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(54) Title: *LACTOBACILLUS ACIDOPHILUS* SURFACE LAYER PROTEIN A (SLPA) AS A THERAPEUTIC AGENT FOR THE TREATMENT OF INFLAMMATORY DISEASES

(57) Abstract: The current invention provides a recombinant bacterium, the recombinant bacterium being genetically modified to decrease or eliminate the display of lipoteichoic acid (LTA), surface layer protein B (SlpB) and surface layer protein X (SlpX) on the surface of said bacterium. Efficacious therapies for a subject suffering from an inflammation mediated disease are also provided. The methods of the current invention comprise administering to a subject in need thereof a therapeutically effective amount of the recombinant *L. acidophilus* cells or a therapeutically effective amount of the isolated surface layer protein A (SlpA) or a non-naturally occurring derivative thereof. The recombinant *L. acidophilus* cells or SlpA isolated from *L. acidophilus* can be in a pharmaceutical composition comprising a pharmaceutically acceptable carrier and/or excipient. In an embodiment of the invention, the pharmaceutical composition is administered orally.



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DESCRIPTION

LACTOBACILLUS ACIDOPHILUS SURFACE LAYER PROTEIN A (SLPA)
AS A THERAPEUTIC AGENT FOR THE TREATMENT OF
INFLAMMATORY DISEASES

CROSS-REFERENCE TO RELATED APPLICATION

This application claims the benefit of U.S. Provisional Application Serial No. 62/068,338, filed October 24, 2014, the disclosure of which is hereby incorporated by reference in its entirety, including all figures, tables and amino acid or nucleic acid sequences.

This invention was made with government support under R01 AI093370 awarded by National Institutes of Health. The government has certain rights in the invention.

BACKGROUND OF THE INVENTION

The gastrointestinal (GI) microbiota plays a critical role in determining the immunologic outcome of various signaling events in host cells via their gene products, exceeding the human genome by a hundredfold (Ley et al., 2006; Qin et al., 2010). As such, the composition of the GI microbiota and host immunity are mutualistic and continuously influence each other (Maslowski and Mackay, 2011; McDermott and Huffnagle, 2014).

Intestinal homeostasis is tightly controlled by regulatory immune mechanisms, which are established by the interactions of the trillions of microbes and their gene products with numerous pattern recognition receptors (PRRs), including C-type lectin receptors (CLRs), such as SIGNR3 (Konstantinov et al., 2008; Osorio and Reis e Sousa, 2011). Disruption of this delicate balance by inimical signals has devastating consequences that may result in intestinal disorders, including inflammatory bowel disease (IBD). When this occurs, highly activated innate cells trigger intestine-infiltrating pathogenic T cell subsets (*e.g.*, Th1, Th17), and regulatory T cells (Tregs) with pro-inflammatory characteristics (Geremia et al., 2014; Khazaie et al., 2012; Neurath, 2014) that ultimately drive tissue destruction and intestinal disease progression. Innate cells (*e.g.*, dendritic cells, macrophages) are the initial targets of either culpable microbes or their gene products, which subsequently affect the regulation/stimulation of intestinal immunity (Atarashi et al., 2013; Ivanov and Honda, 2012; Yang et al., 2014). Given these entwined relationships, it is not surprising that microbial

products have been linked to the pathology of intestinal auto-inflammation (Nicholson et al., 2012). The underlying associations between gut microbes and inflammatory diseases (*e.g.*, IBD) have been well documented; however, the cellular and molecular mechanisms by which intestinal commensal gene product(s) and their molecular receptor(s) impact immune responses remain unclear.

Information regarding the immunobiologic functions of *Lactobacillus acidophilus* surface layer proteins (Slps) is relatively limited. Slps are paracrystalline (glyco) protein arrays that are abundantly present on the cell surfaces of most eubacteria and archaea, including *L. acidophilus* (Johnson et al., 2013). *L. acidophilus* NCFM possesses three Slp-encoding genes: *slpA* (LBA0169), *slpB* (LBA0175), and *slpX* (LBA0512) (Goh et al., 2009). Diverse functional roles have been proposed for Slps, including cell shape determinants, molecular sieves, protective layers against viral infection, anchoring sites for surface-associated enzymes and facilitators of cellular adhesion through PRRs, including C-type lectins (CLECs) (Konstantinov et al., 2008).

CLECs recognize carbohydrate structures on self and non-self antigens (Engering et al., 2002; Osorio and Reis e Sousa, 2011). Eighteen CLECs, including DC-specific ICAM-3-grabbing nonintegrin (DC-SIGN), have been identified on dendritic cells (DCs) and macrophages (MΦs) (Ehlers, 2010; van Kooyk and Geijtenbeek, 2003). DC-SIGN, which was previously shown to bind *L. acidophilus*-SlpA *in vitro* (Konstantinov et al., 2008), is a calcium-dependent carbohydrate-binding protein with specificity for the mannose-containing glycans of microbial surface components and fucose-containing Lewis antigens (Ehlers, 2010). Of the eight murine homologs of DC-SIGN, SIGNR3 (CD209d) exhibits the most biochemical similarity to human DC-SIGN (Powlesland et al., 2006).

SIGNR3 contains a carbohydrate recognition domain (CRD) and signals through a hemi-immunoreceptor tyrosine-based activation motif (hemi-ITAM) pathway (Tanne et al., 2009). Such signaling potentially downregulates the ubiquitously expressed leukotriene A₄ hydrolase (LTA₄H) (Tobin et al., 2010) that catalyzes proinflammatory leukotriene B₄ (LTB₄) synthesis from LTA₄ (Snelgrove et al., 2010), which consequently activates interleukin (IL)-1β production. Here, we identify SlpA as a key effector molecule expressed by *L. acidophilus*, and demonstrate its *in vivo* protective role in murine colitis models. Moreover, we provide evidence that protection by *L. acidophilus*-SlpA is conferred via signaling through a single CLR, namely SIGNR3.

As discussed above, normal gut immune responses dictate that resident innate and adaptive immune cells must coexist with the large number of microbes inhabiting the GI tract while still being able to mount an immune response against invading pathogens. Maintenance of immune homeostasis toward commensal bacteria and their microbial gene products is essential in the prevention of chronic inflammation in the gut. Overt intestinal inflammation is a hallmark of IBD. Current therapies for the management of IBD include antibiotic regimens to prevent the outgrowth and systemic dissemination of pathogenic microorganisms, as well as corticosteroids and immunomodulators to decrease the inflammatory response in the intestines. However, these therapies are not without undesirable and harmful side effects, as antibiotics also deplete the beneficial intestinal microflora, and corticosteroids and immunomodulators act as global immune suppressors, thereby increasing the risk of infection and cancer. Thus, there is a need for identifying new therapeutic agents for the treatment of such diseases.

BRIEF SUMMARY OF THE INVENTION

The current invention provides a recombinant bacterium, for example, a recombinant *Lactobacillus acidophilus*, the recombinant bacterium being genetically modified to decrease or eliminate the display of lipoteichoic acid (LTA), surface layer protein B (SlpB) and surface layer protein X (SlpX) on the surface of said bacterium.

The current invention also provides an efficacious therapy for a subject suffering from an inflammation mediated disease (inflammatory diseases), for example, an autoimmune disease, such as IBD or other inflammatory diseases, such as allergies, ankylosing spondylitis, Crohn's disease, diabetes, Type I diabetes, gastroesophageal reflux disease, Hashimoto's thyroiditis, hyperthyroidism, hypothyroidism, interstitial cystitis (IC), Löfgren's syndrome, lupus erythematosus, myasthenia gravis, multiple sclerosis, osteoarthritis, polymyalgia rheumatica, prostatitis, psoriasis, psoriatic arthritis, Raynaud's syndrome/phenomenon, reactive arthritis (Reiter syndrome), restless leg syndrome, reflex sympathetic dystrophy (RSD), rheumatoid arthritis, scleroderma, Sjögren's syndrome, ulcerative colitis and uveitis. The methods of the current invention comprise administering to a subject in need thereof a therapeutically effective amount of recombinant *L. acidophilus* cells of the current invention or a therapeutically effective amount of purified surface layer protein A (SlpA), for example, SlpA isolated from *L. acidophilus*. In one embodiment, the *L. acidophilus* cells belong to *L. acidophilus* strain NCK2187 which is a bacterium genetically

modified to decrease or eliminate the display of LTA, SlpB and SlpX on the surface. Another embodiment provides for the recombinant expression of SlpA in bacterial cells that are devoid of LTA, SlpB and SlpX expression on the cell surface.

The recombinant bacterial cells (e.g., *L. acidophilus* cells) or SlpA isolated from *L. acidophilus* can be formulated into a pharmaceutical composition comprising a pharmaceutically acceptable carrier and/or excipient (optionally in combination with other therapeutic agents). In an embodiment of the invention, the pharmaceutical composition is administered orally.

BRIEF DESCRIPTION OF THE DRAWINGS

The patent or application file contains at least one drawing executed in color. Copies of this patent or patent application publication, with color drawing(s), will be provided by the Office upon request and payment of the necessary fee.

Figures 1A-1E. *L. acidophilus* NCK2187 strain development and characteristics. A. Agarose gel image illustrating PCR amplicons of *ltaS* (LBA0447), *slpB*, and *slpX* deletions in NCK2187. B. SDS-PAGE gel of 5 M LiCl-purified S-layer fractions from the parental strains, NCK56 and NCK1909; NCK2030 (LTA⁺, SlpA⁺, SlpB⁻, SlpX⁻); and NCK2187 (LTA⁻, SlpA⁺, SlpB⁻, SlpX⁻). C. Protein gel showing predominance of SlpA in NCK2187 and absence of other Slps. D. B6 mice were orally gavaged with 10⁹ CFU erythromycin-resistant NCK56 or NCK2187. Fecal pellets were collected daily and tested for the presence of erythromycin-resistant strains. n = 3 mice/group. Data are representative of five independent experiments and are shown as mean ± SEM. E. Colonic LP cells were co-cultured with NCK56 or NCK2187 (1:1) and secreted cytokines were measured in the supernatants. Data are shown as mean ± SEM. *P<0.05, **P<0.01, ***P<0.001.

Figures 2A-2C. *L. acidophilus* NCK2187 promotes intestinal regulation in steady-state. A. B6 mice were orally gavaged with 10⁹ CFU NCK56 (blue) or NCK2187 (green) on days 0, 3, 6, and 9, or left untreated, and immune responses in the colon analyzed at day 14 by flow cytometry. B-C. FoxP3-GFP mice were treated and evaluated as in (A). C. Regulatory cytokine production in FoxP3-GFP⁺ (green dotted bars) versus FoxP3-GFP⁻ (white bars) cells was measured by intracellular staining and FACS analyses. n = 5 mice/group. Data represent four individual experiments and are shown as mean ± SEM. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001. Black asterisks compare NCK2187 to untreated (PBS) mice, and red asterisks to NCK56-treated mice.

Figures 3A-3D. *L. acidophilus* NCK2187 and its SlpA protect against pathogenic T-cell induced colitis. *RagI*^{-/-} mice were injected with 10⁶ CD4⁺CD45RB^{hi} T cells, and then orally gavaged with NCK56 (red), NCK2187 (green), or SlpA (blue), 1 and 3 days after transfer, and subsequently once a week for 4 consecutive weeks, or left untreated (magenta). A group of mice was co-transferred with CD4⁺CD25⁺ T cells as a positive control for protection (Tregs; gray). Colitis severity was determined in part by weight loss, diarrhea scores, and FOB (A). (See Tables 2-4 for statistical analyses results.) B-C. Colitis scores based on histopathology and gross morphology of the colons were also used as measures of disease. Scale bar = 200 μm. D. Circulating levels of proinflammatory cytokines were measured in the sera of the mice transferred and treated as mentioned above, or sham adoptive transferred (white bars). n = 5 mice/group. Data represent three individual experiments and are shown as mean ± SEM. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001. Black asterisks compare NCK2187 to PBS-treated adoptively transferred mice, and red asterisks to NCK56-treated mice.

Figures 4A-4D. *L. acidophilus* NCK2187 and its SlpA protect intestinal barrier function and prevent dysbiosis in pathogenic T-cell induced colitis. *RagI*^{-/-} mice were injected with 10⁶ CD4⁺CD45RB^{hi} T cells, and then orally gavaged with NCK56 (red), NCK2187 (green), or SlpA (blue), 1 and 3 days after transfer, and subsequently once a week for 4 consecutive weeks, or left untreated (magenta). A group of mice was co-transferred with CD4⁺CD25⁺ T cells as a positive control for protection (Tregs; gray). A. Colonic expression of tight junction-associated genes *Cldn3* and *Ocln*, determined by RT-PCR, as well as passive transepithelial absorption of FITC-dextran, were used as measures of epithelial barrier integrity. Sham adoptive transferred *RagI*^{-/-} mice (white bars) were used as baseline controls in some cases. n = 5 mice/group. Data represent three individual experiments and are shown as mean ± SEM. *P<0.05, **P<0.01. Black asterisks compare NCK2187 to PBS-treated adoptively transferred mice, and red asterisks to NCK56-treated mice. B. UniFrac analyses were used to calculate distances between the microbial communities of the different samples (week 7) and three-dimensional scatterplots were generated by using principal coordinate analyses (PCoA). Gray dots = CD4⁺CD45RB^{hi} T cells + PBS; yellow dots = CD4⁺CD45RB^{hi} T cells + NCK56; green dots = CD4⁺CD45RB^{hi} T cells + NCK2187; blue dots = CD4⁺CD45RB^{hi} T cells + SlpA; red dots = CD4⁺CD45RB^{hi} T cells + Tregs. Each dot represents the fecal microbiota data of an individual mouse. C. Changes in the relative abundance of different phyla were also analyzed. (See Table 5 for

statistical analyses among the different groups.) Dark blue = Actinobacteria; green = Bacteroidetes; red = Proteobacteria; yellow = Verrucomicrobia; aqua = Firmicutes; purple = Tenericutes; black = Others. D. Comparison of microbial communities at family or order levels. The heat map depicts the relative value in individual mice. n = 5-6 mice/group.

Figures 5A-5D. *L. acidophilus* NCK2187 and its SlpA bind to murine SIGNR3 to induce regulatory signals. A. B6 mice were orally gavaged with 10^9 CFU NCK56 or NCK2187 and the colonic gene expression of C-type lectin receptors were measured by RT-PCR. Each box represents an individual mouse; n = 4. Data represent three individual experiments and are shown as mean $\pm$ SEM. B. Binding of SlpA to various hFc fusion proteins were analyzed by flow cytometry. Gray tinted line = SlpA-coated beads only; orange = SlpA-coated beads + secondary antibody; green = SlpA-coated beads + control fusion protein; blue = SlpA-coated beads + SIGNR1-hFc; red = SlpA-coated beads + SIGNR3-hFc. Binding assays results were confirmed five independent times. C. IL-1 β production by colonic DCs of naïve WT B6 GF or *Signr3*^{-/-} mice treated with NCK56 (blue) or NCK2187 (green) on days 0, 3, 6, and 9, or left untreated (black), as determined by flow cytometry. D. Frequency of colonic FoxP3⁺ Tregs in KO mice treated with NCK56 or NCK2187 was measured by flow cytometry. n = 5 mice/group. Data represent four individual experiments and are shown as mean $\pm$ SEM. *P<0.05. Black asterisks compare NCK2187 to untreated (PBS) mice, and red asterisks to NCK56-treated mice.

Figures 6A-6H. *L. acidophilus* NCK2187 and its SlpA do not protect against DSS-induced colitis in *Signr3*^{-/-} mice. WT or *Signr3*^{-/-} (KO) mice were orally gavaged with NCK56, NCK2187 or SlpA at days -1 and -3, and 3% DSS was given in the drinking water. Mice were gavaged with bacteria or purified SlpA an additional 2 times and monitored for disease progression. Colitis severity was determined in part by weight loss (A). (See Table 6 for statistical analyses from WT mice.) B-C. Colitis scores based on histopathology, and gross morphology of the colons were also used as measures of disease. Scale bar = 200 μ m. n = 5 mice/group. Empty bars = WT; lined bars = KO; white bars = untreated; purple bars = DSS; red bars = DSS + NCK56; green bars = DSS + NCK2187; blue bars = DSS + SlpA. D. Colonoscopies were performed in the different groups with a Multi-Purpose Rigid™ Telescope attached to a TELE PACK X. E. Mean relative colonic expression of tight junction-associated genes in WT mice. F. Fecal albumin levels in WT mice as a measure of intestinal permeability. G. UniFrac analyses were used to calculate distances between the microbial communities of the different samples (Day 10) and three-dimensional scatterplots

were generated by using PCoA. Light gray: WT + DSS; green: WT + DSS + NCK56; brown: WT + DSS + NCK2187; blue: WT + DSS + SlpA; aqua: KO + DSS; red: KO + DSS + NCK56; yellow: KO + DSS + NCK2187; purple: KO + DSS + SlpA. n = 4-6 mice/group. H. Species richness and microbial diversity in DSS-treated mice. Top: The Chao richness index was used as a measure of species richness. Bottom: The Shannon diversity index was used to estimate microbial diversity for each group. Data are shown as mean $\pm$ SEM. *P<0.05, **P<0.01, ****P<0.0001.

Figures 7A-7C. *L. acidophilus* NCK2187 and its SlpA do not prevent immune infiltration and activation during DSS-induced colitis in *Signr3*^{-/-} mice. WT or *Signr3*^{-/-} (KO) mice were orally gavaged with NCK56, NCK2187 or SlpA at days -1 and -3, and 3% DSS was given in the drinking water. Mice were gavaged with bacteria or purified SlpA an additional 2 times, and immunity analyzed by flow cytometry at day 10. A. Representative plots indicate the frequency of neutrophils in the colons of untreated or DSS-treated WT (left) and *Signr3*^{-/-} mice (right). B-C. Colonic DCs and MΦs were analyzed by flow cytometry for the production of IL-1β (B), and colonic Tregs were evaluated for co-expression of RORγt⁺ (C). n = 5 mice/group. Gray tinted line = isotype control; black = untreated; purple = DSS; red = DSS + NCK56; green = DSS + NCK2187; blue = DSS + SlpA. Data are shown as mean $\pm$ SEM, and are representative of three different experiments. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.

Figures 8A-8B. *L. acidophilus* NCK2187 and its SlpA in pathogenic T-cell induced colitis. *Rag1*^{-/-} mice were injected with 10⁶ CD4⁺CD45RBhi T cells, and then orally gavaged with NCK56 (red), NCK2187 (green), or SlpA (blue), 1 and 3 days after transfer, and subsequently once a week for 4 consecutive weeks, or left untreated (magenta). A group of mice was co-transferred with CD4⁺CD25⁺ T cells as a positive control for protection (Tregs; gray). A. Colonic expression of *Ltb4r1*, *Ltb4r2*, and *Il1b* were determined by RT-PCR. n = 5 mice/group. Data represent three individual experiments and are shown as mean $\pm$ SEM. *P<0.05, **P<0.01. Black asterisks compare NCK2187 to PBS-treated adoptively transferred mice, and red asterisks to NCK56-treated mice. B. Colonoscopies were performed in the specified groups with a Multi-Purpose Rigid™ Telescope attached to a TELEPACK X.

Figure 9. *L. acidophilus* NCK2187 and its SlpA cannot normalize sera cytokine levels upon DSS-induced colitis in *Signr3*^{-/-} mice. WT or *Signr3*^{-/-} mice were orally gavaged with NCK56, NCK2187 or SlpA at days -1 and -3, and 3% DSS was given in the

drinking water. Mice were gavaged with bacteria or purified SlpA an additional 2 times. Cytokine levels were measured in the sera at the endpoint of the experiment. $n = 4$ mice/group. Empty bars = WT; lined bars = KO; white bars = untreated; purple bars = DSS; red bars = DSS + NCK56; green bars = DSS + NCK2187; blue bars = DSS + SlpA. Data are shown as mean $\pm$ SEM. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Figures 10A-10B. Dextran sodium sulfate (DSS) treatment and oral gavage regimens. (A) DSS-induced colitis prevention study; mice were orally gavaged with NCK56, NCK2187 or SlpA at the specified time points. 3% DSS was given in the drinking water from days 0 to 5. (B) DSS-induced colitis therapy. 3% DSS was given in the drinking water from days 0 to 5. Mice were then orally gavaged with NCK56, NCK2187, or SlpA at the circled time points.

Figures 11A-11E. *L. acidophilus*-SlpA protects against DSS-induced colitis. C57BL/6 mice were orally gavaged with NCK56, NCK2187 or SlpA, and 3% DSS was given in the drinking water. Colitis development in the mice was monitored by measuring weight loss (A), diarrhea development (B), presence of fecal occult blood (FOB) (C), gross morphology of the colons (D), and histopathology (E). * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ representing the specified group by its color compared with PBS.

Figures 12A-12E. *L. acidophilus*-SlpA reverses DSS-induced colitis. C57BL/6 mice were given 3% DSS in the drinking water, and were then orally gavaged with NCK56, NCK2187 or SlpA. Colitis development in the mice was monitored by measuring weight loss (A), diarrhea development (B), presence of fecal occult blood (FOB) (C), gross morphology of the colons (D), and histopathology (E). * $P < 0.05$ and ** $P < 0.01$ representing the specified group by its color compared with PBS.

Figures 13A-13E. *L. acidophilus*-SlpA protects against pathogenic T cell-induced colitis. *Rag*^{1-/-} mice were injected with 10^6 CD4⁺CD25⁻ T cells, and were then orally gavaged with NCK56, NCK2187, or SlpA, 1 and 3 days after transfer, and subsequently once a week for 4 consecutive weeks. Colitis development in the mice was monitored by measuring weight loss (A), diarrhea development (B), presence of fecal occult blood (FOB) (C), gross morphology of the colons (D), and histopathology (E). * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ representing the specified group by its color compared with PBS.

Figures 14A-14B. NCK2187 induces the generation of Tregs in healthy controls. C57BL/6 mice were orally gavaged with 10^9 CFU NCK56 (WT), NCK2187 (SlpA⁺), or left untreated; immune responses were analyzed by flow cytometry 3 days-post gavage. When

compared to untreated or NCK56-treated mice, NCK2187 led to an expansion of total Tregs in the mesenteric lymph nodes (MLN; Figure 14A) and spleens (Figure 14B) of the mice tested. **P<0.01 and ***P<0.001 compared with PBS.

Figures 15A-15C. *L. acidophilus*-SlpA isolation by NaCl. *L. acidophilus*-SlpA was isolated and purified using NaCl. **A.** SDS-PAGE containing 2.5 µg of LiCl- and NaCl-isolated SlpA stained with Coomassie blue to visualize the purified protein. **B-C.** Mass spectrometry data analyzed on the Scaffold (Searle 2010) platform showed 97 unique spectra with 55 unique peptides with the possibility of two proteins (**B**). The predicted protein gi|58336516 (SlpA) shows 54% coverage whereas gi|362076610 (SlpB) reveals only 18% of coverage (highlighted portion. **C**). The regions of SlpB matching the generated peptides are common between SlpA and SlpB (shown in the red box, **C**), and no single unique peptide from SlpB was identified.

Figures 16A-16E. NaCl-purified SlpA is not toxic to mice. **A-E.** C57BL/6 mice were treated orally every other day with SlpA (0, 150, 300, 600 µg/100µL per mouse), for a total of four times. One-week later, mice were sacrificed and a whole blood chemistry profile was generated for each mouse with a comprehensive metabolic chemistry panel, using a VetScan V2S analyzer. All animal experiments were performed under the guidelines of the Animal Welfare Act and the Public Health Policy on Humane Care, and with approval by the Institutional Animal Care and Use Committee (IACUC protocol 201406559) at the University of Florida.

Figures 17A-17E. Generated mAb BM1 recognizes *L. acidophilus*-SlpA. C57BL/6 mice were immunized once a week for 3 months with 100 µg of SlpA, and 300 µg of heat-killed *Lactobacillus gasseri* as adjuvant. Polyclonal sera were tested for recognition of isolated SlpA by Western Blot (WB), and splenic cells from SlpA-reactive mice were fused with Sp2/0 myeloma cells at a ratio of 7:1. Hybridomas were seeded on semi-solid medium for clone selection and screening. Subsequently, clones were screened by ELISA for SlpA reactivity. Reactive clones were isotyped and all IgM secretors removed. Clone BM1 (IgG) was selected for its ability to recognize SlpA by WB (**A**), flow cytometry (**B**), confocal microscopy (**C**), and ELISA (**D, E**). **A.** *L. acidophilus*-SlpA detection by WB with BM1. 100 ng of purified SlpA, 10⁸ CFU *L. acidophilus* (*L. a.*), 10⁸ CFU *L. reuteri* (*L. r.*), or 100 ng of BSA. Proteins separated by SDS-PAGE were transferred onto a PVDF membrane and detected by BM1. **B.** *L. acidophilus*-SlpA detection with BM1 by flow cytometry. Carboxylated Dynabeads were coated with purified SlpA and reactivity of BM1 mAb

confirmed by Canto II flow cytometry. Data were analyzed by FlowJo. Experiments were performed at least three times with similar trends. **C.** *L. acidophilus*-SlpA detection with BM1 by confocal microscopy. RAW 264.7 cells were pulsed for 1 or 3 hrs with NaCl purified SlpA (10 µg/mL). Subsequently, cells were fixed and stained with BM1 mAb for detection by confocal microscopy. Cells were incubated with BM1 mAb, overnight. Cells were washed and subsequently incubated with a secondary antibody (Alexa Fluor 488 anti-mouse IgG1, 1:100) for 4 hrs. Nuclei were stained with DAPI (15 min) and visualized by a Zeiss confocal microscope. **D.** *L. acidophilus*-SlpA detection with BM1 by ELISA. ELISA plates were coated with 500 ng of purified SlpA overnight, and binding by BM1 was tested thereafter. **E.** Germ-free (GF) mice were orally treated with 10^9 CFU *L. acidophilus*, 150 µg of SlpA, or left untreated. Fecal pellets from these mice were used to coat ELISA plates; BSA was used as a negative control. BM1 by ELISA. ELISA plates were coated with 500 ng of purified SlpA overnight, and binding by BM1 was tested thereafter. **E.** Germ-free (GF) mice were orally treated with 10^9 CFU *L. acidophilus*, 150 µg of SlpA, or left untreated. Fecal pellets from these mice were used to coat ELISA plates; BSA was used as a negative control. BM1 mAb only bound to plates coated with feces derived from treated mice. All animal experiments were performed under the guidelines of the Animal Welfare Act and the Public Health Policy on Humane Care, and with approval by the Institutional Animal Care and Use Committee (IACUC protocol 201406559) at the University of Florida. ***denotes statistical significance $p<0.01$, *** $p<0.001$.

BRIEF DESCRIPTION OF THE SEQUENCES

SEQ ID NO: 1: Amino acid sequence of phosphoglycerol transferase protein from *L. acidophilus* (Genbank Accession No. AAV42337.1).

SEQ ID NO: 2: Amino acid sequence of SlpB protein from *L. acidophilus* (Genbank Accession No. YP_193105).

SEQ ID NO: 3: Amino acid sequence of SlpX protein from *L. acidophilus* (Genbank Accession No. YP_193425).

SEQ ID NO: 4: Amino acid sequence of SlpA protein from *L. acidophilus* (Genbank Accession No. P35829).

SEQ ID NO: 5 to 46: The list of primer sequences for Real-Time PCR analyses (see Table 1).

SEQ ID NO: 47 to 55: SlpA protein sequences as discussed below.

DETAILED DISCLOSURE OF THE INVENTION

The current invention provides a bacterium lacking on its surface LTA, SlpB and SlpX. The current invention also provides SlpA as an effector molecule expressed by bacteria, for example, *L. acidophilus* and its *in vivo* protective role in inflammation mediated diseases (inflammatory diseases), for example, inflammation mediated diseases of the gastrointestinal tract such as IBD or other inflammatory diseases, such as allergies, ankylosing spondylitis, Crohn's disease, diabetes, Type I diabetes, gastroesophageal reflux disease, Hashimoto's thyroiditis, hyperthyroidism, hypothyroidism, Irritable Bowel Syndrome (IBS), interstitial cystitis (IC), Löfgren's syndrome, lupus erythematosus, myasthenia gravis, multiple sclerosis, osteoarthritis, polymyalgia rheumatica, prostatitis, psoriasis, psoriatic arthritis, Raynaud's syndrome/phenomenon, reactive arthritis (Reiter syndrome), restless leg syndrome, reflex sympathetic dystrophy (RSD), rheumatoid arthritis, scleroderma, Sjögren's syndrome, ulcerative colitis and uveitis.

A bacterium lacking LTA and methods of preparing such bacterium are described in US Patent Application Publication 20130224153, the contents of which are incorporated by reference herein in its entirety, particularly, paragraphs [0031] to [0034]. In addition to modifications required to decrease or eliminate display of LTA on the surface, the current invention provides a bacterium further modified to decrease or eliminate the display of SlpB and SlpX on the surface. In some embodiments, bacterial cells lacking LTA expression on the cell surface are genetically modified to decrease or eliminate SlpB and SlpX expression on the cell surface. Such cells can be genetically modified to expression SlpA and used in the methods disclosed herein. Yet other embodiments utilize bacterial cells genetically modified to express SlpA but which lack LTA expression on the cell surface and also lack genes encoding SlpB and SlpX or orthologs of SlpB and SlpX (i.e., protein homologs that are present within different species and have very similar or identical function). Non-limiting examples of such bacterial cells include those that lack genes encoding phosphoglycerol transferase protein (Genbank Accession No. AAV42337.1; SEQ ID NO: 1), SlpB and SlpX or orthologs of phosphoglycerol transferase (Genbank Accession No. AAV42337.1), SlpB and SlpX. For example, the cells do not express SEQ ID NO: 2 or the SlpB polypeptides associated with Uniprot access numbers Q48508, C2HR61, Q5FMK0, Q8GFE5, J9W284, J9W905, B1H0V4, H6VTN5, Q09FL7, V7HZR4, S4NDQ7, S4NKH4, S4NL65 and S4NQU9 and SEQ ID NO: 3 or the SlpX polypeptides associated with SlpX protein are

provided by Uniprot access numbers C2HMW6, S6E4Y8, S6DRU6, S6DL03, S6E593, S6DQJ3, F0TJ46, Q5FLN0, D4YUC6, R5ZGF3, E4SM72, F2M2V8, C2KB60, D5H1S0, I7KQ44, U6FUJ7, U6FJC0, U6F914, U6F7V6, U6F834, U4QN79, U4QA33, F3MP54, F0NWR2, F0NVR1, F6CEM8, F6CBQ1, I7JYF2 and C2ELK0.

5 In certain embodiments, the phosphoglycerol transferase protein comprises SEQ ID NO: 1, SlpB protein comprises the amino acid sequence of SEQ ID NO: 2 and SlpX protein comprises the amino acid sequence of SEQ ID NO: 3. Accordingly, in addition to the modifications required to reduce the surface display of LTA, the bacterium of the current invention has been further genetically modified to decrease or eliminate the expression of a
10 polypeptide comprising amino acid sequence of SEQ ID NO: 1, comprising amino acid sequence of SEQ ID NO: 2 and a polypeptide comprising the amino acid sequence of SEQ ID NO: 3. In an embodiment, in addition to the modifications required to reduce or eliminate the surface display of LTA, the bacterium of the current invention has decreased or eliminated expression of a polypeptide comprising the amino acid sequence having at least 70%, 80%,
15 90%, 95%, 97%, 98%, or 99% sequence identity to the amino acid sequence of SEQ ID NO: 1, at least 70%, 80%, 90%, 95%, 97%, 98%, or 99% sequence identity to the amino acid sequence of SEQ ID NO: 2 and a polypeptide comprising the amino acid sequence having at least 70%, 80%, 90%, 95%, 97%, 98%, or 99% sequence identity to the amino acid sequence of SEQ ID NO: 3.

20 Certain examples of phosphoglycerol transferase protein from *L. acidophilus* (Genbank Accession No. AAV42337.1) protein are provided by Uniprot access numbers Q5FLT7, A0A0D5MGR2, F3MQQ2, U6F845, U6FPM2, U6FK51, A8YTT6, U6F7B9, C7XM54, E3R4H1, C2KGR7, D0DKS2 and K1NT37 (each of which is hereby incorporated by reference in their entireties). Additional examples of phosphoglycerol transferase proteins
25 are well known to a person of ordinary skill in the art and such embodiments are within the purview of the current invention.

Certain examples of SlpB protein are provided by Uniprot access numbers Q48508, C2HR61, Q5FMK0, Q8GFE5, J9W284, J9W905, B1H0V4, H6VTN5, Q09FL7, V7HZR4, S4NDQ7, S4NKH4, S4NL65 and S4NQU9 (each of which is hereby incorporated by
30 reference in their entireties). Additional examples of SlpB proteins are well known to a person of ordinary skill in the art and such embodiments are within the purview of the current invention.

Certain examples of SlpX protein are provided by Uniprot access numbers C2HMW6, S6E4Y8, S6DRU6, S6DL03, S6E593, S6DQJ3, F0TJ46, Q5FLN0, D4YUC6, R5ZGF3, E4SM72, F2M2V8, C2KB60, D5H1S0, I7KQ44, U6FUJ7, U6FJC0, U6F914, U6F7V6, U6F834, U4QN79, U4QA33, F3MP54, F0NWR2, F0NVR1, F6CEM8, F6CBQ1, I7JYF2
5 and C2ELK0 (each of which is hereby incorporated by reference in their entireties). Additional examples of SlpX proteins are well known to a person of ordinary skill in the art and such embodiments are within the purview of the current invention.

The genetic modifications resulting in decreased or eliminated expression of the polypeptides include, but are not limited to, deleting the entire coding region of the gene or a
10 portion of the coding nucleotide sequence, introducing a frame shift mutation, a missense mutation, an insertion, by introducing a stop codon or a combinations of any of the aforementioned mutations. Additional mutations which would lead to decreased, or eliminated, expression of a polypeptide of interest and methods of introducing such mutations into a bacterium are well known to a person of ordinary skill in the art and such embodiments
15 are within the purview of the claimed invention. In one embodiment of the invention, the *upp*-countersselective knockout strategy (described in Goh et al., 2009) which is hereby incorporated by reference in its entirety) was used to generate in-frame deletions in the *slpB* and *slpX* genes of *L. acidophilus* NCK2030 to produce *L. acidophilus* NCK2187.

In a certain embodiment, the lactic acid bacterium is a surface layer protein
20 expressing *Lactobacillus*. These bacterial cells may also be referred to as probiotic bacterial cells. Non-limiting examples of such lactic acid bacteria include, but are not limited to, *L. acidophilus*, *L. amylolyticus*, *L. amylovorus*, *L. brevis*, *L. brevis* ssp *gravesensis*, *L. buchneri*, *L. crispatus*, *L. gallinarum*, *L. gigeriorum*, *L. helveticus/suntoryeus*, *L. hilgardii*, *L. kefiranoferiens*, *L. pasteurii*, *L. lactis* and *L. ultunensis*.

25 An embodiment provides a composition comprising the recombinant bacterium of the current invention and a pharmaceutically acceptable carrier and/or excipient.

The bacteria of the current invention provide protective role in inflammation mediated diseases, for example, inflammation mediated diseases of gastrointestinal tract such IBD. Accordingly, certain embodiments of the current invention provide methods of treating
30 and/or preventing an inflammation mediated disease of the gastrointestinal system in a subject, the method comprising, administering to the subject a therapeutically effective amount of the bacterium of the current invention. In one embodiment, the bacterium is orally administered to the subject.

In certain embodiments of the invention a subject is a mammal. Non-limiting examples of a mammal treatable according to the methods of the current invention include mouse, rat, dog, guinea pig, cow, horse, cat, rabbit, pig, monkey, ape, chimpanzee, and human. Additional examples of mammals treatable with the methods of the current invention are well known to a person of ordinary skill in the art and such embodiments are within the purview of the current invention.

For the purposes of the current invention, a probiotic food refers to a food which contains microorganisms associated with beneficial effects to humans and animals upon ingestion of the probiotic food. Non-limiting examples of probiotic food include yogurt, fermented vegetable, kefir, sauerkraut, miso soup, pickle, tempeh and kimchi.

For the purposes of this invention the term “inflammation mediated disease” or “inflammatory disease” refers to a disease characterized by a dysregulation of the normal immune response. Inflammation mediated diseases (inflammatory diseases) can cause organ damage, and are associated with increased morbidity and/or mortality. An example of immune dysregulation is the inappropriate activation of inflammatory cytokines, such as IL-12, IL-6 or TNF alpha, whose actions lead to pathological consequences.

For the purposes of this invention the terms “treatment, treating, treat” or equivalents of these terms refer to healing, alleviating, relieving, altering, remedying, ameliorating, improving, or affecting the condition or the symptoms of a subject suffering with a disease, for example, a gastrointestinal disorder. The subject to be treated can be suffering from or at risk of developing the disorder, for example, a gastrointestinal disorder, including, for example, an IBD or be at risk of developing an IBD. When provided therapeutically, the bacterium is provided at (or shortly after) the onset of a symptom. The therapeutic administration of the substance serves to attenuate any actual symptom.

For the purposes of this invention, the terms “preventing, preventive, prophylactic” or equivalents of these terms are indicate that the recombinant bacterium is provided in advance of any disease symptoms and are a separate aspect of the invention (i.e., an aspect of the invention that is distinct from aspects related to the terms “treatment, treating, treat” or equivalents of these terms which refer to healing, alleviating, relieving, altering, remedying, ameliorating, improving, or affecting the condition or the symptoms of a subject suffering with an inflammatory disease, for example, a gastrointestinal disorder). The prophylactic administration of the recombinant bacterium serves to prevent or attenuate any subsequent symptoms or disease.

By “therapeutically effective dose,” “therapeutically effective amount”, or “effective amount” is intended to be an amount of a recombinant bacterium disclosed herein or the amount of SlpA that, when administered to a subject, decreases the inflammatory response, or reduces any increase in an inflammatory response as compared to untreated subjects.

5 “Positive therapeutic response” refers to, for example, improving the condition of at least one of the symptoms of an inflammatory disorder.

An effective amount of the therapeutic agent is determined based on the intended goal. The term “unit dose” refers to a physically discrete unit suitable for use in a subject, each unit containing a predetermined quantity of the therapeutic composition calculated to

10 produce the desired response in association with its administration, *i.e.*, the appropriate route and treatment regimen. The quantity to be administered, both according to number of treatments and unit dose, depends on the subject to be treated, the state of the subject and the protection desired. Precise amounts of the therapeutic composition also depend on the judgment of the practitioner and are peculiar to each individual. Generally, the dosage of

15 recombinant bacteria will vary depending upon such factors as the patient's age, weight, height, sex, general medical condition and previous medical history. In specific embodiments, it may be desirable to administer the bacterium in the range of about 10^4 to about 10^{12} CFU, 10^5 to 10^{11} CFU, 10^6 to 10^{10} CFU, 10^8 to 10^{10} CFU or 10^8 to 10^{12} CFU.

In some embodiments of the invention, the method comprises administration of

20 multiple doses of the bacterium. The method may comprise administration of 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 35, 40, or more therapeutically effective doses of a composition comprising the bacterium as described herein. In some embodiments, doses are administered over the course of 1 day, 2 days, 3 days, 4 days, 5 days, 6 days, 7 days, 10 days, 14 days, 21 days, 30 days, or more than 30 days. The frequency and duration of administration of

25 multiple doses of the compositions is such as to reduce or prevent an inflammatory response and thereby treat or prevent a gastrointestinal disorder. Moreover, treatment of a subject with a therapeutically effective amount of the recombinant bacterium of the invention can include a single treatment or can include a series of treatments. It will also be appreciated that the effective dosage of a bacterium used for treatment may increase or decrease over the course

30 of a particular treatment. Changes in dosage may result and become apparent from the results of diagnostic assays for detecting inflammation known in the art and described herein.

The present invention also includes combinations of the recombinant bacteria with one another, and/or with one or more other agents useful in the treatment of an inflammation

mediated disease of the GI tract. For example, bacteria of the invention may be administered in combination with effective doses of conventional anti-inflammatory agents, such as sulfasalazine, cyclosporine, prednisone, methylprednisone, budesonide, mesalamine, azathioprine, TNF inhibitors, methotrexate, or 6-mercaptopurine, a corticosteroid, infliximab or combinations thereof, for treatment of inflammation mediated diseases of the GI tract. The term “administration in combination” refers to both concurrent and sequential administration of the active agents. The combination therapies are of course not limited to the agents provided herein, but include any composition for the treatment of inflammatory disorders.

In certain embodiments, the inflammation mediated disease treated according to the current invention is IBD. Non-limiting examples of IBD include Crohn’s disease or ulcerative colitis. Additional examples of IBD are well known to a person of ordinary skill in the art and such embodiments are within the purview of the current invention. As discussed above, the disclosed methods and compositions are aimed at improving the condition of at least one of the symptoms of an inflammatory disorder, such as IBD.

The current invention also provides SlpA as an effector molecule expressed by bacteria and which provide protective role in inflammation mediated diseases, for example, inflammation mediated diseases of gastrointestinal tract such IBD. Accordingly, certain embodiments of the current invention provide an isolated bacterial SlpA protein or a non-naturally occurring protein derivative thereof.

An example of bacterial SlpA protein is provided by a protein having the sequence of SEQ ID NO: 4 or a protein having at least 95% sequence identity to the sequence of SEQ ID NO: 4. Certain other examples of SlpA protein are provided by Uniprot access numbers C2HR60 (SEQ ID NO: 47), P35829 (SEQ ID NO: 48), G1UE81 (SEQ ID NO: 49), Q9Z4J9 (SEQ ID NO: 50), H6VTN4 (SEQ ID NO: 51), Q09FM2 (SEQ ID NO: 52), L7YE91 (SEQ ID NO: 53), K8DVK7 (SEQ ID NO: 54) and F0NUB7 (SEQ ID NO: 55) (each of which is hereby incorporated by reference in its entirety). Additional examples of SlpA proteins are well known to a person of ordinary skill in the art and such embodiments are within the purview of the current invention for use in formulation of the compositions disclosed herein as well as the methods of using such compositions for the treatment of inflammatory diseases.

For the purposes of this invention a “non-naturally” occurring protein derivative indicates that the protein derivative is different than the its naturally occurring counterpart in some manner. Certain examples of modifications which can distinguish a non-naturally

occurring protein derivative from its naturally occurring counterpart include mutations in the amino acid sequences (e.g., point mutations or the introduction of one or more glycosylation site into the protein), non-naturally occurring post-translational modifications (e.g. glycosylation or phosphorylation patterns), attachment to the protein of extraneous molecules (e.g. molecular labels, such as radioisotopes or fluorescent labels, polyethyleneglycol (PEG), etc.). Additional examples of such modifications are well known to a person of ordinary skill in the art and such embodiments are within the purview of the current invention.

In one embodiment, the non-naturally occurring SlpA protein derivative according to the current invention comprises a molecular label conjugated to a bacterial SlpA protein, for example, SlpA protein having the sequence of SEQ ID NO: 4 or the protein having at least 95% sequence identity to the sequence of SEQ ID NO: 4. The label can be a radiolabel, fluorescent label, affinity label, targeting label.

In another embodiment, the non-naturally occurring SlpA protein derivative according to the current invention comprises a protein having one or more mutations in the naturally occurring sequence of a bacterial SlpA protein, for example, SlpA protein having a sequence of SEQ ID NO: 4 or having a sequence at least 95% identical to the sequence of SEQ ID NO: 4. In certain embodiments, the non-naturally occurring SlpA protein derivative comprises about 1 to about 20 mutations, about 3 to about 15 mutations, or about 5 to about 10 mutations. In another embodiment, the mutations do not negatively affect the ability of the non-naturally occurring SlpA protein derivative of the current invention of protecting against inflammation mediated diseases, for example, inflammation mediated diseases of gastrointestinal tract such IBD.

An embodiment of the current invention also provides a composition comprising the non-naturally occurring SlpA protein derivative and a pharmaceutically acceptable carrier and/or excipient.

The pharmaceutically acceptable carrier and/or excipient comprise substances, such as an inert vehicle, or pharmaceutical acceptable adjuvants, preservatives *etc.* Examples pharmaceutically acceptable substances are well known to a person of ordinary skill in the art and such embodiments are within the purview of the current invention.

The pharmaceutical composition may be a liquid formulation or a solid formulation. When the pharmaceutical composition is a solid formulation it may be formulated as a tablet, a sucking tablet, a chewing tablet, a chewing gum, a capsule, a sachet, a powder, a granule, a coated particle, a coated tablet, an enterocoated tablet, an enterocoated capsule, a melting

strip or a film. When the pharmaceutical composition is a liquid formulation it may be formulated as an oral solution, a suspension, an emulsion or syrup. Said composition may further comprise a carrier material independently selected from, but not limited to, the group consisting of lactic acid fermented foods, fermented dairy products, resistant starch, dietary
5 fibers, carbohydrates, proteins, and glycosylated proteins.

Pharmaceutical compositions, as disclosed herein, can be formulated in accordance with standard pharmaceutical practice (see, *e.g.*, Remington: The Science and Practice of Pharmacy (20th ed.), ed. A. R. Gennaro, Lippincott Williams & Wilkins, 2000 and Encyclopedia of Pharmaceutical Technology, eds. J. Swarbrick and J. C. Boylan, 1988-1999,
10 Marcel Dekker, New York) known by a person skilled in the art. Pharmaceutical composition according to the invention may also be formulated to release active agents substantially immediately upon administration or at any predetermined time or time period after administration.

For oral administration, the composition can be formulated into conventional oral
15 dosage forms such as tablets, capsules, powders, granules and liquid preparations such as syrups, elixirs, and concentrated drops. Non-toxic solid carriers or diluents may be used which include, for example, pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharine, talcum, cellulose, glucose, sucrose, magnesium, carbonate, and the like. For compressed tablets, binders, which are agents which impart cohesive qualities to
20 powdered materials are also necessary. For example, starch, gelatin, sugars such as lactose or dextrose, and natural or synthetic gums can be used as binders. Disintegrants are also necessary in the tablets to facilitate break-up of the tablet. Disintegrants include starches, clays, celluloses, algin, gums and cross-linked polymers. Moreover, lubricants and glidants are also included in the tablets to prevent adhesion to the tablet material to surfaces in the
25 manufacturing process and to improve the flow characteristics of the powder material during manufacture. Colloidal silicon dioxide is most commonly used as a glidant and compounds such as talc or stearic acids are most commonly used as lubricants.

Pharmaceutical composition can also be formulated as a food composition, a dietary supplement, a functional food, a medical food or a nutritional product as long as the required
30 effect is achieved, *i.e.* treatment and/or prevention of an inflammatory disorder of the gastrointestinal tract. Said food composition may be chosen from the group consisting of beverages, yogurts, juices, ice creams, breads, biscuits, crackers, cereals, health bars, spreads and nutritional products. The food composition may further comprise a carrier material,

wherein said carrier material is chosen from the group consisting of lactic acid fermented foods, fermented dairy products, resistant starch, dietary fibers, carbohydrates, proteins and glycosylated proteins.

Accordingly, the current invention provides a method of treating or preventing an inflammation mediated disease of the gastrointestinal system in a subject, the method comprising, administering to the subject a therapeutically effective amount of the composition comprising the SlpA protein or a non-naturally occurring SlpA derivative. In certain embodiments, the composition is orally administered to the subject. SlpA protein or a non-naturally occurring SlpA derivatives can be administered or formulated in combination with effective doses of conventional anti-inflammatory agents, such as sulfasalazine, cyclosporine, prednisone, methylprednisone, budesonide, mesalamine, azathioprine, TNF inhibitors, methotrexate, or 6-mercaptopurine, a corticosteroid, infliximab or combinations thereof, for treatment of inflammation mediated diseases of the GI tract. Non-limiting examples of such diseases include IBD, for example, Crohn's disease or ulcerative colitis.

Yet another aspect of the invention relates to a method of purifying SlpA comprising growing SlpA expressing bacterial cells, pelleting said bacterial cells from culture medium containing said bacterial cells, resuspending said bacterial cells in 5M NaCl for a period of 5 minutes to 24 hours, preferably between 30 minutes and two hours, to form an extraction composition, removing bacterial cells from said extraction composition by centrifugation to form a composition containing SlpA and dialyzing or filtering said composition using a dialysis bag or ultrafiltrating device having a molecular weight cut-off of 30 kDa to reduce the salt content of said composition containing SlpA. The method can further comprise the precipitation of SlpA protein in said dialyzed composition comprising SlpA (for example, with 1M NaCl or another suitable precipitating agent). The method can also further comprise the washing of said precipitated SlpA with water or a buffer and lyophilization of said washed SlpA.

In some embodiments of the purification methodology, the SlpA expressing bacterial cells do not express LTA, SlpB or orthologs thereof or SlpX or orthologs thereof. Alternatively, the SlpA expressing bacterial cells do not express phosphoglycerol transferase or orthologs thereof, SlpB or orthologs thereof or SlpX or orthologs thereof.

Thus, the bacterial cells, in some embodiments: a) express a protein that has the amino acid sequence of SEQ ID NO: 4 or the protein has the amino acid sequence at least 70%, 80%, 90%, 95%, 97%, 98%, or 99% identical to the sequence of SEQ ID NO: 4; and b)

do not express (i) a polypeptide comprising the amino acid sequence of SEQ ID NO: 1 or a polypeptide having at least 70%, 80%, 90%, 95%, 97%, 98%, or 99% sequence identity to the amino acid sequence of SEQ ID NO: 1; (ii) a polypeptide comprising the amino acid sequence of SEQ ID NO: 2 or a polypeptide comprising the amino acid sequence having at least 70%, 80%, 90%, 95%, 97%, 98%, or 99% sequence identity to the amino acid sequence of SEQ ID NO: 2; and (iii) a polypeptide comprising the amino acid sequence of SEQ ID NO: 3 or a polypeptide having at least 70%, 80%, 90%, 95%, 97%, 98%, or 99% sequence identity to the amino acid sequence of SEQ ID NO: 3. In certain aspects of the invention, these bacterial cells are *Lactobacillus* strain, such as a *Lactobacillus* strain is selected from the group consisting of *L. acidophilus*, *L. amylolyticus*, *L. amylovorus*, *L. brevis*, *L. brevis* ssp *gravesensis*, *L. buchneri*, *L. crispatus*, *L. gallinarum*, *L. gigeriorum*, *L. helveticus/suntoryeus*, *L. hilgardii*, *L. kefiranoferiens*, *L. pasteurii*, *L. lactis* and *L. ultunensis*.

MATERIALS AND METHODS

Mice

C57BL/6 (B6), and B6 recombination-activating gene 1-deficient (*Rag1*^{-/-}) mice were purchased from Jackson Laboratories (Bar Harbor, ME). Germ-free (GF) B6 mice were obtained from the National Gnotobiotic Rodent Resource Center at the University of North Carolina and maintained in the GF facilities at the University of Florida (UF). The mouse strain 031934-UCD, C57BL/6-*Cd209*^{tm1.1Cfz}/Mmucd (*Signr3*^{-/-}) was provided by the NIH-sponsored Mutant Mouse Regional Resource Center (MMRRC) National System and was backcrossed at the Max Planck Institute of Colloids and Interfaces, Berlin, Germany. Genotyping of the *Signr3* gene in WT and *Signr3*^{-/-} mice was performed according to a protocol provided by the Consortium for Functional Glycomics. Dr. L. Morel (UF) contributed the FoxP3-GFP mice. Mice were bred in-house in the animal facility at the College of Veterinary Medicine, UF. Mice were maintained under specific pathogen-free, *Helicobacter*-free conditions and used at 6-8 weeks of age in accordance with the Animal Welfare Act and the Public Health Policy on Humane Care. Procedures were approved by UF's Institutional Animal Care and Use Committee (IACUC).

Bacterial Strains

The *upp*-counterselective knockout strategy was used to generate an in-frame deletion of the phosphoglycerol transferase gene within NCK2030 ($LTA^+ SlpB^- SlpX^- SlpA^+$), resulting in the generation of NCK2187 ($LTA^- SlpB^- SlpX^- SlpA^+$) (Goh et al., 2009). Wild-type *L. acidophilus* NCFM (NCK56), and NCK2187 were propagated anaerobically in MRS broth (Difco, BD, Franklin Lakes, NJ) at 37°C for 15 hrs. In preparation for oral treatment, bacteria were washed twice with sterile PBS, and the number of colony-forming units (CFU) were estimated by measuring the optical density at 600 nm. The concentration of each *L. acidophilus* strain was accordingly adjusted. To determine the clearance kinetics of the different *L. acidophilus* strains, groups of mice (n=3) were orally gavaged with erythromycin-resistant (Em^r) NCK56 or NCK2187 (1×10^9 CFU/100 μ L/mouse). Fecal pellets were collected before gavage and every day thereafter for up to 7 days. Each fecal pellet was then resuspended in 10% MRS (0.2 g/2 mL). The homogenized material was serially diluted and plated onto MRS agar containing Em (2 μ g/mL). The daily average excreted *L. acidophilus* was quantified. For the oral gavage of mice, each mouse received either 1×10^9 CFU of NCK56 or NCK2187 in 100 μ L of PBS. Mice enrolled in steady-state studies were orally gavaged every 3 days for a total of 4 times, and immune changes analyzed at day 14. The gavage schedule was determined based on the clearance kinetics of the bacterial strains.

Surface Layer Protein A Isolation

SlpA was purified from NCK2187 with LiCl. Cultures from 18 h grown NCK2187 were pelleted at 5,000 rpm for 10 min. Bacterial pellets were washed with cold PBS and re-pelleted before extraction. Pellets were resuspended in 5 M LiCl or 5M NaCl (Sigma-Aldrich, St. Louis, MO), gently stirred for 30 min, and the bacteria removed by centrifugation (13,000 rpm, 15 min). Supernatants were dialyzed against distilled water overnight using a dialysis bag with a cut-off molecular weight of 30 kDa for salt removal. The protein precipitate was dissolved in 1 M LiCl or 1M NaCl and pelleted at 13,000 rpm for 30 min. The SlpA protein preparation was washed with water a minimum of three times before lyophilization (FreeZone, Labconco, Kansas City, MO). Freeze-dried SlpA was stored at 4°C until used. SDS-PAGE gels and proteomics analyses were used to confirm SlpA purity. For oral gavage, mice were given 150 μ g of SlpA in 300 μ L PBS.

Lamina Propria Leukocyte (LPL) Preparation

Colonic lamina propria cells were isolated, as previously described (Lightfoot et al., 2014). Freshly isolated colons were cut into 0.5 cm sections and intraepithelial lymphocytes removed with a digestion buffer consisting of HBSS (GIBCO, Life Technologies, Grand Island, NY) containing 5 mM EDTA (GIBCO, Life Technologies) and 10 mM HEPES (GIBCO, Life Technologies), for (20 min, 37°C). Remaining colon tissues were digested in DMEM (GIBCO, Life Technologies) supplemented with 0.25 ng/mL Collagenase Type VII (Sigma-Aldrich), 0.125 U/mL Liberase TM Research Grade (Roche Applied Science, Indianapolis, IN), 10 mM HEPES, 0.1 M CaCl₂ (Sigma-Aldrich), and 5% FBS (GIBCO, Life Technologies). Three digestions of 10 min each at 37°C were performed. Single cell suspensions obtained were combined and stained for flow cytometry-based analyses or used for *ex vivo* studies.

Ex vivo Stimulation of Colonic LPLs

Isolated colonic LPLs were co-cultured with NCK56 or NCK2187 for 12 h at 37°C. Supernatants were then collected and stored at -80°C for later cytokine analyses using Bio-Plex Pro Mouse Cytokine Immunoassay kits (Bio-Rad, Hercules, CA). Activation phenotypes of DCs were analyzed by flow cytometry using the appropriate antibodies to quantify expression levels of MHC-II molecules and costimulatory markers.

Flow Cytometry and Antibodies

Colonic LPLs were stained as described previously (Lightfoot et al., 2014). Colonic LPLs were stained with LIVE/DEAD Aqua Dead Cell Stain Kit (Molecular Probes, Life Technologies). Washed cells were incubated with Mouse Fc Blocking Reagent (Miltenyi Biotec, Auburn, CA) per the manufacturer's instructions before staining with combinations of the following antibodies or their corresponding isotype controls: CD45 (30-F11), CD11c (N418), CD11b (M1/70), CD11b (M1/70), F4/80 (BM8), GR1 (RB6-8C5), I-A/I-E MHCII (2G9), CD3 (145-2C11), CD4 (RM4-5), CD8 (53-607), Pro-IL-1 β (NJTEN3)/Rat IgG1, κ , IFN γ (XMG1.2)/Rat IgG1, κ , IL-17A (TC11-18H10.1)/Rat IgG1, κ , IL-10 (JES5-16E3)/Rat IgG2b, κ , LAP (TGF- β 1, TW7-16B4)/Mouse IgG1, κ , FoxP3 (FJK-16A)/Rat IgG2a, κ , ROR γ t (AFKJS-9)/Rat IgG2a, κ . For the detection of intracellular cytokines, cells were fixed and permeabilized with BD Cytofix/Cytoperm (BD Biosciences). Colonic T cells were stimulated with phorbol 12-myristate 13-acetate (PMA) (50 ng/mL) and ionomycin (2.5 μ g/mL) in the presence of Brefeldin A (Biolegend) for 2.5 h. The Transcription Factor

Fixation/Permeabilization kit from eBioscience was used for FoxP3 staining. After staining, a BD LSRFortessa (BD Biosciences) cell analyzer was used to acquire fixed cells. Data were analyzed with FlowJo software (Tree Star, Ashland, OR).

Antibodies and their corresponding isotype controls were purchased from eBioscience (San Diego, CA), Biolegend (San Diego, CA), BD Pharmingen, or R&D Systems (Minneapolis, MN).

T cell-Induced Colitis

In preparation for the adoptive transfer of CD45RB^{hi} CD4⁺ T cells into *Rag1*^{-/-} mice, spleen and mesenteric lymph node (MLN) single cell suspensions obtained from healthy B6 mice were pooled and incubated in AffiniPure Goat Anti-Mouse IgG (H+L)-coated cell culture plates (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) at 37°C for 1 h. CD4⁺ T cells were isolated from non-adherent cells using the CD4⁺ T cell Isolation Kit II (MACS, Miltenyi Biotec, San Diego, CA), and CD25⁺ CD4⁺ T cells were then depleted by positive selection (MACS, Miltenyi Biotec). Bound CD25⁺ CD4⁺ T cells were collected and injected into the regulatory T cells (Tregs) group. The resulting cell suspensions after negative and positive selection was consistently comprised of >98% CD25⁻ CD45RB^{hi} CD4⁺ T cells. *Rag1*^{-/-} mice were orally gavaged once with NCK56, NCK2187, or SlpA prior to the adoptive transfer of T cells by intraperitoneal injection (i.p.). One day later, the mice were orally gavaged once more, and once a week for 4 consecutive weeks thereafter (**Fig. 3A**). Colitis progression was monitored by determining mouse weight loss, diarrhea development, and fecal occult blood (FOB) presence throughout the study. Stool consistency was scored as follows: 0 = normal, 2 = pasty, 4 = watery with perianal staining.

DSS-Induced Colitis

WT and *Signr3*^{-/-} mice were treated with 3% DSS in the drinking water for 5 days (made fresh every 2-3 days) to induce colitis. Mice were monitored for disease progression through day 10 after treatment as described above. For prevention studies, mice were orally gavaged with NCK56, NCK2187, or SlpA at days -3 and -1, then every other day after 3% DSS treatment, for a total of 5 gavages (2 before, and 3 after 3% DSS).

Histopathology

Colitis scores in T cell- and DSS-induced colitis were determined by histopathology. Tissues were fixed, sectioned, and stained with hematoxylin and eosin (Histology Tech Services, Gainesville, FL). Stained sections were analyzed blindly by a boarded veterinary pathologist. Colitis was graded based on 7 parameters (0–17) as previously described (Cheng et al., 2014).

FITC-Dextran Intestinal Permeability Assay

Passive transepithelial absorption of FITC-labeled dextran (Sigma-Aldrich) *in vivo* was used to determine intestinal barrier function as previously described (Napolitano et al., 1996). Mice were gavaged with FITC-dextran, MW 4,000 (60 mg/100 g body weight). Blood was collected retro-orbitally after proper anesthetization; mice were sacrificed after blood collection. Fluorescence intensity in the serum was measured with a fluorimeter (485 nm excitation, 519 nm emission). FITC-dextran concentrations in the mouse sera were determined from standard curves generated by serial dilution of FITC-dextran using blank subtraction in the test samples using sera from mice that were not gavaged with the permeability tracer.

Colonoscopy of DSS- and T cell-Induced Colitis Mice

Macroscopic damage in the colons of *Rag1*^{-/-}, WT, and *Signr3*^{-/-} mice was visualized with a Multi-Purpose Rigid Telescope attached to a TELE PACK X (Karl Storz–Endoscope, Germany). Mice were fasted for 2-4 h, and subsequently the colons of the living subjects were imaged under appropriate anesthetic conditions.

Real-time PCR and 16S Ribosomal DNA Sequencing

Colonic tissues from *Rag1*^{-/-}, WT, and *Signr3*^{-/-} mice were isolated and processed for changes in gene expression as previously described (Lightfoot et al., 2014). Microbiome analyses were performed on the Illumina Miseq (Illumina, Inc., San Diego) as outlined previously (Lightfoot et al., 2014). Primers used, as well as their sequences, are listed in the Table 1 below showing the list of primer sequences for Real-Time PCR analyses.

Table 1. List of primer sequences for Real-Time PCR analyses		
Gene name	Sequence (5'-3')	SEQ ID NO:
111b	Forward AAGGAGAACCAAGCAACGAC	5
	Reverse GAGATTGAGCTGTCTGCTCA	6

Ocln	Forward GCTGTGATGTGTGTGAGCTG	7
	Reverse GACGGTCTACCTGGAGGAAC	8
Cd209a	Forward TCTGGA TTCAGT AGCTTCACAGG	9
	Reverse GGGTCAGTTCTTGGT AGACA TTC	10
Cd209b	Forward TTGA TGGTCAGCGGCAGCAGG	11
	Reverse TCAGCAGGAGCCCAGCCAAGA	12
Cd209c	Forward CTGGAATGACTCTGTCAATGCC	13
	Reverse GCCA TCTGCCTTCA TGCTTCA	14
Cd209d	Forward GGGCCCAACTGGTCATCATA	15
	Reverse AGCGTGTAAGCTGGGTGAC	16
Cd209e	Forward CCACA TTCCCCTGGTGTG	17
	Reverse CAGAGGCGACAGAGTCTATCA	18
Cd209f	Forward CTCTTTGGGCCTCTTTTGTGCT	19
	Reverse AGTATGCACGAATCCTGGAGA	20
Cd209g	Forward GGCCTCAGCGATCACAGAAG	21
	Reverse ACAACGGCTGTCATTCCATTTA	22
Muc2	Forward GTGTGGGACCTGACAATGTG	23
	Reverse ACAACGAGGTAGGTGCCATC	24
Muc3	Forward GCCGTGAATTGTATGAACGGA	25
	Reverse CGCAGTTGACCACGTTGACT A	26
Tjp1	Forward AGGACACCAAAGCATGTGAG	27
	Reverse GGCATTCTCTGCTGGTT ACA	28
Tjp2	Forward ATGGGAGCAGTACACCGTGA	29
	Reverse TGACCACCCTGTCA TTTTCTTG	30
Tjp3	Forward TCGGCATAGCTGTCTCTGGA	31
	Reverse GTTGGCTGTTTTGGTGCAGG	32
Cldn1	Forward TCCTTGTTCCGGCTATGTGTC	33
	Reverse GGCATGCACCTAAGAATCAG	34
Cldn2	Forward GGCTGTTAGGCACATCCAT	35
	Reverse TGGCACCAACATAGGAATC	36
Cldn3	Forward AAGCCGAATGGACAAAGAA	37
	Reverse CTGGCAAGTAGCTGCAGTG	38
Cldn5	Forward GCAAGGTGTATGAATCTGTGCT	39
	Reverse GTCAAGGT AACAAAGAGTGCCA	40
Cldn8	Forward GCCGGAATCA TCTTCTTCA T	41
	Reverse CA TCCACCAGTGGGTTGT AG	42
Hsp25	Forward GGTTGCCCGATGAGTGGTC	43
	Reverse CTGAGCTGTCGGTTGAGCG	44
Hsp72	Forward CTCCCTCTTGCGTTGCCTC	45
	Forward ACCCGCAGT AAT AGCCA TCTG	46

C-type lectin receptors, SIGNR1 and SIGNR3, were fused to the Fc part of human IgG1 (SIGNR1-hFc and SIGNR3-hFc) as previously described (Eriksson et al., 2013). Briefly, the extracellular regions of murine SIGNR1 and SIGNR3 were amplified and ligated into the expression vector pFUSE-hIgG1-Fc2 (Invivogen, Toulouse, France) for expression in CHO-S cells. Expression in CHO cells was driven by an hEF1-HTLV promoter and secretion into the culture supernatant was mediated by an external IL2 signal sequence (IL2ss). Binding of SlpA-coated beads (Dynabeads MyOne Carboxylic Acid, Life Technologies) to fusion proteins was analyzed by flow cytometry.

Statistical Analyses

Representative data indicate mean $\pm$ SEM. Significance was determined by two-tailed unpaired *t*-tests for two group comparisons (GraphPad Prism v6.0d for Mac OS X, La Jolla, CA). Statistical significance for differences in weight loss, diarrhea score, and FOB score was calculated using multiple unpaired *t*-tests correcting for multiple comparisons with the Holm-Sidak method in Prism v6.0d.

All patents, patent applications, provisional applications, and publications referred to or cited herein are incorporated by reference in their entirety, including all figures and tables, to the extent they are not inconsistent with the explicit teachings of this specification.

Following are examples which illustrate procedures for practicing the invention. These examples should not be construed as limiting. All percentages are by weight and all solvent mixture proportions are by volume unless otherwise noted.

EXAMPLE 1 – NCK2187 PROMOTES INTESTINAL IMMUNE REGULATION IN STEADY-STATE

Transient colonization of the colon with NCK2025 (LTA⁻) significantly mitigated chemical and T cell-mediated colitis (Mohamadzadeh et al., 2011). Additionally, NCK2025 significantly abated inflammation-promoting polyposis in *Apc*^{lox468} x TS4-Cre mouse model, where protection correlated with the regulation of innate and T cell-induced inflammation (Khazaie et al., 2012). Thus, the controlled inflammation may result from the crosstalk between NCK2025-SlpA and intestinal cells. To test this hypothesis, the *upp*-counterselective gene replacement strategy was used to generate in-frame deletions in the *slpB* and *slpX* genes of NCK2030. The LTA⁻ derivative was created by a deletion of the

phosphoglycerol transferase gene (Mohamadzadeh et al., 2011) in NCK2030, resulting in NCK2187, which expresses only SlpA (**Fig. 1A-C**).

To demonstrate that the newly generated NCK2187 transiently colonizes the gut, the clearance kinetics of both the erythromycin-resistant NCK56 and NCK2187 strains were determined in C57BL/6 (B6) mice that were orally treated once with 10^9 CFU/mouse. Data show that mice cleared both NCK56 and NCK2187 after 3 days, indicating that the deletion of LTA, SlpB, and SlpX in NCK2187 did not alter its transient passage through the GI tract when compared to its WT parent (**Fig. 1D**).

To investigate the activation of colonic DCs when co-cultured with NCK56 or NCK2187, colonic cells were obtained from naïve B6 mice. While such intestinal cell-bacterial co-cultures did not significantly change the expression of DC costimulatory molecules (e.g., CD40) (not shown) or IL-10, only NCK56 elevated the levels of IL-1 β , IL-6, IL-12, and TNF- α (**Fig. 1E**).

Next, naïve mice were orally gavaged with NCK56 or NCK2187 and colonic immune responses analyzed. Treatment with NCK2187 significantly increased the frequency of colonic FoxP3⁺ Tregs when compared to both untreated (PBS) and NCK56-treated mice (**Fig. 2A**). Moreover, IL-17A⁺ and IFN γ ⁺ CD4⁺ T cells were significantly reduced by NCK2187 treatment (**Fig. 2A**). NCK2187-treated FoxP3-GFP mice also exhibited higher numbers of colonic IL-10⁺ TGF- β 1⁺ Tregs than NCK56-treated and untreated mice (**Fig. 2B, 2C**). Collectively, oral treatment with this novel *L. acidophilus* strain induced colonic regulatory immune responses.

EXAMPLE 2 – PROTECTIVE PROPERTY OF NCK2187 AND ITS SLPA AGAINST INFLAMMATION AND DYSBIOSIS

To elucidate the consequences of the immunoregulatory responses observed above during inflammation, *Rag1*^{-/-} mice adoptively transferred with CD45RB^{hi} CD4⁺ T cells were orally treated with NCK56, NCK2187, its purified SlpA, or PBS (**Fig. 3A**). Untreated (PBS) and NCK56-treated mice with adoptively transferred T cells developed severe colitis as demonstrated by weight loss, bloody diarrhea, shortening of the colon, and increased damage of the colon (**Fig. 3A-C, Fig. 8A**). Furthermore, the levels of systemically induced proinflammatory IL-1 β , IL-6, TNF- α , IFN γ , G-CSF, and MIP-1 α were significantly enhanced in the sera of these groups of mice (**Fig. 3D**). In contrast, similar to the Treg co-transferred

mice, NCK2187 and its purified SlpA significantly protected *Rag1*^{-/-} mice from T cell-induced colitis (Tables 2-4).

Table 2. Statistical analysis of weight loss curves in T cell-induced colitis.

Week	Weight Loss (<i>p</i> -value)					
	vs. + PBS			vs. + NCK56		
	+ Tregs	+ NCK2187	+ SlpA	+ Tregs	+ NCK2187	+ SlpA
1	1.0	1.0	1.0	1.0	1.0	1.0
2	0.0601	0.1714	0.1224	0.5588	0.9927	0.8543
3	0.2331	0.0237	0.1943	0.5119	0.0556	0.5386
4	0.0653	0.0778	0.1229	0.1194	0.1220	0.2526

Table 3. Statistical analysis of diarrhea score curves in T cell-induced colitis.

Week	Diarrhea Score (<i>p</i> -value)					
	vs. + PBS			vs. + NCK56		
	+ Tregs	+ NCK2187	+ SlpA	+ Tregs	+ NCK2187	+ SlpA
1	1.0	1.0	1.0	1.0	1.0	1.0
2	1.0	1.0	1.0	1.0	1.0	1.0
3	1.0	1.0	1.0	1.0	1.0	1.0
4	0.0231	0.0024	0.0014	0.2100	0.0592	0.0287
5	0.1139	0.1100	0.507225	0.0101	0.0094	0.0749
6	0.0011	<0.0001	0.0046	0.0011	<0.0001	0.0031
7	<0.0001	<0.0001	0.0008	<0.0001	<0.0001	0.0003

Table 4. Statistical analysis of fecal occult blood (FOB) score curves in T cell induced colitis.

Week	FOB Score (<i>p</i> -value)					
	vs. + PBS			vs. + NCK56		
	+ Tregs	+ NCK2187	+ SlpA	+ Tregs	+ NCK2187	+ SlpA
1	1.0	1.0	1.0	1.0	1.0	1.0
2	1.0	1.0	1.0	1.0	1.0	1.0
3	1.0	1.0	1.0	1.0	1.0	1.0
4	0.0730	0.0176	0.2532	0.0721	0.0175	0.2479
5	0.0227	0.0008	0.0733	0.0009	<0.0001	0.0027
6	0.0014	0.0015	0.0009	0.0001	0.0001	<0.0001
7	<0.0001	<0.0001	<0.0001	<0.0001	0.0003	<0.0001

NCK2187- and SlpA-treated mice gained weight throughout the course of the study and did not develop significant bloody diarrhea in the way that the PBS and NCK56 groups did (Fig. 3A). Furthermore, cecal and colonic atrophy due to pathogenic inflammation was not observed in these mice, as the tissue destruction and immune cell infiltration associated with T cell-induced colitis were significantly abrogated in NCK2187 and SlpA treated groups (Fig. 3B and 3C). Accordingly, systemic inflammation was significantly reduced in these groups of mice (Fig. 3D).

The genes encoding the receptors for LTB₄, Ltb4r1 and Ltb4r2 were significantly down-regulated in the colons of NCK2187- and SlpA-treated mice (Fig. 8B), which may have contributed to the reduced expression of colonic *Il1b* (Fig. 8B).

T cell-induced colitis resulted in intestinal epithelial erosions and ulcerations in mice that did not receive NCK2187 or purified SlpA (Fig. 3C). Indeed, the colonic expression of tight junction associated genes was significantly downregulated in PBS- and NCK56-treated *Rag1*^{-/-} mice (Fig. 4A).

Furthermore, FITC-dextran permeability assays confirmed that these mice were suffering from a dysfunctional intestinal barrier (Fig. 4A). Accordingly, NCK2187 and SlpA significantly protected barrier integrity and function (Fig. 4A). An immunologically and anatomically weakened intestinal epithelial barrier during acute intestinal inflammation

allows luminal bacteria to interact with the intestinal mucosae and the infiltrating immune cells, initiating inflammatory responses directed against the gut commensals and introducing dysbiosis. To elucidate the status and composition of the microbiota in the T cell-induced colitis model (week 7), we analyzed the microbial communities in the colons of the different experimental groups and found that the severity of colitis was associated with significant changes in the microbiota (Fig. 4B-D, Table 5). UniFrac analyses revealed that fecal bacterial diversity in PBS- and NCK56 treated *Rag1*^{-/-} mice was modified in such a way that these groups were found to cluster separately from each other and from the protected mice (Fig. 4B). Conversely, SlpA-, NCK2187-, and Treg-treated groups clustered together and showed similar phyla distributions (Fig. 4B and 4C). Induced colitis in PBS- and NCK56-treated groups resulted in a significant contraction of members of the *Bacteroidetes* phyla (Fig. 4C). Additionally, the normally underrepresented *Verrucomicrobia* phyla was increased in these colitogenic mice (Fig. 4C), suggesting a shift in the intestinal milieu and the substrates available in the inflamed colon, which may promote previously underrepresented microbial communities so that they dominate the population. Alterations in the microbial composition were also manifested at lower taxonomic levels: NCK2187-, SlpA-, and Treg-treated groups once again showed similar relative abundance and distribution of several unclassified genera (Fig. 4D).

Table 5. Analysis of phyla distribution in the fecal microbiota after T cell-induced colitis.

Phylum	p-value						Mean Value (%)				
	vs. + PBS			vs. + NCK56							
	+ Tregs	+ NCK2187	+ SlpA	+ Tregs	+ NCK2187	+ SlpA	+ PBS	+ NCK56	+ Tregs	+ NCK2187	+ SlpA
Actinobacteria	0.1818	0.1595	0.1812	0.2663	0.2188	0.2854	1.84	1.16	0.79	0.82	0.80
Bacteroidetes	0.0010	0.0129	0.0276	<0.0001	0.0009	0.0029	12.70	7.44	31.32	25.30	24.87
Firmicutes	0.0053	0.0714	0.1022	<0.0001	0.0010	0.0043	80.43	88.89	66.45	73.35	72.61
Proteobacteria	0.2836	0.3284	0.0673	0.1508	0.1364	0.1955	0.14	1.15	0.26	0.21	0.42
Tenericutes	0.0494	0.4280	0.1102	0.0015	0.1215	0.0208	0.54	0.24	1.17	0.49	1.22
Verrucomicrobia	0.0244	0.0249	0.0263	0.0796	0.0834	0.0934	3.79	1.13	0.01	0.06	0.08

EXAMPLE 3 – *L. ACIDOPHILUS*-SLPA BINDING TO SIGNR3 PROMOTES COLONIC REGULATORY IMMUNE RESPONSES

Symbiotic bacteria and their gene products dictate the nature of innate responses via their sensing receptors (Ivanov and Honda, 2012; Yang et al., 2014); however such stimulatory signals must be regulated by other receptors to avoid intestinal inflammation. As previously stated, SIGNR3 exhibits the most biochemical similarity to human DC-SIGN. We screened all known murine SIGNR1-8 and found that *Signr1* and *Signr3* genes are differentially activated in the colonic tissue of mice orally treated with NCK2187 (Fig. 5A), prompting us to evaluate the binding of SlpA to SIGNR1 and SIGNR3.

Subsequently, the corresponding extracellular domain of SIGNR1 and SIGNR3 were fused to the Fc portion of human IgG₁ (SIGNR1-hFc, SIGNR3-hFc) and then transiently expressed in Chinese hamster ovary (CHO)-S cells (data not shown). Data demonstrate that while expressed SIGNR3-hFc bound to purified SlpA coated onto charged beads, SIGNR1-hFc, DCAR-hFc (control protein), hFc, and the secondary rat anti-human Fc antibody alone did not, suggesting SlpA-binding specificity to SIGNR3 (Fig. 5B).

To clarify the role of SlpA:SIGNR3 binding and signaling *in vivo*, we first orally treated WT and *Signr3*^{-/-} mice with our bacterial strains and analyzed the immunologic responses induced in steady-state. While NCK2187 treatment led to reduced IL-1β in both conventional (data not shown) and germ-free (GF) B6 mice (Fig. 5C, left), no anti-inflammatory effects were observed in NCK2187-treated *Signr3*^{-/-} mice (Fig. 5C, right). Furthermore, the Treg-inducing properties of NCK2187 (Fig. 2A and 2B) were abrogated in *Signr3*^{-/-} mice (Fig. 5D). These data strongly suggest that NCK2187 delivers immunoregulatory signals via its interaction with SIGNR3.

EXAMPLE 4 – NCK2187 AND ITS SLPA CANNOT PREVENT DEXTRAN SULFATE SODIUM (DSS) INDUCED COLITIS IN THE ABSENCE OF SIGNR3 SIGNALING

Previous reports have highlighted the role of specific CLRs in experimental colitis. For instance, mice lacking *Signr1* expression are less susceptible to induced colitis (Saunders et al., 2010), while mice deficient in *Dectin1* and *Signr3* exhibit exacerbated disease (Eriksson et al., 2013; Iliev et al., 2012). To further investigate SlpA:SIGNR3 signaling in disease conditions, DSS-treated WT and *Signr3*^{-/-} mice were orally gavaged with NCK56, NCK2187, or SlpA, and monitored for disease progression.

Consistent with the T cell-induced colitis model, disease progression and severity were significantly reduced in WT mice orally treated with NCK2187 or purified SlpA (Fig. 6 and Table 6); however, NCK2187 and SlpA did not confer any protection in *Signr3*^{-/-} mice (Fig. 6). Measurements included weight loss, histopathology-based colitis scores, evaluation of gross mucosal damage, and immune cell recruitment and activation (Figs. 6 and 7).

Disease progression and inflammation were associated with alterations in intestinal barrier integrity and the composition of the gut microbiota (Figs. 6E-6H). Several gut permeability markers were evaluated by RT-PCR. The restoration of claudins (*Cldn1*, *Cldn3*, and *Cldn5*) in WT mice treated with NCK2187 or SlpA, indicate that NCK2187 and SlpA were capable of promoting intercellular tight junctions (Fig. 6E).

Accordingly, only NCK2187 and SlpA treatments of WT mice prevented increased fecal albumin levels seen with protein-losing enteropathies after DSS treatment (Fig. 6F). On the other hand, no positive effects by NCK2187 or SlpA on barrier integrity were noted in *Signr3*^{-/-} mice (data not shown). In terms of microbiota composition, protected WT mice (NCK2187- and SlpA-treated) clustered together in UniFrac analyses, while diseased untreated and NCK56-treated WT mice clustered separately (Fig. 6G).

Conversely, the microbial communities of all DSS-treated *Signr3*^{-/-} mice formed a single cluster, indicating that dysbiosis was uniformly distributed independent of treatment group (Fig. 6G). Furthermore, richness and diversity, analyzed by the Chao Richness index and Shannon Diversity index, respectively, were maintained in NCK2187- and SlpA-treated WT mice, while no such effects could be observed in *Signr3*^{-/-} mice (Fig. 6H).

Table 6. Statistical analysis of weight loss curves in DSS-induced colitis in WT mice.

Day	Weight Loss (p-value)			
	vs. + DSS alone		vs. + NCK56	
	+ NCK2187	+ SlpA	+ NCK2187	+ SlpA
1	1.0	1.0	1.0	1.0
2	0.6705	0.7567	0.2844	0.7430
3	0.9668	0.7071	0.0862	0.0580
4	0.3974	0.5816	0.8394	0.2873
5	0.6360	0.2916	0.3444	0.1472
6	0.0087	0.0181	0.0042	0.0098
7	<0.0001	0.0001	<0.0001	<0.0001
8	<0.0001	<0.0001	<0.0001	<0.0001
9	<0.0001	0.0011	<0.0001	0.0035
10	<0.0001	0.0023	<0.0001	0.0359

Induced immune responses in the colons of DSS-treated mice were analyzed to determine differences, if any, among the treatment groups. Neutrophilic infiltration in the colons of NCK2187- and SlpA-treated SIGNR3-sufficient mice that were given DSS decreased to nearly PBS-treated control mice levels; while, in contrast, an even higher frequency of infiltrating neutrophils was detected in *Signr3*^{-/-} mice after the induction of colitis, irrespective of the treatment group analyzed (Fig. 7A). Similarly, the number of IL-1 β -producing DCs and macrophages was significantly decreased with NCK2187 and SlpA treatment in WT mice; however, no changes were observed among the different treatment groups in the absence of SlpA:SIGNR3 signaling (Fig. 7B).

Pathogenic inflammation can result in proinflammatory FoxP3⁺ROR γ t⁺ Tregs (Hovhannisyan et al., 2011; Khazaie et al., 2012). While no major change in the total number of FoxP3⁺ Tregs was measured among the WT and *Signr3*^{-/-} KO groups, the quality of these Tregs was significantly altered. A large number of FoxP3⁺ cells co-expressed ROR γ t after DSS treatment in both WT and *Signr3*^{-/-} mice (Fig. 7C). However, in accordance with the protection observed, NCK2187 and SlpA treatment prevented the generation of FoxP3⁺ROR γ t⁺ Tregs only in WT mice but not *Signr3*^{-/-} mice (Fig. 7C). Correspondingly, the levels

of circulating cytokines in the sera (IL-1 β , IL-10, IL-17A, IFN γ and TNF- α) were rebalanced only in NCK2187- and SlpA-treated WT mice but not in *Signr3*^{-/-} mice (Fig. 9).

Collectively, these clinical and immunologic data provide robust evidence in support of an immunoregulatory role for *L. acidophilus* SlpA that is highly dependent on intact
5 SIGNR3 signaling.

EXAMPLE 5 – EFFECTIVE DOSE FOR PREVENTION OF DSS-INDUCED COLITIS BY *L. ACIDOPHILUS* SLPA

To test the required dose of purified SlpA for efficacy in the prevention of colitis, DSS-induced colitis model was employed. 6 oral treatments with 150 μ g SlpA/mouse, and
10 only 4 oral gavages of 10⁹ CFU NCK2187 were sufficient to prevent weight loss in mice given 3% DSS in the drinking water for 5 days (Fig. 11A). See Fig. 10A for graphical summary of the treatment regimen. For the DSS-induced colitis prevention studies, age and sex-matched C57BL/6 mice were gavaged twice with NCK56 (WT), NCK2187, or purified
15 SlpA, prior to being given 3% DSS in the drinking water. At the start of the treatment, the mice were gavaged twice more with the different bacterial strains or 4 more times with SlpA to compensate for the ability of NCK56 and NCK2187 to persist in these animals for 3 days post-gavage (Fig. 1D). Mice treated with NCK2187 or SlpA developed only a mild form of colitis and recovered significantly faster than DSS-alone or NCK56-treated mice (Fig. 11).
20 Collectively, these data suggest that NCK2187 and SlpA are suitable treatments for the prevention of colitis.

EXAMPLE 6 – REVERSAL OF DSS-INDUCED COLITIS BY *ACIDOPHILUS* SLPA

Having tested the protective capacity of *L. acidophilus* SlpA, its ability to ameliorate established colitis was tested. For these studies, mice were orally gavaged with varying bacteria strains or purified SlpA once signs of colitis were noted, *i.e.*, diarrhea and fecal occult blood (see Fig. 10B for treatment regimen). Mice receiving either NCK2187 or SlpA recovered significantly faster than NCK56-treated or DSS-alone treated mice (Fig. 12).
30 These results indicate that NCK2187 and SlpA are efficacious in ameliorating existing colitis.

EXAMPLE 7 – PREVENTION OF T CELL-INDUCED COLITIS BY *L. ACIDOPHILUS* SLP A

The regulatory effects of *L. acidophilus* SlpA in a chronic inflammatory model of colitis, namely, the pathogenic T cell transfer model was tested. Immunodeficient *RagI*^{-/-} mice were injected with regulatory T cell (Treg)-depleted CD4⁺ splenic cells (CD4⁺CD25⁻), then left untreated or gavaged with NCK56, NCK2187, or purified SlpA (150µg/mouse), and monitored for the onset of colitis. Recipient mice were gavaged twice with their corresponding treatments at days 1 and 3 after the transfer, then once a week for the next 4 weeks, for a total of 6 gavages. An additional group receiving Tregs was used as a positive control for the prevention of colitis. Oral treatment with 10⁹ CFU NCK2187 was as effective as the Tregs in the prevention of weight loss in recipient *RagI*^{-/-} mice (Fig. 13A). Purified SlpA was also found to be protective in recipient *RagI*^{-/-} mice (Fig. 13); however, given the chronic nature of the model, a higher dose of SlpA may be needed to reach the level of protection observed with NCK2187.

EXAMPLE 8 – NCK2187 PROMOTES THE GENERATION OF FoxP3⁺ REGULATORY T CELLS (TREGS)

To gain a better understanding of the protective mechanisms induced by *L. acidophilus* SlpA that may explain the aforementioned protection, we orally gavaged healthy control mice with either NCK56 or NCK2187, and analyzed the frequency of Tregs locally and systemically. Compared to untreated or NCK56 treated mice, NCK2187 induced the generation of Tregs (Fig. 14). Taken together, these data suggest that *L. acidophilus* SlpA triggers important regulatory signaling cascades that may enable the restoration of intestinal homeostasis in experimental models of colitis.

EXAMPLE 9 –

The human GI tract harbors trillions of microbes, most of which are bacteria (Qin et al., 2010), and are critical determinants to the health of the host (Nicholson et al., 2012; Subramanian et al., 2014). This is especially true in the case of IBD, given the intimate association of the gut microbiota and their gene products with the adjacent colonic tissue (Hold et al., 2014; Huttenhower et al., 2014). Early experiments suggest that susceptibility to pathogenic intestinal inflammation in experimental colitis depends upon the presence of enteric antigens (Kuhn et al., 1993), and were later supported by human studies, which

demonstrated that an imbalance in the commensal bacterial composition, termed dysbiosis, is a defining characteristic of patients suffering from IBD (Frank et al., 2007; Sokol et al., 2006). Accordingly, a major focus in the field has been the identification of effector bacterial strains that influence the immune system (Ahern et al., 2014), and thus, may be employed to reprogram undesired immune responses, both locally and systemically.

Search for microbes with immunoregulatory properties at the strain level and not merely at the species level is warranted. Certain embodiments of the current invention identify specific bacterial molecule-host receptor interactions that may account for the responses induced by effector bacterial strains. For example, oral treatment using a *L. acidophilus* strain lacking the gene responsible for LTA biosynthesis significantly reduced pathogenic inflammation in the GI tract, thereby promoting the mitigation of induced colitis (Mohamadzadeh et al., 2011) and the ablation of colonic polyposis (Khazaie et al., 2012). The bacteria lacking LTA and their uses are described in US Patent Application Publication 20130224153, the contents of which are incorporated by reference herein in its entirety. However, a need still remains for identifying other bacterial strains and/or agents useful for treatment of inflammatory diseases, such as colitis or inflammatory bowel disease.

To address this need, the current invention provides bacteria with systematically deleted genes for the construction of novel bacterial strains, for example, NCK2187, and the assignment of roles to *slp* candidate genes that are responsible for SlpA, SlpB, and SlpX protein expression (Goh et al., 2009). This molecular approach to targeting genes in *L. acidophilus* defined the functional role of SlpA and demonstrated that SlpA affects intestinal innate cells and conventional T cell subset activation, including Tregs, in steady-state and murine colitis models.

As seen in Figures 3 and 4, NCK2187 and purified SlpA not only mitigated T cell-induced colitis by significantly reducing inflammation, but also protected the composition of the microbiota and intestinal barrier function. Additionally, systemic immune responses were also altered, whereupon the levels of proinflammatory cytokines, including IL-1 β , whose detrimental role in IBD was recently demonstrated (Coccia et al., 2012), decreased significantly. These data suggests the involvement of the IL-1 β signaling axis in intestinal protection. Accordingly, gene-screening results, along with SlpA binding to SIGNR3, highlight the involvement of SIGNR3 in the process of tempering highly activated gut immune responses. Additional data regarding SIGNR3 engagement using *Signr3*^{-/-} mice

clarified the role of this signaling molecule in induced immune regulation, as was also documented in the *Leishmania infantum* murine model (Lefevre et al., 2013).

These data reflect that the SlpA:SIGNR3 interaction significantly reduces the high affinity receptors for LTB₄ in T cell transferred *Rag1*^{-/-} mice. Downregulation of LTB₄ and/or its receptors is critical in preventing inflammasome activation, which otherwise results in increased IL-1β (Lefevre et al., 2013). Interestingly, interrupting the interaction between SlpA and SIGNR3 resulted in hyperactive immunity and the production of IL-1β in *Signr3*^{-/-} mice under inflammatory conditions. Such dysregulated immune responses in *Signr3*^{-/-} mice promoted neutrophil infiltration and significantly affected the function of colonic Tregs, which reverted toward proinflammatory FoxP3⁺ RORγt⁺ Tregs, all of which significantly contributed to pathologic inflammation, a condition seen in IBD progression.

In contrast, balanced immunity was restored in WT mice that were treated with NCK2187 or SlpA. Induced colonic inflammation in WT mice that were treated with NCK56, but not in NCK2187- or SlpA-treated mice, and in KO mice, regardless of treatment, resulted in microbial dysbiosis and barrier dysfunction, another hallmark of IBD.

As such, the current invention indicates that the interaction of SlpA with SIGNR3 can impact the status of innate and T cell polarization in induced colitis. Also, effective modulation of these cellular and molecular factors may significantly modify pathogenic inflammation that results in colitis, and would therefore restore intestinal homeostasis by rebalancing deteriorated immunity, the composition of the gut microbiota, and mucosal barrier function.

EXAMPLE 10 – A PROCESS FOR ISOLATOIN AND PURIFICATOIN OF SLPA FROM LACTOBACILLUS ACIDOPHILUS

It is estimated that over 1 million individuals in the U.S. suffer from IBD (Kappelman et al., 2007; Kappelman et al., 2013). Additionally, ample evidence indicates that dysfunctional immune responses are potentially elicited by gut dysbiosis (Major et al., 2014). To specifically determine the effects of SlpA and its binding to SIGNR3 on intestinal cells and the consequences thereafter, the *upp* counter-selective knockout strategy (Mohamadzadeh et al., 2011a) was used to generate a new strain of *L. acidophilus*, called NCK2187, which expresses only SlpA. Our data show that SlpA plays a critical role in controlling immune responses upon its interaction with SIGNR3, resulting in the diminution of induced colitis, protection of intestinal barrier integrity, and sustenance of the gut bacterial

composition. To build upon these observations, we have optimized the purification of SlpA to investigate its physiological effects when orally administered to mice, and evaluated whether this protein could resist the harsh condition of the gastrointestinal milieu, both important factors that may facilitate the feasibility of potential clinical trials.

Isolation and Detection of *L. acidophilus* Surface Layer Protein A

S-layers are paracrystalline (glyco) protein arrays that are present in abundance on the cell surface of a subset of eubacteria and archaea. We first sought to improve the process of SlpA isolation and purification. For this purpose, we used sodium chloride (NaCl) (5 M) as discussed in the Materials and Methods section. To avoid non-SlpA protein contamination in our isolation, we employed the LTA-, SlpB-, and SlpX-deficient *L. acidophilus* NCK2187 strain. Visualization of the isolated protein by SDS-PAGE showed a single protein band of the expected size (46 kDa, Fig. 15A). An automated mass spectrometry microbial identification system that uses Matrix Assisted Laser Desorption Ionization Time-of-Flight technology (MALDI-TOF) indicated 97 unique spectra and 55 unique peptides generated post-trypsinization of the protein isolate, which identified two possible proteins [gi|58336516 (SlpA) and gi|362076610 (SlpB)] (Fig. 15B). Further analyses revealed that the peptides generated cover 54% of SlpA and 18% of SlpB (highlighted, Fig. 15C). However, the coverage region of SlpB is shared between SlpA and SlpB (red box, Fig. 15C), and no single unique peptide from SlpB was identified. MALDI-TOF data were analyzed on Scaffold1 (Searle, 2010).²⁶ Therefore, mass spectrometry and SDS-PAGE analyses clearly demonstrated that the identity of the purified SlpA protein was retained whether purified by NaCl or by LiCl (Fig. 15).

To assess potential toxicity of the isolated SlpA, groups of C57BL/6 mice were then orally gavaged with SlpA (0, 150, 300, 600 µg/100µL per mouse) every other day for a total of four times. Subsequently, the blood chemistry profiles of these animals were analyzed. Obtained data demonstrated that oral treatment of the mice with varying doses of SlpA did not significantly alter whole blood biochemical values in these animals (Fig. 16). Changes in enzyme activity or concentration of other analytes in the blood were used as metrics of tissue damage or physiologic stress. Various parameters were measured, including total protein and albumin; the concentration of globulins is a calculation based on the aforementioned measurements (Fig. 16A). Function of the urinary system was evaluated by measuring blood levels of urea nitrogen (BUN) and creatinine, which are normally excreted by the kidneys

(Fig. 16B). Any evidence of hepatocyte injury was assessed by measuring the activity of the hepatocellular leakage enzyme, alanine aminotransferase (ALT) (Fig. 16C). The production and activity of alkaline phosphatase (ALP), which is associated with biliary epithelial cells and canalicular membranes of cells in the liver (Center, 2007),²⁷ can be seen with solubilization of hepatocyte membranes due to increased bile salts and release of membrane blebs with cellular injury (Fig. 16D) (Thrall *et al.*, 2012).²⁸ The electrolytes, sodium, potassium, calcium, and phosphorus, were also measured to gauge any changes in hydration status, excretional activity, or global cellular damage within the treated mice (Fig. 16E). No statistical differences were found in any of the parameters when comparing the controls and those mice receiving varying doses of SlpA administration, indicating no evidence of toxicity with oral treatment with SlpA in these animals.

We then elected to generate a specific monoclonal antibody against purified SlpA (Bergeron *et al.*, 2009; Simrell *et al.*, 1979).^{29, 30} Thus, groups of C57BL/6 mice were immunized with purified SlpA with *L. gasseri* as an adjuvant for 3 months (every week/100 µg of SlpA). Subsequently, spleen cells were derived to generate hybridoma cells producing monoclonal antibody (mAb) recognizing SlpA. As seen in Fig. 17, the antibody derived from one of our hybridoma cell clones, BM1, recognized SlpA by Western blot (Fig. 17A). Furthermore, this mAb also recognized SlpA on the surface of SlpA-coated beads, and on SlpA-pulsed RAW 264.7 macrophages (Figs. 17B-C, respectively). As we previously noted significant immunomodulatory effects by purified SlpA in the colon, these data suggested that SlpA dissolved in PBS may resist the hostile acidic milieu of the upper gastrointestinal tract and/or enzymatic degradation within the intestinal lumen. To verify this, we established an ELISA using the mAb, BM1, that can detect SlpA (Fig. 17D). Data clearly show that using this developed ELISA, SlpA can be detected in the fecal samples from mono-associated germ-free B6 mice (Fig. 17E), indicating that SlpA can potentially resist the harsh conditions of the gastrointestinal system. These data may be useful for initiating Phase I clinical trials using NaCl-purified SlpA to demonstrate its ability to potentially downregulate induced colonic inflammation in man.

Concluding Remarks

To gain further insights into the physiological effects of SlpA, studies have been performed to elucidate the feasibility of Phase I clinical trials using this protein. It appears that SlpA using the newly employed purification method does not elicit potential toxicity

when administered orally to animals, and that the structural epitope(s) of this bacterial protein can still be recognized by the mAb generated in our laboratory even after it is excreted through the feces. Nonetheless, further mechanistic studies, such as local and peripheral, targeted and untargeted metabolomics in treated animals, are required to demonstrate the role of SlpA on the host physiology, as well as its effects on other intestinal immune cells, including epithelial cells, colonic B cells, which mount critical humoral immune responses (e.g., IgA), and innate lymphoid cells (ILCs) in steady state and colonic disease.

It should be understood that the examples and embodiments described herein are for 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 the scope of the appended claims. In addition, any elements or limitations of any invention or embodiment thereof disclosed herein can be combined with any and/or all other elements or limitations (individually or in any combination) or any other invention or embodiment thereof disclosed herein, and all such combinations are contemplated with the scope of the invention without limitation thereto.

SEQ ID NO: 1 (Genbank Accession No. AAV42337.1)

1 MERTKSFFKW LTQTKLGFFT IVLVLFWLKT YYYILTKFNL GAVGPMQQFL LLINPIPSGM
 61 LLLGIGLFFK GRKSYWIILI IDFLTLWLFL SNILYYREFS NFLSFSIIKT SGSTSDNLGK
 121 SIAGITLASD FLAFLDIAVI IALLATKVIK MDVRPLKLV SLLIEFLALS LMGLNLLMAQ
 181 KDRSGLLTRT FDNMYIVKYL GINEYAIYDG YKTAQTS AQM AKANVSDLKS VRNYLNANKV
 241 KPNPEYTGVA KGKNVLVIHL ESFQQFLIGY KWKGKEVTPN LNKIYHQDIT ISFDNFFNQV
 301 GQGKTSDAEM MLENSLYGLQ SGSAMSTYGT SNTFESAPAI LHQQAGYT TA VMHGGAGSFW
 361 NRNNAYKSFG YQYFMPLSFY ENKPSYYIGY GLKDKIFFDQ SIKYIERLPQ PFYLMITVT
 421 NHYPYDIDKK NQSI AKTNTG DETVDGYVQT AHYLDQAIGE LMSWMKKTGL DKKTLIVFYG
 481 DHYGISGNHH KAS AQLLKKK SFNDFDNLQF QRVPLMFHMK GLKGGINHTY GGEIDVLPTL
 541 LNLLGIKDS D TIQFGYDLLS KNAPQIVAQR NGDFITPEYS KVGSDYYYTK TGKRIKPNKK
 601 LKAELTAISN TVTTQLSLSD RVINGNLLRF YRPKWFTKVK PKDYDYNKEP SLKRLFNDPS
 661 KTS LWYQNHK KTTQKDFKTD APELKK

SEQ ID NO: 2 (Genbank Accession No. YP_193105)

1 MKKNLRIVSA AAAALLAVAP VAASAVSTVN AAAVNAIAVG GSATPLPNNS DVQISSSVAG
 61 VTTKNGSSYT NGRISGSINA SYNGT SYSAN FSSSNAGVVV STPGHTELSG EQINGLEPGS
 121 AVTVTLRDGV SFNFGSTNAN KTITLAFPKN VSAAGLADAN KVSATSETSV DAGKTIQVKT
 181 DKNGVVSFGS AQVLNVKVVE TSDVRAVSFY DIQTGKTVEN GTLSIVAGSN ARANVQEIVN
 241 AFNAKYQASQ LNNANSNANV RLTDNNAQAV ATMLRAQNID VDAQGYFTAP ASLSLTFHAE
 301 STQNNETAQL PVTVSVTNGK EVTPSTVDVS SKRIMHNAYY YDKDAKRVGT DSVKRYNSVS
 361 VLPNTTTING KAYYQVVEN G KAVDKYINAA NIDGKTRTLK HNAYVYASSK KRANKVVLKK
 421 GEVVTTYGAS YTFKNGQKYY KIGDNTDKTY VKVANFR

SEQ ID NO: 3 (Genbank Accession No. YP_193425)

1 MKKNRKMLGL AAATLLAVAP VATSVVPVQA DTAVNVGSAA GTGANTTNTT TQAPQNKPYF
 61 TYNNEIIGEA TQSNPLGNV RTTISFKSDD KVS DLISTIS KAVQFHKNN S ASGENTVINE
 121 NDFINQLKAN GVTVKTVQPS NKNEKAYEAI DKVPSTSFNI TLSATGDNNQ TATI QIPMVP
 181 QGASTPTDTT QNPQINWTKG GQAQSSSLNG QVFQVAVGSN FNPLNFTNSN GENIIVSAQQ
 241 SKNNTTFASI EATSNPVNTS EAGRYYNVTL TATGNTGKKT TATYTVLITS SQKQTLYGNG
 301 ESTISTYSIY GNNVLSNSTT FKG DQVYVS DQTKTVGGVS YSQVSPKSKN DANSSNIWVK
 361 TSALVKPAGD TNVKTYPVMV DSRAYDKNGN YLGHMYAYD NIDIVPTVVT INGKTY YKVA
 421 NKDEYVRVTN ITGNQRTLKH NAYIYWSSYR RTPGTGKMYR GQTVTTYGPQ MKFKNGKKYY
 481 RIEGCRNNNK RYIKAVN FY

SEQ ID NO: 4 (Genbank Accession No. P35829)

1 MKKNLRIVSA AAAALLAVAP VAASAVSTVS AATTINASSS AINTNTNAKY DVDVTPSVSA
 61 VAANTANNT PAIAGNLTGTI SASYNGKTYT ANLKADTENA TITAAGSTTA VKPAELAAGV
 121 AYT VTVNDVS FNF GSENAGK TVTLGSANSN VKFTGTNSDN QTETNVSTLK VKLDQNGVAS
 181 LTNVSIANVY AINTTDNSNV NFYDVTSGAT VTNGAVSVNA DNQGGVNVAN VVAAINS KYF
 241 AAQYADKKLN TRTANTEDAI KAALKDQKID VNSVG YFKAP HTFTVNVKAT SNTNGKSATL
 301 PVVVTVPNVA EPTVASVSKR IMHNAYYYDK DAKRVGTDSV KRYNSVSVLP NTTTTINGKTY
 361 YQVVEN GKAV DKYINAA NID GTRTLKHNA YVYASSKKRA NKVVLKKGEV VTTYGASYTF
 421 KNGQKYKIG DNTDKTYVKV ANFR

SEQ ID NO: 47 Uniprot Access Number C2HR60

AATTINASSSAINTNTNAKYDVDVTPSVS AAVAANTANNTPAIAGNLTGTISAS YNGK
 TYTANLKADTENATITAAGSTTAVKPAELAAGVAYTVTVNDVS FNF GSENAGKTVTLGSA
 NSNVKFTGTNSDNQTETNVSTLKV KLDQNGVASLTNVSIANVY AINTTDNSNVNFYDVTS
 GATVTNGAVSVNADNQGQVNVANVVAAINS KYFAAQYADKKLNTRTANTEDAIKAALKDQ
 KIDVNSVG YFKAPHTFTVNVKATSNTNGKSATLPVVVTVPNVAEPTVA

SEQ ID NO: 48 Uniprot Access Number P35829

MKKNLRIVSAAAAALLAVAPVAASAVSTVSAATTINASSSAINTNTNAKYDVDVTPSVSA
VAANTANNTPAIAGNLTGTISASYNKTYTANLKADTENATITAAGSTTAVKPAELAAGV
AYTVTVNDVSFNFGESENAGKTVTLGSANSNVKFTGTNSDNQETENVSTLKVKLDQNGVAS
LTNVSIAINVYAINTTDNSNVNFYDVTSGATVTNGAVSVNADNQGVNVANVVAAINSKEYF
AAQYADKKLNTRTANTEDAIKAALKDQKIDVNSVGYFKAPHTFTVNVKATSNTNGKSATL
PVVVTVPNVAEPTVASVSKRIMHNAYYYDKDAKRVGTDSVKRYNSVSVLPNTTTINGKTY
YQVVENGKAVDKYINAANIDGTRKRTLKHNAVYVYASSKKRANKVVLKKGEVVTTYGASYTF
KNGQKYYKIGDNTDKTYVKVANFR

SEQ ID NO: 49 Uniprot Access Number G1UE81

MFGGRKIMQSSSLKKSLEYLGLAALSFAGVAAVSTTASAKSYATAGAYTTLKTDATKRNEA
TGTNALYTKPGTVKGAKVVASKATMAKLASSKKSADYFRAYGVKTTNRGSVYYRVVSMDG
KYRGYVYGGKSDTAFAGGIKSADTTTTATTPTRTTGYLLKDVSKNTLWTAPKNTQYKASK
VSLYGVKSTDTFKVDSAATKTREGSLYYHVTDTQNTSVSGWIYAGKGYVAGATTQDLGGL
SLTMSDAAATSDNSVKVYRASGSQVGTATWVTAAAGTKAGATVGTAVNAAGVKLADFFV
TNSLPSGYTTTGTVD TASATYGNTVYVDVTAAATSKVQLVADNVNDNTASTTNAVAGVLA
NGAKLSSSDLSATLKEAGIKALTGTKEAIGATNLATISGAFDTAEINGSKTYAANGDA
YHYVFTYEPANFANDNRLATYGDTLTASFKAFLTCKGAPSASSSNSSWIA

SEQ ID NO: 50 Uniprot Access Number Q9Z4J9

MKKNLRIVSAAAAALLAVAPVAASAVSVNAADNTVATTTNTANTVINADGTAINTPADAK
YDVDVTPNLTATAASTVNGQTINGSI TGNITASYNGQSYTGTLDTKNGKVSADSKGTAV
TDFSKLTNGSYT VTVSGVSFNFGTANANKTITLGSKNSNVKFAGADGKFADTVKVELGQN
GTLTTPISVQVSNVNALDLSNANGVNFYNASNGSQVTGKSVNVTAGLIGRLNVSTVASEI
LKNCAAYQVSNGKPVSQLPDQKAVVADVNAALKAANI PVDNAGWFTAPI SLSVNVKASSS
INGVGCYFTCTVNVANGKDMTVPSQSKTIMHNAYYYDKDAKRVGTDKLTTRYNSVTVMNT
TTINGKAYYEVIENGKATGKF INADNIDGTRKRTLKHNAVYVYKTSKKRANKVTLKKGTEVT
TYGGTYTFKNGKQYYKIGNNTDKTYVKASNF

SEQ ID NO: 51 Uniprot Access Number H6VTN4

MKKNLRIVSAAAAALLAVAPVAAAGVSSVTASSIEFVGSSNSSLLPEVNDHTVNFGINFN
AIGAYGNVPSSVSATAEVTINGQKTTINLPENQKSYIYYATTNESVDASKLVAGQKYYTG
INNASLNLGSPNHDKDITLEGSNVSFKTNDSDPYTKTLKVNTDKNGVISNLSIKSANFDA
VDVNNARTVSFYDADTGNIVTSGALEINAGPNAQMNVTILAKFEQKYQAAQLNNAGTTN
NVSYNNDLISTTPADLAAQLKAGYSVDNNGYFTAKHSFTVNFSKSGQNGYTTTTPVTV
TVPNVAEETVPSQIRTVMHNAFFYDKNGKRVGSDKVTRYNSATVAMNTTTIIGKAYYEVI
ENGKATGKF INAANIDGTRKRTLKHNAVYVYKSSKKRANKVVLKKGETVVTYGGAYTFKNGK
QYYKIGNNTDKTYVKVANF

SEQ ID NO: 52 Uniprot Access Number Q09FM2

MKKNLRIVSAAAAALLAVAPVAASAVSVNAASSSAVQTATNIGTVLPLTDGSTVNVKPN
SLNTSAYEGVKANISVSFSATVDGTTATSNFTPNASTIELWKNEKNKVTQVTYLQQVTSS
NAGATYQVKMTQVGLNFGSQNANKKVTTLTFPEGDMFKTADTSLAQSHVVKLDQNGTITLP
EVVMNVTAKDFANPAVVNWNATNAVSTGNIELFAGSDAGKMNVAVQVVSATEKKYHAS
NYGTKANQESSTISYTNLKDALKAMNVDVDAQGWVAPKSF TFMNTAKANNNDASSTLA
VTVSVPNGKDMTVPSQSKTVMHNAFFYDKNGKRVGSDKVTRYNSATVAMNTTTINGKAYY
EVIENGKATGKF INAANIDGTRKRTLKHNAVYVYKSSKKRANKVVLKKGETVVTYGGAYTFK
NGKQYYKIGNNTDKTYVKVSNF

SEQ ID NO: 53 Uniprot Access Number L7YE91

SVSESKDTVNVTPSF TLTSAIPAKGV PATLQGSIEASLNGTSVTADVADVAKDVTLT DGN
KTVYSYNERENKVDNNLSAVEASKEYTMTLSGVGFSFGKANAGKTLTFKLPKNVKVNDTS
5 NDVKVSLDQYGNATNLKFVISNIKAYDSANTNAVVSFYAAKSGLVATQGSYMTLADENGNL
NVNTLLDKLKGKYEAMQFKDSKFETVNVNTTADDVKAELEKAGIKVDAANNFEAPDTFTV
TLNAKSDVNGKTASLPVVVTVPNGKSTVVPSQSKTIMHNAYYYDKDAKRVGTDKVTRYNA
VTVAMNTTKLANGISYYEVIEN

SEQ ID NO: 54 Uniprot Access Number K8DVK7

ADSAINANTNAKYDVDVTPSISAI AAVAKSDTMPAIPGSLTGSI SASYNGKSYTANLPKD
SGNATITDSNNNTVKPAKLEADKAYTVTVPDVSFNFGSENAGKVITIGSANPNVTFTKKT
GDQPASTVKVTL DQDGVAKLSSVQIKNVY AIDTTYNSNVNFYDVTTGAI VTTGAVSIDAD
15 NQGQLNITSVVAAINS KYFAAQYDKKQLTNDVTFDTETAVKDALKAQKIEVSSVGYFKAP
HTFTVNVKATSNKNGKSATLPVTVPNVADPVVPSQSKTIMHNAYFYDKDAKRVGTDKV
TRYNTVTVAMNTTKLANGISYYEVIENGKA

SEQ ID NO: 55 Uniprot Access Number F0NUB7

MDHVS KGFVHYRLLSHAEP MAYYIFYISRRKDHMKKNLRIVSAAAAALLAVAPVAATAMP
20 VNAATTINADSAINANTNAKYDVDVTPSISAI AKVTGSATIPGSLTGSI SASYNGKSYTA
NLPKDSGNATIADKHGPNV KPADLEADKAYTVTVPDVSFNFGSENAGKEITIGSANQNV T
FTTKDSQSGSTVSGSTVKVTL DQDGVAKLSSVQIKDVY AIDTTYNSNVNFYDVTTGAI V T
TGAVSIDADNQGQLNTASVVA AISKYFAAQYADKNLTSDNVTYNIETAVKDALKAQKIE
VSSVGYFKAPHTFTVNVKATSNKNGKSATLPVTVPNVADPVVPSQSKTIMHNAYFYDK
25 DAKRVGTDKVTRYNTVTVAMNTTKLANGISYYEVIENGKATGKYINADNIDGTRTLKHN
AYVYKTSKKRANKVVLKKGTEVTTYGGSYKFKNGKKYYKIGADTKKTYVRVENFD

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CLAIMS

We claim:

1. An isolated bacterial SlpA protein or a non-naturally occurring protein derivative thereof.
2. The isolated bacterial SlpA protein of claim 1, wherein the protein has the amino acid sequence of SEQ ID NO: 4 or 47-55 or the protein has the amino acid sequence at least 95% identical to the sequence of SEQ ID NO: 4 or 47-55.
3. The non-naturally occurring SlpA protein derivative according to claim 1 or 2, wherein the non-naturally occurring protein SlpA derivative comprises:
 - a) a molecular label conjugated to the non-naturally occurring SlpA protein,
 - b) one or more mutations in the naturally occurring sequence of the SlpA protein, or
 - c) a multimer of SlpA protein.
4. The non-naturally occurring protein derivative of SlpA according to claim 3, wherein the molecular label is a radiolabel, fluorescent label, affinity label or targeting label.
5. The multimer of SlpA protein according to claim 3, wherein the multimer is a dimer, trimer, tetramer, pentamer, hexamer, heptamer, octamer, nonamer or decamer.
6. The multimer of SlpA protein according to claim 3, wherein the multimer comprises more than ten SlpA molecules.
7. A composition comprising the naturally or non-naturally occurring SlpA protein derivative of claims 1-7 and a pharmaceutically acceptable carrier and/or excipient, said composition optionally containing sulfasalazine, cyclosporine, prednisone, methylprednisone, budesonide, mesalamine, azathioprine, TNF inhibitors, methotrexate, or 6-mercaptopurine, a corticosteroid, infliximab or combinations thereof.

8. A method of treating an inflammation mediated disease of the gastrointestinal system in a subject, the method comprising, administering to the subject a therapeutically effective amount of the composition of claim 7.

9. The method of claim 8, wherein the composition is orally administered to the subject.

10. The method of claim 8, wherein the inflammation mediated disease is IBD.

11. The method of claim 10, wherein the IBD is Crohn's disease or ulcerative colitis.

12. A recombinant bacterium, the recombinant bacterium being:

a) genetically modified to decrease or eliminate the display of lipoteichoic acid (LTA), surface layer protein B (SlpB) and surface layer protein X (SlpX) on the surface of said bacterium and genetically modified to express surface layer protein A (SlpA) on its surface; or b) lacking expression of LTA, SlpB and SlpX on the cell surface and genetically modified to express surface layer protein A (SlpA) on its surface.

13. The recombinant bacterium of claim 12, wherein said recombinant bacterium has been genetically modified to:

a) express a protein that has the amino acid sequence of SEQ ID NO: 4 or the protein has the amino acid sequence at least 70%, 80%, 90%, 95%, 97%, 98%, or 99% identical to the sequence of SEQ ID NO: 4; and decrease or eliminate the expression of:

(i) a polypeptide comprising the amino acid sequence of SEQ ID NO: 1 or a polypeptide having at least 70%, 80%, 90%, 95%, 97%, 98%, or 99% sequence identity to the amino acid sequence of SEQ ID NO: 1;

(ii) a polypeptide comprising the amino acid sequence of SEQ ID NO: 2 or a polypeptide comprising the amino acid sequence having at least 70%, 80%, 90%, 95%, 97%, 98%, or 99% sequence identity to the amino acid sequence of SEQ ID NO: 2; and

- (iii) a polypeptide comprising the amino acid sequence of SEQ ID NO: 3 or a polypeptide having at least 70%, 80%, 90%, 95%, 97%, 98%, or 99% sequence identity to the amino acid sequence of SEQ ID NO: 3;
- b) express a protein that has the amino acid sequence of SEQ ID NO: 4 or a protein that has the amino acid sequence at least 70%, 80%, 90%, 95%, 97%, 98%, or 99% identical to the sequence of SEQ ID NO: 4 and lacks genes encoding SEQ ID NO: 1, 2 and 3 or orthologs thereof.

14. The recombinant bacterium of claim 12 or 13, wherein said recombinant bacterium is selected from the group consisting of *L. acidophilus*, *L. amylolyticus*, *L. amylovorus*, *L. brevis*, *L. brevis* ssp *gravesensis*, *L. buchneri*, *L. crispatus*, *L. gallinarum*, *L. gigeriorum*, *L. helveticus/suntoryeus*, *L. hilgardii*, *L. kefiranoferiens*, *L. pasteurii*, *L. lactis* and *L. ultunensis*.

15. The recombinant bacterium of claim 12 or 13, wherein said bacterium is a lactic acid bacterium.

16. The recombinant bacterium of claim 15, wherein said lactic acid bacterium is a *Lactobacillus*.

17. The recombinant bacterium of claim 16, wherein said recombinant bacterium is *L. acidophilus* or *L. lactis*.

18. A probiotic food comprising the recombinant bacterium of claims 12-17.

19. The probiotic food of claim 18, wherein the food is yogurt, fermented vegetable, kefir, sauerkraut, miso soup, pickle, tempeh or kimchi.

20. A method of treating and/or preventing an inflammation mediated disease of the gastrointestinal system in a subject, the method comprising, administering to the subject a therapeutically effective amount of the recombinant bacterium of claims 12-17 or a composition comprising said recombinant bacterium.

21. The method of claim 20, wherein the recombinant bacterium is orally administered to the subject.

22. The method of claim 20, wherein the inflammation mediated disease is inflammatory bowel disease (IBD).

23. The method of claim 22, wherein the IBD is Crohn's disease or ulcerative colitis.

24. A method of purifying SlpA comprising growing SlpA expressing bacterial cells, pelleting said bacterial cells from culture medium containing said bacterial cells, resuspending said bacterial cells in 5M NaCl for a period of 5 minutes to 24 hours to form an extraction composition, removing bacterial cells from said extraction composition by centrifugation to form a composition containing SlpA and dialyzing or filtering said composition using a dialysis bag or ultrafiltrating device having a molecular weight cut-off of 30 kDa to reduce the salt content of said composition containing SlpA.

25. The method of claim 24, wherein said SlpA expressing bacterial cells do not express LTA, SlpB or orthologs thereof or SlpX or orthologs thereof.

26. The method of claim 24, wherein said SlpA expressing bacterial cells do not express phosphoglycerol transferase or orthologs thereof.

27. The method of claims 24, 25 or 26, wherein said bacterial cells:

a) express a protein that has the amino acid sequence of SEQ ID NO: 4 or the protein has the amino acid sequence at least 70%, 80%, 90%, 95%, 97%, 98%, or 99% identical to the sequence of SEQ ID NO: 4; and:

b) do not express (i) a polypeptide comprising the amino acid sequence of SEQ ID NO: 1 or a polypeptide having at least 70%, 80%, 90%, 95%, 97%, 98%, or 99% sequence identity to the amino acid sequence of SEQ ID NO: 1; (ii) a polypeptide comprising the amino acid sequence of SEQ ID NO: 2 or a polypeptide comprising the amino acid sequence having at least 70%, 80%, 90%, 95%, 97%, 98%, or 99% sequence identity to the amino acid sequence of SEQ ID NO: 2; and (iii) a polypeptide comprising the amino acid sequence of

SEQ ID NO: 3 or a polypeptide having at least 70%, 80%, 90%, 95%, 97%, 98%, or 99% sequence identity to the amino acid sequence of SEQ ID NO: 3;

28. The method of claims 24-37, wherein said bacterial cells are a *Lactobacillus* strain.

29. The method of claim 28, wherein said *Lactobacillus* strain is selected from the group consisting of *L. acidophilus*, *L. amylolyticus*, *L. amylovorus*, *L. brevis*, *L. brevis* ssp *gravesensis*, *L. buchneri*, *L. crispatus*, *L. gallinarum*, *L. gigeriorum*, *L. helveticus/suntoryeus*, *L. hilgardii*, *L. kefiranoferiens*, *L. pasteurii*, *L. lactis* and *L. ultunensis*.

30. The method of any preceding claim, said method further comprising the precipitation of SlpA protein in said dialyzed composition comprising SlpA.

31. The method of claim 30, said wherein SlpA is precipitated with 1M NaCl.

32. The method of claim 31, said method further comprising the washing of said precipitated SlpA with water or a buffer.

33. The method of claim 33, said method further comprising the lyophilization of said washed SlpA.

34. A method of treating an inflammatory disease in a subject, the method comprising, administering to the subject a therapeutically effective amount of the composition of claim 7.

35. The method of claim 34, wherein the composition is orally administered to the subject.

36. The method of claims 34-35, wherein the inflammatory disease is selected from the group consisting of allergies, ankylosing spondylitis, Crohn's disease, diabetes, Type I diabetes, gastroesophageal reflux disease, Hashimoto's thyroiditis, hyperthyroidism, hypothyroidism, Irritable Bowel Syndrome (IBS), interstitial cystitis (IC), L fgren's

syndrome, lupus erythematosus, myasthenia gravis, multiple sclerosis, osteoarthritis, polymyalgia rheumatica, prostatitis, psoriasis, psoriatic arthritis, Raynaud's syndrome/phenomenon, reactive arthritis (Reiter syndrome), restless leg syndrome, reflex sympathetic dystrophy (RSD), rheumatoid arthritis, scleroderma, Sjögren's syndrome, ulcerative colitis and uveitis.

37. A method of treating an inflammatory disease in a subject, the method comprising administering to the subject a therapeutically effective amount of the recombinant bacterium of claims 12-17 or a composition comprising said recombinant bacterium.

38. The method of claim 37, wherein the recombinant bacterium is orally administered to the subject.

39. The method of claims 37-38, wherein the inflammatory disease is selected from the group consisting of allergies, ankylosing spondylitis, Crohn's disease, diabetes, Type I diabetes, gastroesophageal reflux disease, Hashimoto's thyroiditis, hyperthyroidism, hypothyroidism, Irritable Bowel Syndrome (IBS), interstitial cystitis (IC), Löfgren's syndrome, lupus erythematosus, myasthenia gravis, multiple sclerosis, osteoarthritis, polymyalgia rheumatica, prostatitis, psoriasis, psoriatic arthritis, Raynaud's syndrome/phenomenon, reactive arthritis (Reiter syndrome), restless leg syndrome, reflex sympathetic dystrophy (RSD), rheumatoid arthritis, scleroderma, Sjögren's syndrome, ulcerative colitis and uveitis.

40. A composition comprising the recombinant bacterium of claims 12-17 and a pharmaceutically acceptable carrier and/or excipient and, optionally, sulfasalazine, cyclosporine, prednisone, methylprednisone, budesonide, mesalamine, azathioprine, TNF inhibitors, methotrexate, or 6-mercaptopurine, a corticosteroid, infliximab or combinations thereof.

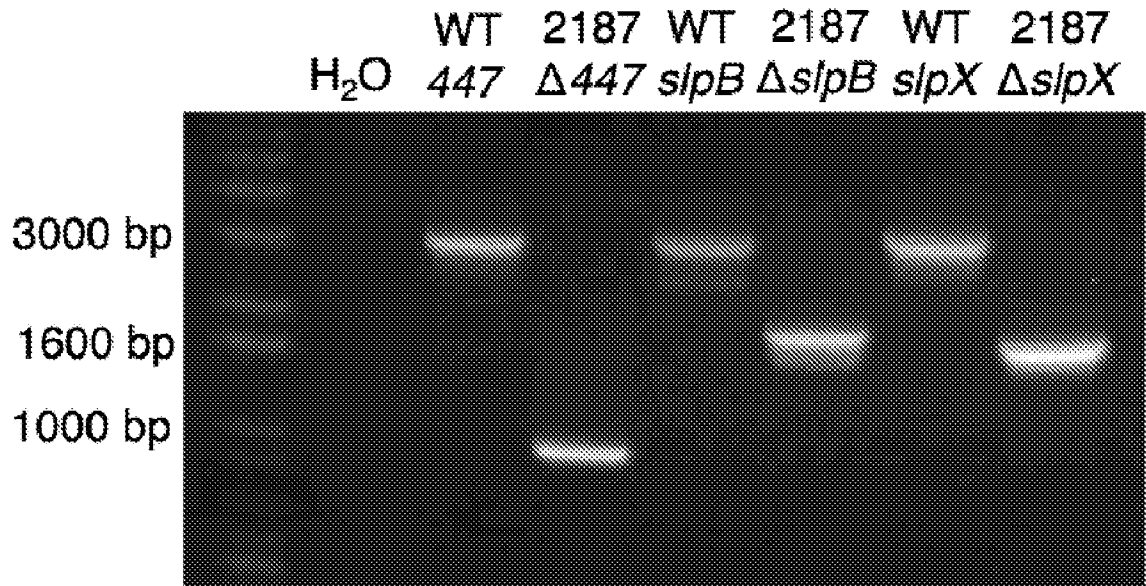


FIG. 1A

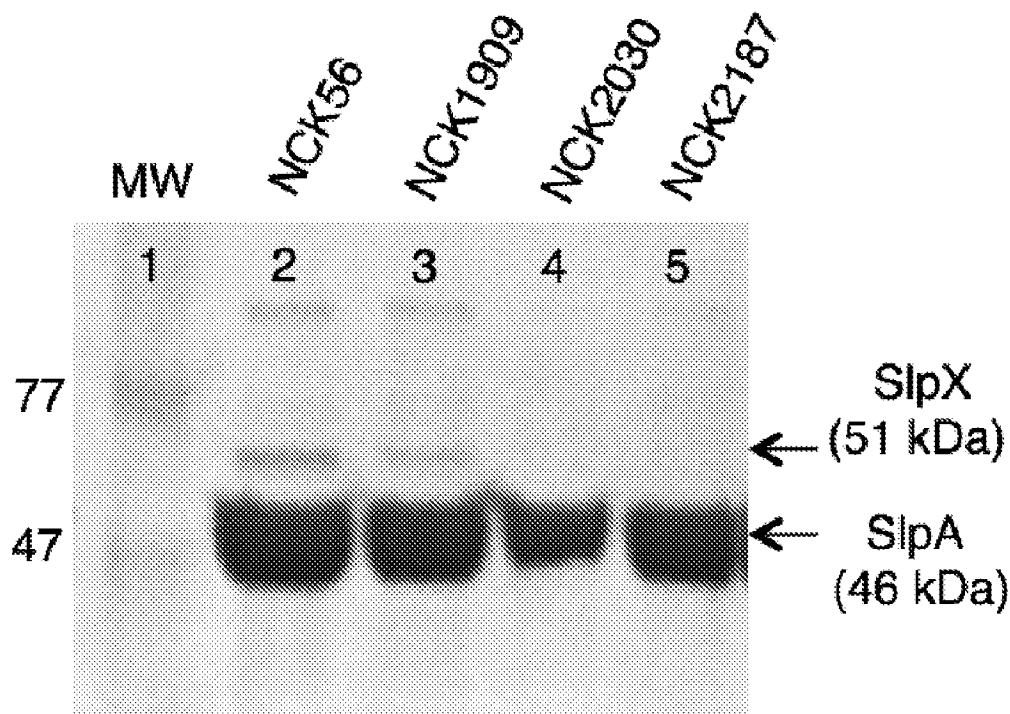


FIG. 1B

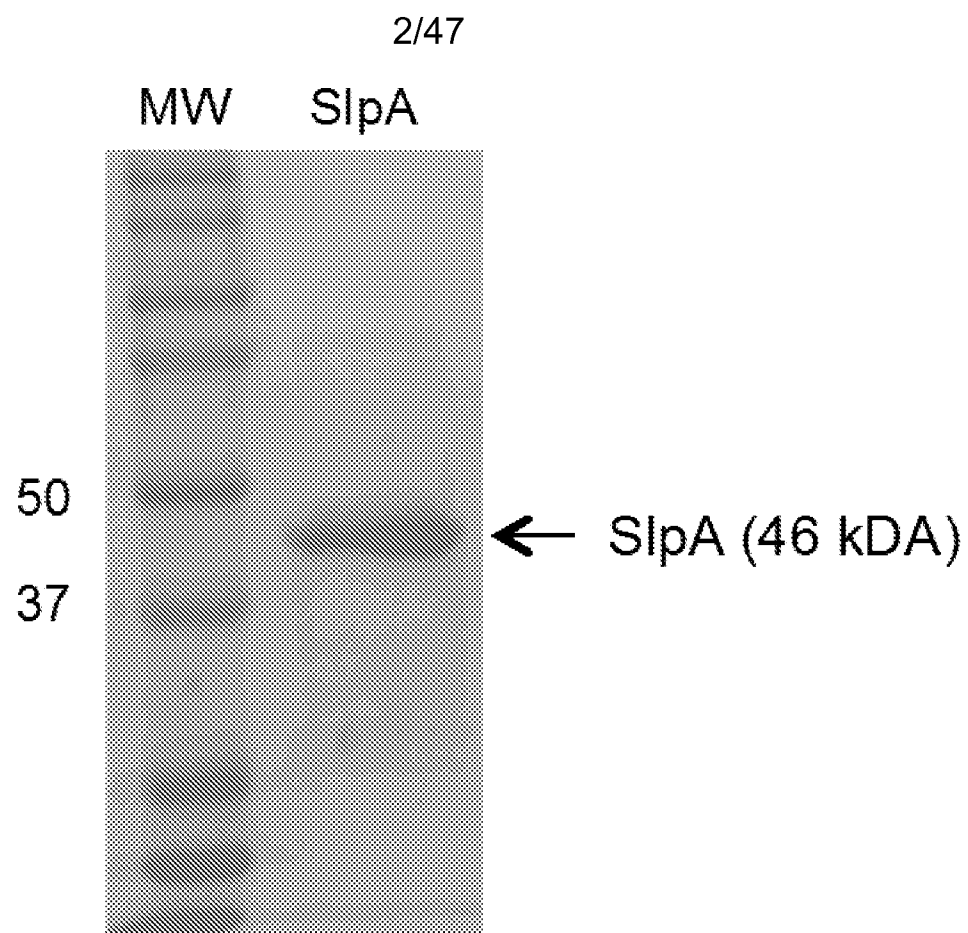


FIG. 1C

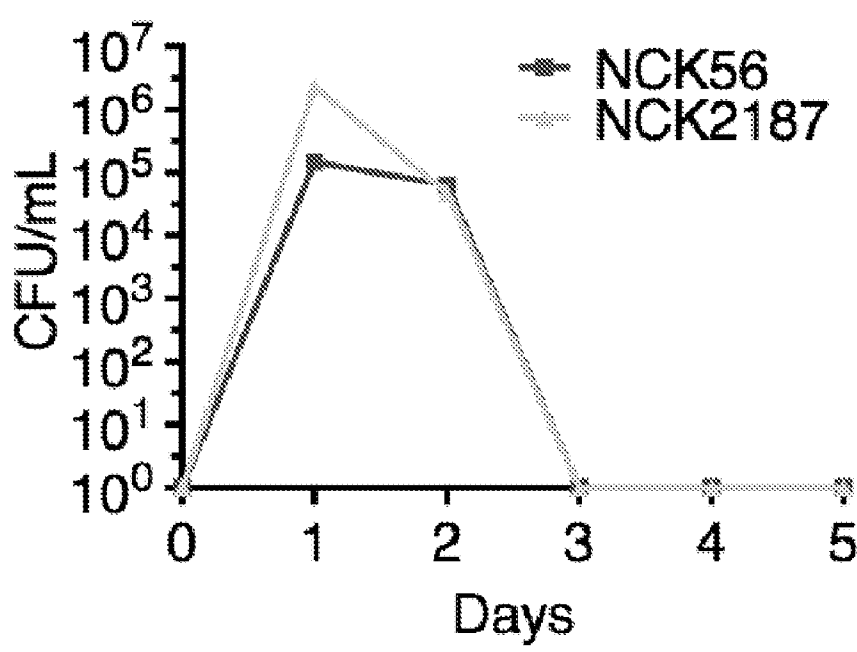


FIG. 1D

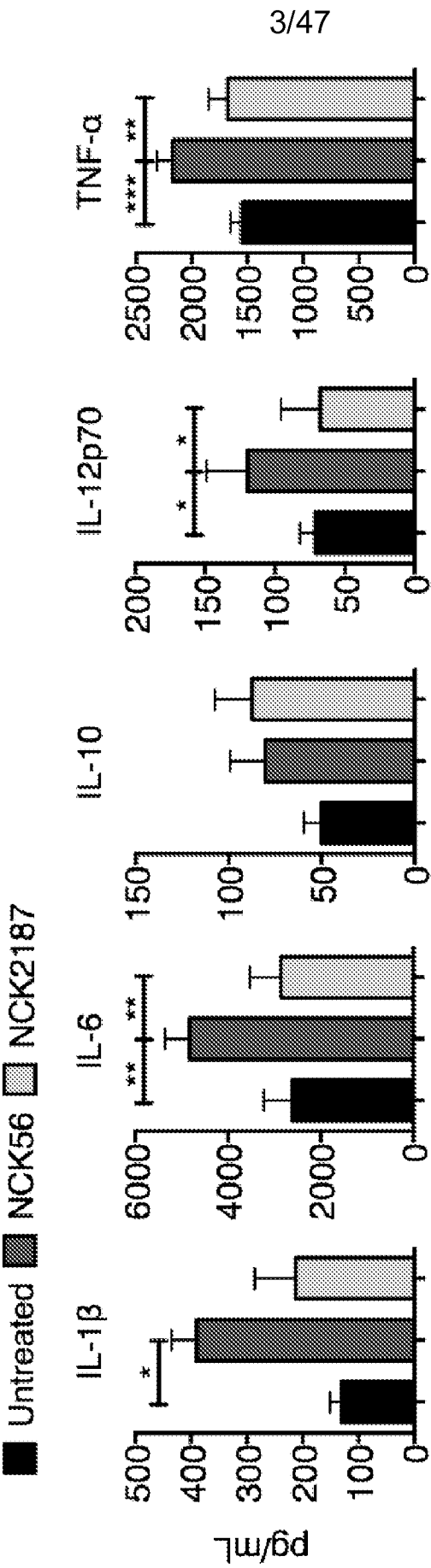


FIG. 1E

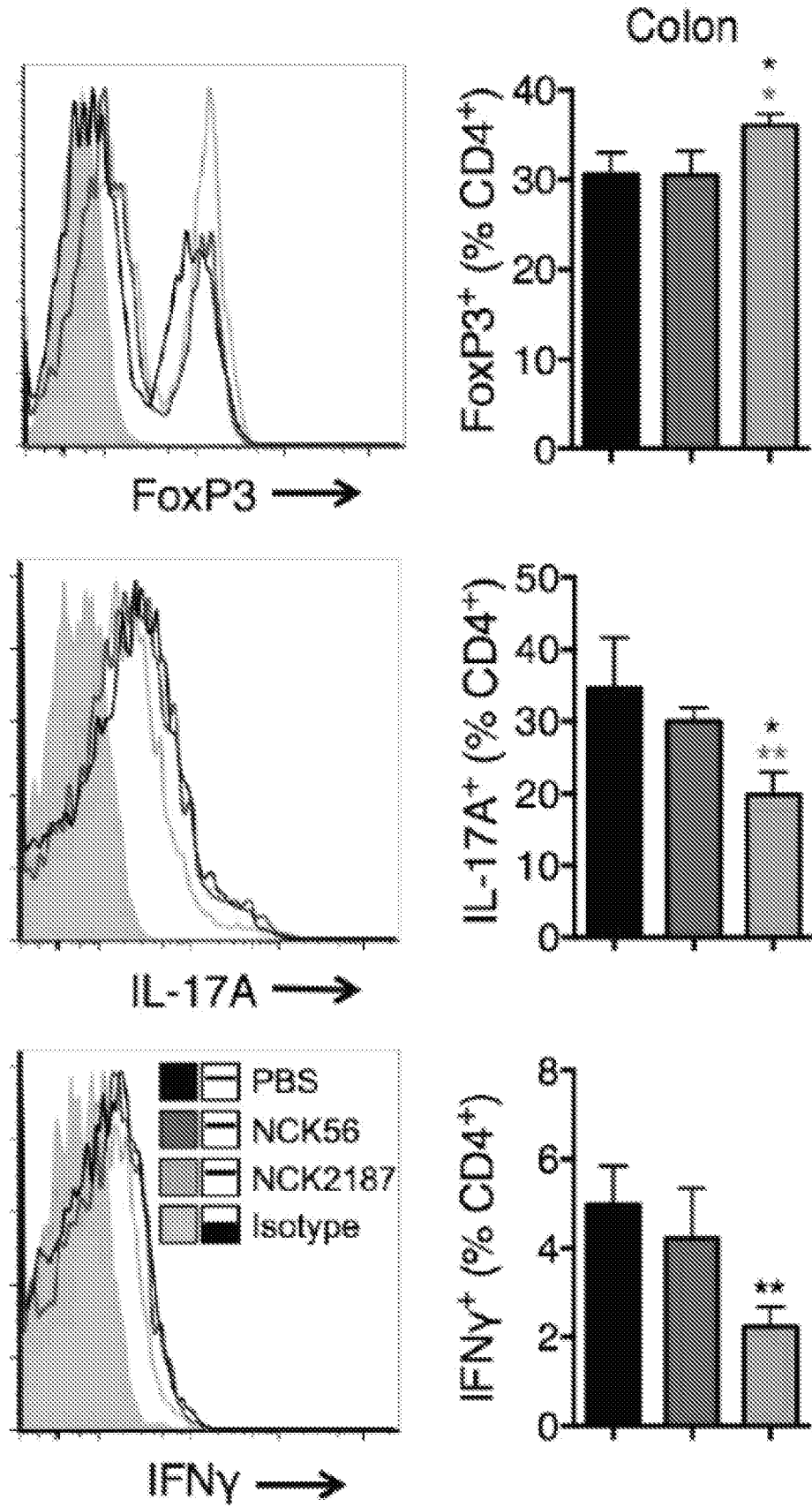
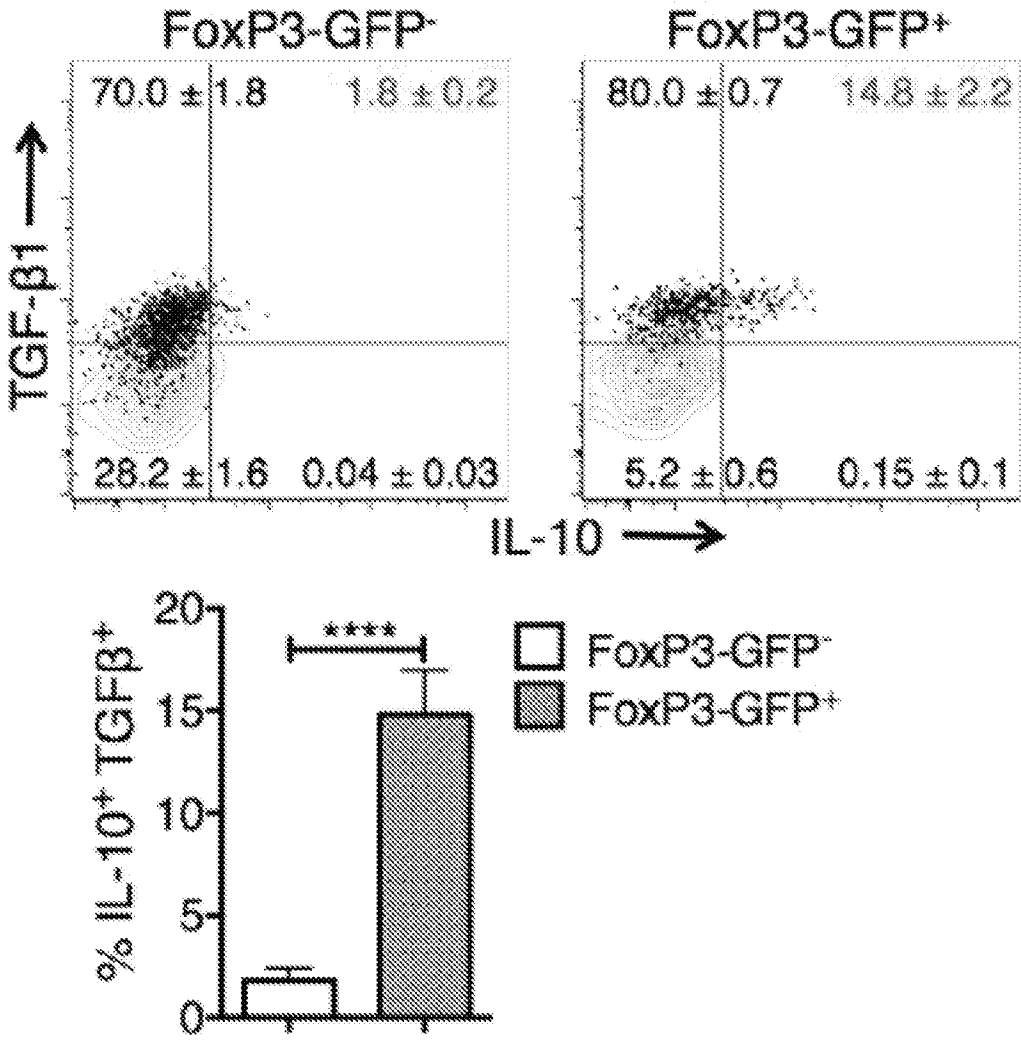
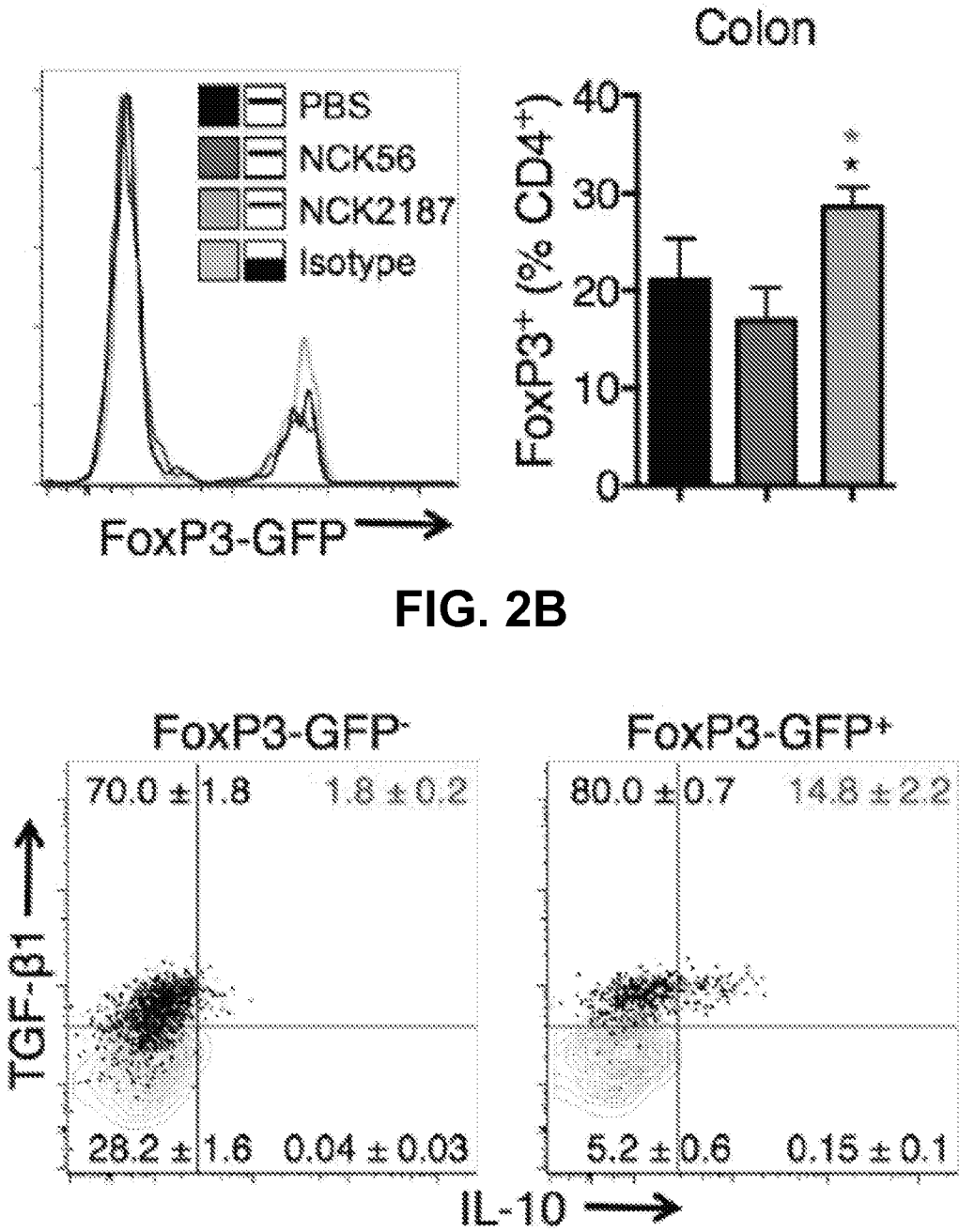
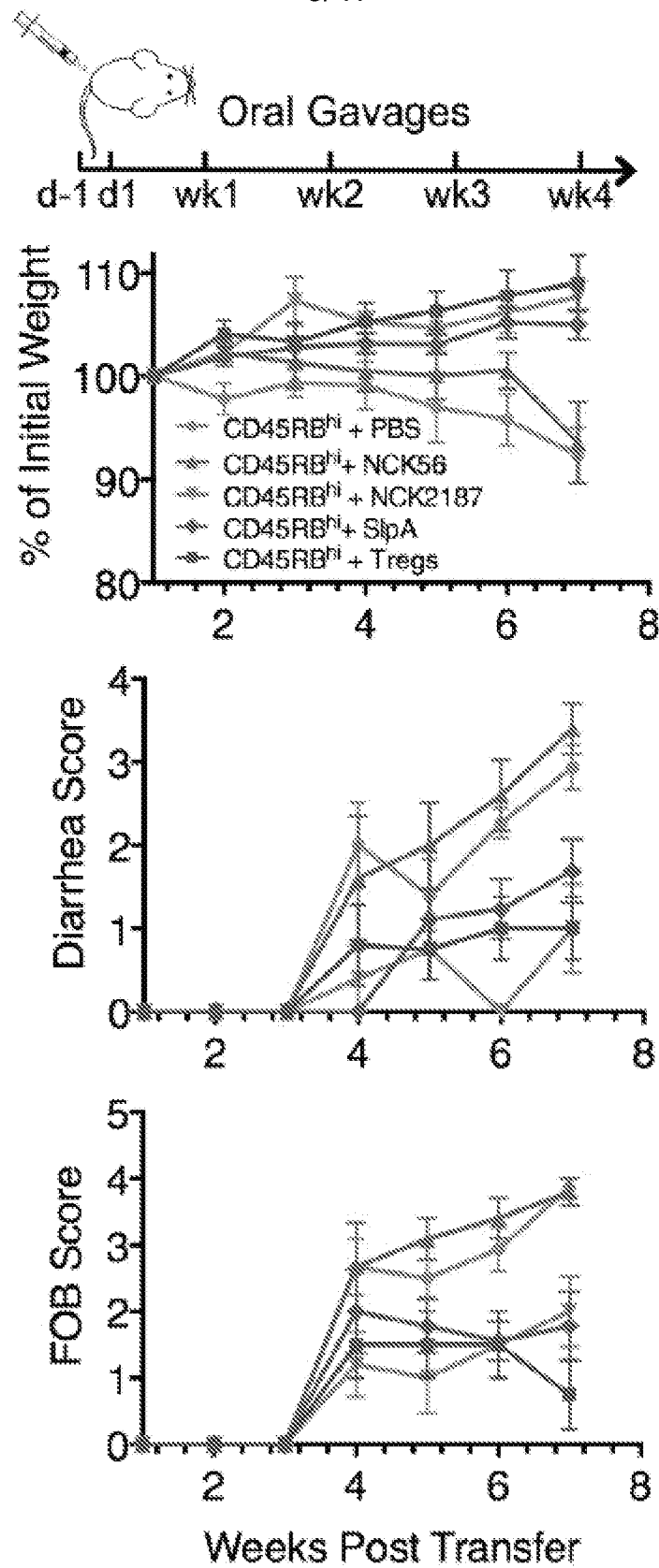


FIG. 2A



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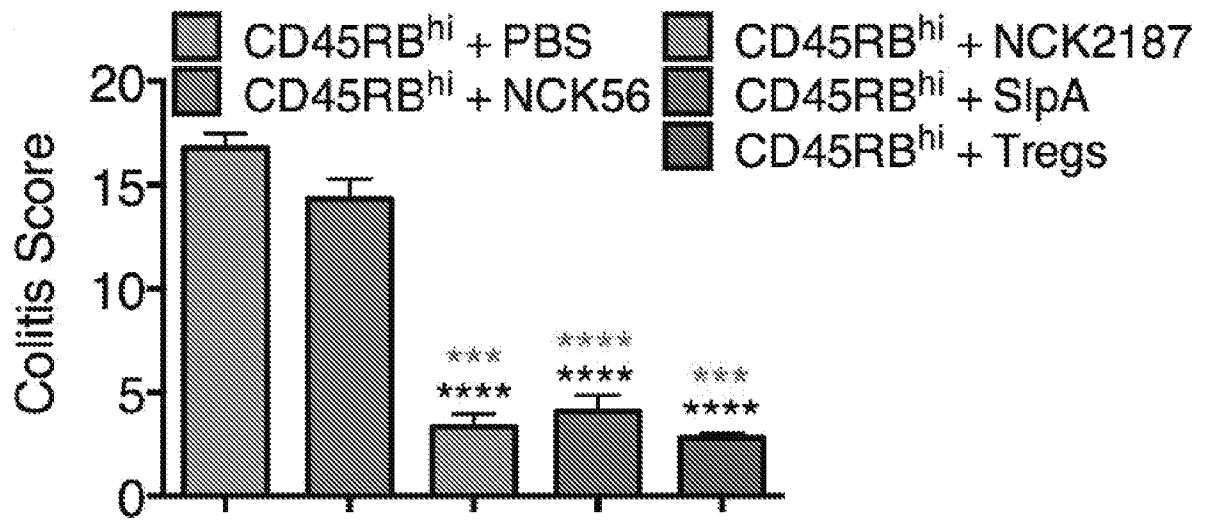


FIG. 3B

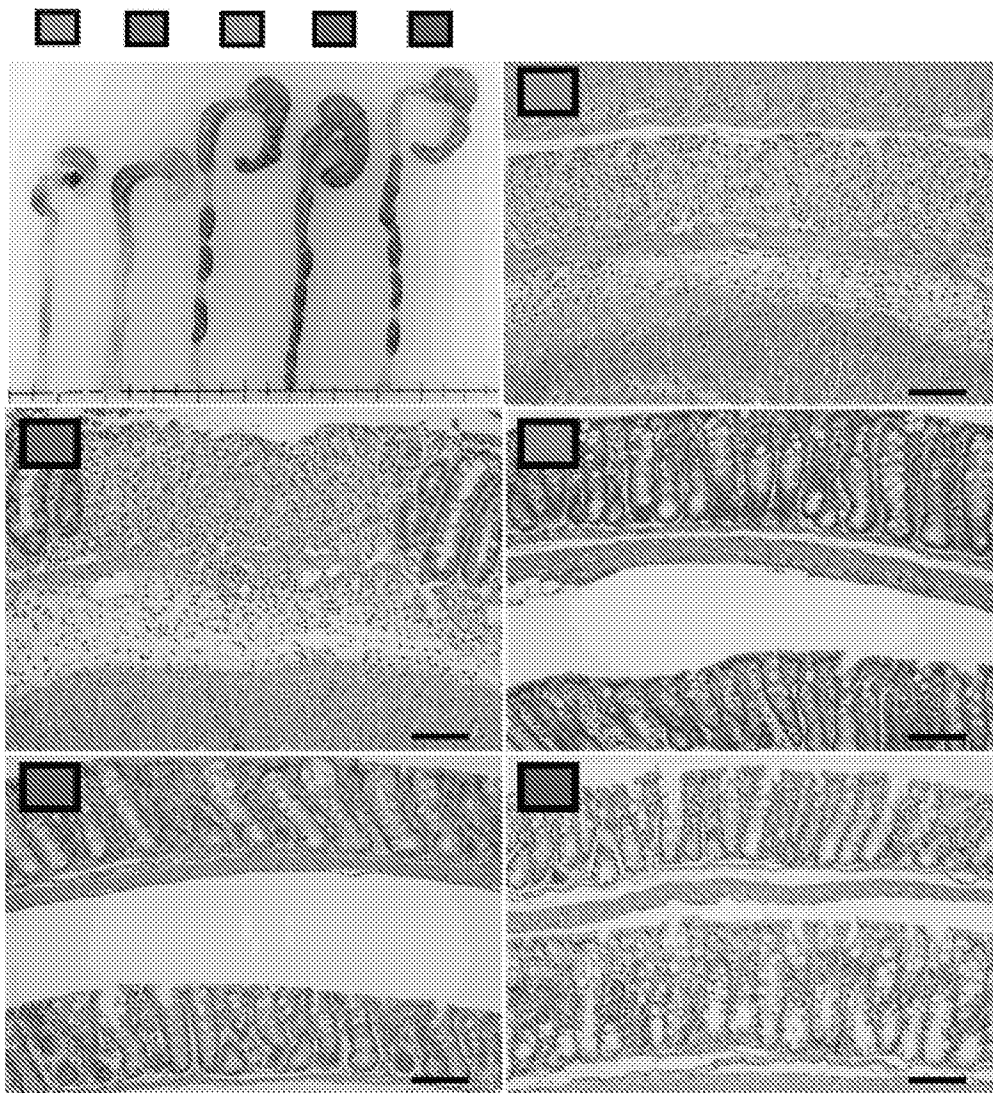


FIG. 3C

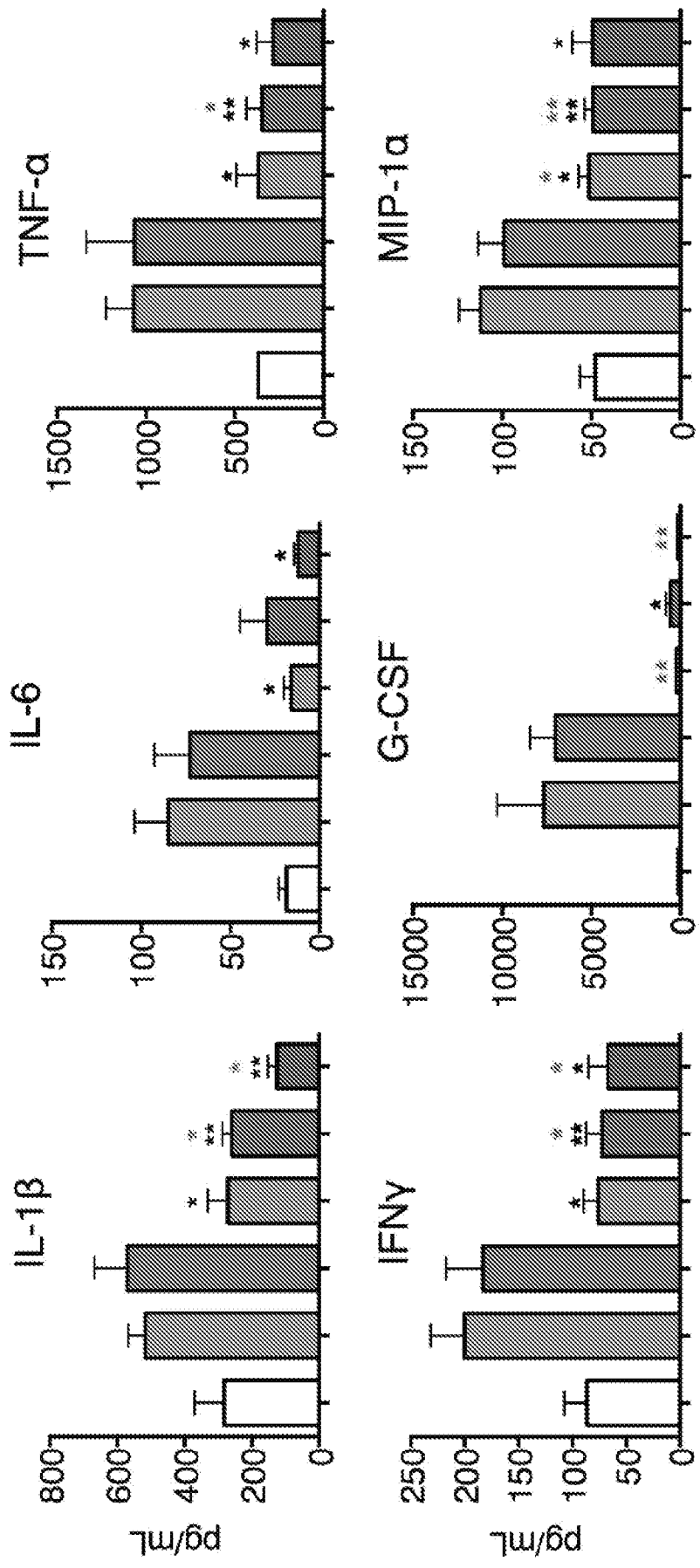


FIG. 3D

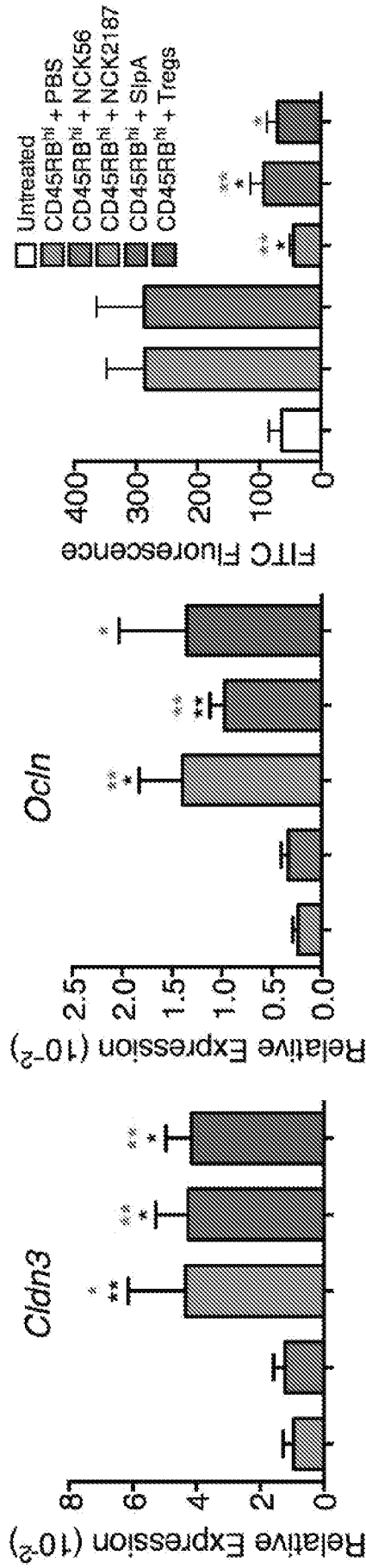


FIG. 4A

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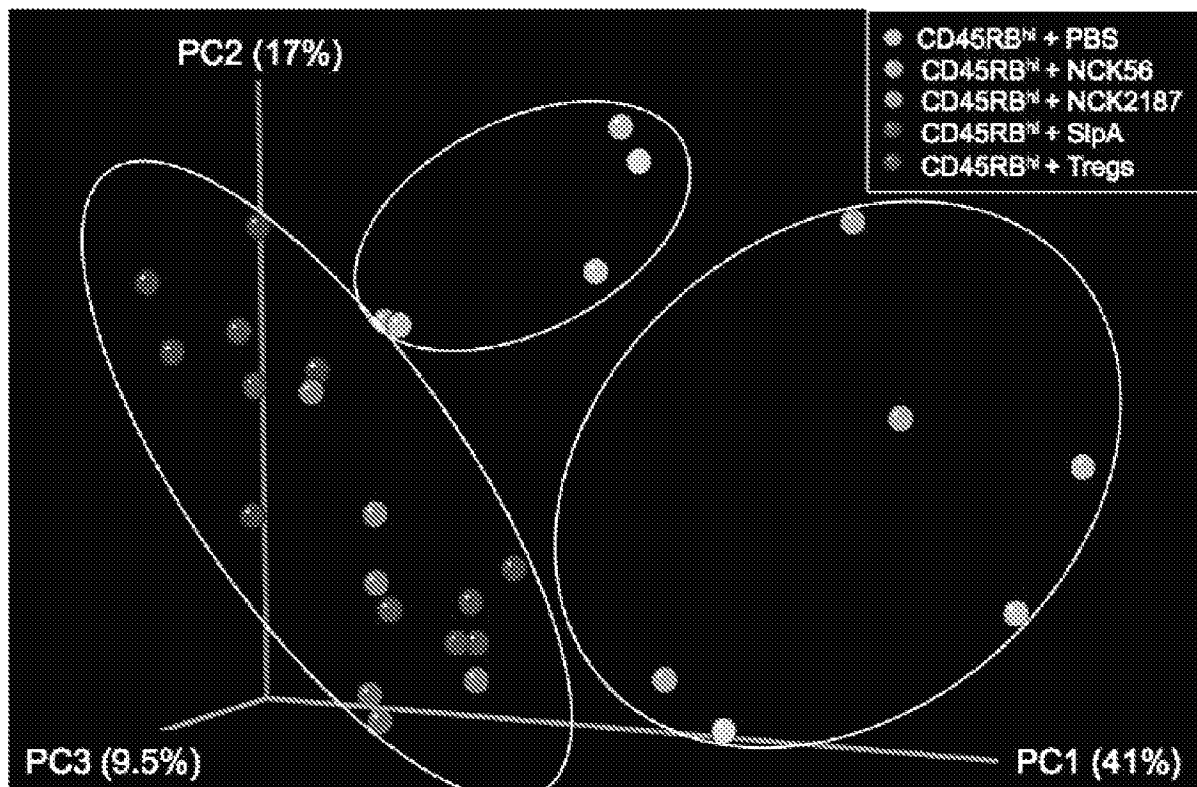


FIG. 4B

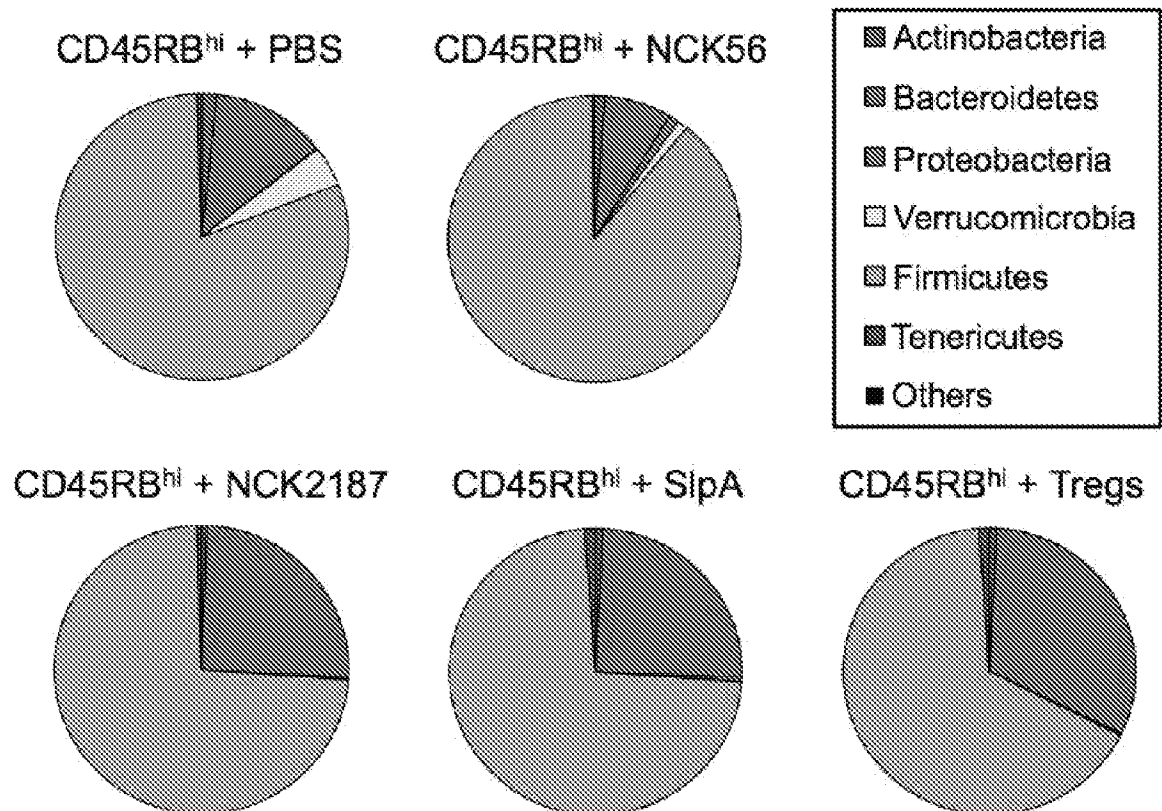


FIG. 4C

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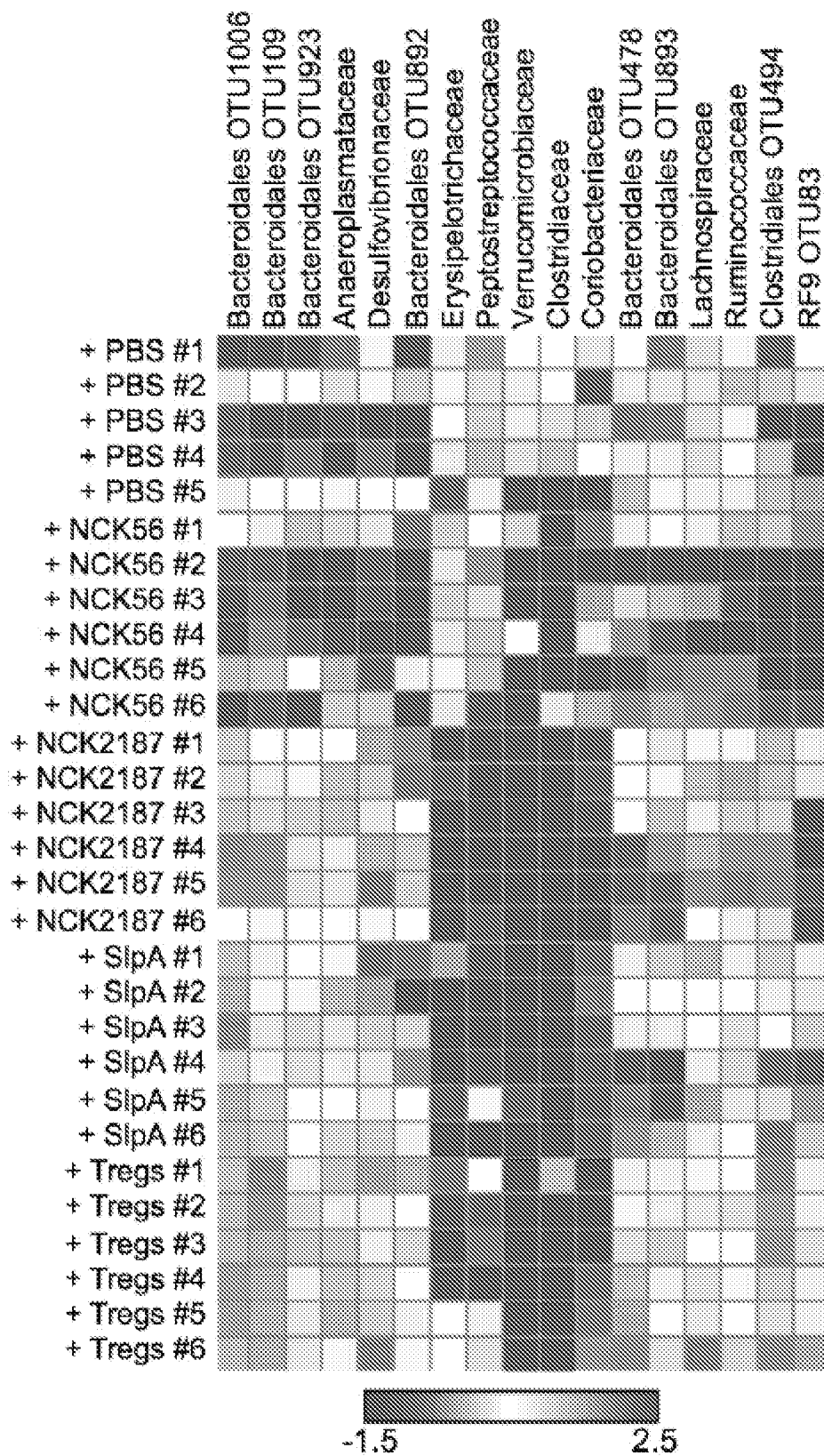


FIG. 4D

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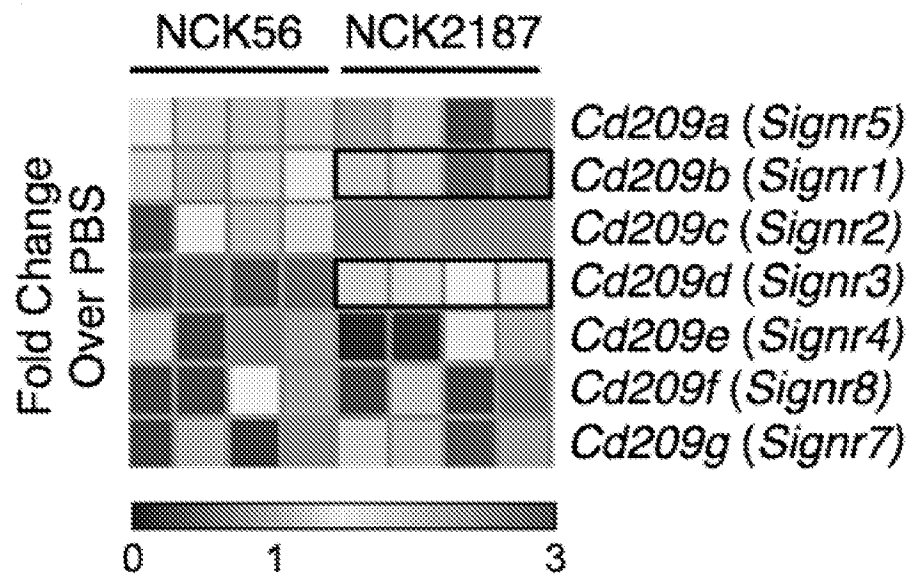


FIG. 5A

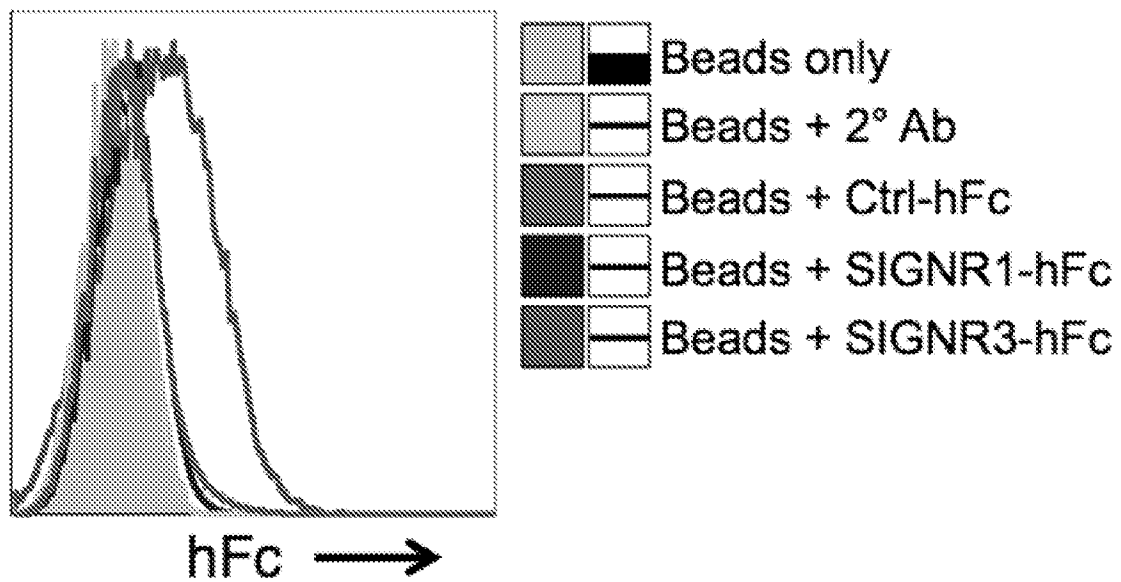


FIG. 5B

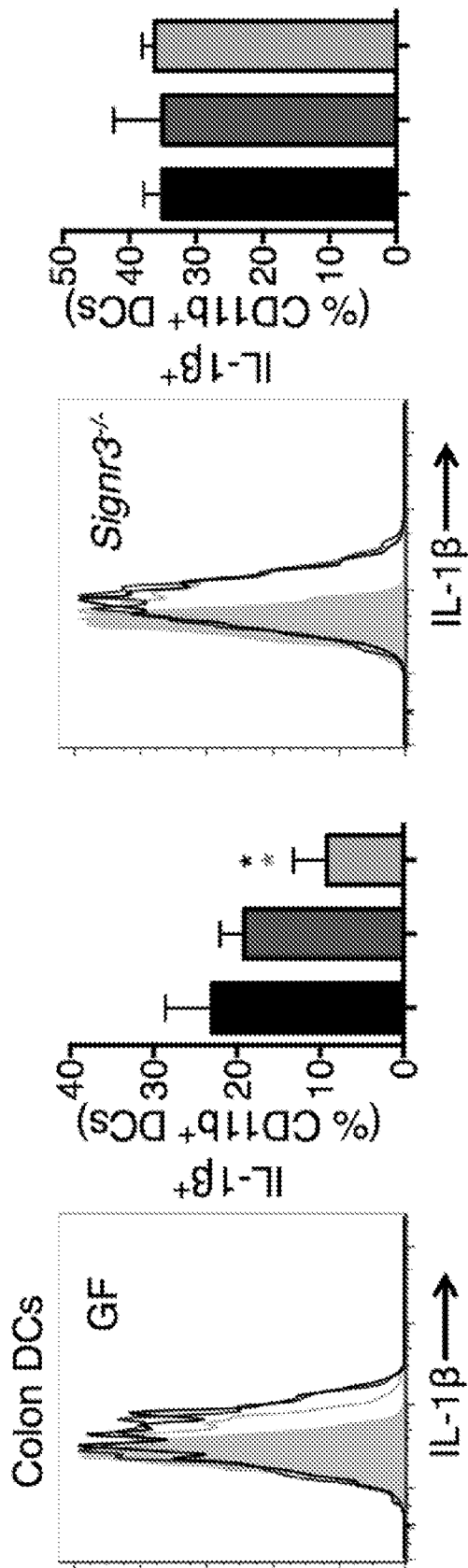


FIG. 5C

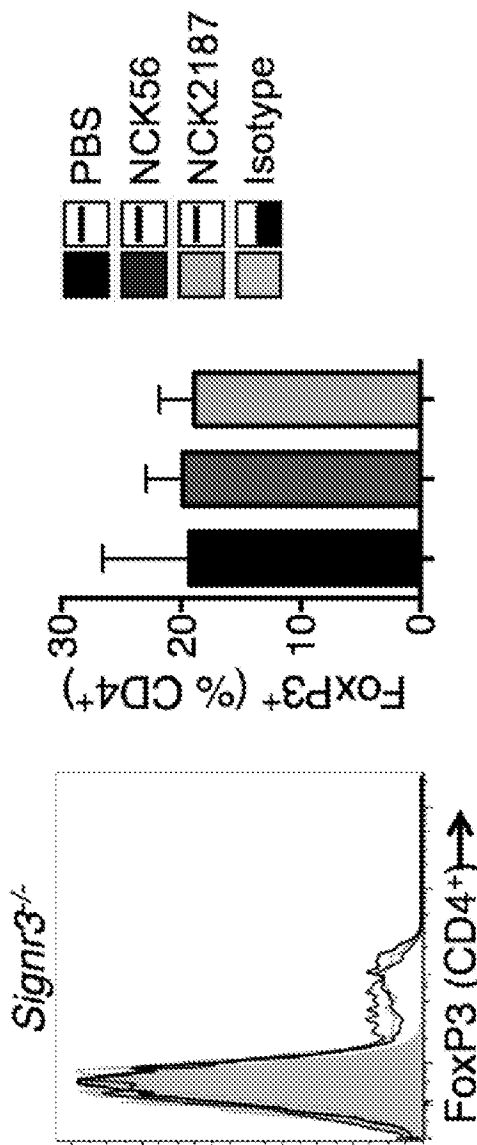


FIG. 5D

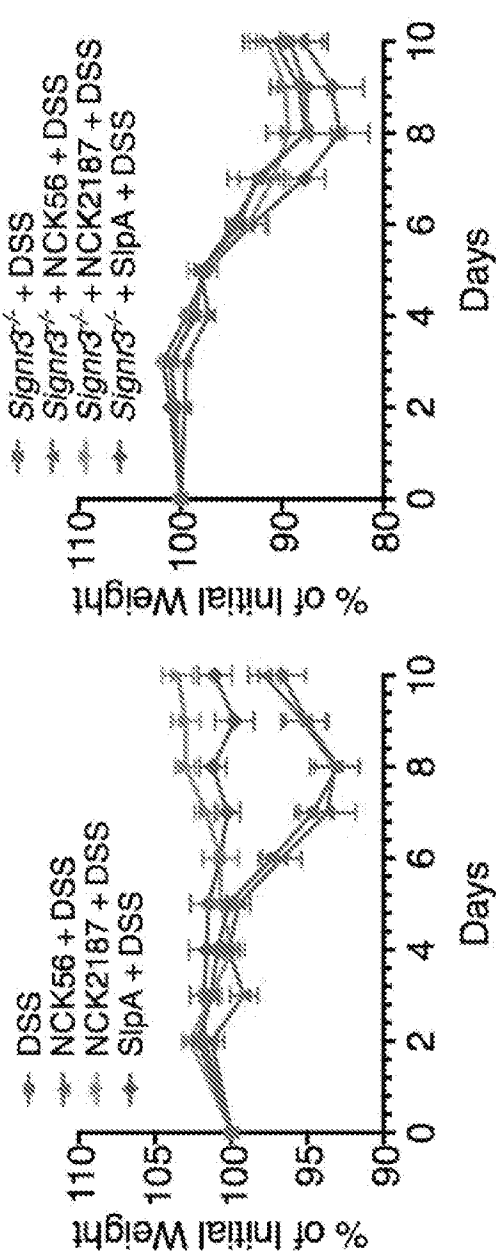


FIG. 6A

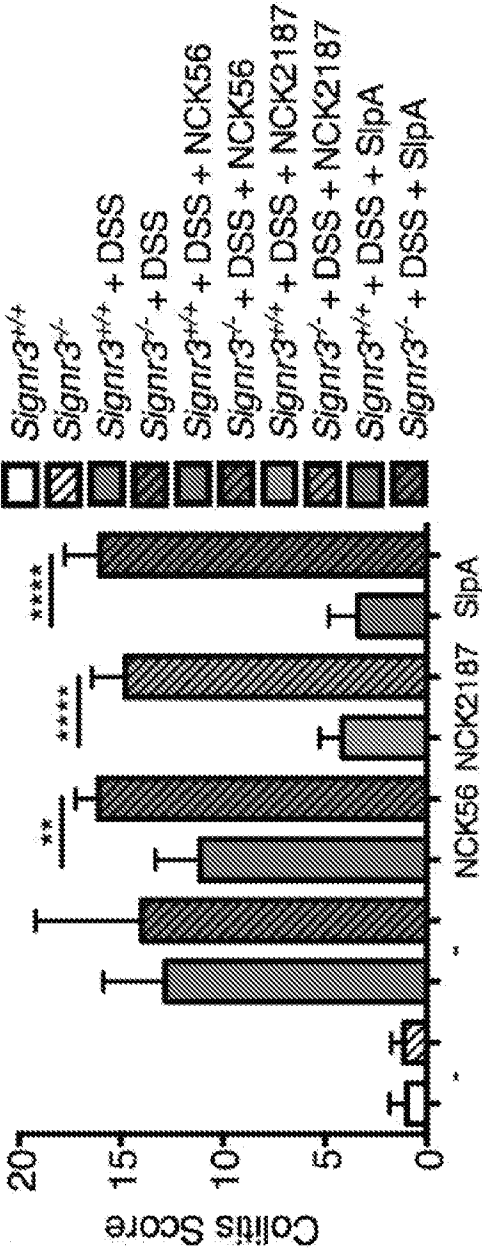


FIG. 6B

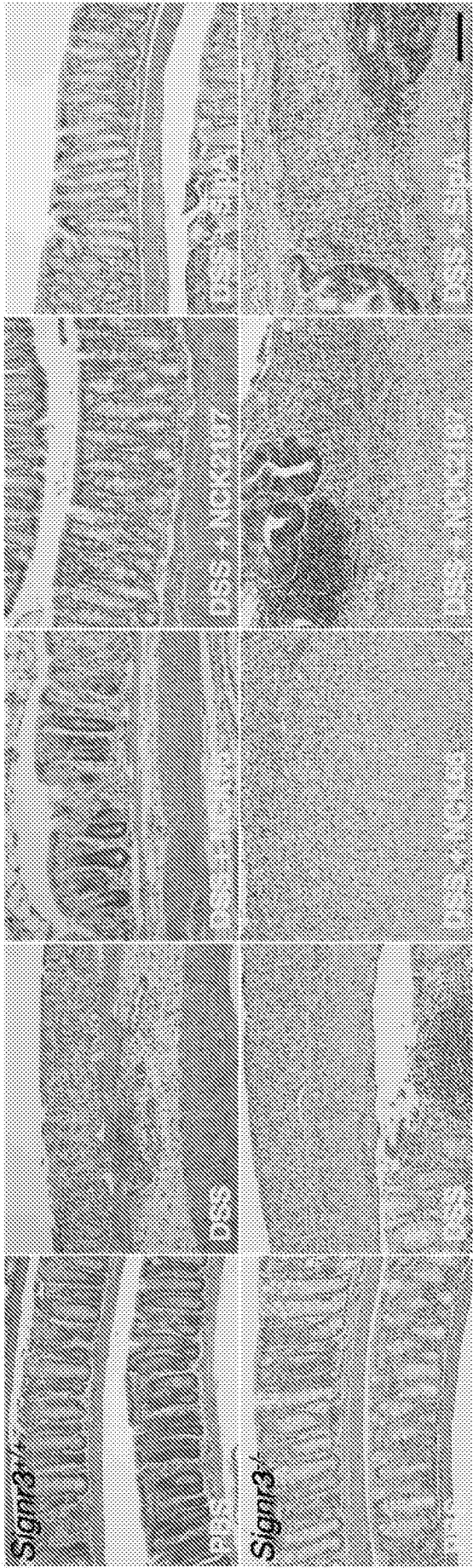


FIG. 6C

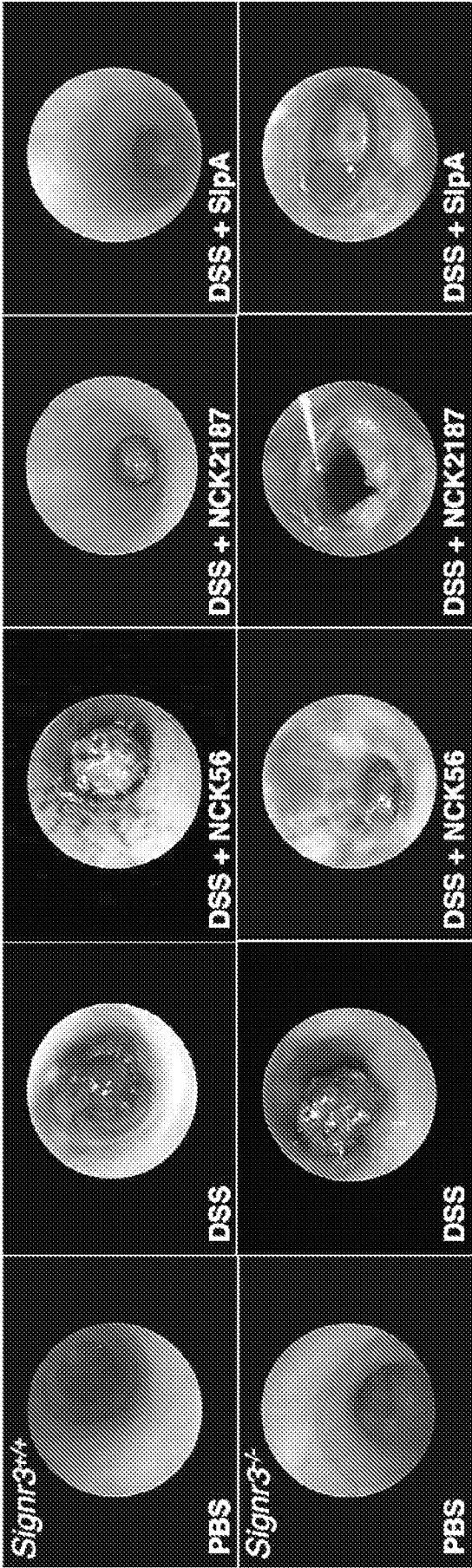


FIG. 6D

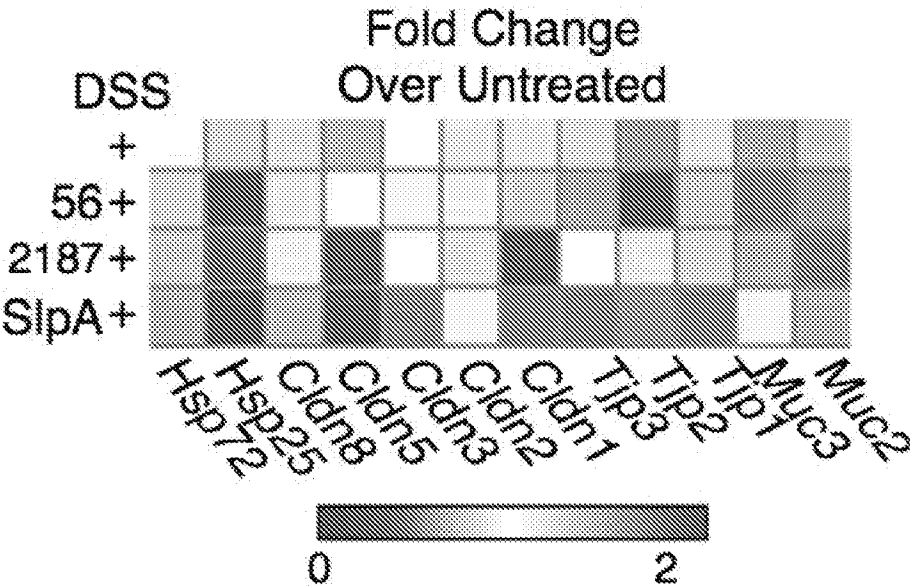


FIG. 6E

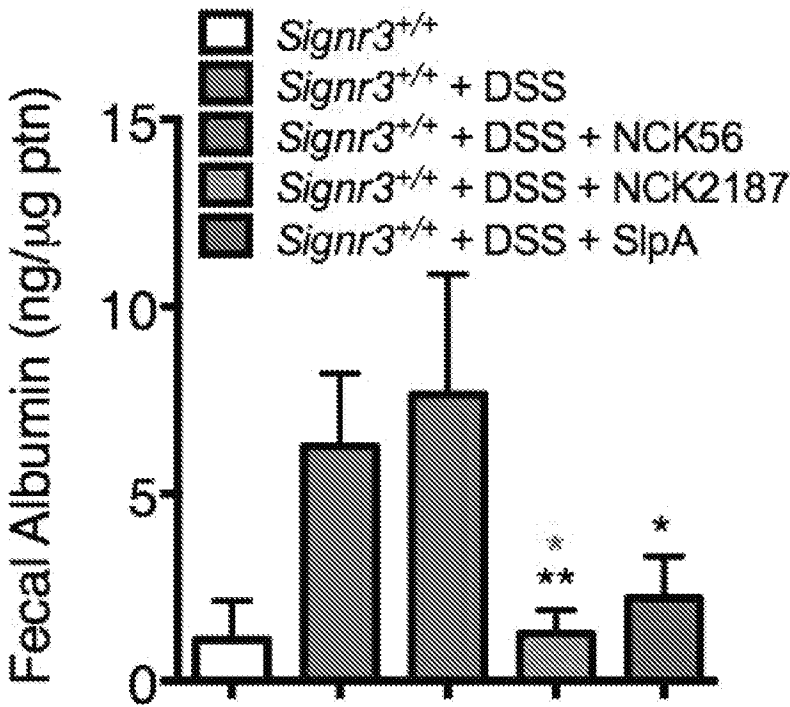
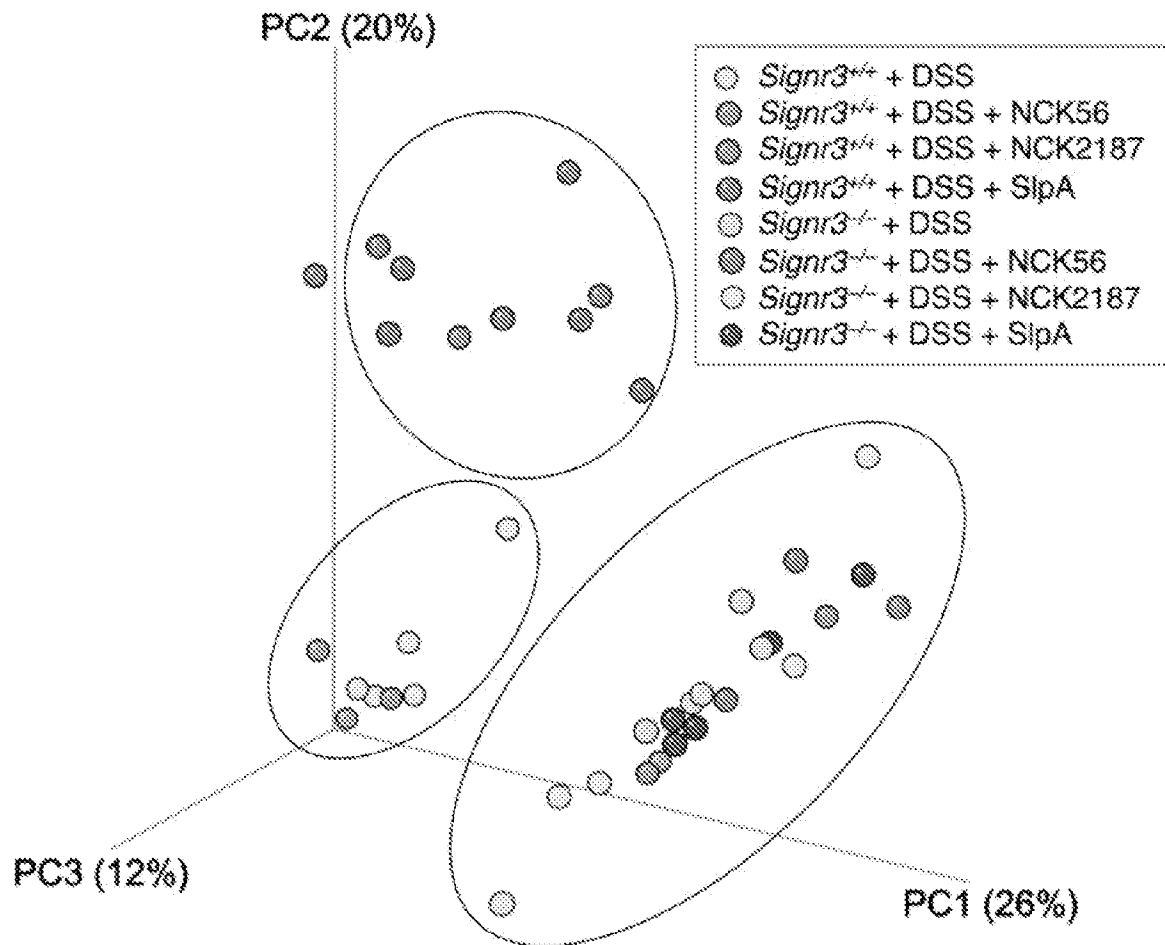


FIG. 6F

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**FIG. 6G**

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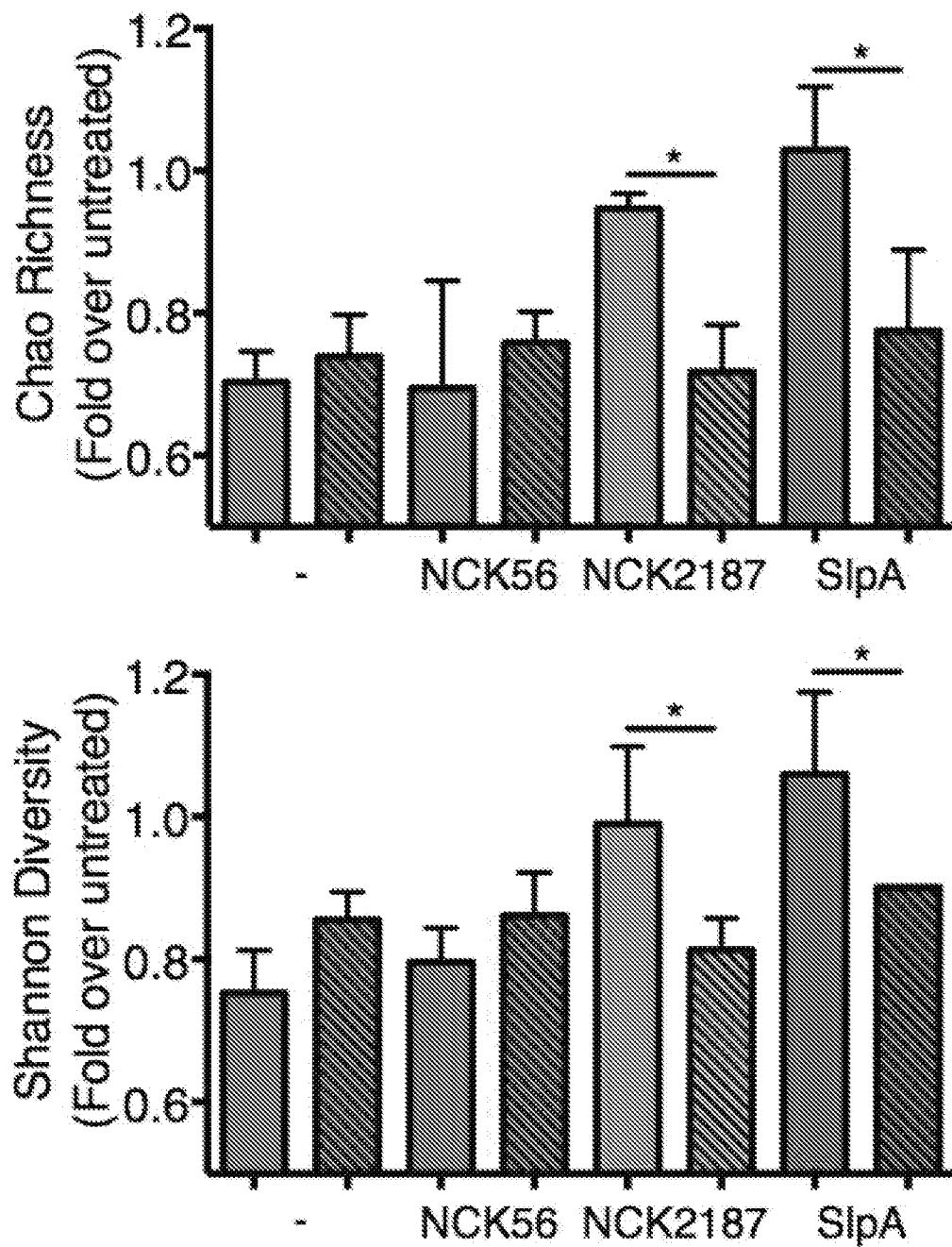


FIG. 6H

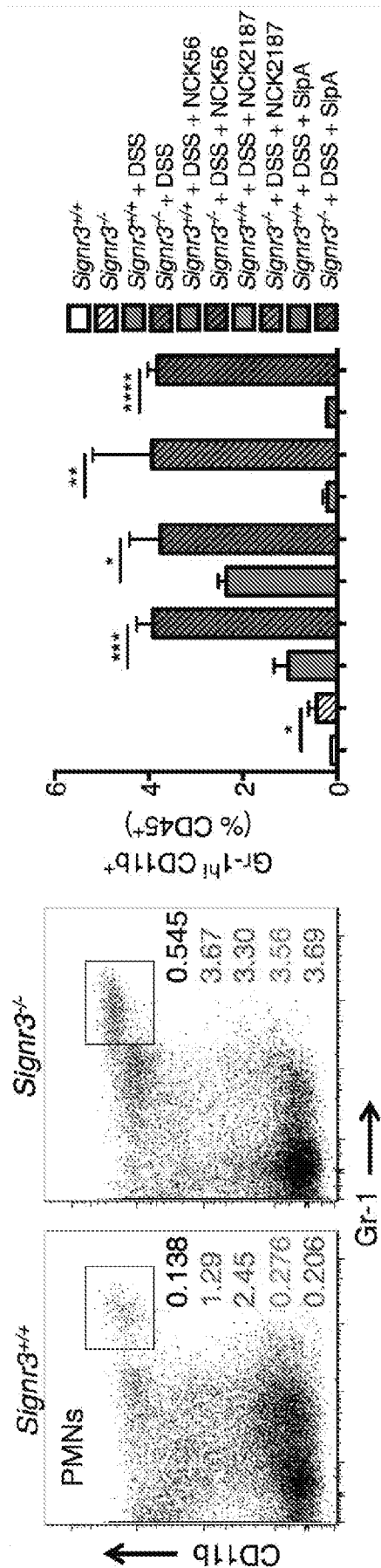


FIG. 7A

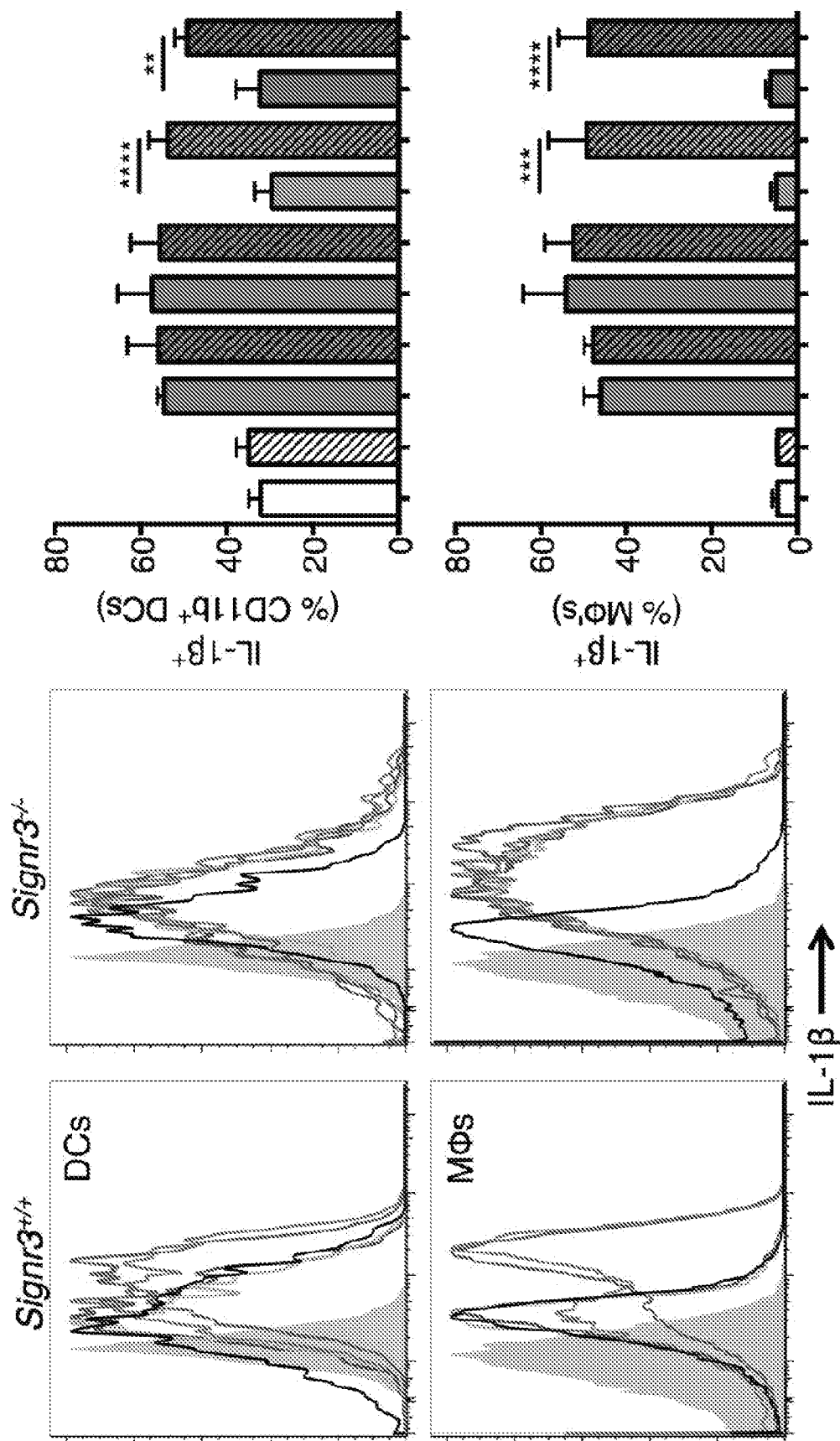


FIG. 7B

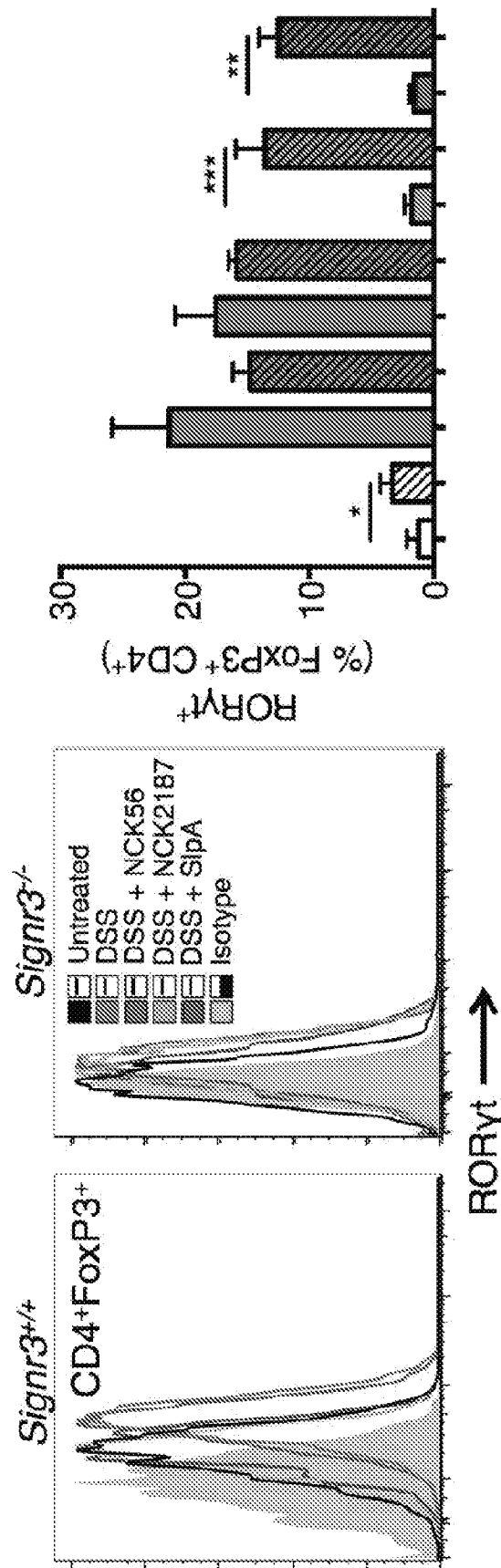


FIG. 7C

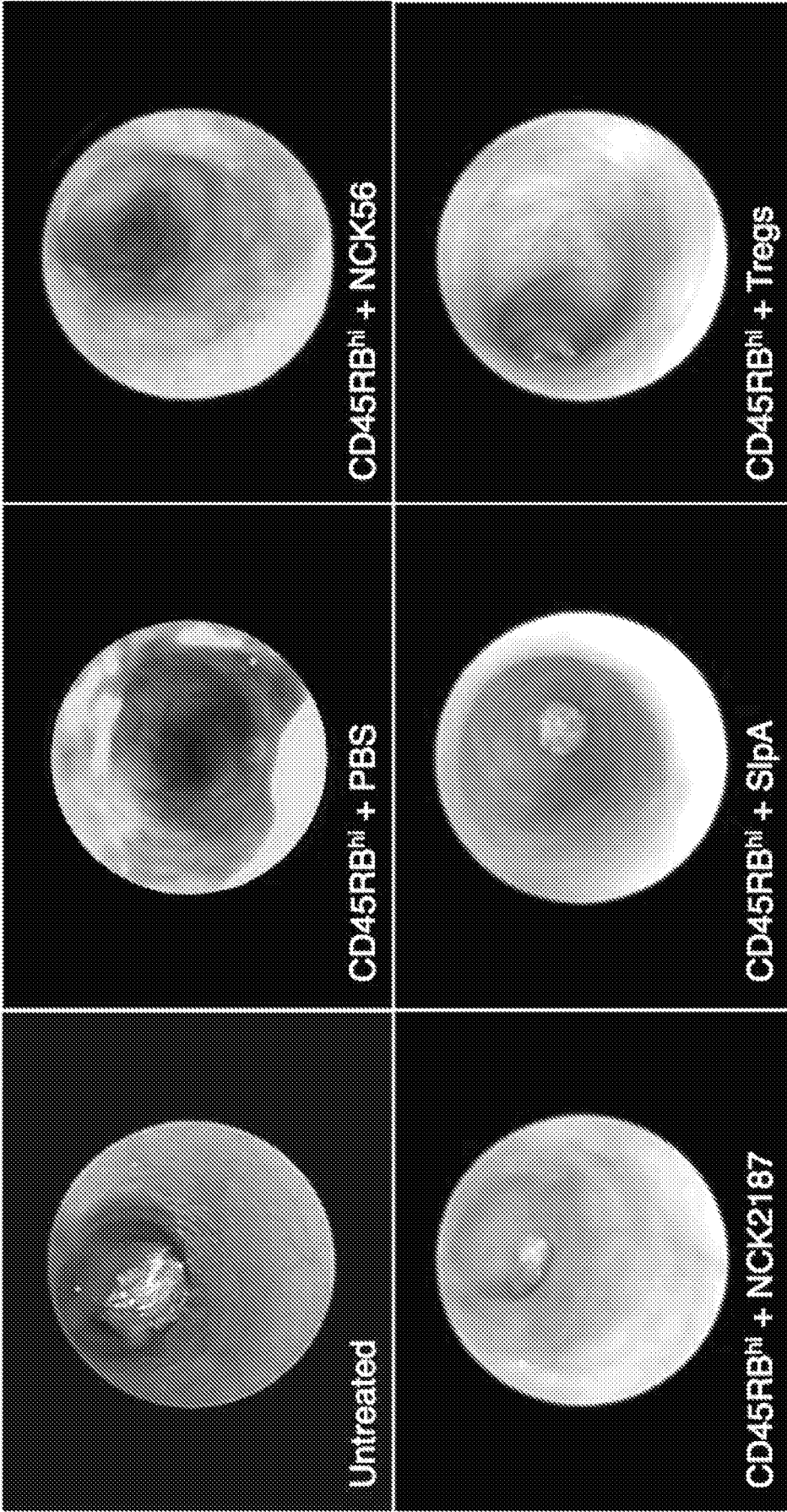


FIG. 8A

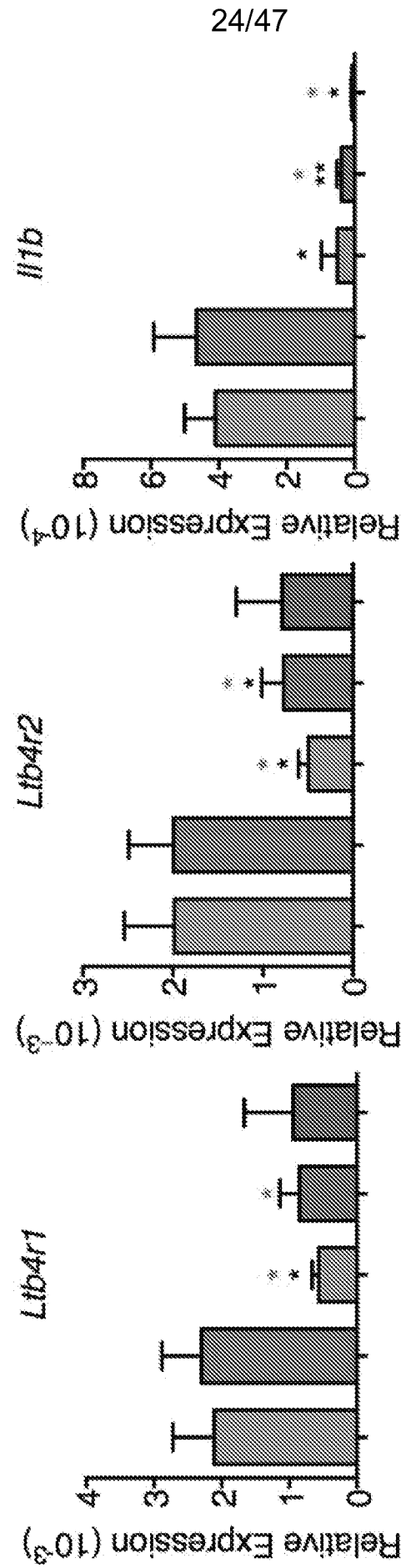


FIG. 8B

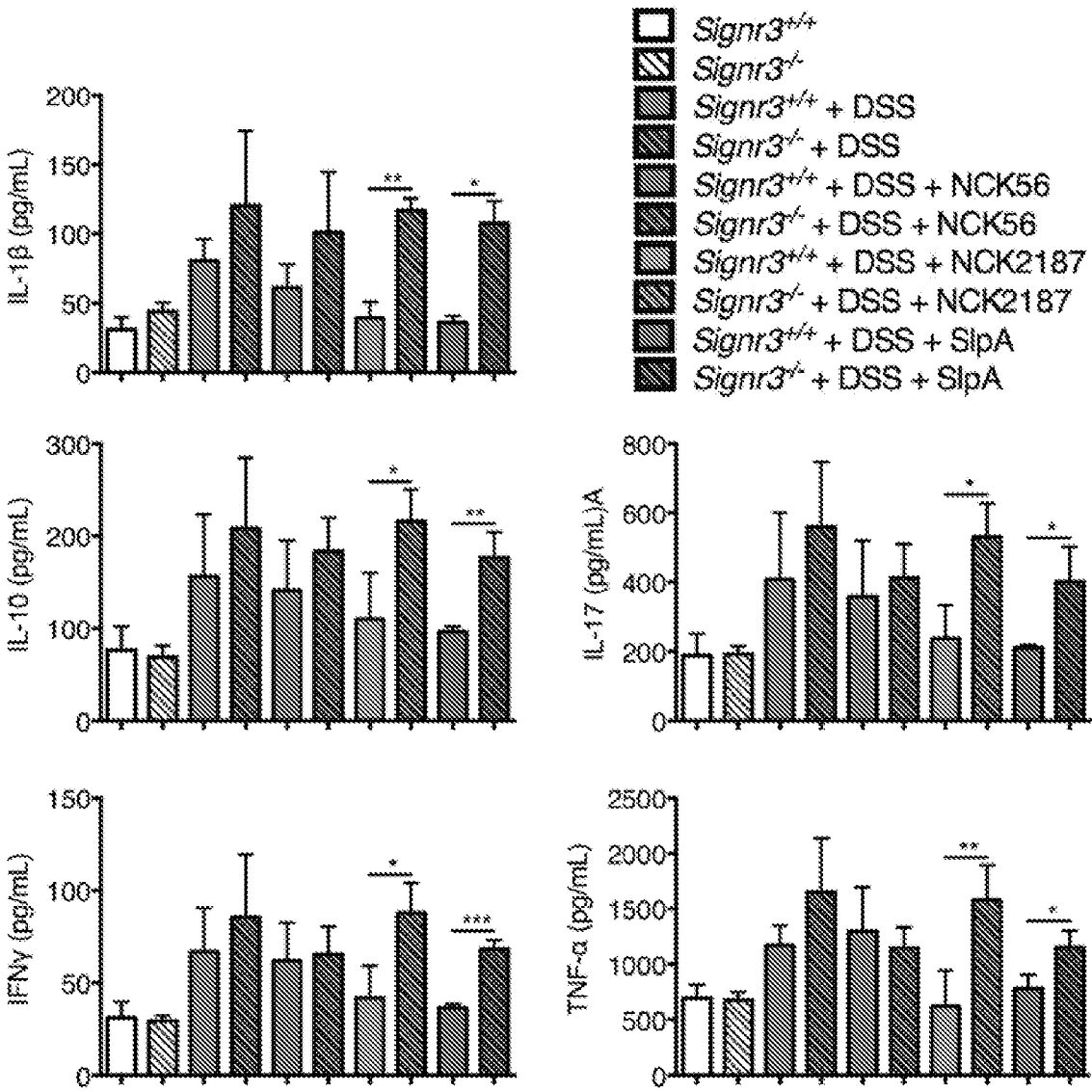
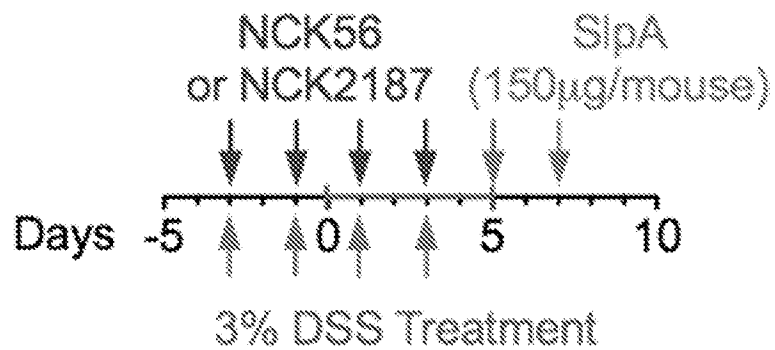
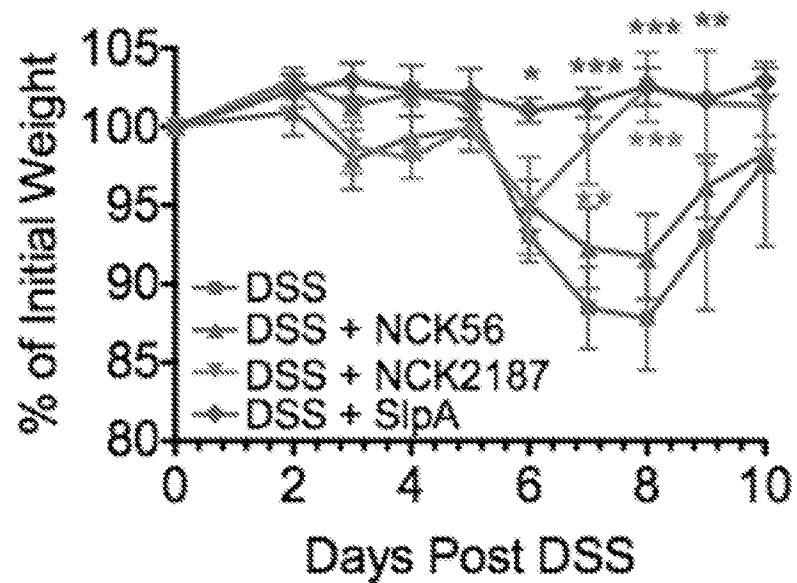
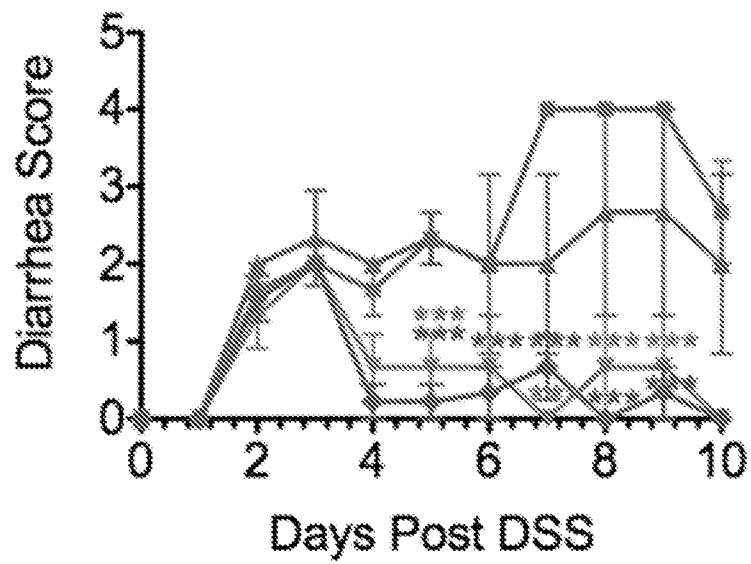
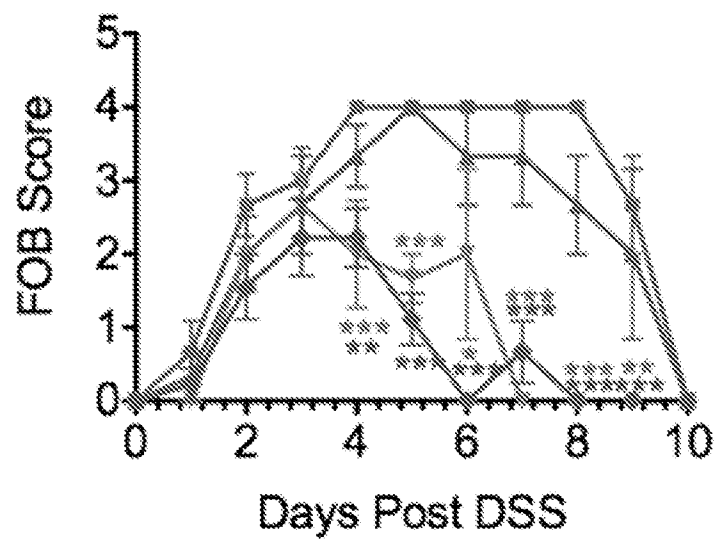


FIG. 9

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**FIG. 10A****FIG. 10B****FIG. 11A**

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**FIG. 11B****FIG. 11C**

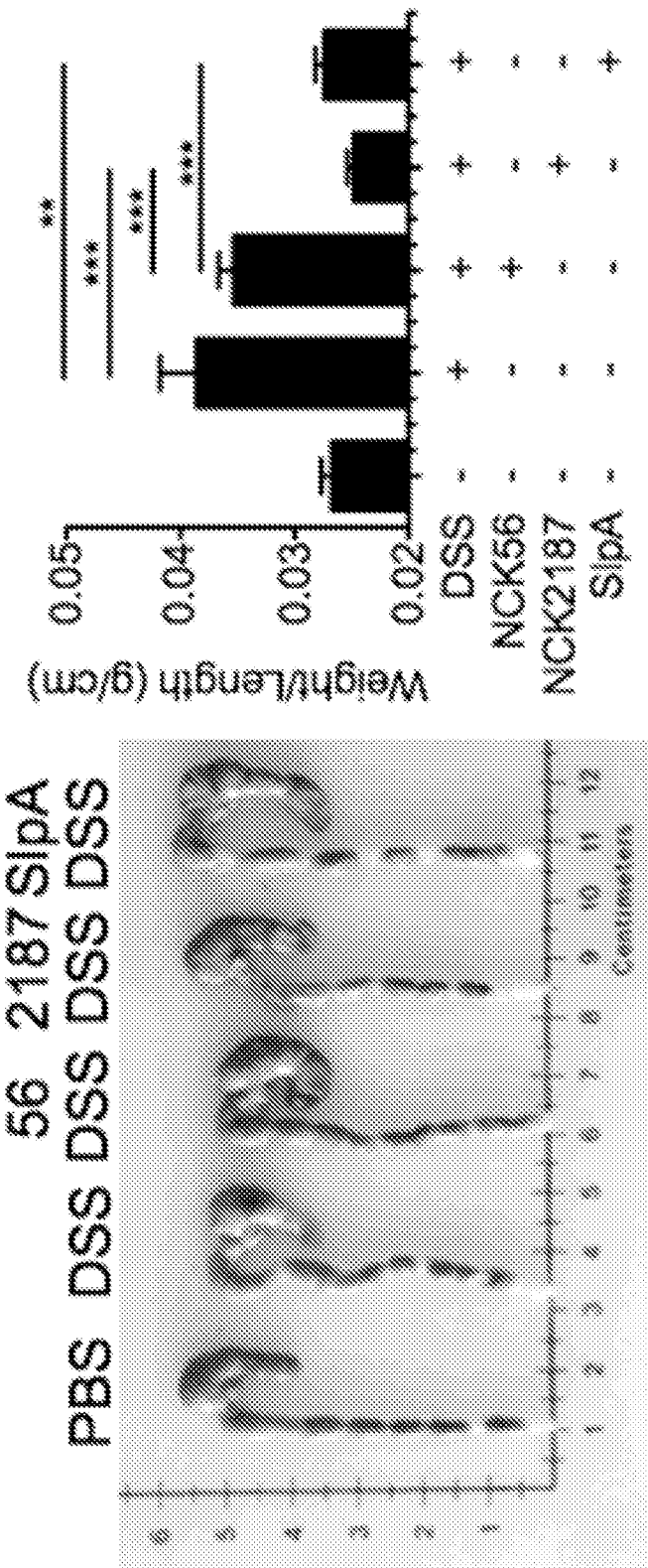


FIG. 11D

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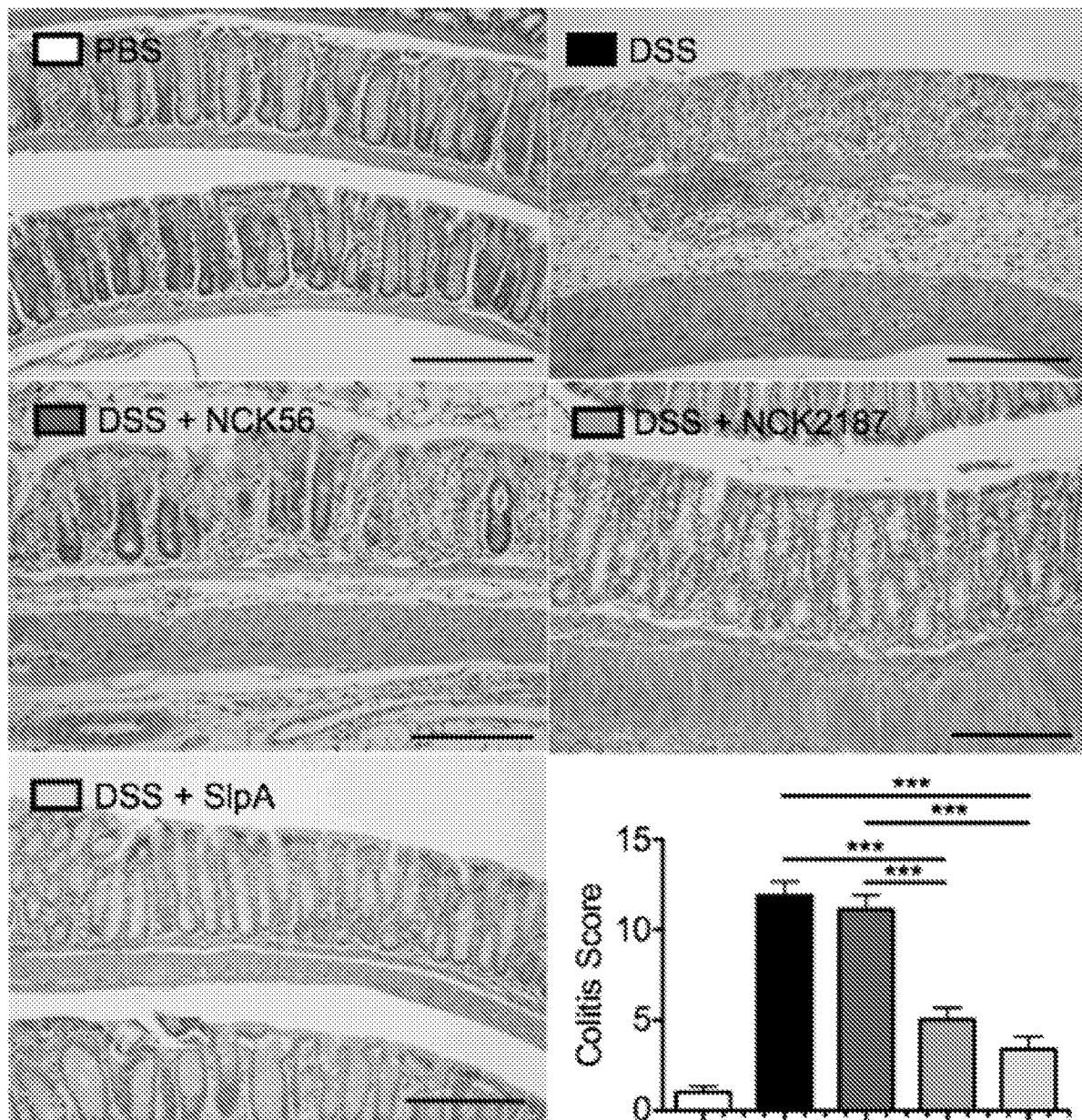
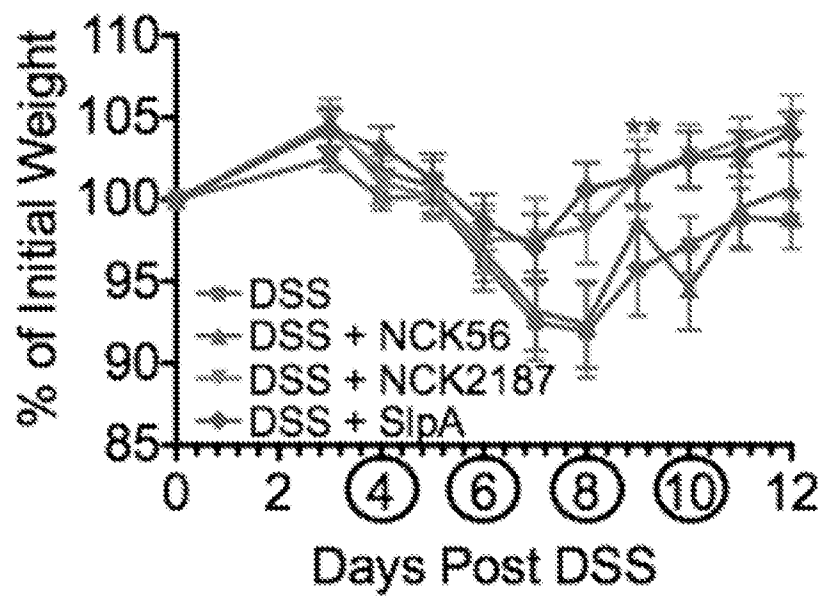
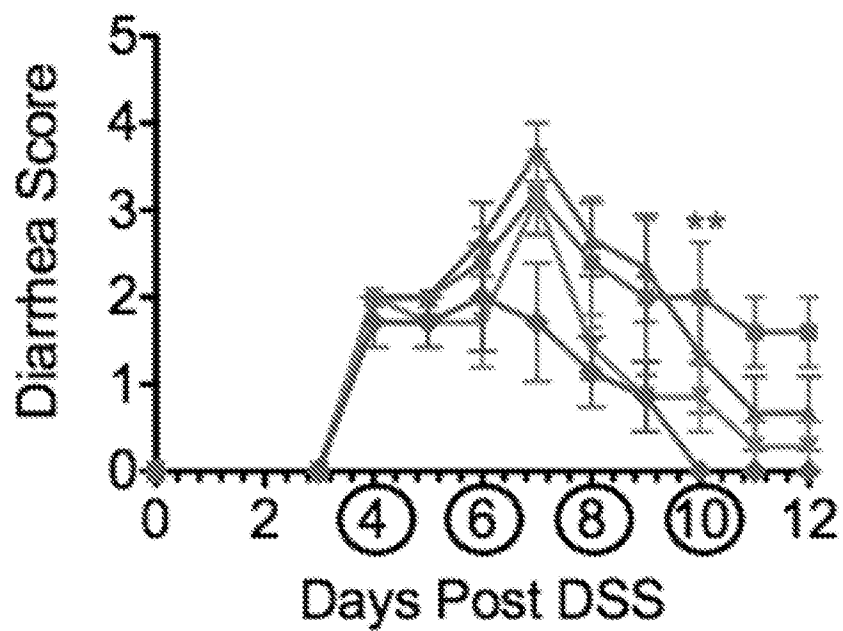
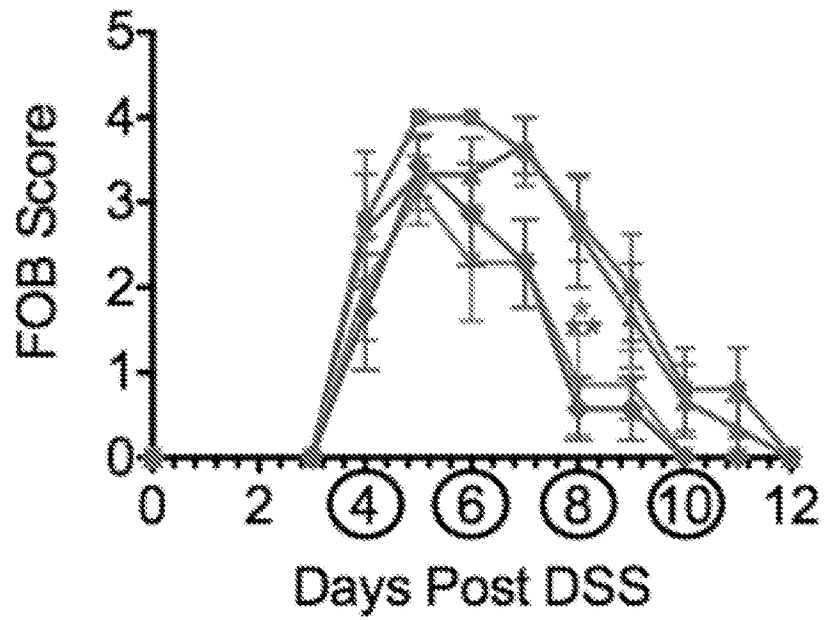


FIG. 11E

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**FIG. 12A****FIG. 12B**

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**FIG. 12C**

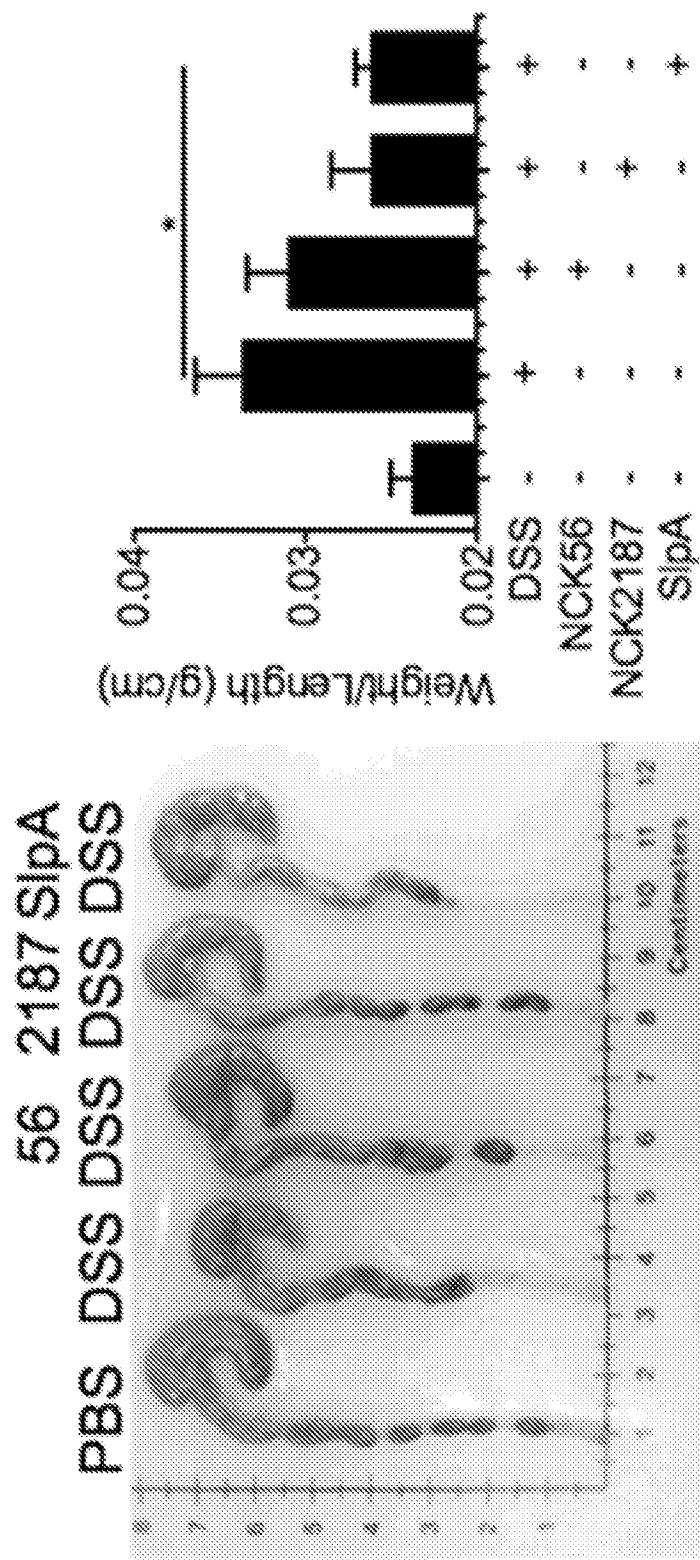


FIG. 12D

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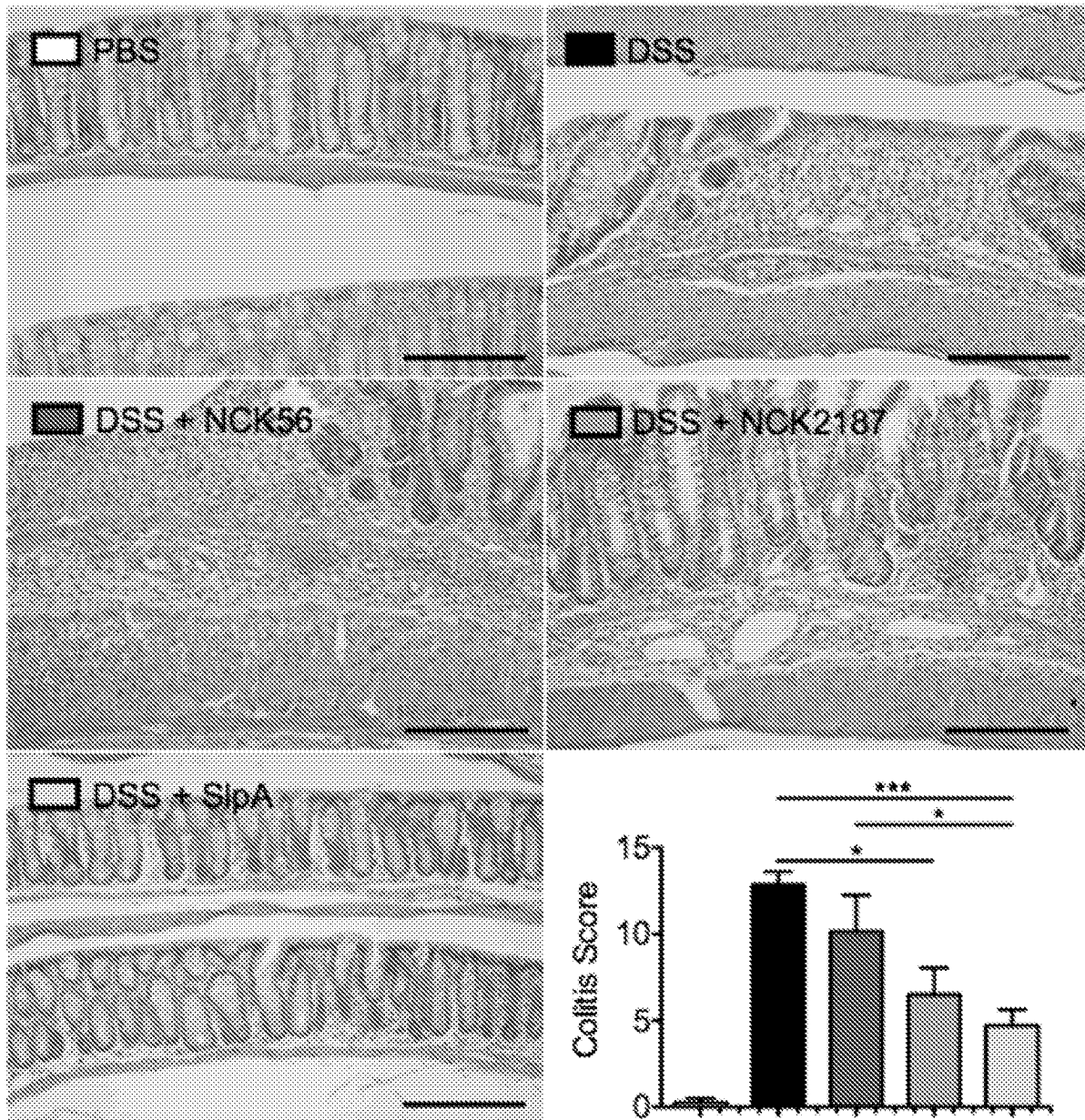


FIG. 12E

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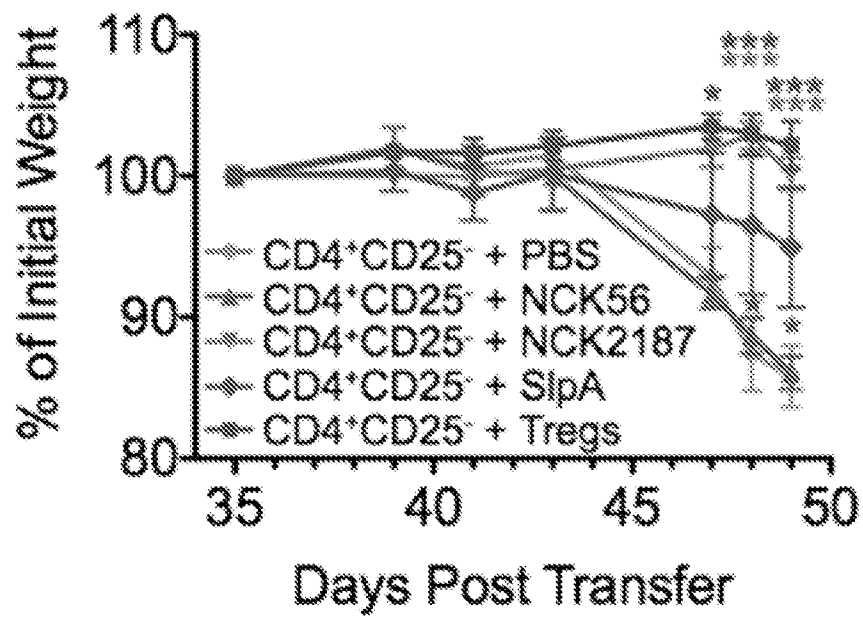


FIG. 13A

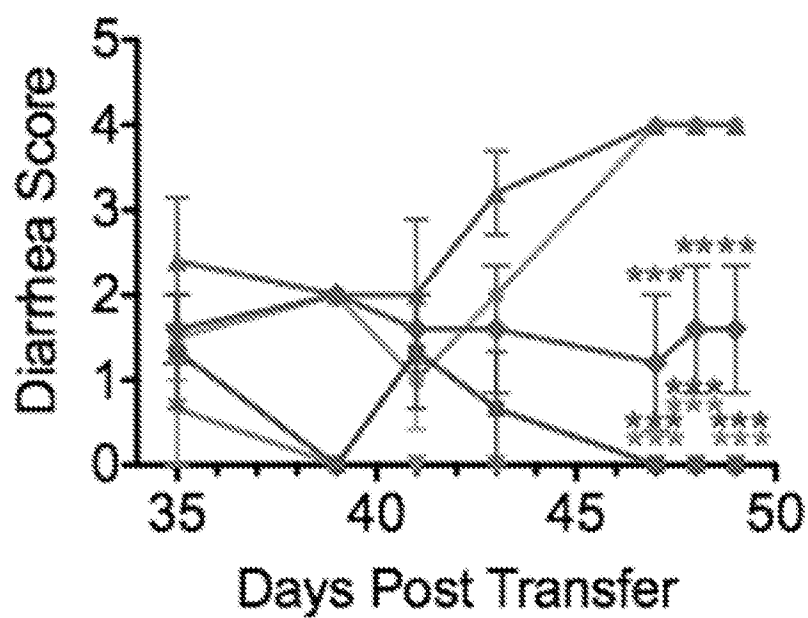


FIG. 13B

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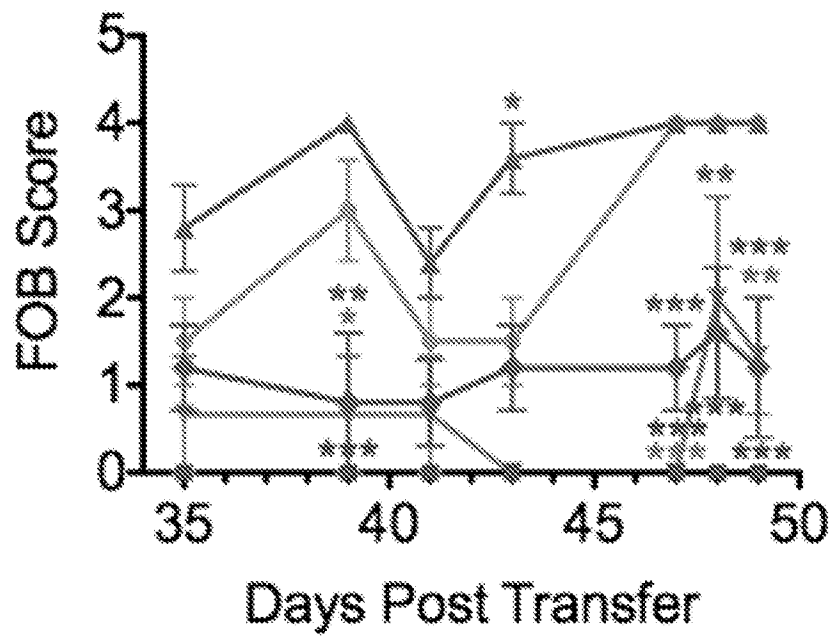


FIG. 13C

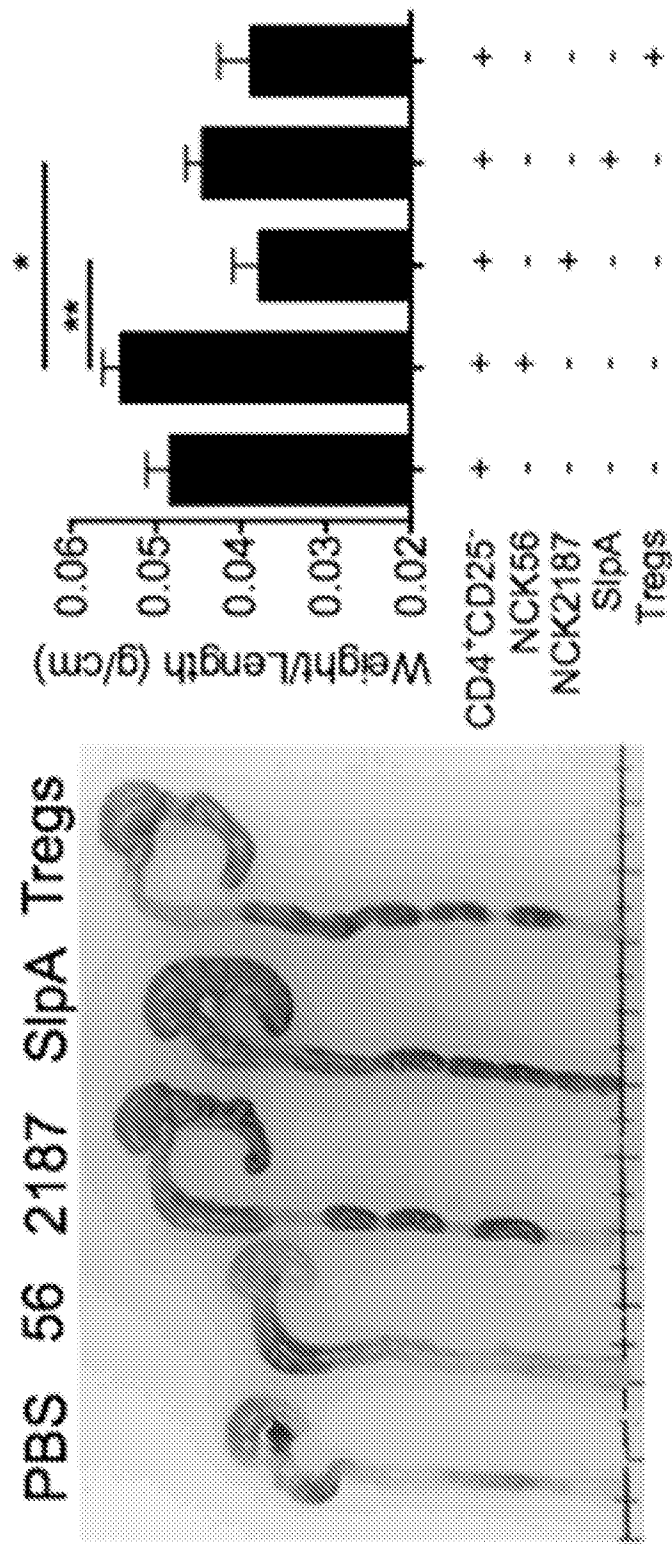


FIG. 13D

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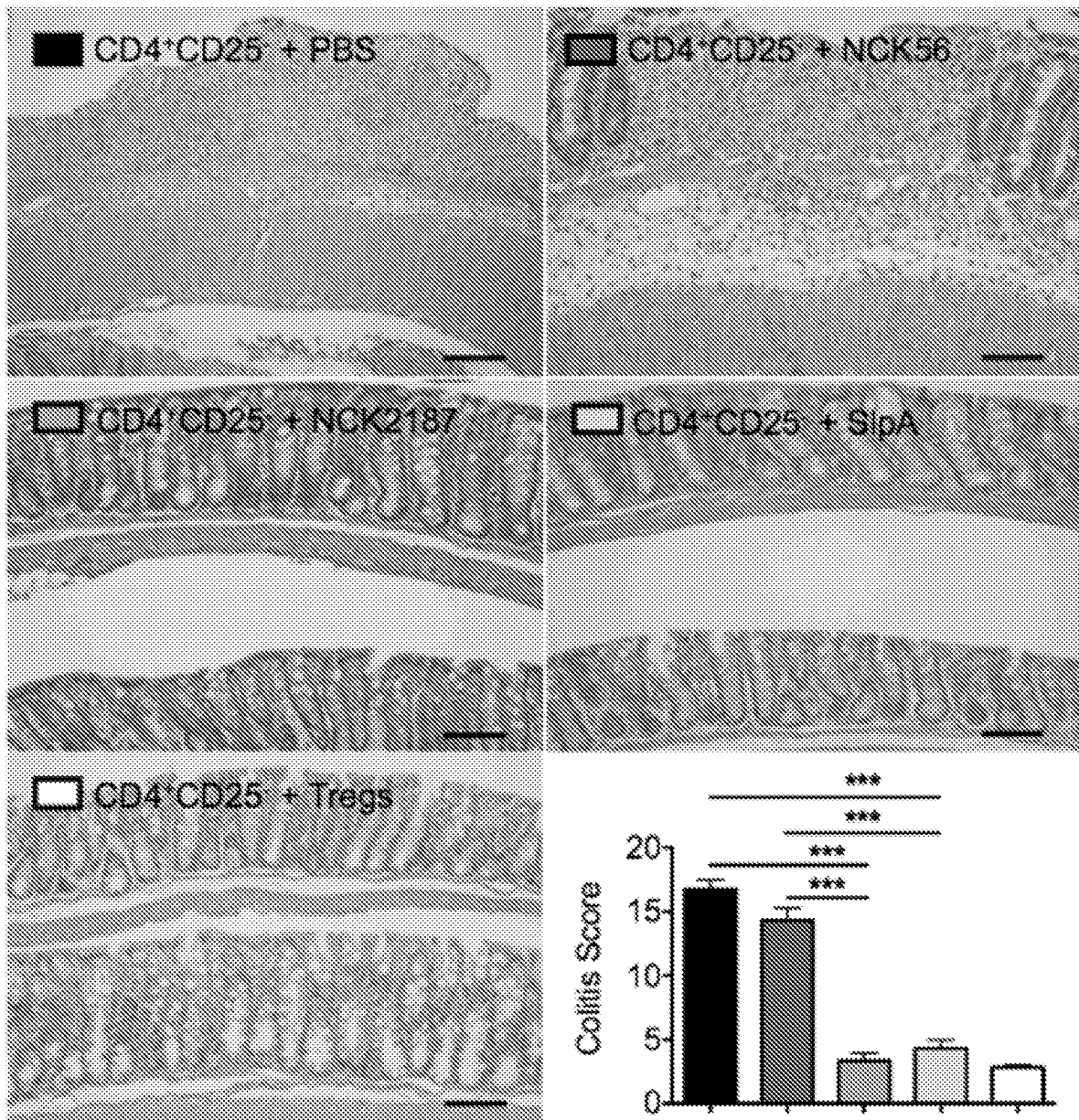


FIG. 13E

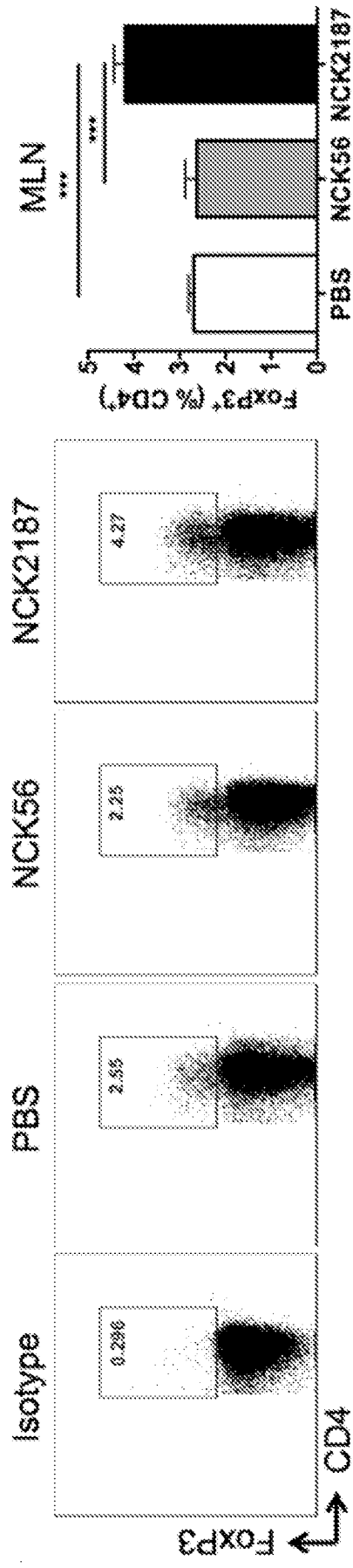


FIG. 14A

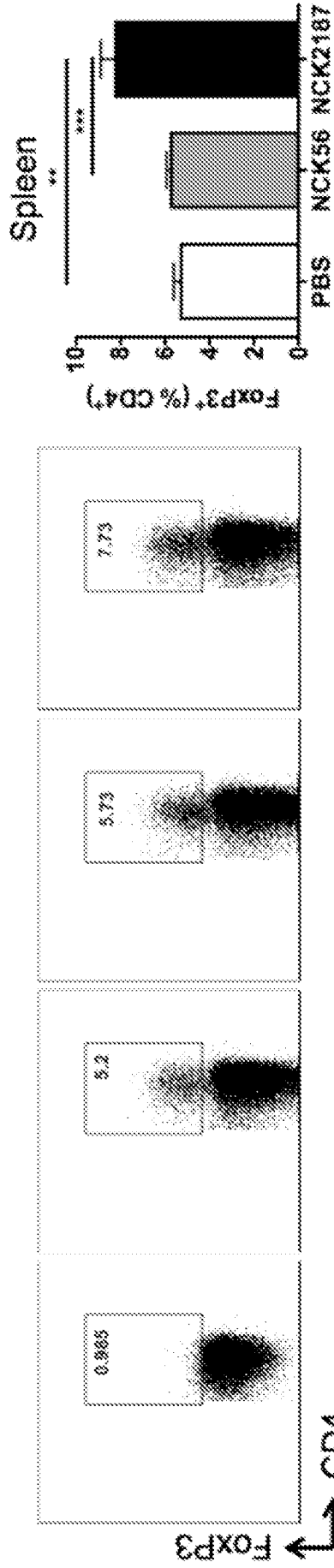


FIG. 14B

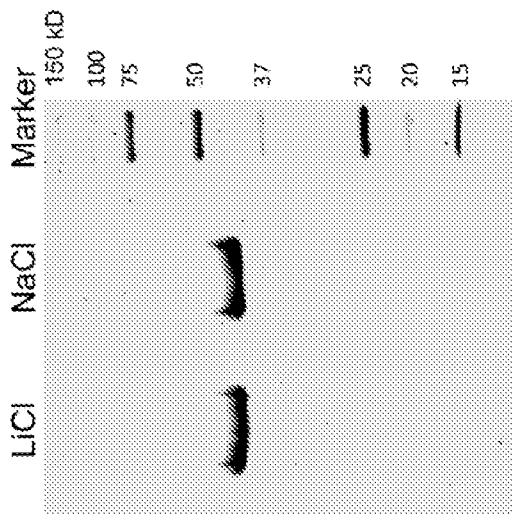


FIG. 15A

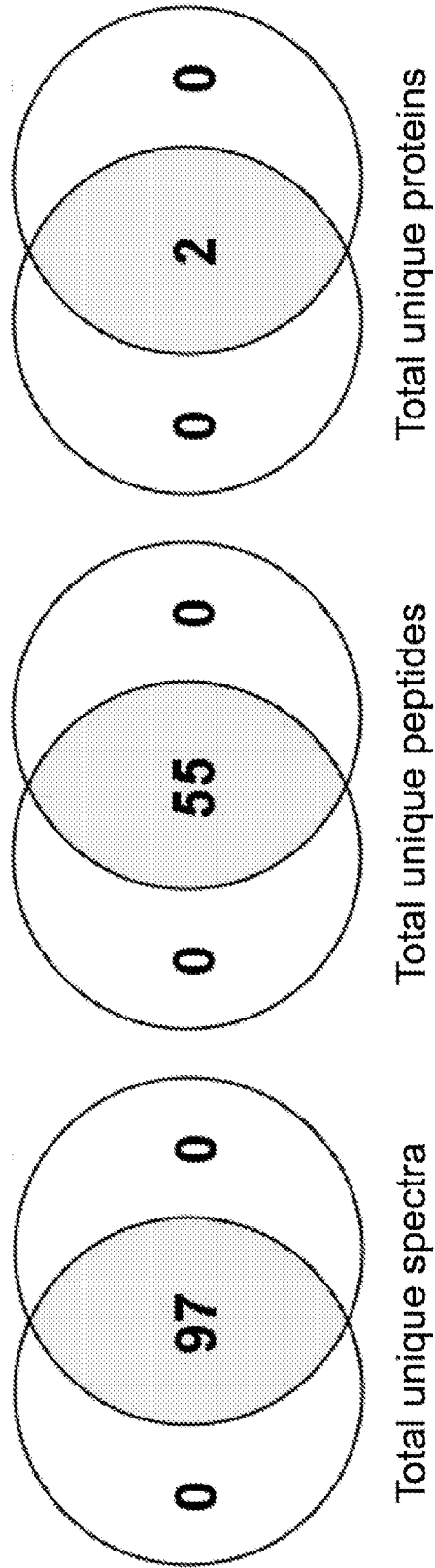


FIG. 15B

gi|58336516 (100%), 46,569.0 Da
S-layer protein [Lactobacillus acidophilus NCFM]
29 exclusive unique peptides, 49 exclusive unique spectra, 218 total spectra, 238/444 amino acids (54% coverage)

MKKNLRIVSA	AAAALLAVAP	VAAASAVSTVS	AATTINASSS	AINTNTNAKY	DVDVTPSVSA	VAANTANNTP	AIAGNLTGTI
SASYNGKTYT	ANLKADTENA	TITAAGSTTA	VKPAELAAGV	AYTVTVNDVS	FNFGSENAGK	TVTILGSANS	VKFTGTSDN
QTETNVSTLK	VKLDQNGVAS	LTNVSIANVY	AINTDNSNV	NFYDVTSGAT	VTNGAVSVNA	DNQGQVNVAN	VVAAINSKYF
AAQYADKKLN	TRTATEDAI	KAALKDQKID	VNSVGYFKAP	HTFTVNVKAT	SITIGKSATL	PVVVTVPNVA	EPTVASVSKR
IHHAYYYDK	DAKRVGTDVS	KRYNSVSVLP	TTTTIIGKTY	YQVVEGKAV	DKYINAAID	GTKRTLKHA	YVYASSKKRA
NKVVLKKGEV	VTTYGASYTF	KGGKYYKIG	DTDKTYVKV	ANFR			

gi|362076610 (100%), 49,364.6 Da
surface layer protein [Lactobacillus acidophilus]
1 exclusive unique peptides, 2 exclusive unique spectra, 118 total spectra, 85/467 amino acids (18% coverage)

MKKNLRIVSA	AAAALLAVAP	VAAASAVSTVS	AADATTTTAA	TTTTNKPTVD	LTGAGAVTNA	AKTVTVTPNF	TLTAAIAKDG
KVTASATLQG	TITASLNGTS	VTANVIDAAK	GITLKNSGY	TTIYKYDANT	NTTENNLGKW	NEKTNDVYVK	AGNDYQVELT
GVGFSFGSAN	ANKEVSLKLP	SNVTVGVKD	NKVTLDDQYGN	VTNLTFIVKD	IKAYDATNTS	AVQFYNTNSG	LIESKATYMA
LADNNGNLNV	NTLLNGLNKQ	YKAVQLQNGE	LKDVTVTTTA	ADLTAELTKA	GIKVNAAGDF	EAPASFTATL	TAKSEVNGKV
ATLPVTVTVP	NGKVTTVDSV	SKRIMHNAYF	YDKDAKRVGT	DSVKRYASVS	VLPNTTTING	KAYYQVVENG	KAVDKYI
NIDGTKRTLK	HAYYVYASSK	KRANKVVLKK	GEVVTTYGAS	YTFKGGKYY	KIGNTDKTY	VKVANFR	

FIG. 15C

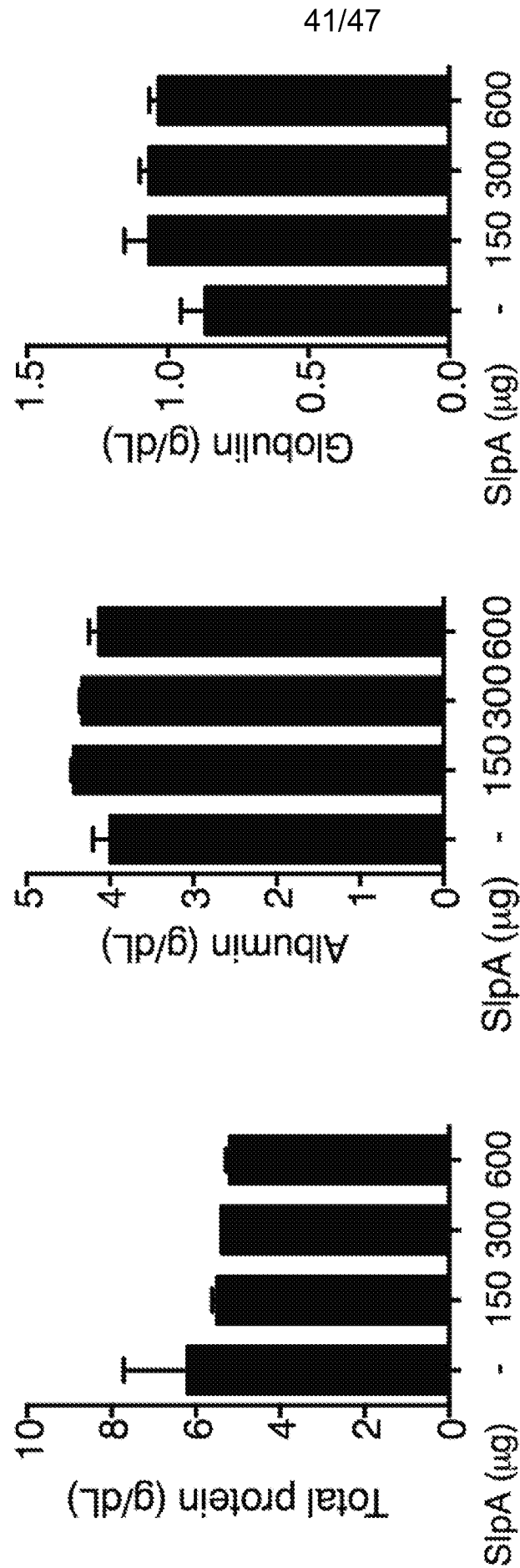


FIG. 16A

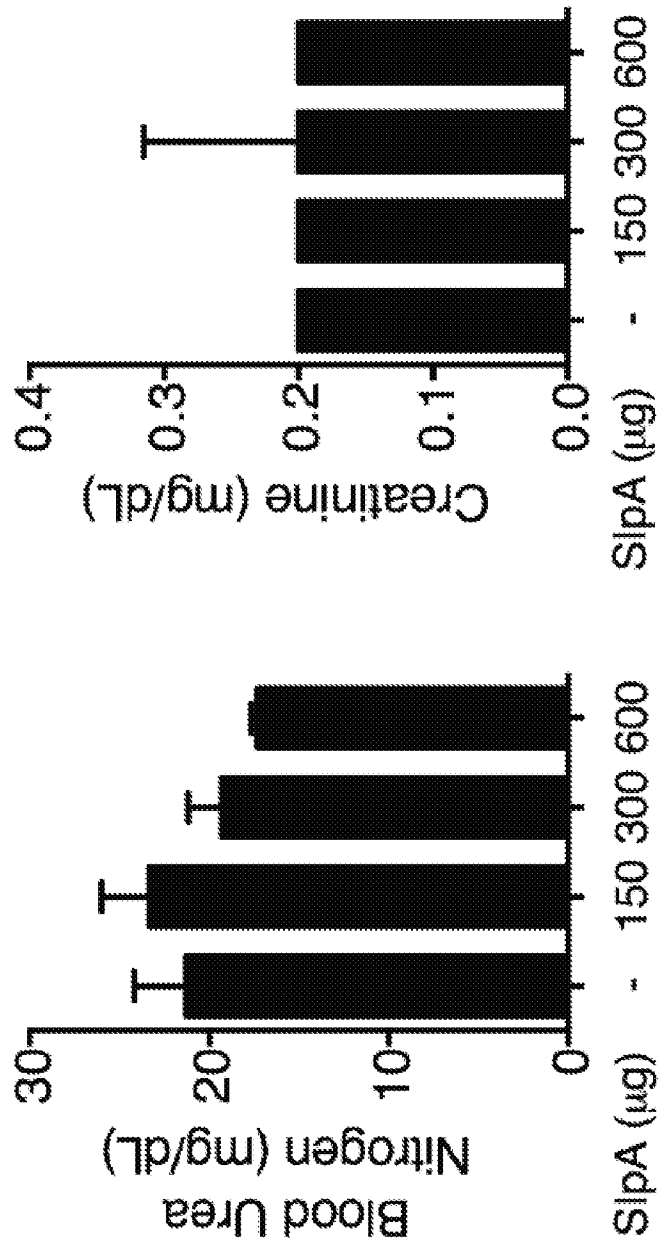


FIG. 16B

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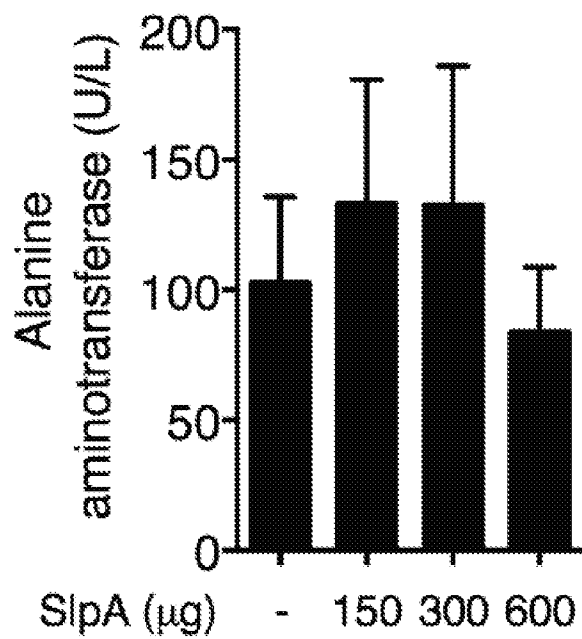


FIG. 16C

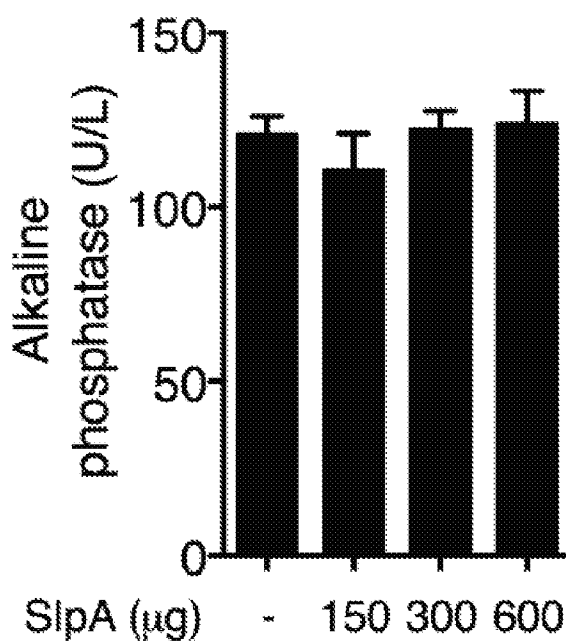


FIG. 16D

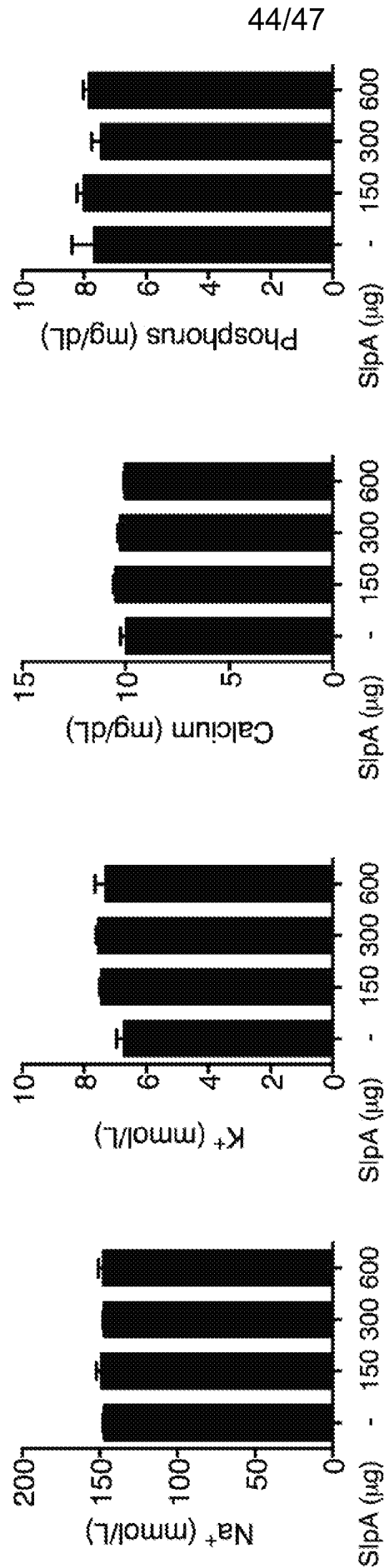


FIG. 16E

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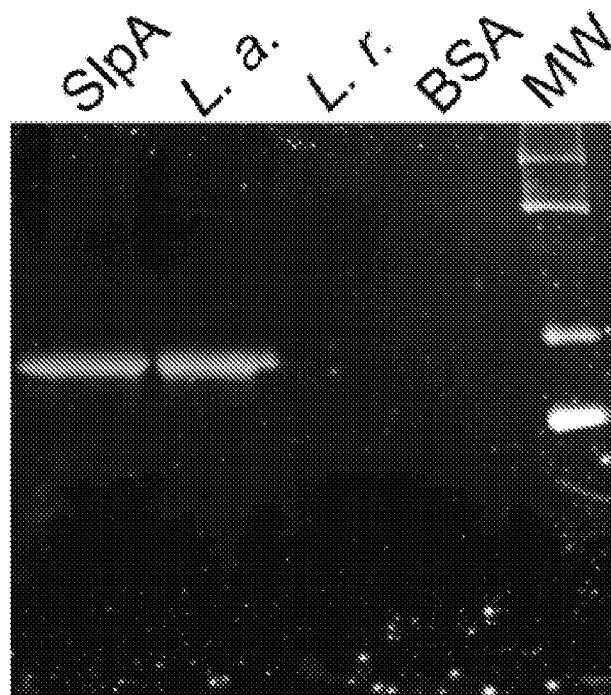


FIG. 17A

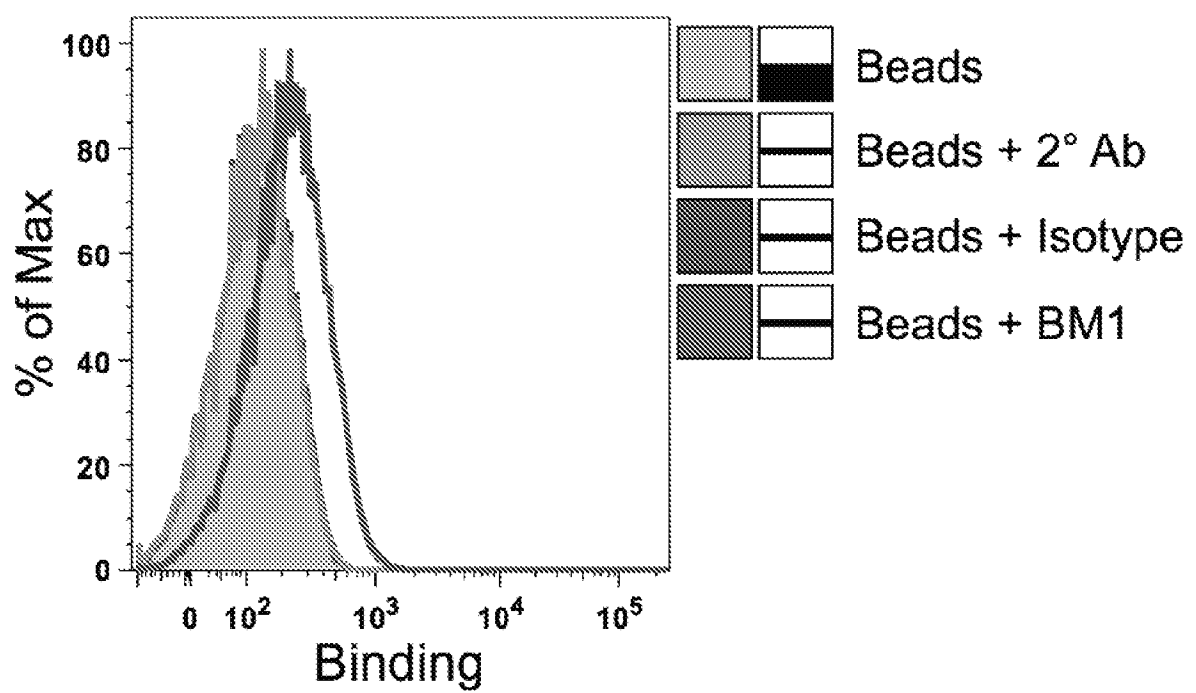


FIG. 17B

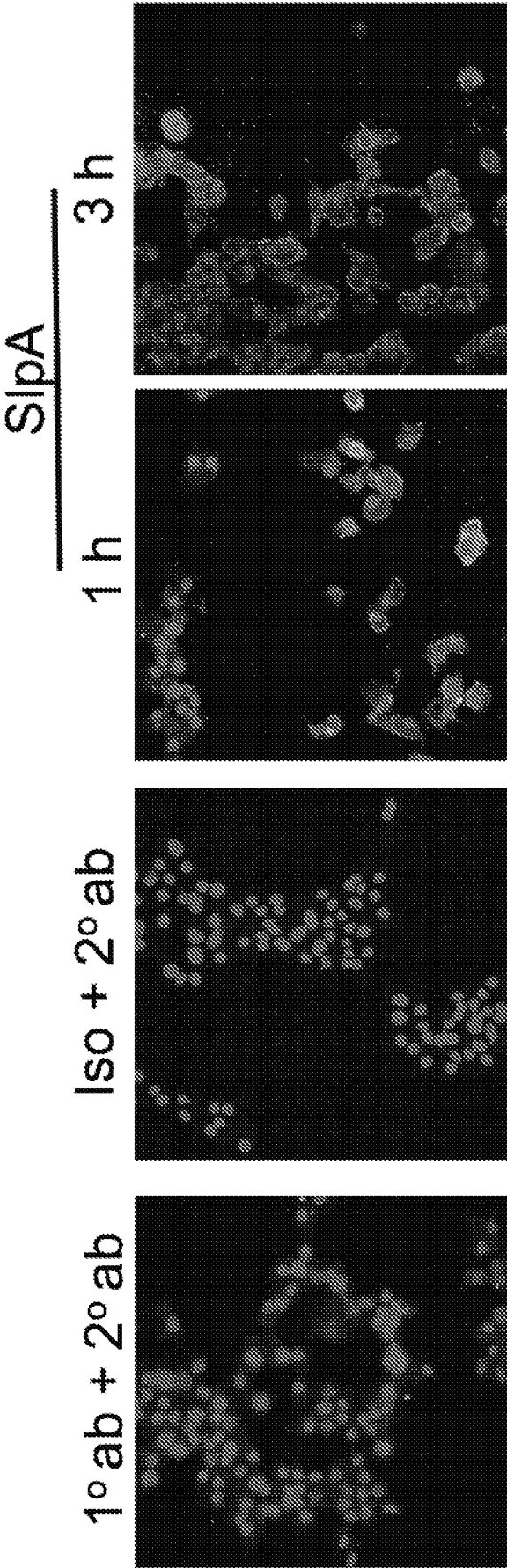


FIG. 17C

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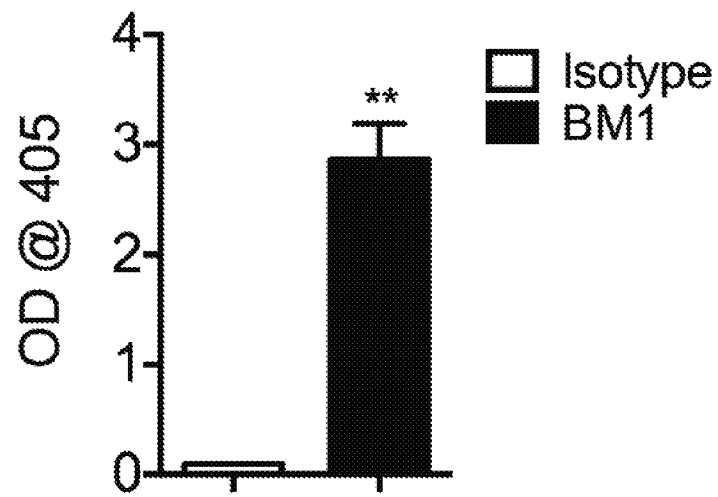


FIG. 17D

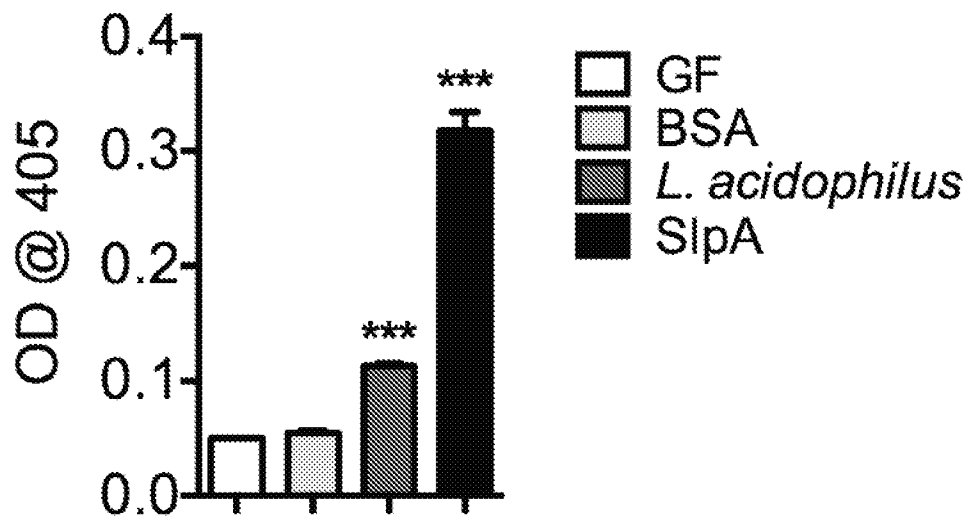


FIG. 17E

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 2015/055620

A. CLASSIFICATION OF SUBJECT MATTER

*C07K 14/335 (2006.01)**C12N 1/21 (2006.01)**A61K 38/16 (2006.01)**A61P 1/00 (2006.01)*

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)

C07K 14/335, 14/195, C12N 1/21, A61K 38/16, A61P 1/00, A61K 38/00

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)

PatSearch (RUPTO internal), EMBL, NCBI, PAJ, Espacenet, DWPI, Patentscope, USPTO, CIPO (Canada PO), SIPO DB, Freepatentsonline, FASTA, NucleotideBLAST

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X Y	US 7348420 B2 (NORTH CAROLINA STATE UNIVERSITY) 25.03.2008, abstract, col. 1-6, 16, 18, 20, 35-36, 82-83, example 3, claims	1-4, 7-11, 34-36 5-6, 12-23, 25-27, 37-40
Y	LIGHTFOOT Yaima L. et al. Tailoring gut immune responses with lipoteichoic acid-deficient <i>Lactobacillus acidophilus</i> , <i>Frontiers in immunology</i> , 2013, Vol.4, Article 25, pp.1-6	12-23, 25, 37-40
Y	WANG FU-JUN et al. Tandem multimer expression and preparation of hypoglycemic peptide MC6 from <i>Momordica charantia</i> in <i>Escherichia coli</i> , <i>Appl Biochem Biotechnol</i> , 2012, 166, pp.612-619	5-6



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents:	"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
"A" document defining the general state of the art which is not considered to be of particular relevance	"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
"E" earlier document but published on or after the international filing date	"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
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&" document member of the same patent family
"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	

Date of the actual completion of the international search

21 January 2016 (21.01.2016)

Date of mailing of the international search report

31 March 2016 (31.03.2016)

Name and mailing address of the ISA/RU:

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Telephone No. (495)531-65-15

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 2015/055620

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X Y	US 2012/0308994 A1 (ROCHE DIAGNOSTICS OPERATIONS, INC.) 06.12.2012, example 1, paragraphs [0113] - [0114]	24 25-27

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 2015/055620

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☒ Claims Nos.: 28-33
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.