over TGF- β_1 . Although the function of milk-borne TGF- β has not yet been fully elucidated, orally-ingested TGF- β may promote gut barrier function, immune tolerance, and mucosal repair in the neonatal gastrointestinal tract. TGF- β_2 suppresses macrophage cytokine expression and mucosal inflammatory responses in the developing human intestine, and in preclinical models, it has been shown that enterally-administered TGF- β_2 can protect against intestinal injury similar to NEC. Oral administration of TGF- β can protect mouse pups against experimental NEC.

As described below, it was discovered that preterm human milk contains biologically-relevant amounts of transforming growth factor-beta (TGF- β), most of which is in an inactive state and requires 'activation' before it becomes bioavailable or and/or bioactive. Further, neuraminidases, e.g., sialidase, in milk and in the intestinal epithelium were found to activate TGF- β in milk. Finally, the addition of sialidase to preterm milk was found to significantly increase bioavailability and/or bioactivity of TGF- β and its ability to suppress inflammatory responses in cellular systems.

In certain embodiments are provided a composition comprising a neuraminidase and employing a neuraminidase to catalyze the conversion of latent or inactive TGF- β in milk to its active form, thereby increasing TGF- β bioactivity and treating, reducing the risk for developing, and/or preventing necrotizing enterocolitis and gastrointestinal allergies in an infant or toddler. This activation may suitably be accomplished by mixing an effective amount of one or more neuraminidases with milk or formula prior to enteral administration, e.g., oral feeding, to the infant or toddler in an effective amount, or by separate enteral administration to the infant or toddler of a milk or formula and a composition comprising one or more neuraminidases in an effective amount to activate TGF- β in the intestinal lumen. In certain embodiments, the milk is preterm milk having little or no TGF- β activity but containing latent TGF- β .

As defined herein, "neuraminidase" (sialidase) refers to any of the family of enzymes that catalyzes the hydrolysis of terminal acylneuraminic acid residues from glycoproteins and oligosacchaccharides. Any recombinant neuraminidase and

neuraminidase from probiotic culture supernatants and which catalyzes the conversion of latent TGF- β to active TGF- β in milk is suitable for use in the method and composition of this invention. Recombinant neuraminidase may be cloned from any suitable organism, i.e., any organism encoding a neuraminidase, including, for example, *Clostridium perfringens*, *Arthrobacter ureafaciens*, *Vibrio cholerae*, *Streptococcus pneumoniae*, *Lactobacillus* species, and the like. Recombinant neuraminidase is commercially available from a variety of sources including New England Biolabs, Alfa Aesar; Roche Applied Sciences and Sigma Aldrich.

Representative probiotic species include, for example, *Lactobacillus* species, *Bifidobacterium* species and *Streptococcus* species. For a list of commercial sources of probiotic species, see, P.S.M. Yeung, et al., Species Specific Identification of Commercial Probiotic Strains, J. Dairy Sci., 2002, 85: 1039-1051.

Compositions for administration according to the invention may be prepared by dissolving or suspending an effective amount of one or more neuraminidases in a physiologically acceptable diluent such as water. Liquid preparations for oral use may also contain suitable antimicrobial preservatives, antioxidants and other excipients such as dispersing, suspending, thickening, emulsifying, buffering, wetting, solubilizing, stabilizing, flavoring and sweetening agents and colors.

In certain embodiments, compositions are prepared by combining an effective amount of one or more neuraminidases with human or non-human mammalian milk, with infant formula that contains biologically relevant amounts of TGF- β or by mixing milk with human milk- derived human milk fortifier that comprises one or more neuraminidases. Human milk may be obtained from an infant's mother or from suppliers of donor human milk. Bovine milk and other non-human mammalian milk are readily commercially available.

Infant formula is defined by the U.S. Federal Food, Drug and Cosmetic Act (FFDCA) as "a food which purports to be or is represented for special dietary use solely as a food for infants by reason of its simulation of human milk or its suitability as a complete or partial substitute for human milk". Infant formulas are widely available in both powdered forms for mixing with water and in ready to use liquid form. The composition of infant formula is designed to be roughly based on a human mother's milk at approximately one to three months postpartum, although there are significant differences in the nutrient content of these products. Commercially available infant formulas typically contain purified cow's milk whey and casein, a

blend of vegetable oils, lactose, a vitamin-mineral mix, and other ingredients depending on the manufacturer. Because infant formulas typically do not contain biologically important amounts of active TGF- β , in certain embodiments, infant formulas could be supplemented with an effective amount of latent TGF- β .

5 Accordingly, in an aspect, this invention is infant formula comprising an effective amount of latent/active TGF- β and an effective amount of one or more neuraminidases.

Infant formula comprising neuraminidase according to this invention may be prepared by combining effective amount of one or more neuraminidases and powder
10 or liquid infant formula at the point of manufacture, or by adding an effective amount of one or more neuraminidases to commercially available powdered formula before or after mixing with water; or by adding an effective amount of one or more neuraminidases to commercially available liquid infant formula.

Human milk fortifiers are used for supplementation of human milk with energy,
15 protein, vitamins and minerals, particularly for use with premature infants who are not able to process the volume of milk necessary to provide the required nutrients. Milk fortifiers typically are composed of fats, protein sources, carbohydrates, vitamins, zinc, iron and electrolytes. Milk fortifiers are widely commercially available in powdered form for adding to human milk prior to feeding. Milk fortifiers comprising
20 neuraminidase according to this invention may be prepared by adding an effective amount of one or more neuraminidases to a milk fortifier at the point of manufacture; by adding to commercially available milk fortifier before mixing with milk; or by adding to milk prior to mixing with the milk fortifier. Accordingly, in another aspect, this invention is human milk comprising human milk fortifier and an effective amount
25 of one or more neuraminidases.

In certain embodiments is provided a method of treating, reducing the risk of developing, or preventing necrotizing enterocolitis and gastrointestinal allergies in an infant or toddler comprising orally administering to the infant or toddler an effective amount of a composition comprising one or more neuraminidases. As discussed in
30 detail herein, the method of this invention is particularly suitable for treating premature infants at risk of developing necrotizing enterocolitis. The neuraminidases may be administered as an oral composition in addition to milk or formula feedings or by feeding human milk, non-human mammalian milk, e.g., bovine, sheep, goat, or

camel milk, or infant formulas supplemented with an effective amount of the neuraminidase(s). The human milk may also comprise a human milk fortifier.

As used herein, "infant" typically refers to a human child between birth and about 12 months. "Premature infant" generally refers to a baby born before 37 completed weeks of gestation. "Toddler" means a child between the ages of about one and three.

"Treat," "treating," "treatment," and the like mean eliminating, reducing, relieving, reversing, and/or ameliorating necrotizing enterocolitis and gastrointestinal allergies and/or symptoms associated therewith in an infant or toddler. Although not precluded, treating a disease or condition does not require that the disease, condition, or symptoms associated therewith be completely eliminated, including the treatment of acute or chronic signs, symptoms and/or malfunctions. As used herein, the terms "treat," "treating," "treatment," and the like may include "preventing," which refers to reducing the probability of developing a disease or condition, or of a recurrence of a previously-controlled disease or condition, in a subject who does not have, but is at risk of or is susceptible to, developing or redeveloping a disease or condition. The term "treat" and synonyms contemplate administering a therapeutically effective amount of one or more neuraminidases to a subject in need of such treatment. A treatment can be orientated symptomatically, for example, to reduce or suppress symptoms. Treatment duration may vary from a relatively short period of time, e.g., a single treatment, to medium term treatments, to long-term treatments, e.g., within the context of a maintenance therapy.

The terms "therapeutically effective amount" or "effective amount" refer to an amount of neuraminidases that, when orally administered to an infant or toddler, is (are) sufficient to efficaciously deliver the neuraminidases into the gastrointestinal tract of an infant or toddler in an amount sufficient to activate at least a portion of latent TGF- β in milk or formula consumed by the infant or toddler effective to treat or prevent necrotizing enterocolitis or gastrointestinal allergies in the infant or toddler. The effective amount of neuraminidases may be determined by one of skill in the art taking into account naturally-occurring variation in neuraminidase structure and function. In an aspect of the invention, the effective amount is in the range of about 1 to about 10 mU (micro units) of neuraminidase per mL of liquid.

In certain embodiments is provided a method of treating or preventing necrotizing enterocolitis and gastrointestinal allergies in an infant or toddler comprising administering to the infant or toddler an effective amount of an oral composition comprising one or more neuraminidases.

5 In certain embodiments, the oral composition comprises human milk, non-human mammalian milk or infant formula and one or more neuraminidases.

In certain embodiments, the infant is a premature infant delivered prior to 37 completed weeks of pregnancy.

10 In certain embodiments, the human milk is preterm or term or banked human milk.

In certain embodiments, the human milk further comprises human milk fortifier.

In certain embodiments, the neuraminidases are recombinant neuraminidases

15 In certain embodiments, the neuraminidases are obtained from probiotic culture supernatants.

The foregoing may be better understood by reference to the following experiments, which are presented for purposes of illustration and are not intended to limit the scope of the invention.

20 EXPERIMENTAL

Design/Methods: Mothers who delivered between 23 0/7 and 31 6/7 weeks or at ≥ 37 weeks of gestation provided milk samples at serial time points. TGF- β bioactivity and NF- κ B signaling was measured using specific reporter cells and in murine intestinal tissue explants. TGF- β_1 , TGF- β_2 , and TGF- β_3 and various TGF- β 25 activators were measured by real-time PCR, enzyme immunoassays, or established enzymatic activity assays.

Human milk samples: Milk samples were collected at Evanston Hospital, Evanston, IL during the period Oct 2008-Sept 2010, at University of Texas at San Diego, TX 30 during Jan 2009-Jan 2011, and de-identified samples were received at University of Illinois at Chicago (UIC) after approval by the local Institutional Review Board at each site. Mothers who delivered between 23 0/7 and 31 6/7 weeks or at ≥ 37 weeks

of gestation were enrolled after informed consent. Mothers who delivered prior to term provided 2-5 mL milk samples at 3 time points after delivery: within 48 hours (colostrum), on day 6-7 (1 week), and on day 30-31 (1 month). Mothers who delivered at term provided samples within the 1st week. Donor human milk samples were purchased from the Mother's Milk Bank, Austin, TX. Human milk-derived milk fortifier was donated by the manufacturer (Prolacta Bioscience, Monrovia, CA). All samples were centrifuged at $13,000 \times g$ for 10 min at 4°C. After gently removing the fat layer, the aqueous fractions and the cell pellets were harvested and stored separately. Samples were stored at -80°C until testing and were transported to UIC on dry ice. Freeze-thaw cycles were minimized.

Human milk samples used in to evaluate activation of TGF- β by *Lactobacillus* supernatants were collected from mothers who delivered prior to 32 weeks gestation (n=20).

Animals: Murine intestine was harvested after euthanasia from fetuses on embryonic day 18.5 (E18.5), pups on postnatal day 2 (P2) and P10, and adult mice (N = 6 animals per group). Studies were approved by Institutional Animal Care and Use Committee at UIC.

Enzyme-linked immunosorbent assays: TGF- β_1 , TGF- β_2 , TGF- β_3 , and thrombospondin-1 (THBS-1) concentrations were measured in the aqueous fraction of milk samples using commercially-available ELISA kits (R&D Systems). Optical densities and standard concentrations were log-transformed and a linear equation was obtained (acceptable $r^2 \geq 0.95$). Analyte concentrations in test samples were calculated by regression. The linear standard range of measurement of all 4 assays was 31.2-1000 pg/mL.

TGF- β Bioactivity: TGF- β bioactivity was measured in milk samples by using one of two luciferase reporter cell lines: (1) mink lung epithelial cells (MLEC) stably-transfected with a luciferase reporter construct containing the TGF- β -responsive plasminogen activator inhibitor-1 (PAI-1) gene promoter (14); and (2) RAW 264.7 cells stably-transduced with a luciferase lentiviral construct containing a Smad-response element (Cignal SRE luc reporter kit, Qiagen, Valencia, CA). Bioactive

TGF- β and total TGF- β bioactivity in milk samples was measured by adding milk samples in the native state or after heat-treatment at 80°C $\times$ 5 min (to activate latent TGF- β), diluted 1:1 in serum-free media. After 16 h, cell lysates (M-PER reagent, Thermo Scientific, Rockford, IL) were used to measure luciferase activity using a commercially-available kit (GloMax multi-detection system, Promega, Madison, WI). In some experiments, a neuraminidase inhibitor, N-Acetyl-2, 3-dehydro-2-deoxyneuraminic acid (50 μ M, pre-determined optimum; Sigma, St. Louis, MO) was added. All assays were performed in triplicate.

- 10 **Cell survival and cytotoxicity** Cell survival and cytotoxicity were measured by DAPI staining and XTT assay (Roche applied science).

Reverse transcriptase-real-time polymerase chain reaction (RT-qPCR): Primers (Table 1) were designed using the Beacon Design software (Bio-Rad, Hercules, CA). A standard reverse transcriptase reaction and SYBR green-based method was used to measure mRNA expression and normalized data against glyceraldehyde 3-phosphate dehydrogenase (GAPDH) or the 18S ribosomal RNA gene. Groups were compared by the $2^{-\Delta\Delta CT}$ method (15, 16).

- 20 **Gelatinase zymography:** Gelatinolytic activity of matrix metalloproteinase-2 (MMP-2) and MMP-9 was measured in milk samples by zymography. Milk-borne gelatinases were separated electrophoretically using 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (gel co-polymerized with 1 mg/mL gelatin), and then renatured, stained with Coomassie blue, and then destained using established methods (17). Zymography detects MMP-2 and MMP-9 activity as clear bands over a blue background. Quantification was done by densitometric analysis.

Immunohistochemistry: De-identified human mammary tissues from biopsies and healthy margins of surgically-resected neonatal intestinal tissues from preterm (24-27 weeks' gestation) and term neonates were immunostained as previously described (18). Briefly, tissue sections were deparaffinized and antigen retrieval was achieved using the EZ-AR solution (Biogenex, San Remon, CA). Sections were treated with 20 μ g/mL proteinase-K (Promega, Madison, WI) $\times$ 10 min, blocked using the Super-block T20 buffer (Thermo Scientific) $\times$ 30 min, and then incubated

overnight at 4°C with an primary anti-human TGF- β_2 or Neu-1 (Santa Cruz Biotechnology, Santa Cruz, CA). Secondary staining was performed with Alexa Fluor 488-conjugated secondary antibody (Invitrogen, San Diego, CA) $\times$ 30 min at room temperature. Nuclear staining was obtained with 4', 6-diamidino-2-phenylindole (DAPI; Calbiochem, San Diego, CA). Fluorescence imaging was performed using a Zeiss LSM 710 confocal microscope.

Western blots: TGF- β_2 was immunoprecipitated from milk samples using a standard protocol (19), separated in non-denaturing polyacrylamide gel electrophoresis, and immunoblotted as previously described (7). Smad2 and phospho-Smad2 expression was measured in ex planted intestinal tissue from 10-day-old C57/BL6 mouse pups after treatment with milk samples. The intestine was opened longitudinally, rinsed gently in PBS, and cut into 3-5 mm square explants that were placed in serum-free RPMI 1640 media in 5% CO₂ at 37°C. Some explants were treated with equal volumes of milk samples or phosphate-buffered saline added 1:1 (v/v) $\times$ 25 min. After treatment, explants were washed and homogenized in ice-cold lysis buffer (T-PER reagent containing protease and phosphatase inhibitors, Thermo Scientific, Rockford, IL).

Enzymatic activity assays: Plasmin activity was measured in human milk using a commercially-available fluorometric assay (Anaspec, Inc., Fremont, CA). The assay measures plasmin activity using a substrate that releases the yellow-green fluorophore 7-amido-4-trifluoromethylcoumarin (AFC), which can be quantified at excitation/emission=380 nm/500 nm. Data are expressed as the final concentrations of AFC (μ M).

Neuraminidase activity was measured in human milk and murine intestinal tissue using the Amplex Red neuraminidase assay kit (Invitrogen). This assay is based on the detection of H₂O₂ generated by galactose oxidase oxidation of desialiated galactose, the end result of neuraminidase action. In the presence of horseradish peroxidase, H₂O₂ reacts with the Amplex Red reagent to generate the red oxidation product, resorufin, which can be read spectrophotometrically at 571/585 nm. The assay has a sensitivity of 0.2 mU/mL.

NF- κ B activation: NF- κ B activation was measured using RAW 264.7 cells stably-transfected with the pNF- κ B/SEAP plasmid, which expresses the secreted alkaline phosphatase (SEAP) protein under the control of the NF- κ B promoter (NF- κ B SEAPorter Assay Kit, Imgenex, San Diego CA). RAW 264.7 reporter cells were
5 treated with milk samples mixed 1:1 with serum-free media for 24 h and culture supernatants were assayed for SEAP activity per manufacturer's protocol. Briefly, 250 μ l culture supernatant was first diluted 1:10 in assay buffer and then equal volumes of the diluted sample and H₂O were incubated at 65°C $\times$ 30 minutes to inactivate endogenous alkaline phosphatase. Then, 100 μ L of a colorigenic substrate
10 (p-nitrophenyl phosphate, 1 mg/mL) was added to each well and incubated $\times$ 1 h. Absorbance was read at 405 nm after 30 min and 1 h. The linear range of measurement of the assay was 3.1–200 ng/mL.

Preparation of *Lactobacillus* species supernatants. *Lactobacillus* species
15 supernatants were prepared by growing *L. acidophilus*, *L. rhamnosus*, *L. plantarum* and *L. casei* overnight in MRS broth at 37°C in a 5% CO₂-95% O₂ incubator. The next day, bacteria were spun down by centrifuging at 3,000 rpm for 10 min. For in vitro studies, culture supernatants were separated from spun-down bacteria, filtered through a 0.22- μ m filter, and mixed with cell culture media for further use. In these studies,
20 predetermined standard curves showed that an optical density (600 nm) of 1 indicated the presence of 10⁹ cfu/mL in the media. *L. rhamnosus* culture supernatants were fractioned using molecular weight (MW) cut-off filters (Millipore). Bacterial neuraminidase antibody (Thermo scientific) was used to immunoprecipitate *L. rhamnosus* neuraminidase, which was further analyzed by Western blots.

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Statistical methods: Statistical analysis was performed using the Sigma Stat 3.1.1 software (Systat, Point Richmond, CA). Parametric data were depicted using bar diagrams, whereas non-parametric data were shown using Tukey-Koopman box-whisker plots. For PCR data, crossing-threshold ($\Delta\Delta$ CT) values for genes with $\geq$ 2-
30 fold change were compared by the Mann-Whitney U test. In all tests, $p < 0.05$ was accepted as significant.

RESULTS

Preterm human milk shows minimal TGF- β bioactivity in the native state but contains a large pool of latent TGF- β which can be readily activated. Using MLEC reporter cells, TGF- β bioactivity was first measured in preterm human milk from mothers who delivered between 23 0/7 and 31 6/7 weeks and provided milk samples within 48 h (colostrum), after 1 week, and 1 month after delivery (n = 50 mothers per group). TGF- β bioactivity in colostrum, 1-week, and 1-month samples of native preterm milk was median 296 nM (range 20-874), 224 nM (range 6-1322), and 574 nM (range 121-6771), respectively. After heat-treatment, the bioactivity at the three time points was measured as 368 nM (range 169-1658), 3818 nM (range 12-17823), and 1727 nM (range 6-11068), respectively (**Fig. 1A**). Taken together, these data showed low levels of bioactive TGF- β in preterm milk in the native state. However, a substantial pool of latent TGF- β was detected in 1-week and 1-month samples that could be activated by heat-treatment.

To determine whether preterm human milk contains less bioactive TGF- β than term milk, preterm and term milk samples received within the 1st week after delivery (n = 20 mothers in each group) were compared. Preterm milk contained less bioactive TGF- β in the native state (**Fig. 1B i**) but after heat-treatment to activate latent TGF- β , showed significantly more TGF- β bioactivity than term milk (**Fig. 1B ii**). These data showed that preterm milk not only showed less TGF- β bioactivity than term milk in absolute terms, but also contained less bioactive TGF- β when expressed as a proportion of the total pool (latent + active) of milk-borne TGF- β (**Fig. 1B iii**). Based on these findings, it was hypothesized that preterm milk is relatively deficient in TGF- β -activating mechanisms present in term milk. In view of emerging clinical evidence indicating that donor human milk and a human milk-derived human milk fortifier may provide modest protection against NEC (20, 21), TGF- β bioactivity was measured in donor milk (n = 10 donors) and the human milk-derived fortifier (n = 10 samples). Similar to fresh preterm milk, donor milk and human milk-derived fortifier showed minimal TGF- β bioactivity in the native state but revealed substantial TGF- β bioactivity after heat-treatment (**Fig. 1C, D**).

Preterm human milk contains a larger pool of TGF- β_2 than term milk. Specific ELISA was used to compare TGF- β_1 , TGF- β_2 , and TGF- β_3 concentrations in preterm vs. term milk. Each of these ELISAs includes an acid-activation step to activate

latent TGF- β and therefore, measures the sum of active and latent form of the specific TGF- β isoform. A significantly larger pool of active and latent TGF- β_2 was detected in preterm milk than in term milk (median 8837 pg/mL, range 1771-15849 pg/mL in preterm milk vs. median 4125, range 982-10927 pg/mL in term milk; $p = 0.04$). These findings are consistent with higher levels of total TGF- β bioactivity detected in heat-treated preterm (vs. term) milk; **Fig. 1B ii**). The concentrations of TGF- β_1 and TGF- β_3 in preterm and term milk samples were comparable (TGF- β_1 : median 110, range: undetectable-227 pg/mL in preterm milk vs. median 152, range: undetectable-318 pg/mL in term milk; $p = 0.88$; TGF- β_3 : median 37, range: undetectable-48 pg/mL in preterm milk vs. median 83, range: undetectable-95 pg/mL in term milk; $p = 0.89$). These data are depicted in **Fig. 2A**; a consistent y-scale was used in the 3 boxplots to highlight the predominance of TGF- β_2 among the 3 TGF- β isoforms in milk.

To confirm that preterm milk contains less bioactive TGF- β_2 than term milk, TGF- β_2 was immunoprecipitated from milk samples, and active and latent TGF- β_2 was resolved by non-denaturing PAGE ($n = 10$ samples per group). Consistent with bioactivity measurements in MLEC reporter cells, native preterm milk expressed less active TGF- β_2 (14 kDa) than term milk (**Fig. 2B i**). Immunoprecipitation of TGF- β_2 depleted most TGF- β bioactivity from milk samples, further confirming that TGF- β_2 is the predominant isoform of TGF- β in milk (**2B ii**).

We next investigated whether the observed differences in the concentrations of TGF- β isoforms in preterm vs. term milk resulted from differential expression of these isoforms in milk cells. In RT-qPCR assays, a significant difference in mRNA expression of TGF- β_1 , TGF- β_2 , or TGF- β_3 was not detected in the cellular fractions of preterm and term milk (**Fig. 2C**). Immunohistochemistry was performed on human mammary tissue to identify the cellular source of milk-borne TGF- β_2 . TGF- β_2 was immunolocalized to epithelial cells (**Fig. 2D**) and was more prominent on the apical aspects of mammary epithelial cells (inset), which is consistent with a vectorial discharge pattern into mammary glands (22).

Low neuraminidase activity in preterm milk can explain lower TGF- β bioactivity in preterm than in term milk. Next, differences between preterm and term milk were evaluated to identify TGF- β activator(s) that could potentially explain the differences in 'pre-activated' TGF- β in preterm vs. term milk. Pro-TGF- β_2 lacks the

tripeptide motif arginyl-glycyl-aspartic acid and is therefore, not sensitive to integrin-dependent activation (14). Current evidence indicates a role for the glycoprotein THBS-1, proteases such as plasmin, MMP-2, and MMP-9, and glycoside hydrolases such as neuraminidase (13, 23).

5

Plasmin activity was comparable in preterm and term milk (**Fig. 3A**). THBS-1 mRNA was expressed at significantly lower levels in the cellular fractions of preterm milk (compared to cells in term milk, fold change 0.02 ± 0.01 , $p < 0.05$; data not depicted). However, there was no difference in the THBS-1 protein concentrations in
10 preterm vs. term milk (**Fig. 3B**). Preterm and term milk showed considerable variability in MMP-2 and MMP-9 mRNA/enzyme activity; the two groups did not show a significant difference (**Fig. 3C, D**).

Preterm milk showed significantly less neuraminidase activity than term milk (**Fig. 3E**). In human mammary tissue, neuraminidase-1, a membrane-associated
15 protein in this enzyme family (24), was immunolocalized to epithelial cells (**Fig. 3F**). Neuraminidase-1 immunoreactivity was more prominent along the apical aspects of epithelial cells (inset), indicating that the protein is likely discharged into the mammary glands.

In addition to the activators of TGF- β_2 , cellular fractions of preterm and term
20 milk were compared for mRNA expression of ITGAV (integrin chain α_V), ITGB6 (integrin chain β_6), ITGB8 (integrin chain β_8), and Thy-1/CD90, genes involved in integrin-mediated activation of TGF- β_1 and TGF- β_3 . Cells in preterm milk expressed less ITGB8 but the other analytes were comparable (**Fig. 3G**).

25 **Fetal/neonatal intestine is less efficient than the mature intestine at in situ activation of milk-borne TGF- β .** To confirm that milk-borne neuraminidase activity and the levels of pre-activated TGF- β may not be biologically-relevant if there was adequate capacity for in situ activation of orally-ingested TGF- β in the neonatal intestine the highly-conserved nature of TGF- β across mammalian species was
30 exploited: each of the TGF- β isoforms in humans and mice shows >98% amino acid sequence homology and shows cross-species reactivity (12, 25). To estimate the capacity of the neonatal intestine for in situ activation of milk-borne TGF- β , preterm human milk was treated with tissue lysates from murine neonatal (P10) and adult

intestine and measured TGF- β bioactivity after 16 h. As depicted in **Fig. 4A** intestinal tissue from pups was less efficient than from adults at TGF- β activation.

To confirm the contribution of intestinal neuraminidase expression to the ability of intestinal tissue to activate milk-borne TGF- β preterm human milk was treated with tissue lysates from murine neonatal (P10), and in some experiments, added a neuraminidase inhibitor (N-Acetyl-2, 3-dehydro-2-deoxyneuraminic acid). The neuraminidase inhibitor blocked the effect of intestinal tissue lysates on TGF- β activation (**Fig. 4B**), indicating that neuraminidase has a major contribution in the in situ activation of milk-borne TGF- β in the intestine.

To show that neuraminidase expression in the intestine is developmentally-regulated and increases with maturation. To investigate this hypothesis, neuraminidase activity in the murine intestine on E18.5, P2, P10, and at 6-8 weeks after birth was measured. Consistent with this hypothesis, low neuraminidase activity was detected in the fetal intestine, which increased after birth through adulthood (**Fig. 4B**).

Finally, to validate the findings of maturational increase in intestinal neuraminidase expression in the human developing intestine, immunohistochemistry was performed for neuraminidase-1 on histologically-intact preterm (25-28 weeks' gestation) and term intestinal tissue that was resected during surgery for bowel obstruction (n = 3 per group). Neuraminidase-1 was immunolocalized in epithelial cells in a discrete, punctate pattern along the apical membrane (**Fig. 4C**). Consistent with findings in the murine intestine, preterm human intestine showed patchy neuraminidase-1 immunoreactivity, which was weaker than in the term intestine.

Addition of bacterial neuraminidase to preterm human milk increased TGF- β bioactivity. As depicted in **Fig. 5A**, *Clostridium perfringens* neuraminidase increased TGF- β bioactivity in preterm milk in a dose-dependent fashion (as measured in the MLEC reporter cells). Similar results were obtained in RAW 264.7 SRE reporter cells (data not shown).

Previous studies indicated that the porcine fetal/neonatal intestine expresses TGF- β receptors at much lower levels than the adult intestine (26). Because data depicted in **Fig. 5A** were obtained in reporter cell lines, it was confirmed that neonatal intestine is not already saturated for TGF- β signaling and is capable of responding to increased TGF- β bioactivity in neuraminidase-treated preterm milk.

Towards this goal, ex planted murine intestinal tissue was treated with preterm human milk supplemented with *C. perfringens* neuraminidase. Similar to the porcine intestine (26), TGF- β receptor 2 (TGF β -R2), which is a key, rate-limiting gene in TGF- β signaling (6), was expressed at lower levels in fetal/neonatal murine intestine
5 than in the adult intestine (**Fig. 5B**, inset). However, neuraminidase treatment increased Smad2 phosphorylation in the explants (**Fig. 5B**), indicating that exposure to additional milk-borne TGF-bioactivity can increase TGF- β effects in the neonatal intestine.

Biological relevance of increased TGF- β bioactivity was confirmed in
10 neuraminidase-treated preterm human milk. Because TGF- β is a potent inhibitor of macrophage inflammatory responses (6), it was demonstrated that neuraminidase treatment can improve the ability of preterm human milk to suppress NF- κ B signaling in macrophages. To show these effects, RAW264.7 NF- κ B/SEAP reporter cells were treated with preterm and term human milk for 24 h before treating with LPS. As
15 depicted in **Fig. 5C**, addition of *C. perfringens* neuraminidase (10 mU) to preterm human milk increased its ability to suppress LPS-induced NF- κ B activation, to levels comparable to term milk.

Supernatants from *L. rhamnosus* were more effective in activating milk-borne TGF- β than supernatants from *L. acidophilus*, *L. plantarum* and *L. casei* cultures. *L.*
20 *rhamnosus* supernatants were more effective in activating TGF- β than live or heat-killed *L. rhamnosus* bacteria, indicating that activity was mediated via a secreted component. When fractionated by MW, TGF- β activating activity in the *L. rhamnosus* supernatants was present in the 30-50 kDa fractions. Significant neuraminidase activity was detected in these fractions, which activity was enriched by
25 immunoprecipitation by a generic anti-bacterial neuraminidase antibody, detected in Western blots as a discrete band, and was blocked by addition of a neuraminidase inhibitor. In contrast to *Clostridium perfringens* neuraminidase, which induced apoptosis in IEC6 epithelial cell cultures, *L. rhamnosus* neuraminidase was well-tolerated, indicating that it may have a better toxicity profile than other bacterial
30 neuraminidases.

Preterm human mil was found to contain a large pool of latent TGF- β that can be activated by adding culture supernatants from *Lactobacillus* species. FIG. 6A Bar diagram (means $\pm$ SE) shows TGF- β bioactivity in preterm human milk in the native

state, after heat-treatment, and with added culture supernatants from *L. acidophilus*, *L. rhamnosus*, *L. plantarum* and *L. casei* bacterial cultures (109 cfu/mL). TGF- β bioactivity was measured using a reporter cell line (mink lung epithelial cells stably transfected with a luciferase reporter construct containing the TGF- β -responsive plasminogen activator inhibitor-1 gene promoter). *L. rhamnosus* and *L. plantarum* supernatants (1:25 dilution) significantly increased TGF- β bioactivity in preterm milk. FIG. 6B shows TGF- β bioactivity in preterm human milk treated with live, or heat-killed *L. rhamnosus* (108-109 cfu/mL), or with culture supernatants from 24h cultures of *L. rhamnosus* (109 cfu/mL, diluted 1:25). Treatment with culture supernatants was more effective in activating TGF- β than live or heat-killed bacteria; FIG. 6C shows TGF- β bioactivity in preterm human milk after treatment with various fractions of *L. rhamnosus* culture supernatants restricted by molecular weight (>100 kDa, 50-100 kDa, 30-50 kDa, <30kDa, and <10kDa). The 30-50 kDa fractions of the *L. rhamnosus* culture supernatants were most effective in activating milk-borne TGF- β .

L. rhamnosus culture supernatants were found to contain high levels of neuraminidase activity. FIG. 7A shows neuraminidase activity in bacterial culture supernatants of Lactobacillus species, measured by the Amplex red neuraminidase assay. FIG. 7B shows that addition of the neuraminidase inhibitor (50 μ M) blocked the effect of *L. rhamnosus* culture supernatants in activating milk-borne TGF- β . FIG. 7C shows that neuraminidase activity in *L. rhamnosus* culture supernatants could be enriched by immunoprecipitation using an antibody against the bacterial neuraminidase domain.

Neuraminidase activity in *L. rhamnosus* culture supernatants was detected in the 30-50 kDa fractions. FIG. 8A shows neuraminidase activity in various fractions of *L. rhamnosus* culture supernatants restricted by molecular weight (>100 kDa, 50-100 kDa, 30-50 kDa, <30kDa, and <10kDa). The 30-50 kDa fractions of the *L. rhamnosus* culture supernatants contained high levels of neuraminidase activity. FIG. 8B is a Western blot confirming the presence of neuraminidase in the 30-50 kDa fractions, with the bar diagram (means $\pm$ SE) showing densitometric data.

L. rhamnosus-derived neuraminidase does not induce cell death in IEC6 intestinal epithelial cells. FIG. 9 shows that *L. rhamnosus*-derived neuraminidase (5-10 mU) did not affect cell survival in IEC6 cells, which contrasts with the cytotoxicity of similar doses of *C. perfringens*-derived neuraminidase. Cell survival was measured using the XTT in-vitro cytotoxicity assay. Media alone was used as

negative control, whereas acetyl salicylic acid (15mM) was used as positive control in these experiments.

This is believed to be the first study to specifically investigate the effects of preterm delivery on the expression of TGF- β in human milk. It was shown that even
5 though preterm human milk shows minimal TGF- β bioactivity in the native state, there is a large pool of latent TGF- β in preterm milk which can be readily activated. A similar, although slightly lower, expression of TGF- β was detected in donor human milk and a human milk-derived milk fortifier. Neuraminidase was also identified as a key activator of breast milk-borne TGF- β , and showed that the lower levels of pre-
10 activated TGF- β detected in preterm milk can be explained by the relative paucity of neuraminidase in preterm milk. Finally, this deficiency of neuraminidase activity in preterm human milk can be 'corrected' through the addition of neuraminidase of bacterial origin.

It is understood that the examples and embodiments described herein are for
15 illustrative purposes only and that various modifications or changes in light thereof will be suggested to persons skilled in the art and are to be included within the spirit and purview of this application and scope of the appended claims. All patents, patent applications, internet sources, and other published reference materials cited in this specification are incorporated herein by reference in their entireties. Any
20 discrepancy between any reference material cited herein or any prior art in general and an explicit teaching of this specification is intended to be resolved in favor of the teaching in this specification. This includes any discrepancy between an art-understood definition of a word or phrase and a definition explicitly provided in this specification of the same word or phrase.

CLAIMS

What is claimed is:

1. A composition comprising one or more neuraminidases and an excipient pharmaceutically acceptable for enteral administration.
- 5 2. A composition comprising one or neuraminidases and human milk, non-human mammalian milk or infant formula.
3. The composition of claim 2 wherein the neuraminidase is a recombinant neuraminidase.
4. The composition of claim 2 wherein said neuraminidase is from a
10 probiotic culture supernatant.
5. The composition of claim 2 human milk, non-human mammalian milk or infant formula comprises latent TGF- β .
6. The composition of claim 5, wherein the latent TGF- β is converted to active TGF- β .
- 15 7. The composition of claim wherein the human milk is preterm milk.
8. The composition of claim 2 further comprising a milk fortifier.
9. The composition of claim 4, wherein the probiotic supernatant is a *Lactobacillus rhamnosus* supernatant.
10. The composition of claim 9, wherein the supernatant is a 30-50 kDa
20 fraction.
11. Infant formula comprising an effective amount of TGF- β and an effective amount of one or more neuraminidases.
12. Human milk fortifier comprising an effective amount of one or more neuraminidases.
- 25 13. A method of treating or preventing necrotizing enterocolitis and gastrointestinal allergies in an infant or toddler comprising administering to the infant or toddler an effective amount of a composition comprising one or more neuraminidases.
14. The method of claim 13, further comprising administering to the infant
30 human milk, non-human mammalian milk or infant formula comprising latent TGF- β .
15. The method of claim 13, wherein the neuraminidase and the human milk, non-human mammalian milk, or infant formula are administered under conditions that allow the neuraminidase to catalyze conversion of at least a portion of the latent TGF- β to active TGF- β .

16. The method of claim 15, wherein the neuraminidase and the human milk, non-human mammalian milk, or infant formula are co-administered.

17. The method of claim 15, comprising a composition comprising the neuraminidase and human milk, non-human mammalian milk or infant formula.

5 18. The method of claim 13 wherein the infant is a premature infant.

19. The method of claim 14 wherein the human milk is preterm milk.

20. The method of claim 13 wherein the neuraminidase is a recombinant neuraminidase.

21. The method of claim 2 wherein the neuraminidase is from a probiotic
10 culture supernatant.

22. The method of claim 14 wherein the human milk comprises a milk fortifier.

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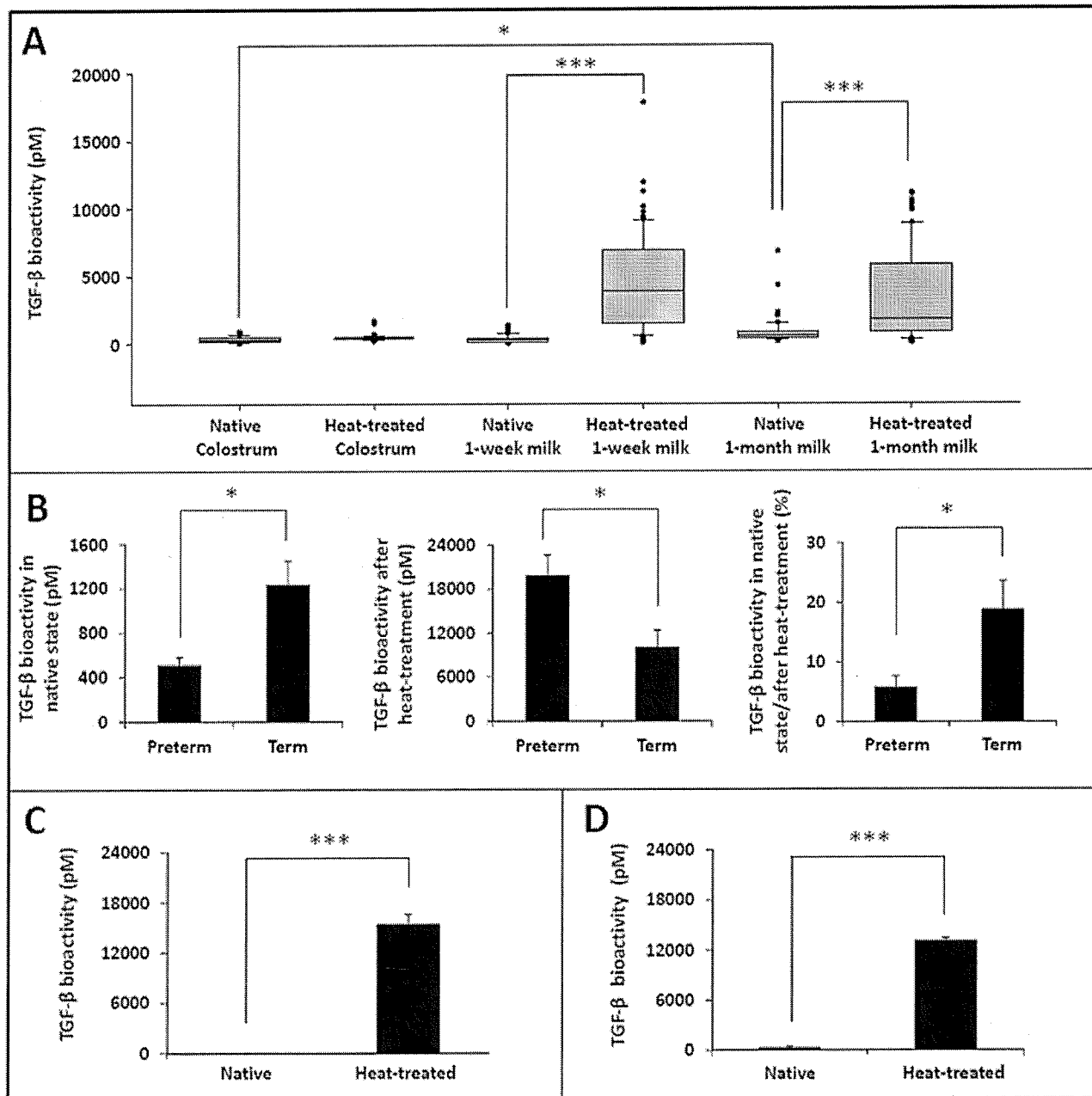


FIG. 1

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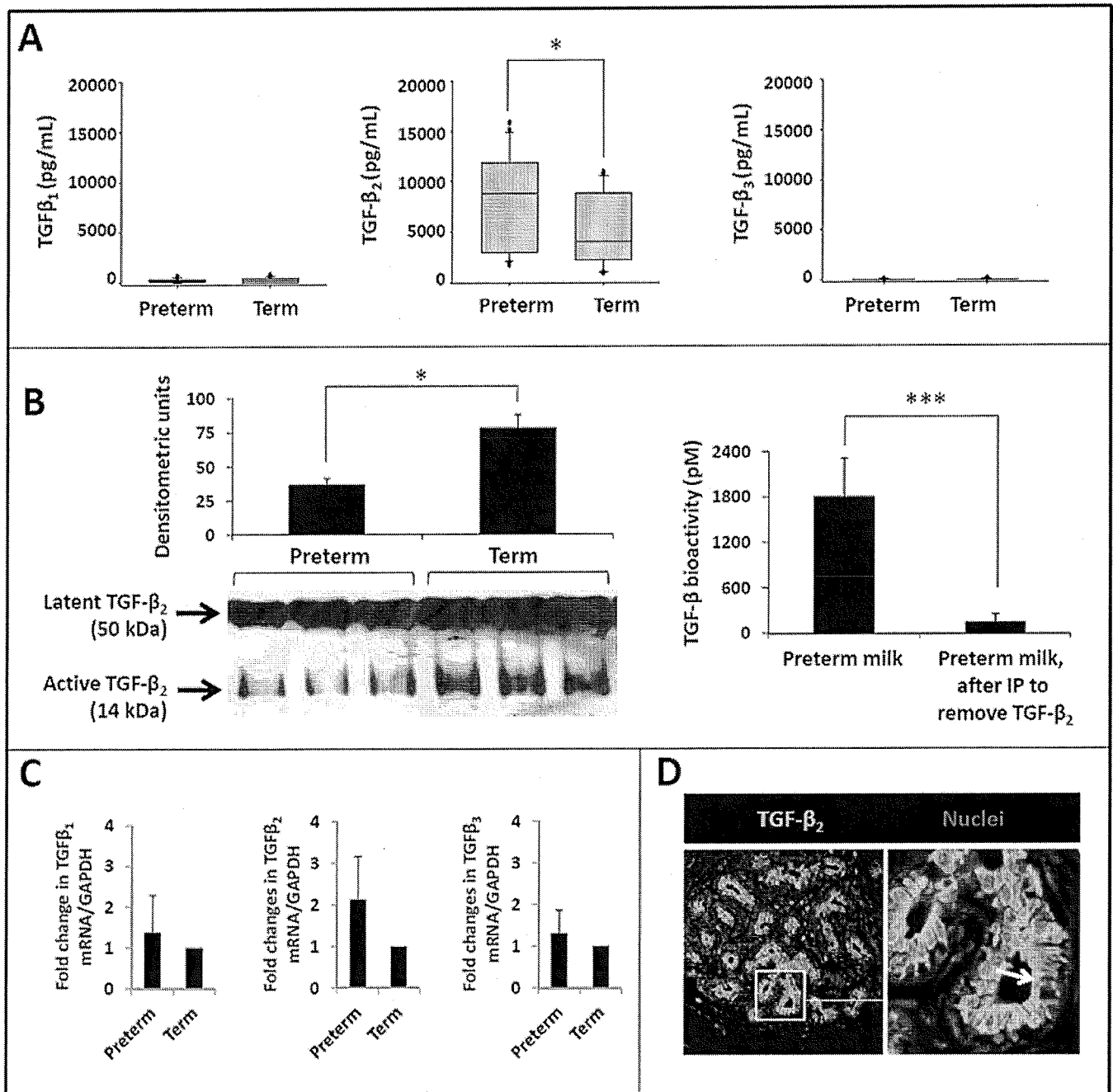


FIG. 2

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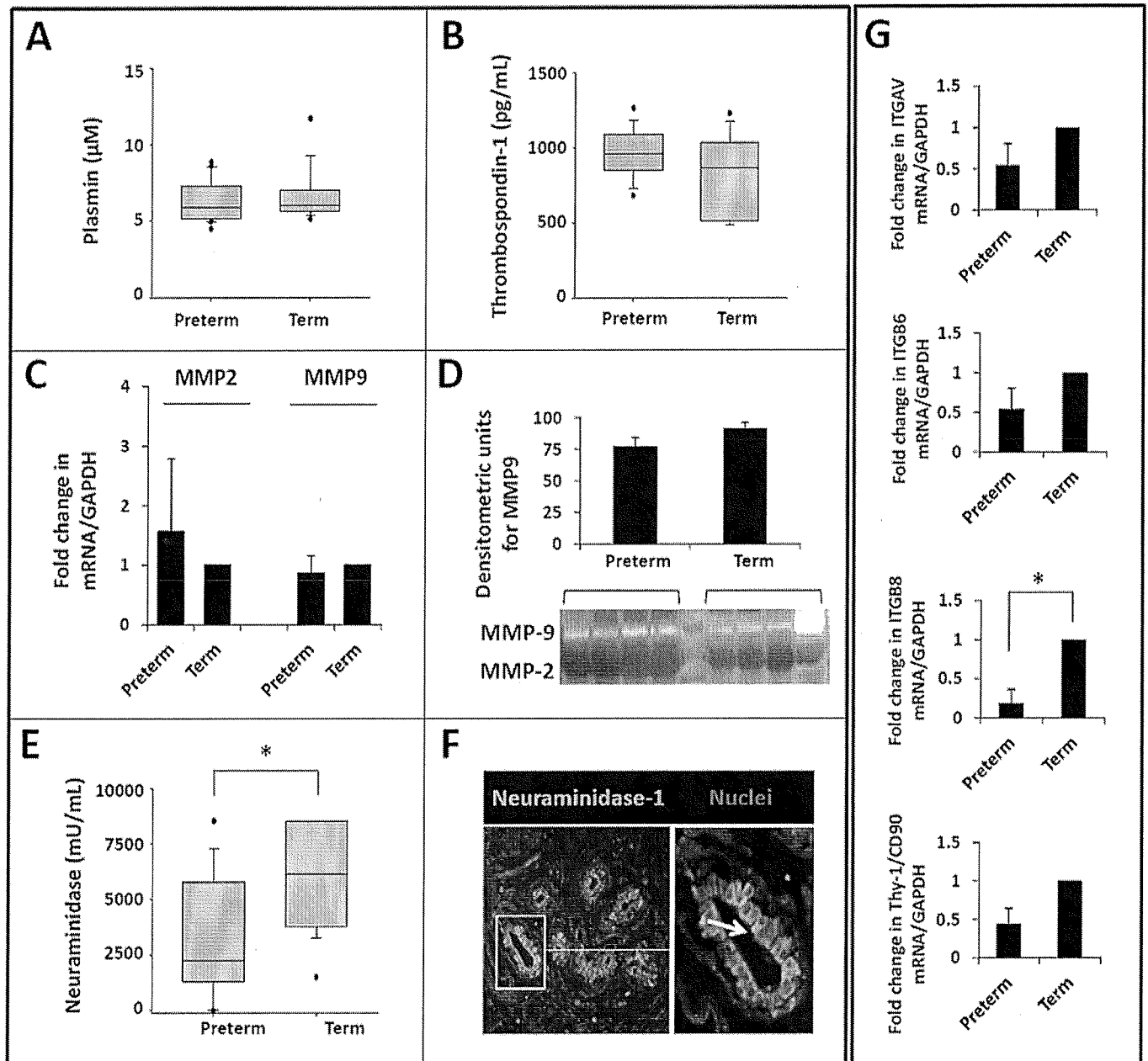


FIG. 3

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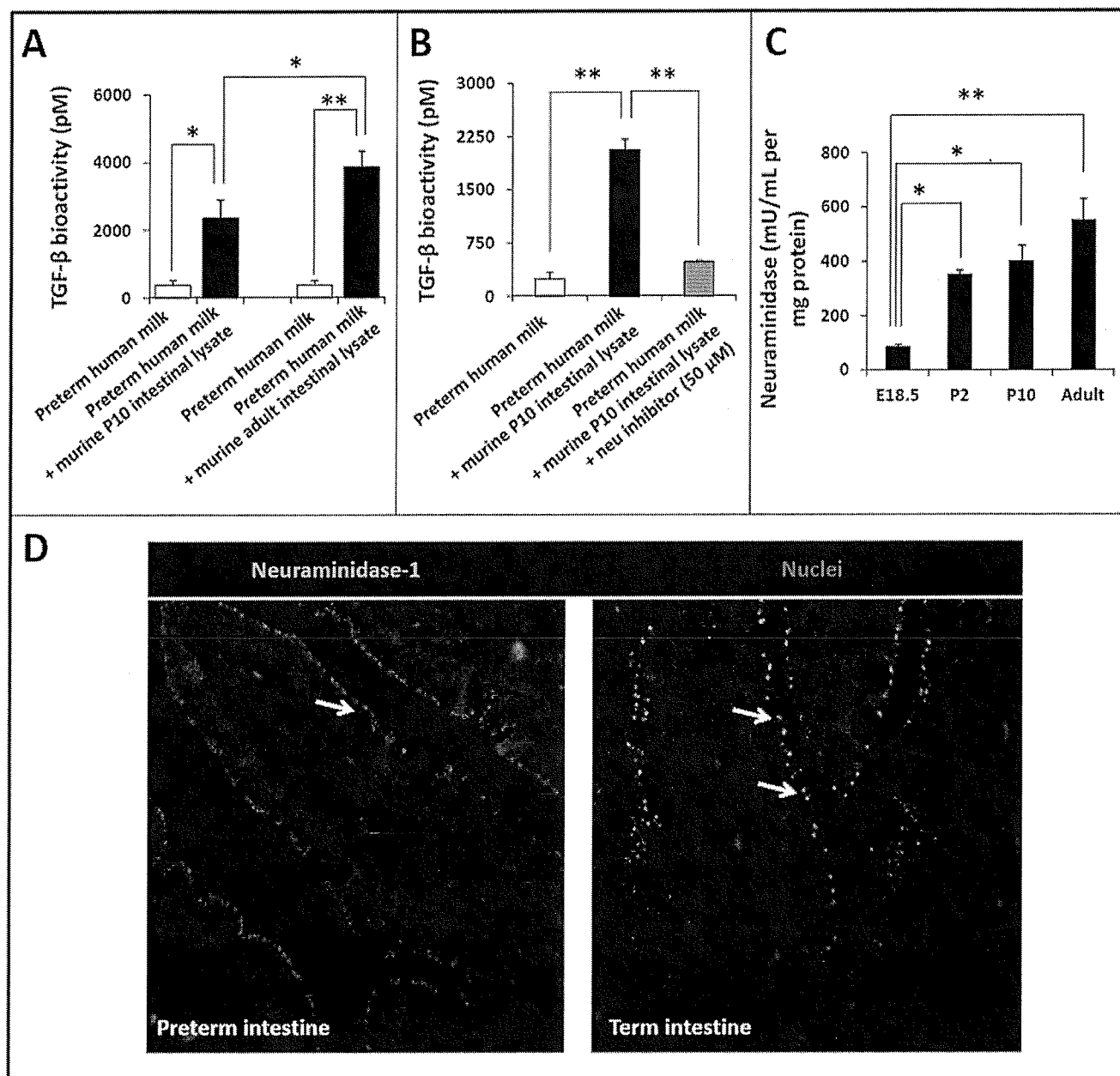


FIG. 4

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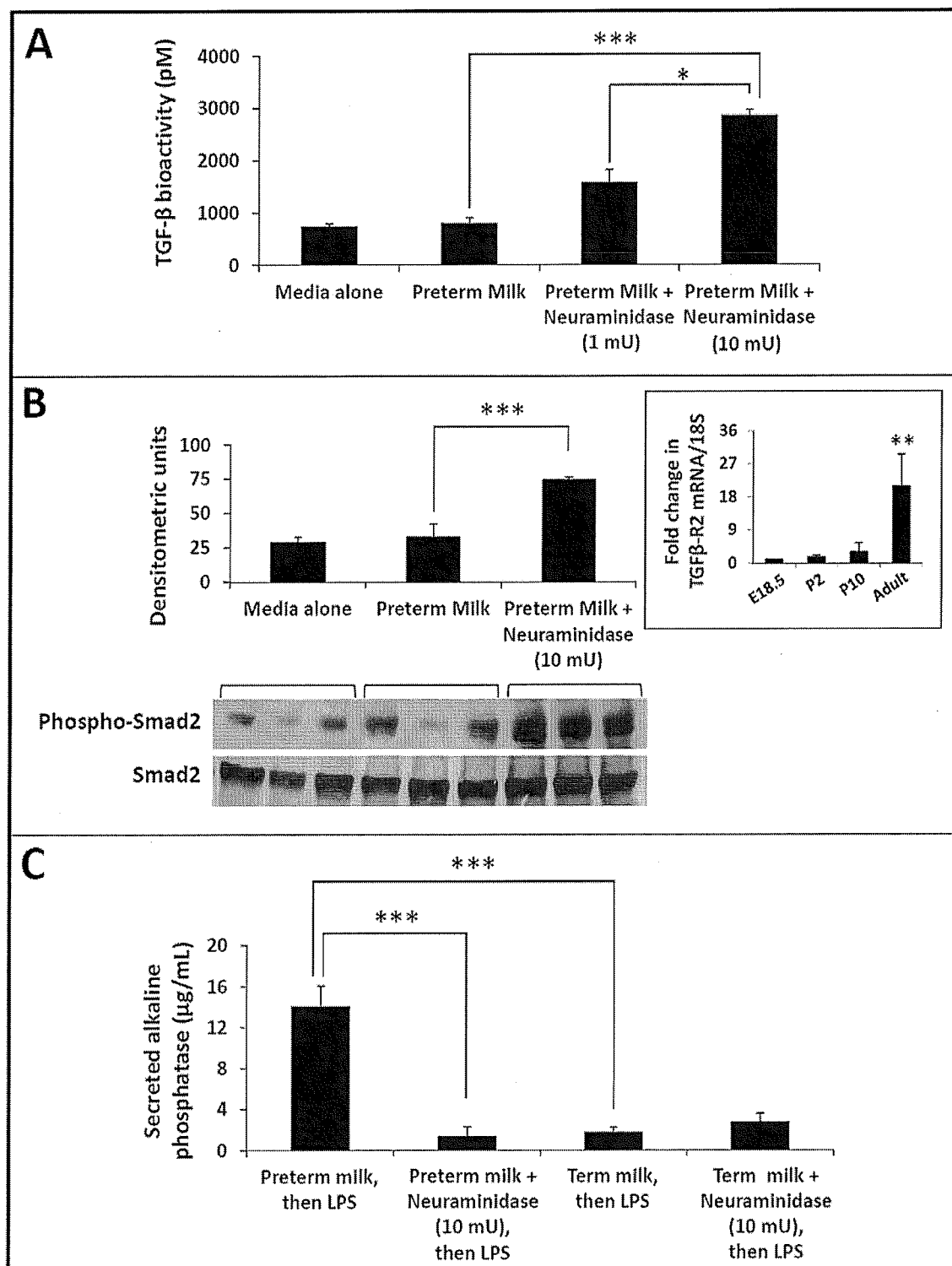


FIG. 5

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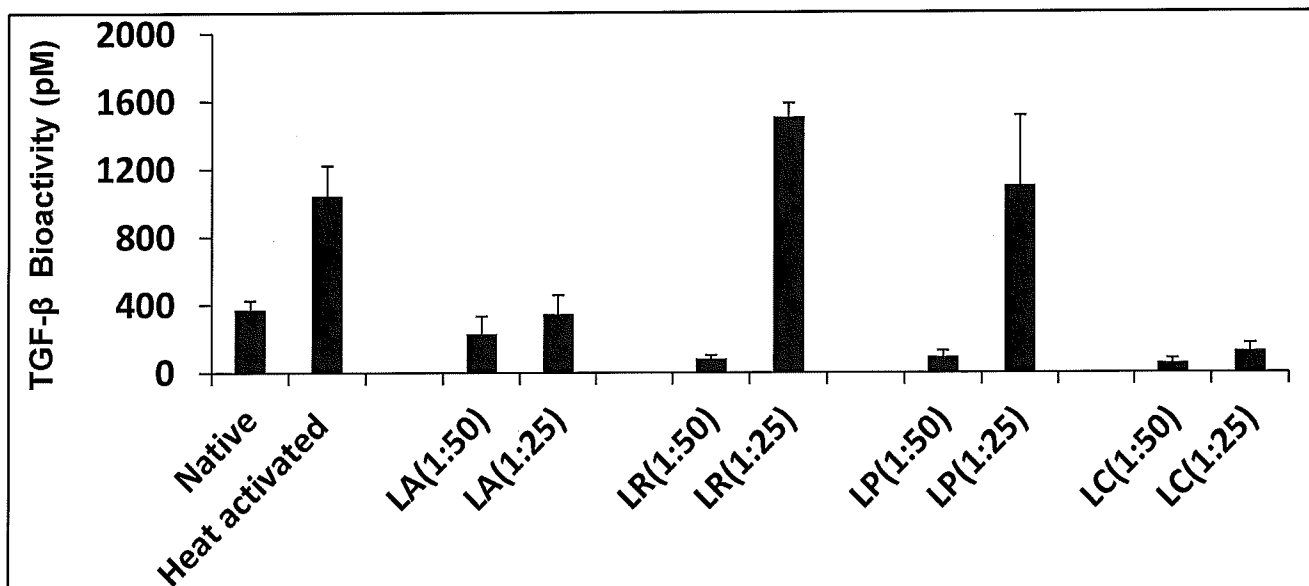


FIG. 6A

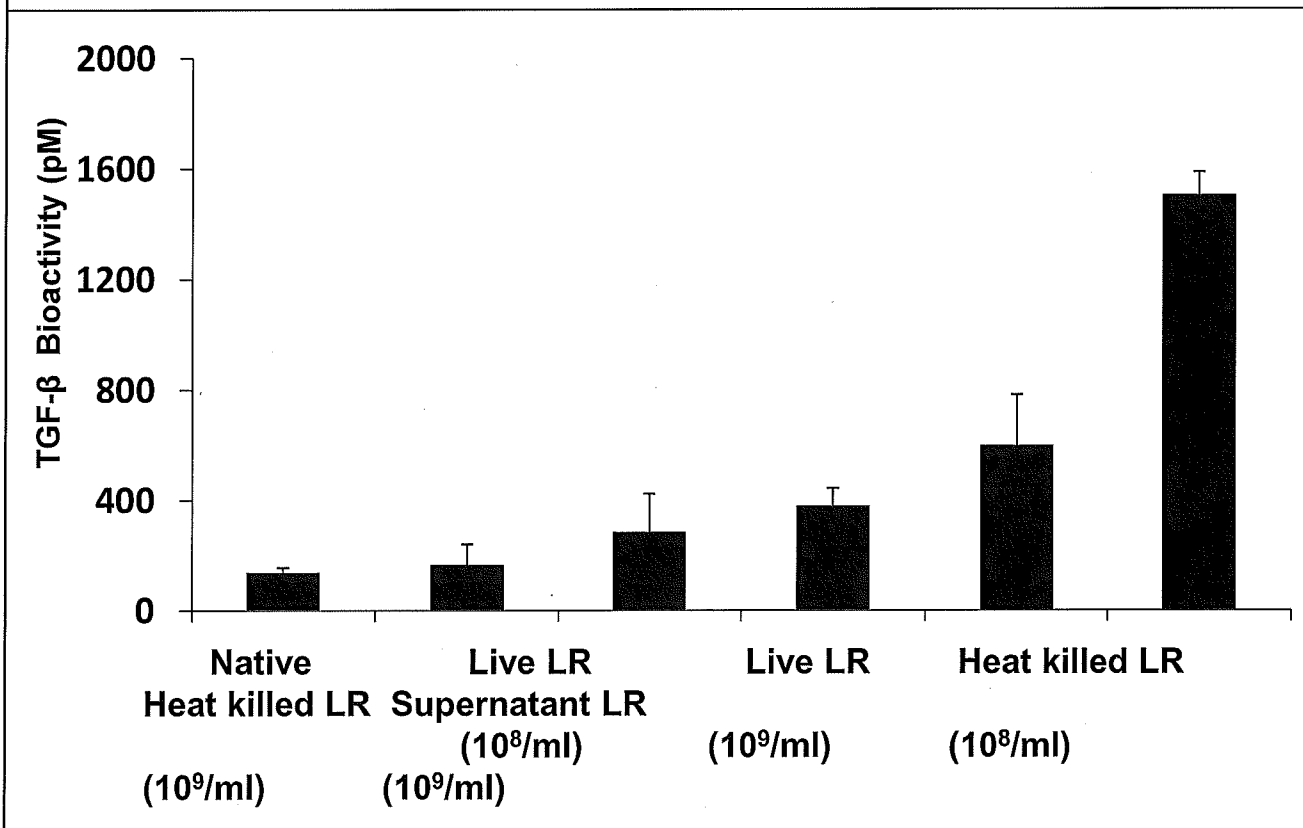


FIG. 6B

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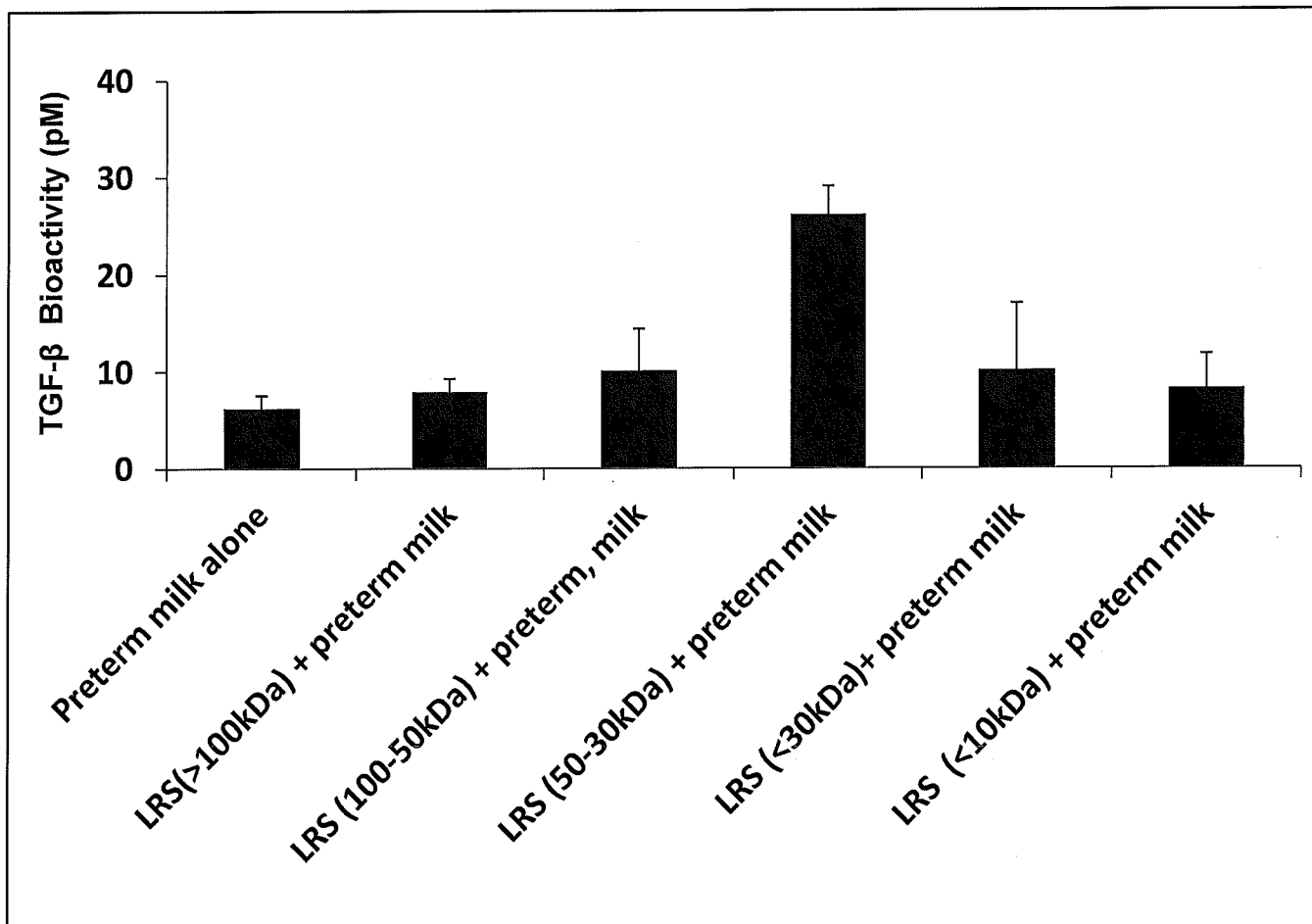


FIG. 6C

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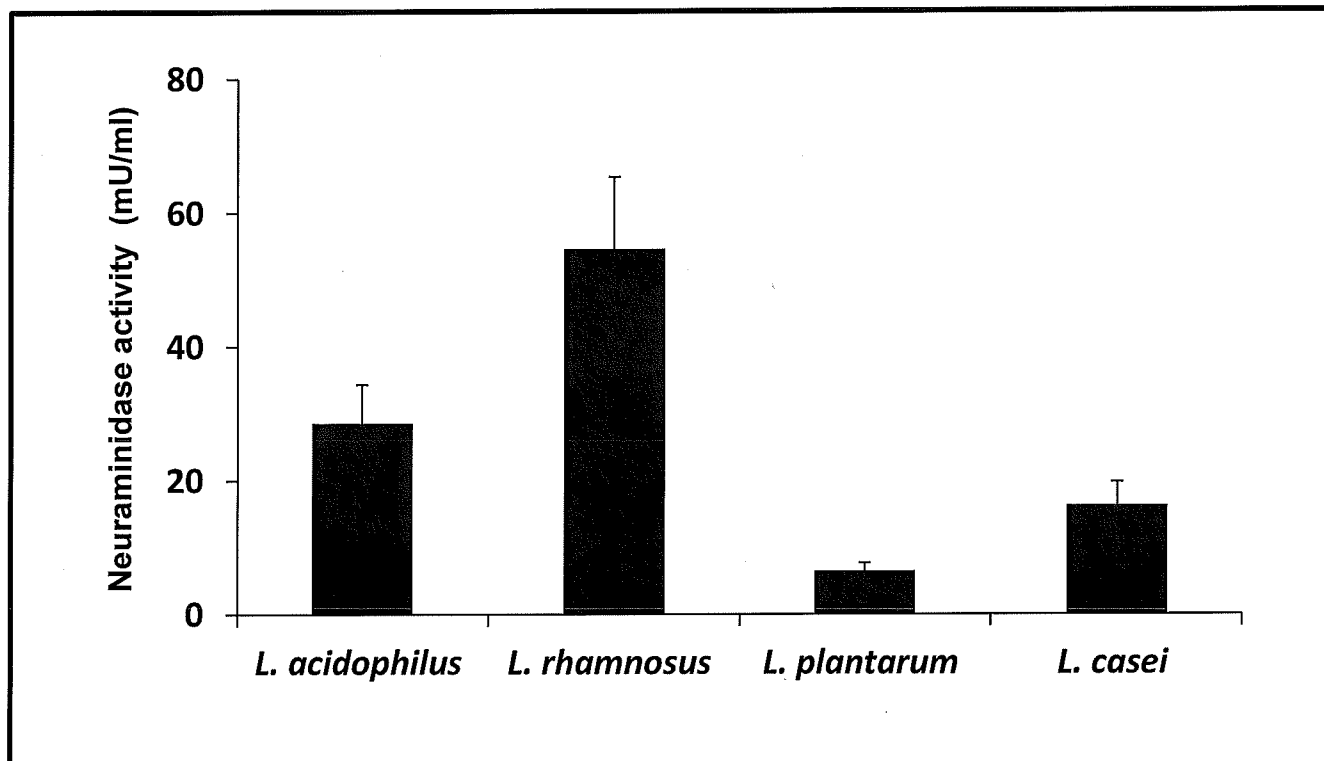


FIG. 7A

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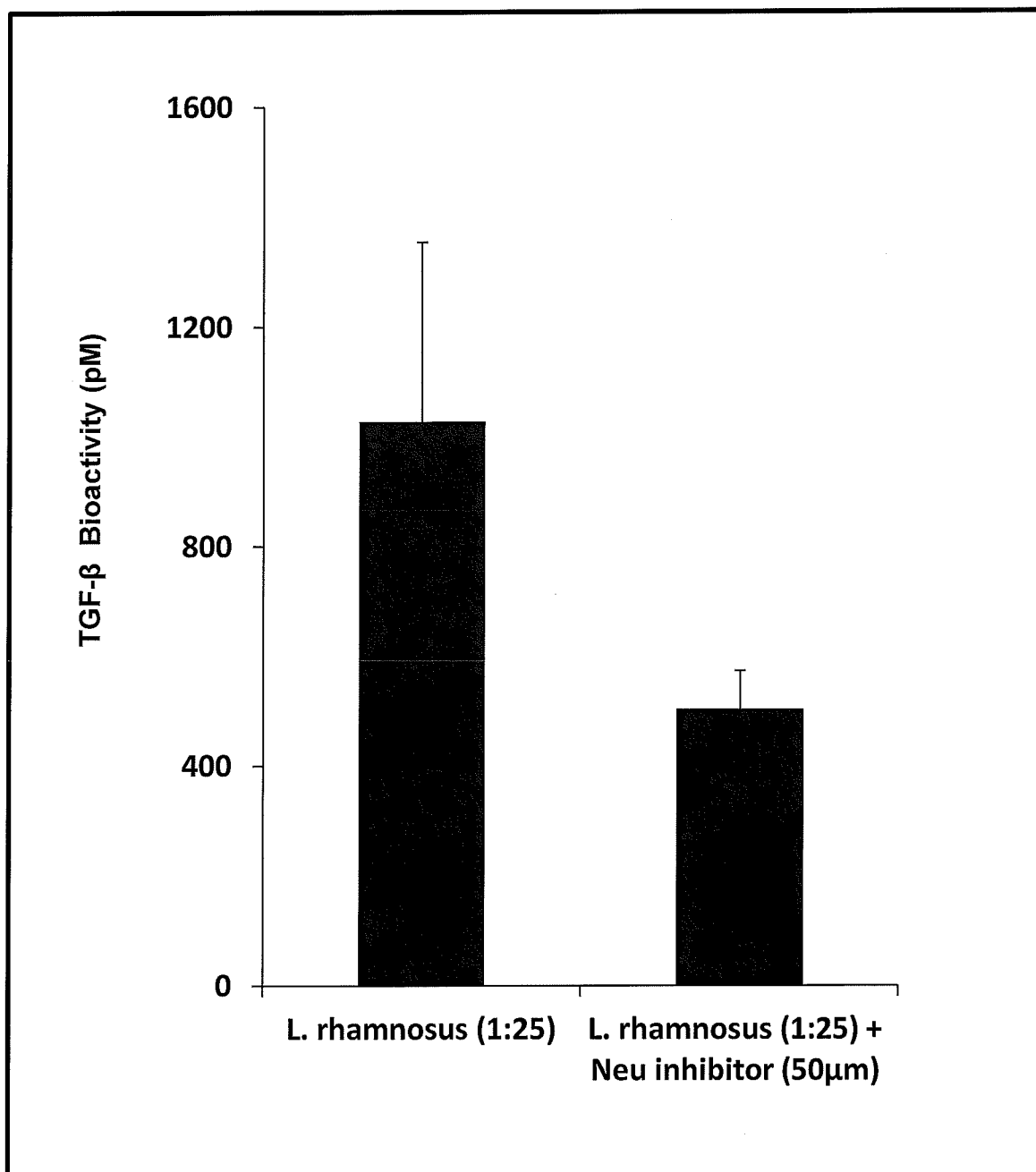


FIG. 7B

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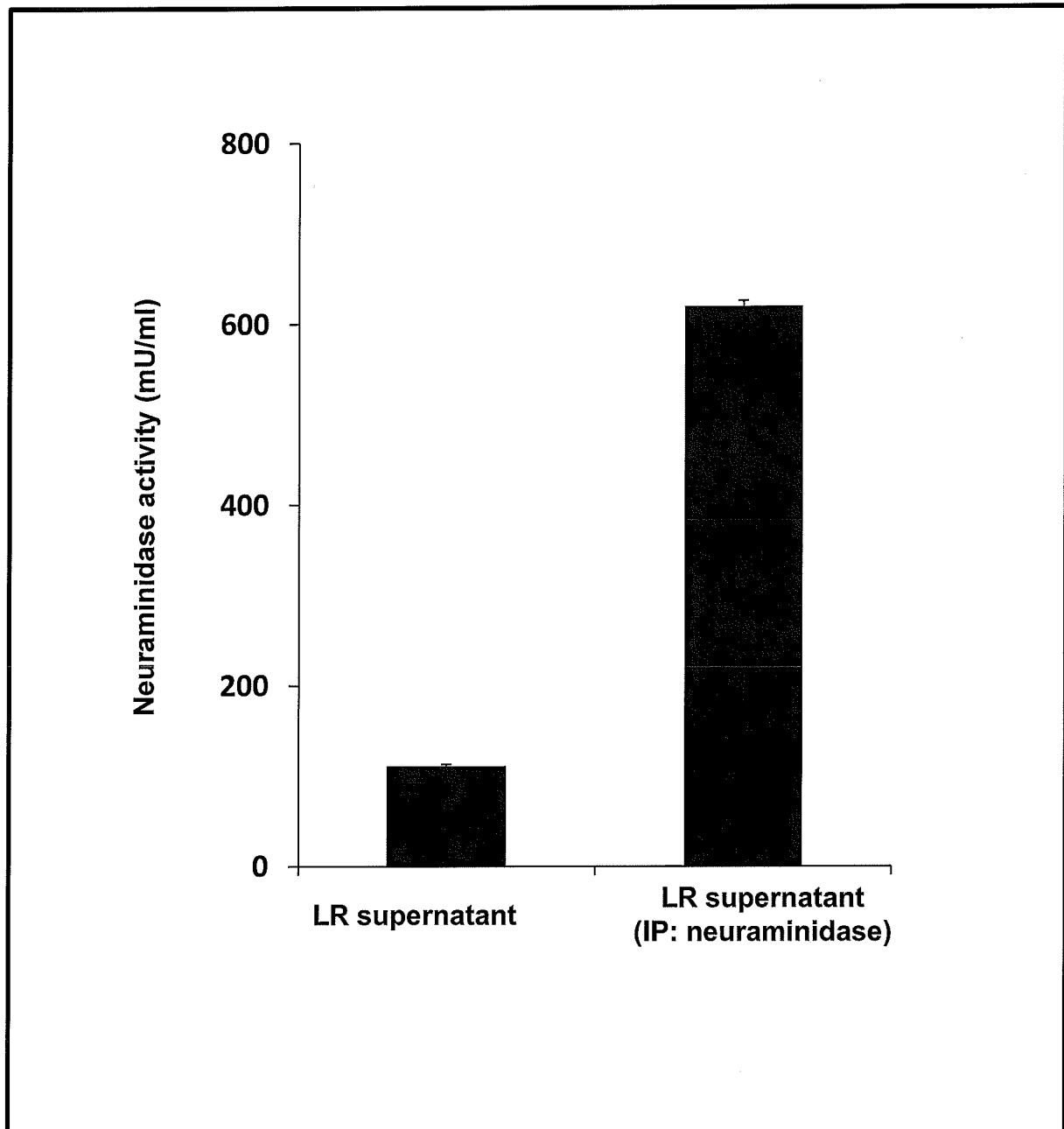


FIG. 7C

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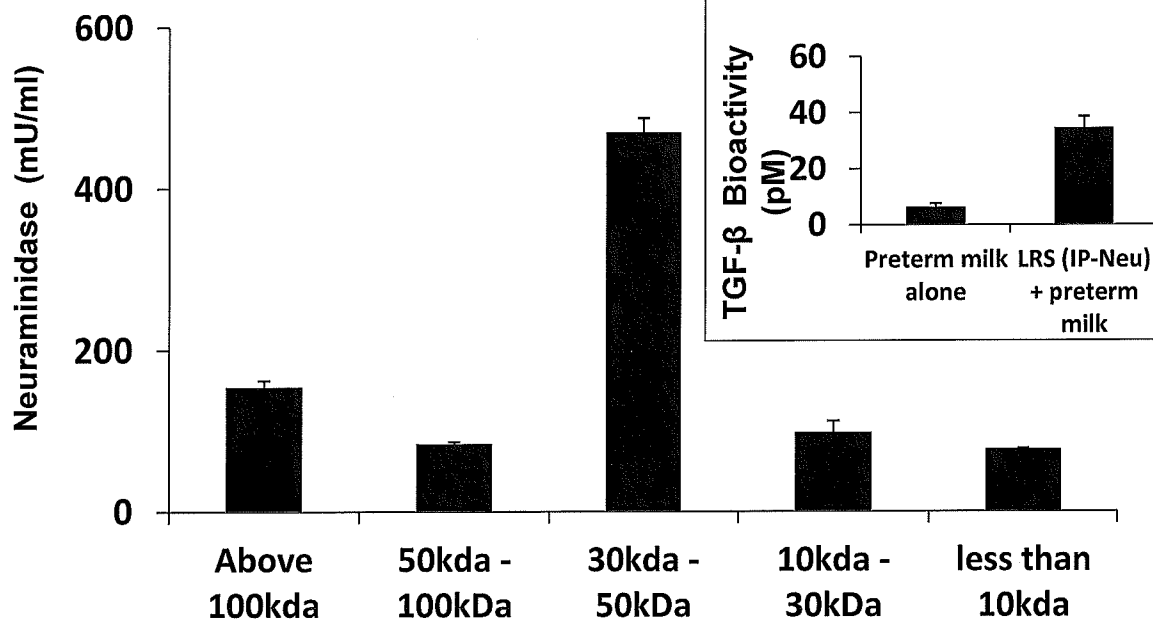


FIG. 8A

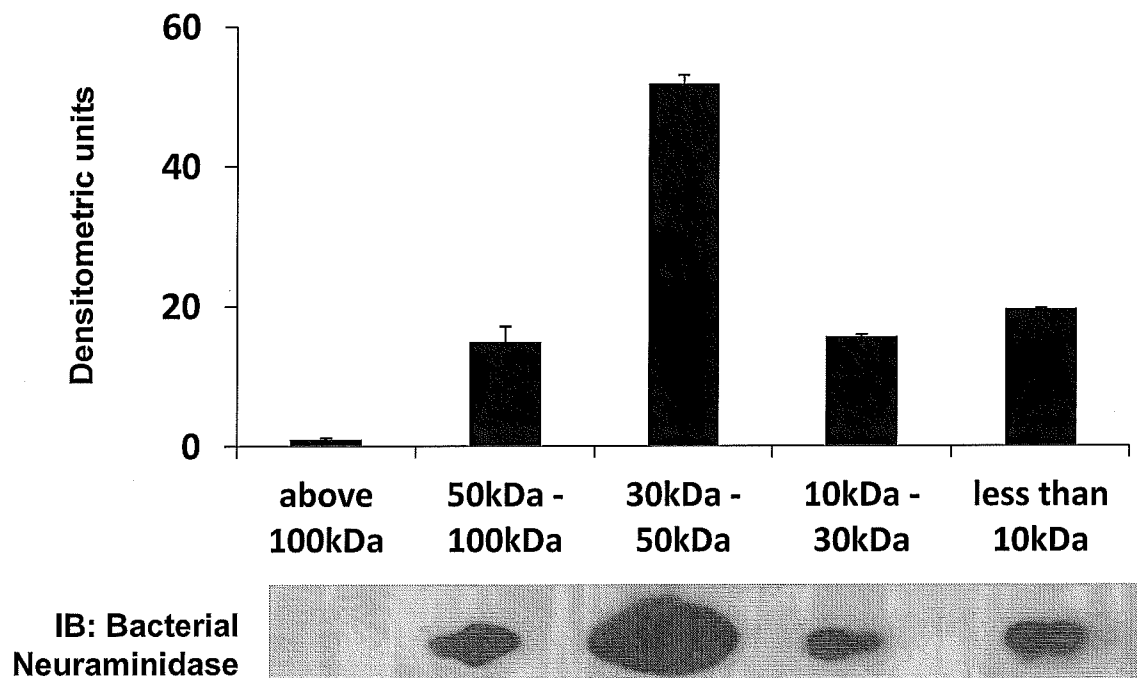


FIG. 8B

SUBSTITUTE SHEET (RULE 26)

12/12

XTT cell survival assay

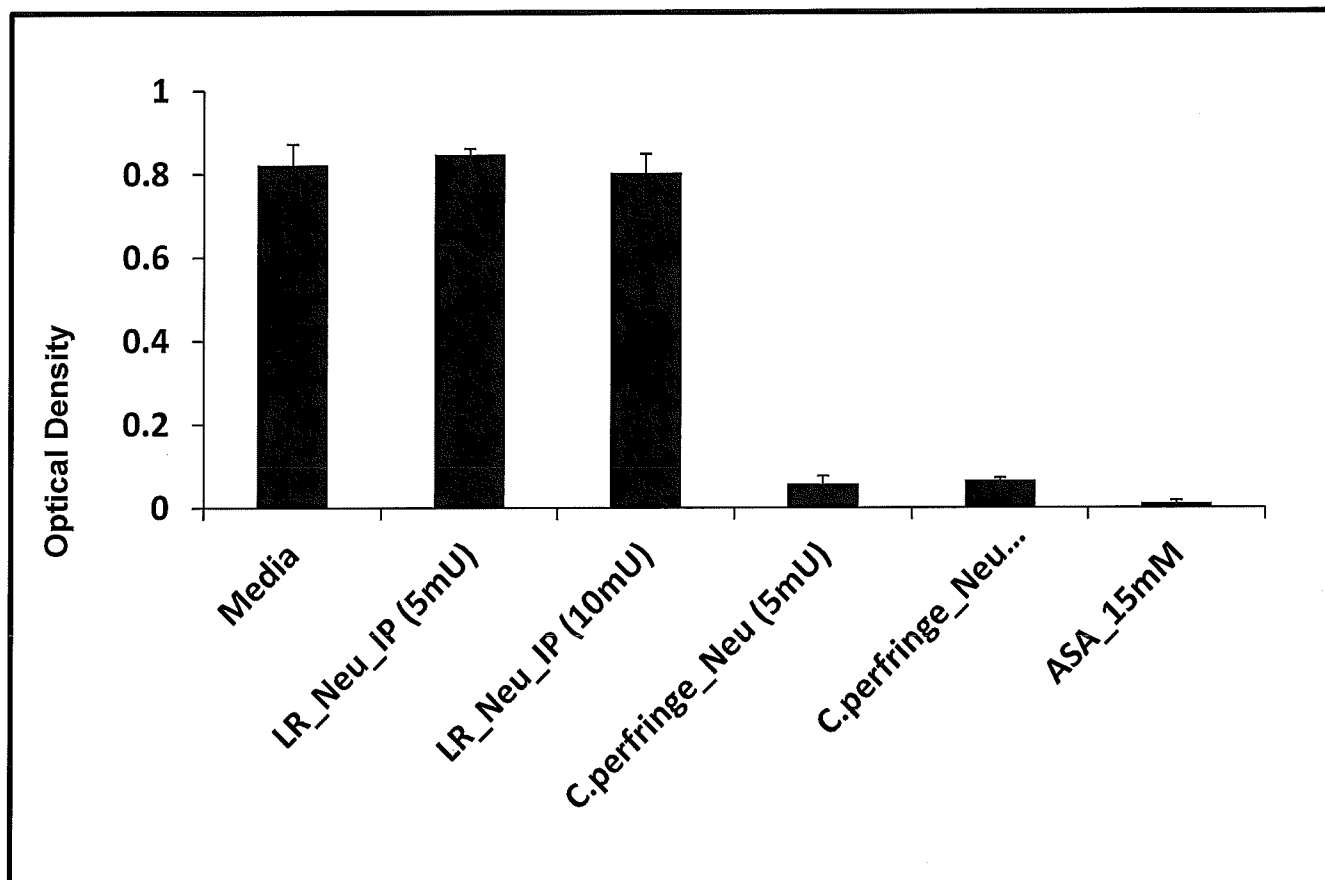


FIG. 9

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2014/015105

A. CLASSIFICATION OF SUBJECT MATTER
INV. A61K38/18 A23L1/29 A61P29/00
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
A23L A61K A61P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-Internal, WPI Data, BIOSIS, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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X	US 6 485 729 B1 (SMITH GAIL EUGENE [US] ET AL) 26 November 2002 (2002-11-26) abstract ----- -/-	1,12



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

18 March 2014

Date of mailing of the international search report

02/04/2014

Name and mailing address of the ISA/

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Authorized officer

Schnack, Anne

INTERNATIONAL SEARCH REPORT

International application No

PCT/US2014/015105

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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Y	CHRISTINA M. CARLSON ET AL: "Transforming Growth Factor-[beta]: Activation by Neuraminidase and Role in Highly Pathogenic H5N1 Influenza Pathogenesis", PLOS PATHOGENS, vol. 6, no. 10, 7 October 2010 (2010-10-07), page e1001136, XP055108172, DOI: 10.1371/journal.ppat.1001136 abstract -----	1-22
X	DATABASE MEDLINE [Online] US NATIONAL LIBRARY OF MEDICINE (NLM), BETHESDA, MD, US; April 1976 (1976-04), SCHAUER R ET AL: "Demonstration of neuraminidase activity in human blood serum and human milk using a modified, radioactively labelled alpha1-glycoprotein as substrate.", XP002721840, Database accession no. NLM964915 abstract & SCHAUER R ET AL: "Demonstration of neuraminidase activity in human blood serum and human milk using a modified, radioactively labelled alpha1-glycoprotein as substrate.", HOPPE-SEYLER'S ZEITSCHRIFT FÜR PHYSIOLOGISCHE CHEMIE APR 1976, vol. 357, no. 4, April 1976 (1976-04), pages 559-566, XP009176976, ISSN: 0018-4888 ----- -/--	1,2,5-7, 12

INTERNATIONAL SEARCH REPORT

International application No

PCT/US2014/015105

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/US2014/015105

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